GENE THERAPY FOR EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS BY DELIVERY OF INHIBITORY CYTOKINES OR CYTOKINE INHIBITORS

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine of University College London

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In memory of Philip Croxford, with love.
ACKNOWLEDGEMENTS

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THANKS everyone!
**ABSTRACT**

Studies have shown that experimental allergic encephalomyelitis, a model for Multiple Sclerosis, can be inhibited by repeated administration of anti-inflammatory cytokines or cytokine neutralising agents. Given the short half-life of many biological agents, administration must be frequent to maintain a therapeutic effect. Gene therapy allows long-term *in vivo* delivery of these biological therapeutic agents. This study has investigated the use of viral and non-viral vectors administered systemically and locally to the central nervous system, to deliver the anti-inflammatory cytokines IL-4, IL-10 TGF-β and IFN-β with the aim of ameliorating EAE. In addition, neutralisation of TNF using soluble TNF receptors and blockade of T-cell costimulation using CTLA4-Ig fusion proteins and anti-B7 antibodies were also studied. This study demonstrates that EAE can be successfully inhibited using gene delivered biological agents by a variety of vectors, either during the priming stage of disease or after disease onset. Local administration of the vector to the CNS increases the efficacy of therapeutic agents even at lower doses than used systemically, and may reduce the systemic side effects seen with standard high-dose protein therapy. The study of currently available vectors under the same conditions and in the same model allows a useful comparison and highlights both advantages and disadvantages of each method. This study provides data on a variety of successful approaches of gene delivery to treat an ongoing CNS autoimmune disease, which hopefully will provide a basis for using this method in humans.
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<tr>
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<tr>
<td>A</td>
<td>Astrocyte</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated Virus</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABH</td>
<td>Antibody High</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2' Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)</td>
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<tr>
<td>Ad</td>
<td>Replication deficient E1 deletion mutant of type 5 human adenovirus</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine Deaminase</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AP</td>
<td>Acute Phase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>B</td>
<td>Bone Marrow-derived lymphocyte</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>Barium Chloride</td>
</tr>
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<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>CDV</td>
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<td>CLC</td>
<td>Cationic Liposome Complex</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>COP-1</td>
<td>Co-Polymer-1</td>
</tr>
<tr>
<td>CREAIE</td>
<td>Chronic Relapsing Experimental Allergic Encephalomyelitis</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
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Fc regions

D11  Day 11
DAB  Diaminobenzidine chromogen
dd   Double Distilled
DHFR  Dihydrofolate Reductase
DMEM  Dulbecco’s Modified Eagle’s Medium
DNA  Deoxyribonucleic Acid
dTNFR  Dimeric Tumour Necrosis Factor Receptor
dTNFR-tsF  Dimeric Tumour Necrosis Factor Receptor expressing temperature-sensitive fibroblasts

E  Endothelial cell
EAE  Experimental Allergic Encephalomyelitis
ECD  Extra-cellular Domain
ELISA  Enzyme Linked Immunoabsorbant Assay
Eo   Eosinophil
F  Fibroblast
F(ab)  Fragment Antigen Binding
FACS  Fluorescent Activated Cell Sorter
Fc   Fragment Crystalline
FCS  Foetal Calf Serum
FDA  Federal Drug Administration
FITC  Fluoroscein Isothiocyanate
GalC  Galactocerebroside
GFAP  Glial Fibrillary Protein
GRO-α  Chemokine Growth Related Oncogene alpha
HBSS  Hanks Balanced Salt Solution
HCl  Hydrochloric Acid
HCC  Human β (CC) Chemokine
H2O2  Hydrogen Peroxide
HLA  Human Leucocyte Antigen
HHV-6  Human Herpes Virus 6
HSP-65  Heat Shock Protein-65
HSV  Herpes Simplex Virus
HSV-tk  Herpes Simplex Virus-thymidine kinase
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<td>HTLV-1</td>
<td>Human T Lymphotropic Retrovirus type 1</td>
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<tr>
<td>i.c.</td>
<td>Intra-cranial</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Inter-cellular Adhesion Molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IGF-1</td>
<td>Insulin-like Growth Factor-1</td>
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<td>i.m.</td>
<td>Intra-muscular</td>
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<tr>
<td>IP</td>
<td>IFN-inducible protein</td>
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<td>i.p.</td>
<td>Intra-peritoneal</td>
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<td>i.v.</td>
<td>Intra-venous</td>
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<tr>
<td>kDa</td>
<td>KiloDalton</td>
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<td>LFA</td>
<td>Leucocyte Functional Antigen</td>
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<td>LTR</td>
<td>Long Terminal Repeats</td>
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<td>m</td>
<td>Murine</td>
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<tr>
<td>M</td>
<td>Macrophage</td>
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<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MAG</td>
<td>Myelin-associated Glycoprotein</td>
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<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
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<tr>
<td>MCP</td>
<td>Monocyte Chemoattractant Protein</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>Microglia</td>
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<td>MIP</td>
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<td>MIU</td>
<td>Million International Units</td>
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<td>MMuLV</td>
<td>Moloney Murine Leukaemia Virus</td>
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<td>MOG</td>
<td>Myelin Oligodendrocyte Glycoprotein</td>
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<td>MRI</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>Magnetic Resonance Spectroscopy</td>
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<td>Multiple Sclerosis</td>
</tr>
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<td>MSRV</td>
<td>Multiple Sclerosis-associated Retrovirus</td>
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</table>
MV  Measles Virus
N   Neutrophil
NGF Nerve growth factor
NK  Natural Killer Cell
NMR Nuclear Magnetic Resonance
NMS Normal Mouse Serum
NSE Neuron-specific Enolase
OCT Ornithine Transcarbamylase
OD  Optical Density
OG  Oligodendrocyte
ON  Onset
PA  Post Acute
Pa  Perivascular astrocyte
PBMC Peripheral Blood Mononuclear Cells
PBS Phosphate Buffer Solution
PCR Polymerase Chain Reaction
PE  Phycoerythrin
PFU Plaque Forming Units
p.i. Post-inoculation
PLP Proteolipid Protein
PML Progressive Multifocal Leukoencephalopathy
PMN Peripheral Mononuclear Cells
PP  Primary Progressive
PVE Post Vaccinal Encephalomyelitis
r  Recombinant
RANTES Regulated upon Activation, Normal T cell Expressed and Secreted
RCV Replication Competent Virus
RL  Relapse
RM  Remission
RSV Rous Sarcoma Virus
RV  Rubella Virus
s.c. Sub-cutaneous
SCH Spinal Cord Homogenate
SEM Standard Error of the Mean
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>SFV</td>
<td>Semliki Forest Virus</td>
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<tr>
<td>SP</td>
<td>Secondary Progressive</td>
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<td>SV5</td>
<td>Simian Virus 5</td>
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<td>SV40</td>
<td>Simian Virus 40</td>
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<td>T</td>
<td>Thymus-derived Lymphocyte</td>
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<td>TC</td>
<td>Tricolor</td>
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<td>TCA</td>
<td>T cell activation gene</td>
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<td>TCR</td>
<td>T Cell Receptor</td>
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<tr>
<td>Tet</td>
<td>Tetracycline</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper lymphocyte</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler's Murine Encephalomyelitis Virus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour Necrosis Factor Receptor</td>
</tr>
<tr>
<td>tsA</td>
<td>Temperature-sensitive Astrocyte</td>
</tr>
<tr>
<td>tsF</td>
<td>Temperature-sensitive Fibroblast</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
</tr>
<tr>
<td>VC</td>
<td>Vascular Smooth Muscle Cell</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
</tr>
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<td>WL</td>
<td>Weight Loss</td>
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CHAPTER ONE

INTRODUCTION
1.1. **MULTIPLE SCLEROSIS**

Multiple sclerosis (MS) is the most common human primary demyelinating disease of the central nervous system (CNS). It affects mainly young adults at diagnosis, between the ages of 20-40 years, with a prevalence of between 5-300 per 100,000 in developed societies. MS occurs more frequently in women than in men (Ransohoff 1992). MS is now widely considered to be an immune-mediated disease of the CNS featuring blood brain barrier (BBB) breakdown, oedema and inflammation. This is probably due to an autoimmune pathogenesis directed at the CNS resulting in the destruction of the myelin sheath surrounding neurons, which causes loss of neuronal function in patients. The aetiology of MS is still unknown, but both genetic and environmental stimuli are considered to play a role in the development of this disorder. The fact that MS is heterogeneous in its onset, symptoms, pathology and disease course highlights the potential problem of those seeking to treat or investigate MS.

Although there are recorded accounts of MS as early as the eighteenth century by Sir Augustus Frederic D'Este, the first observations of the clinical symptoms of MS were made by Robert Carswell (1838) and Jean Cruveillhier (1835-1842). However, the first description of MS as a clinical disease in a living patient was made by Jean Martin Charcot (1877). As well as noting the tremor in patients he also recorded spontaneous remissions in disease progression. Further characterisation of MS symptoms and pathogenesis has been in progress since Charcot's reports.

**1.1.1. Diagnosis of Multiple Sclerosis**

At present there is no specific diagnostic test for MS. Thus MS is not usually diagnosed until the patient has experienced at least two disease episodes involving two areas of the CNS on two separate occasions, at least a month apart (Schumacher et al., 1965). MS also shares symptoms with other diseases so it is necessary to perform numerous tests including neurological examination to exclude other disease possibilities. Lumbar puncture for assessment of oligoclonal immunoglobulin bands in cerebrospinal fluid (CSF) and more recently magnetic resonance imaging (MRI) have been used to aid the diagnosis of MS. MRI detects nuclear magnetic resonance (NMR) of protons present in the lipid and water of tissue and allows the visualisation and determination of CNS structure and the study of MS lesion formation *in vivo*. MRI scans in conjunction with
CSF analysis and clinical examination of the patient were utilised by Poser to define criteria to aid the diagnosis of MS (Poser et al., 1983). A patient with a history of attacks characteristic of MS who also has CNS lesions present identified by MRI, can be diagnosed, by the Poser criteria, with clinically definite MS, or with clinically probable MS, in patients with disease episodes where no MRI has been performed (Poser et al., 1983). MRI may also be of use as a prognostic indicator where individuals who had many white matter lesions at presentation of disease episodes went on to develop MS within three years, whereas those who had normal or near normal white matter had a much lower risk of MS (Ford et al., 1992).

The main pathologic feature of MS is primary demyelination. This term describes the destruction of oligodendrocytes and the myelin sheath surrounding the axons in the CNS with relative sparing of the associated axon. Any region of the CNS containing myelin is susceptible to damage and therefore symptoms of MS are variable between patients and can differ in their severity and duration. Often, initial symptoms include sensory loss, such as blurred vision, hearing problems, numbness or tingling sensations in the limbs (Whitaker and Mitchell, 1997). Over the time course of MS many more physical and psychological symptoms become apparent, including weakness and fatigue, reduced tactile sensations, thermal abnormalities, insensitivity to, or chronic pain, spasms, bladder and sexual dysfunction, and cerebellar and spinal cord dysfunction which can cause limb ataxia and paralysis (Whitaker and Mitchell, 1997).

Various risk factors have been described which are thought to be involved in inducing relapse in MS patients, such as stress, trauma, elevated temperatures, infections and immunisation (reviewed by Whitaker and Mitchell, 1997). However, there is little direct evidence to suggest these factors play a role in the exacerbation or induction of MS. MS exhibits a temporal profile of neurological deficits termed "exacerbations" (relapses) which result in multiple episodes of symptoms. Consequently, symptoms may be resolved and either partial or complete restoration of function may occur, in a period termed remission. Often, remission fails to restore fully the deficit and disease worsens with each new relapse.

1.1.2. Clinical types of MS

A group of five distinct demyelinating diseases have been described under the main title
of multiple sclerosis but which all share the same hallmark, namely primary demyelination. The most prevalent of the five is the *Classic (Charcot)* type which afflicts adults mainly between 20-40 years of age, with a relapsing-remitting disease course spanning several decades before developing into a chronic progressive stage and finally death. Usually the disease course is highly unpredictable and the neurological deficit sometimes worsens with each subsequent relapse. *Acute MS (Marburg)* is common in younger patients and characterised by a relatively short duration of disease course coupled with an early death within 2 years (Marburg 1906). *Schilder* type MS (Schilder 1912) usually presents in children and adolescents. It differs from the classic form in that it features Wallerian degeneration (axonal loss) and variable axonal destruction in the cerebellum and brain stem, as well as the customary demyelinated plaques. However, the disease course follows a similar pattern to the *classic* type. Another variant of MS is the *Devic* type (*neuromyelitis optica*), which is an acute form of MS, which can result in death by 3 months. It is characterised by severe demyelination in both the optic nerve and spinal cord within days or weeks of each other, causing visual impairment and spinal cord abnormalities (Gault 1895). The fifth type is *Balò’s MS (concentric sclerosis)* and is comparatively rare. It is an acute form of the disease and is rapidly progressive, with characteristic irregular concentric patterns of demyelination separated by narrow bands of preserved myelin (Balò 1928). These types of MS all present the essential histological feature of the demyelinated plaque and are grouped together for this reason but it is possible that these diseases have different aetiologies, and disease mechanisms.

There are 5 sub-types of MS based on the temporal profile of neurological deficits and remissions, which are used as standard classification (Lublin et al., 1996). *Primary-progressive (PP)* MS shows progressive disease from the onset of clinical symptoms, and prevails in ~15% of patients. Symptoms steadily worsen and although disability may plateau, the usual course is progressive. *Relapsing-remitting (RR)* MS affects 25% of MS patients and presents as clearly defined exacerbations and remissions with little residual deficit. Remissions may last for long periods of time although clinically “silent” lesions may still be seen by MRI. After many attacks the patient may develop *progressive-relapsing* MS where patients develop clearly defined exacerbations, but where neurological deficit is not restored during remission and the patients symptoms worsen with each subsequent relapse. *Secondary-progressive (SP)* MS exhibits a RR form of disease initially but then exhibits a progressive worsening of disease with little
or no relapses. About 40% of MS patients develop SP MS, usually after 15-20 years of onset. Finally, patients who experience a small number of mild disease symptoms with complete recovery and who exhibit no permanent disability after 10-15 years post-onset are diagnosed as having benign MS, which occurs in about 20-30% of MS patients.

1.1.3. Neuropathology of MS

White matter consists of millions of insulated nerve fibres, coated by myelin sheaths. These restrict the ion flux across the axon membrane allowing the saltatory conduction of action potentials along the axon via the Nodes of Ranvier. In the CNS the production, ensheathing and maintenance of myelin on the axon is undertaken by oligodendrocytes, one of the two macroglial cells in the CNS, and in the periphery by the Schwann cell. Oligodendrocytes can extend up to 50 processes, which encompass many axons compared to the peripheral nerves whereas Schwann cells typically ensheathe only one axon. Lipids constitute 70% of myelin with proteins making up the other 30%, thus making it ideal for insulation of electrical impulses.

Typically in MS, demyelinated lesions with varying levels of mononuclear cell infiltrates are located irregularly in the white matter tracts although lesions can also be present within the grey matter. However, distribution of lesions and lesion load varies between patients. Often, lesions in the brain are roughly symmetrical and have a predilection for periventricular regions (90% of all MS patients) (Lumsden, 1970) particularly around the lateral and fourth ventricles. The spinal cord and optic nerve often show demyelination of nerve fibres, loss of oligodendrocytes and fibrous scarring (sclerosis) due to astrogliosis. In addition in the later stages of MS, atrophy of nerve fibres can lead to axonal loss (Raine, C. S. 1997). In contrast, the peripheral nervous system appears to be relatively unaffected. The brain stem is a commonly affected area and lesions in this area have been correlated to clinical symptoms such as respiratory failure and coma. In contrast to the brain, spinal cord lesions tend to be distributed randomly, often crossing anatomical or functional boundaries, but are twice as likely to be found in the cervical region (Raine, C. S. 1997).

Histological analysis of MS is usually post-mortem, which may be years or decades after onset. The histological appearance of MS lesions depends upon the activity and age of the lesion. Generally, lesions fall into one of four categories: the chronic MS
plaque, the chronic active MS plaque, the acute MS plaque and shadow plaques. The striking feature of an acute MS plaque typically associated with acute or severe chronic progressive MS, is its indistinct margin, due to active demyelination, and also its intense inflammatory response associated around small blood vessels. Finger-like lesions of accumulated leucocytes, projecting along the length of blood vessels have been termed "Dawson's Fingers" after J. W. Dawson (1916). There are many macrophages containing myelin debris throughout the lesion, but in contrast there is little fibrous astrogliosis within the centre of the lesion. These lesions also have a marked decrease in axon numbers associated with acute MS, as well as being highly oedematous due to BBB breakdown. Zones of cells, which are possible precursors to astrocytes and oligodendrocytes, which display glial cell phenotypic markers, are also present and may represent an early phase of remyelination and repair.

Chronic active lesions have a prominent perivascular and parenchymal inflammatory infiltrate consisting of CD4+ and CD8+ T cells and monocytes/macrophages. The plaque margin contains hypertrophic astrocytes and many macrophages involved in demyelination, containing myelin debris stained positive by oil-red-O (Raine, C. S. 1994). Unusually there are both oligodendrocyte hyperplasia and ongoing demyelination within these lesions, suggesting attempted remyelination of demyelinated axons (Raine, C. S. 1981). These lesions are often associated with chronic progressive MS with a short duration.

Chronic MS plaques are the most readily detectable lesions and appear as demyelinated plaques with a sharp edge. However, axons that pass through the lesion are often preserved. Around the edge of the lesion there is often residual activity including oligodendrocyte proliferation, and reactive astrocytes are present. Also within chronic lesions there is a low level of inflammatory cell infiltrate, consisting of lymphocytes, plasma cells and mononuclear cells. Macrophages can be detected in the lesion margin, and "foamy" macrophages, those that contain lipids, can be distributed throughout the lesion and in the surrounding white matter. Macrophages containing myelin debris, are however not present in this type of lesion.

The shadow plaque has a controversial history, being thought to either represent early signs of an active lesion or an area of CNS remyelination. It presents as a diffuse area and myelin staining shows thinly myelinated fibres. There is little inflammatory or
demyelinative activity within the lesion and is currently considered to represent an area of CNS remyelination.

The different types of lesion described may be due to the stage of the lesion development at the time of biopsy. However, recently it has been proposed that the pathologic heterogeneity seen in MS lesions may be dependent upon the individual patient and not necessarily on the stage of lesion development (Lucchinetti et al., 1996). A study on oligodendrocyte pathology in samples, including biopsies, from early disease, acute and chronic active MS showed that lesions in an individual maintained a characteristic pattern of oligodendrocyte pathology regardless of their stage of development or location (Lucchinetti et al., 1996). The study also proposed putative mechanisms whereby each of these 4 characteristic patterns could develop their distinctive pathology. However, currently these pathologic descriptions have not been definitively linked to certain types of MS.

1.1.4. The Cellular Pathogenesis of MS

Major histocompatibility complex (MHC) molecules can determine which protein sequences an individual's immune system responds to and also the type and severity of immune response to those sequences. The upregulation of MHC class II antigen (Ag) expression in the CNS suggests immune involvement in MS. Histological studies in MS tissue have demonstrated large numbers of macrophages present in active lesions as well as microglial cells in the parenchyma, and that these cells have the propensity to express MHC class II Ag (Traugott et al., 1983; Woodroffe, et al., 1986; Hauser, et al., 1986). It has also been suggested that other cells may also express MHC class II Ag, such as endothelial cells and astrocytes.

Other features of MS also suggest immunological involvement in the disease process. Studies have observed perivascular infiltrate consisting of lymphocytes and mononuclear cells present at sites of demyelination during the course of MS (McFarland and Dhib-Jalbut 1989). Many studies have concentrated on the analysis of the cellular infiltrate phenotype in MS lesions, particularly chronic active lesions and acute lesions. Generally it was reported that cytotoxic CD8+ T cells were present in greater numbers in the plaque parenchyma whereas helper CD4+ T cells were present in acute lesions and in the perivascular spaces surrounding the lesion margin, with little T
cell presence in silent lesions (McCallum, et al., 1987; Booss, et al., 1983; Traugott, et al., 1983; Nyland, et al., 1982). The γδ T cell receptor (TCR) expressing subset has been demonstrated to be present in chronic MS lesion parenchyma and is thought to have a cytolytic capacity and may be a possible pathway of oligodendrocyte death (Selmaj, et al., 1991a). B cells are often retained around the perivascular space and are very rarely found in plaques. However, myelin-specific antibodies have been detected in MS lesions (Genain et al., 1999).

One constant feature of MS is the entry of haematogenous inflammatory cells into the CNS resulting in perivascular cuffing. Immune cell extravasation is governed by the interaction of cellular adhesion molecules on inflammatory and endothelial cells. As a consequence inflammatory cells can bind to endothelial cells and enter tissues by diapedesis, following a chemokine gradient (Section 1.4.5).

Developing lesions show gadolinium (small molecular weight tracer)-enhancement in MRI scans and this is normally indicative of BBB breakdown and oedema, with perivascular infiltration of lymphocytes and macrophages (Katz et al., 1993). However, these new lesions, associated with relapsing-remitting and secondary-progressive MS, develop more often than detectable clinical deficit and often enhance for about 2 weeks to 1 month. Often gadolinium-enhanced lesions are asymptomatic and suggest that BBB breakdown may be an initial step in lesion formation. Demyelination and axonal loss may account for the more severe deficit that cannot be reversed. Whereas gadolinium-enhanced MRI lesions cannot distinguish the amount of demyelination present in lesions, proton magnetic resonance spectroscopy (MRS) may allow the assessment of individual lesions and how the amount of demyelination present relates to clinical features of MS (Davie et al., 1994).

1.1.5. Epidemiology of MS

To date there have been over 300 prevalence studies for MS. The distribution of MS worldwide can be divided into 3 zones according to frequency or risk: the high risk zone accounts for a prevalence rate (up to 1996) of 30 or more MS cases per 100,000 population and includes northern and central Europe, Canada and northern USA, New Zealand, southeastern Australia and the former Soviet Union (Kurtzke, J. F. 1997).
Chapter 1 Introduction

Medium frequency areas, with a prevalence of between 5-29 MS cases per 100,000 population include southern USA, southwest Norway, northern Australia, northern Scandinavia and the southwest and far east of Russia (Kurtzke, J. F. 1997) and low risk zones include Asia, Africa, Alaska and Greenland, northern South America and the area around the Caribbean, with a prevalence of below 5 MS cases per 100,000 population. In the United Kingdom the number of MS patients is 100 per 100,000 population.

Caucasians from high or medium risk areas are most prone to MS and the incidence of MS increases in temperate zones with moderate or cold climates, and has been linked to both genetic and environmental factors. Migration studies suggest that migrants from high risk areas moving to low risk areas still retain their original risk factor if they move after the age of 15 years. However there is little data on low to high risk migration although it appears that there is an increase in risk (Kurtzke, J. F. 1997).

The age of acquisition of MS “causal factors” may also play a role in determining the migration effect. Young children are not susceptible to MS or at least are not typically diagnosed with MS, and this would suggest a “latency” or “incubation” period of MS where clinical symptoms are not apparent. Therefore MS may be an acquired disease dependent upon environmental factors such as climate (Laborde, et al., 1988), different infectious agents (Kurtzke et al., 1979) and nutrition, especially regarding the consumption of saturated or unsaturated fats (Swank, R. L. 1951; Millar, J. H. D. 1975). Geographical gradients also provide evidence for environmental factors. Studies have shown that a geographical gradient exists in the southern hemisphere and vice versa in the northern hemisphere, including Australia (Hammond, et al., 1988), New Zealand (Skegg, et al., 1987) and the USA (Kurtzke, et al., 1979). However, genetic susceptibility studies were not carried out in these studies and therefore the results can only be an indication that there maybe some environmental influence dependent on latitude for the onset of MS. Further support for environmental factors can be found in studies from the Faroe Islands, Iceland and the Shetland-Orkney Islands although some studies contradict these findings (reviewed by Kurtzke, J. F. 1997). It has been suggested that these islands have experienced epidemics of MS. The first recorded case of MS on the Faroe Islands was in 1943. A possible source of the epidemic could be the arrival of British Troops in 1941 during the Second World War exposing natives to new “environmental factors”. Iceland has had two increases in the total number of MS cases, and significantly both related to the time of the First and Second World Wars
when Iceland was occupied by the British, Canadians and Americans. These studies suggest that there may be a viral component in the induction of MS although at present no single virus has been found consistently in all MS patients.

Although environmental factors appear to play a major role in the risk of MS, genetic factors are likely to confer different susceptibility to MS for each individual. This has been supported by studies showing the differential susceptibility of different ethnic backgrounds living in the same environment. For example, the Japanese have a very low MS incidence, which is retained in Japanese people migrating to the USA, although the MS incidence is higher in these Japanese than in those who still live in Japan (Waksman, et al., 1984). This is also seen in Hungarian Gypsies who have a lower MS incidence than the general Hungarian population (Palffy et al., 1982).

1.2. AETIOLOGY OF MS

1.2.1. Genetics of MS

Familial studies have provided evidence for a genetic role in MS susceptibility. First-degree family members have an increased risk of MS compared to distant relations. Twin studies have shown that the concordance rate amongst siblings is about 4%, and 3% between parents and children of MS patients (Sadovnick, et al., 1988). Dizygotic twins have ~5% concordance rate which increases up to ~30% in monozygotic twins (Sadovnick, et al., 1993). Studies of adoptees have shown that the risk of MS is genetically determined but that its development depends on environmental factors particular to the geographical location studied (Ebers et al., 1995). MRI has been used to correlate changes in disability with an increase in lesion number and lesion enlargement (Filippi et al., 1995). Interestingly MRI scans on clinically unaffected twins of MS patients revealed that about 40% of monozygotic and dizygotic twins had MRI abnormalities when compared to healthy controls (19%), although only 13% and 9% respectively of twins had lesions typical of MS (Thorpe et al., 1994; Mumford et al., 1994). It was suggested that at least 10% of unaffected twins have "subclinical multiple sclerosis" which can be detected as "silent lesions" in MRI scans (Thorpe et al., 1994).

Susceptibility to many autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes mellitus have been linked to MHC antigens, notably the MHC class
II alleles (Todd et al., 1989; Ronningen et al., 1990). Susceptibility to MS is thought to be conferred at least by the human leucocyte antigen (HLA) or MHC class II alleles, including HLA-DR2 and DR15 haplotypes (Haines et al., 1998; Coraddu et al., 1998; Haegert et al., 1996), DQW1 (Francis et al., 1986), DQA1 (Spurkland et al., 1991), DQB1 (Vartdal et al., 1989) but not HLA-DM (Ristori et al., 1997). However, other studies have demonstrated conflicting results indicating that the DQA1 and DQB1 alleles may play a protective role in MS (Haegert et al., 1996). Conflicting data from susceptibility studies may be due to regional variations and genetic heterogeneity in MS susceptibility conferred by MHC alleles. Three recent complete genome screens were applied to MS patients to try to identify MS susceptibility loci (The Multiple Sclerosis Genetics Group 1996; Ebers et al., 1996; Sawcer et al., 1996). However, apart from a region spanning the MHC, where it was not certain if this represented linkage or linkage disequilibrium with other loci in this region, potential candidate loci from previous studies were not found to be significantly linked to MS. Although the MHC plays an important role, other non-MHC alleles may be involved in MS. These genetic studies suggest that MS is a complex genetic disorder and rather than there being one major susceptibility locus, minor susceptibility loci may have additive and/or epistatic effects and that there may be multiple genetic pathways involved in the susceptibility and control of MS.

1.2.2. Putative Role of Infectious Agents in MS

Epidemiological studies have highlighted the likelihood of environmental factors playing a role in the aetiology of MS, specifically infective agents, such as viruses. Many viruses have been studied as potential triggers of MS, but at present none of these have been identified as significant in MS aetiology. Although different viruses have been isolated from MS patients, none has been found universally in all MS patients or achieved significantly higher virus-specific antibody titres than those found in normal healthy individuals or patients with other neurological disorders (Table 1.1). There are a number of potential mechanisms whereby a virus can cause demyelination of axons either by targeting myelin sheaths or oligodendrocytes. Viral infection of oligodendrocytes can induce a cytopathic effect, causing lysis or inhibition of metabolic activity of oligodendrocytes, which then are no longer capable of remyelinating axons in the CNS. Demyelination could also occur as a response of direct lytic activity of infected cells by immune-mediated antibody responses or cytotoxic/cytolytic T cell
responses. Bystander demyelination describes a process where immune-mediators secreted from an immune response against viral proteins or other non-myelin proteins can cause damage to oligodendrocytes or myelin sheaths in the immediate vicinity. Here lysis of the oligodendrocyte could occur due to the immune-mediators such as humoral response factors, tumour necrosis factor (TNF), proteolytic enzymes and oxygen free radicals. An example of experimental bystander demyelination was observed in a study where heat-inactivated *Bacillus Calmette-Guerin* was injected intracranially and induced a delayed-type hypersensitivity response. The response consisting of mononuclear phagocytes and T cells failed to clear the mycobacterium but resulted in myelin damage (Matyszak et al., 1995). Cytokines produced from this event have the potential to upregulate adhesion and MHC molecules which may result in the increased presentation of autoantigens normally sequestered in the CNS, leading to the activation of quiescent autoreactive T cells and an autoimmune response directed to the CNS. Finally, the host's response to the virus may play a role in the outcome of CNS infection. An aberrant response may result in an inappropriately strong pro-inflammatory response to the virus, or alternatively a weak response may allow the virus to persist allowing a chronic CNS infection. Considering the heterogeneity of MS it is possible that different CNS viral infections may be responsible for different forms of MS and that the genetic susceptibility and immunological status of each individual may also play a role in determining the outcome. Therefore, the way in which viruses interact with CNS cells, and the way in which the immune system responds to persistent viruses may provide more data on this subject.

Other CNS demyelinating diseases, both in animals and humans, are known to be associated with viral agents. In humans these include subacute sclerosing panencephalitis, where high antibody titres to measles virus antigens can be detected in the serum and CSF of patients, as well as measles virus antigens in neurons and glial cells (Meulen, et al., 1997); progressive multifocal leukoencephalopathy (PML), a subacute demyelinating disease, associated with papovavirus, detected in intranuclear inclusion bodies in oligodendroglia within lesions (Padgett, et al., 1976); HTLV-1 associated myelopathy/tropical spastic paraparesis (Osame et al., 1986; Gessain et al., 1985), where HTLV-1 has been detected in the white matter of patients but not in cells in the perivascular lesions, and parainfectious encephalomyelitis which is associated with an acute viral infection of a number of viruses including measles virus, influenza virus and varicella-zoster virus (Meulen, et al., 1997).
Table 1.1 Evidence for Viral Aetiology of MS.

<table>
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<tr>
<th>Virus</th>
<th>Tissue(s) Studied</th>
<th>Evidence for Viral Presence</th>
<th>Reference</th>
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<tbody>
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<td>serum / CSF</td>
<td>neutralising Ab to MV</td>
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</tr>
<tr>
<td></td>
<td>brain tissue</td>
<td>measles virus specific mRNA</td>
<td>Haase 1981</td>
</tr>
<tr>
<td></td>
<td>serum / CSF</td>
<td>no presence</td>
<td>Hall 1982; Godec 1992</td>
</tr>
<tr>
<td>SV5</td>
<td>bone marrow CSF</td>
<td>SV5 virus particles</td>
<td>Field 1972</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>antibodies to SV5</td>
<td>Goswami 1987</td>
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<tr>
<td></td>
<td>CSF</td>
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<td>serum</td>
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<td>Catalano 1972</td>
</tr>
<tr>
<td>RV</td>
<td>serum</td>
<td>antibodies to rubella</td>
<td>Horikawa 1973</td>
</tr>
<tr>
<td>CDV</td>
<td>serum</td>
<td>antibodies to canine distemper virus</td>
<td>Cook 1979</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>serum</td>
<td>antibodies to coronavirus</td>
<td>Salmi 1982</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>CSF</td>
<td>antibodies to <em>gag</em> protein of HTLV-1 but not <em>env</em> protein</td>
<td>Ohta 1986</td>
</tr>
<tr>
<td>HHV-6</td>
<td>brain tissue</td>
<td>present in MS and healthy tissue, only present in OG</td>
<td>Challoner 1995</td>
</tr>
<tr>
<td></td>
<td>PBMC / CSF / brain &amp; spinal cord lesions</td>
<td>no difference to level found in normal healthy tissue and Alzheimer's or Parkinson's tissue</td>
<td>Merelli 1997; Sanders 1996</td>
</tr>
<tr>
<td>MSRV</td>
<td>plasma / CSF</td>
<td><em>pol</em> sequence of MSRV</td>
<td>Perron 1997</td>
</tr>
</tbody>
</table>

MV - Measles Virus; SV5 - Simian Virus 5; HSV - Herpes Simplex Virus; RV - Rubella Virus; CDV - Canine Distemper Virus; HTLV-1 - Human T Lymphotropic Retrovirus type 1; HHV-6 - Human Herpes Virus 6; MSRV - Multiple Sclerosis-associated Retrovirus; OG - oligodendrocytes.
Although none of these diseases follow the temporal characteristics of MS they do suggest a possible role for viruses in the destruction of myelin. In addition to viruses being implicated as aetiological agents in MS, other agents such as bacteria (spirochaetes) have also been suggested (Gay and Dick. 1986)

1.2.3. Putative Role of Autoimmunity in MS

Myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been used to induce EAE, an experimental model of MS (Tuohy et al., 1988; Linnington et al., 1993; Amor et al., 1993; Amor et al., 1994). It is this observation that has suggested that MS may have an autoimmune component, whereby self-reactive T lymphocytes which have escaped clonal deletion in the thymus, become activated by as yet not fully understood mechanisms and cross the BBB and induce the attack of myelin components of the brain and spinal cord. However, no single protein Ag or encephalitogenic epitope has been shown to be consistent with MS pathogenesis. Studies have shown that normal healthy individuals possess a similar frequency of peripheral T cells specific for the myelin antigens MBP and PLP as found in MS patients (Ota et al., 1990; Pette et al., 1990). However, other studies have shown a greater number of reactive MBP-specific T cells responding to 6 different MBP epitopes, in MS patients (Olsson et al., 1992). It has also been suggested that a greater frequency of myelin reactive cells display an activated/primed phenotype in MS patients compared with healthy controls (Scholz et al., 1998).

Recent studies in EAE have shown a variety of T cell specificities during disease which recognise a diversity of epitopes of myelin antigens and suggest that “epitope” or “determinant spreading” may be involved in MS (Miller et al., 1995; Yu et al., 1996a). Interestingly, a particular epitope can induce disease with different characteristics depending on the strain of animal used, and different myelin antigens in the same strain can cause different patterns of pathology (Amor et al., 1996). MBP and PLP are located in compact myelin and lesions in EAE are often seen in the brain stem and spinal cord (Lassmann and Vass 1995), whereas MOG, a surface antigen, produces lesions where thin myelin sheaths are present such as the cerebellum and periventricular white matter (Lassmann and Vass 1995). It is likely that an individuals MHC haplotype will determine which epitopes they will respond to and therefore these factors may account
for the heterogeneity of disease seen in MS. Many studies have concentrated on association studies of MBP to MS, as this was the first antigen to be used in developing EAE. However, there are discrepancies between studies and this is probably a reflection of the difficulty in matching studies with different ethnic backgrounds and therefore different frequencies of allelic expression (Tienari et al., 1994; Boylan et al., 1990; Graham et al., 1993; Rose et al., 1993; Wood et al., 1994). The specificity of T cells for autoantigens may be due to certain disease-linked polymorphisms of the MHC genes. At present studies have failed to consistently find a TCR polymorphism associated with susceptibility to MS. Some studies have shown an association with TCR alpha and beta variable chains (Beall et al., 1989; Oksenberg et al., 1989; Seboun et al., 1989) whereas others have not (Hashimoto et al., 1991; Droogan et al., 1996). Autoantibodies have also been demonstrated in MS, and although they may not play a role in initiation of disease, their ability to fix complement or induce antibody-dependent cellular cytotoxicity, suggest they may be involved in demyelination in MS (Warren et al., 1994). Recently a study has shown anti-MOG autoantibodies present in lesions and attached to myelin debris in lesions (Genain et al., 1999).

1.2.4. Mechanisms of Autoimmunity

The initial mechanism of peripheral activation of autoreactive T cells in MS is unknown but may be due to the dual expression of TCR, activation by a “superantigen” or molecular mimicry. It had been accepted dogma that T cells express one specific TCR composed from variable α and β chains (Vα, Vβ). However, it has been shown that T cells can express 2 alleles from either the Vα or Vβ chain and therefore express 2 TCR of different specificities (Padovan et al., 1993; Padovan et al., 1995). By this mechanism, a dual TCR autoreactive T cell can become activated by recognising a foreign antigen, such as that from a bacterial or viral infection. Superantigens are immunostimulatory molecules produced by bacteria and viruses which bind to the specific Vβ chain subsets of the TCR and MHC molecules outside the binding cleft thereby activating a wide range of T cell clones non-specifically (Kotzin et al., 1993). Although superantigens have not been shown to induce EAE in normal non-transgenic animals, they have been shown to exacerbate disease and induce relapses once disease has been induced (Brocke et al., 1993) suggesting that although they may not play a role in the induction of MS, they may be involved in re-activation of autoreactive cells at
later stages of disease. Studies have shown common sequences between major CNS protein components such as PLP and MBP, and viruses (Jahnke et al., 1985; Souberbielle et al., 1991; Wucherpfennig et al., 1995), whereby autoreactive T cells specific for CNS antigens may be activated by recognising similar sequences present in viruses. This is termed "molecular mimicry". In a Theiler's murine encephalomyelitis virus (TMEV) model, virus specific CD4+ T cells initiated an inflammatory response resulting in demyelination, where after 3-4 weeks, T cell responses to PLP epitope 139-151 could be shown which were not due to cross reactivity to TMEV epitopes (Miller et al., 1997). This phenomenon of epitope or "determinant" spreading may explain the induction of an organ-specific autoimmune response following a persistent viral infection. The pathogenic mechanisms involved in autoimmunity have become targets for the current investigations into treatment for MS.

1.2.5. Treatment of Multiple Sclerosis

Therapy of MS is usually either symptomatic or immunosuppressive (Table 1.2). Although symptomatic treatments do not alter the natural history of MS, they are nevertheless important in improving the quality of life of MS patients. At least 1 in 2 patients suffer from severe pain and this can be effectively treated with amitriptyline. The spasticity associated with MS can be treated with drugs such as baclofen, and inhibition of bladder function with anti-cholinergic drugs can be effective (Azouvi, et al., 1996; Sheriff et al 1998).

At present glucocorticosteroids are used extensively for the treatment of acute exacerbations. Again, these therapies tend not to alter the actual disease course but can reduce recovery time from relapse. The actual mechanism by which they work is unknown but they are thought to be immunosuppressive, to reduce brain oedema (Barkhof et al., 1991) and promote axonal conduction (Smith et al., 1986).

As the pathogenesis of MS appears to involve the immune system, immunosuppressants such as cyclophosphamide, cyclosporin A, methotrexate and cladribine have been investigated although many of these compounds have toxic side-effects (reviewed in Goodkin, D. E. 1997). Although MS is thought to be cell-mediated, antibodies and other soluble factors may play a role in demyelination. Plasma exchange to remove immunoglobulin, has proved successful in other immune disorders such as Guillain-
Barré syndrome and myasthenia gravis. However studies have shown little benefit of plasma exchange in conjunction with oral prednisone and intravenous (i.v.) cyclophosphamide, or oral cyclophosphamide and oral prednisone (The Canadian Cooperative Multiple Sclerosis Study Group, 1991). However, to date there is no data on a comparison of plasma exchange and placebo alone.

1.2.6. Interferons in MS treatment

As MS is thought to have a viral aetiology it was proposed to investigate the use of interferons (IFN), which have antiviral properties. There are 3 main types of interferons; α- and β- IFN’s (leucocyte and fibroblast-expressed respectively) and IFN-γ (immune). Despite IFN-γ exhibiting pro-inflammatory properties such as the upregulation of MHC class II molecules in synergy with TNF, it was administered to MS patients. 7 out of 18 patients with MS had an increased exacerbation rate compared to those of placebo and the study was terminated (Panitch et al., 1987). Despite failing to treat MS patients this study provided evidence for the involvement of pro-inflammatory cytokines in MS pathology.

Despite many therapeutic claims, the first agents to show significant therapeutic benefit in double blind phase III placebo controlled trials were the interferons. IFN-β has been approved for therapy of relapsing-remitting MS in the USA by the Federal Drug Administration (FDA). IFN-α and β can inhibit viral replication and also induce MHC class I molecules which serve to increase viral epitope presentation to CD8+ cells. Interferons also activate natural killer cells to kill virus-infected cells selectively. The first clinical trial with IFN-α showed a slight reduction in exacerbations in patients with relapsing-remitting MS (Knobler et al., 1984). However, 2 subsequent studies showed no significant difference in either relapse rate or disability compared to placebo groups (AUSTIMS Research Group 1989; Camenga et al., 1986). Recently a study using higher doses of recombinant IFN-α reduced exacerbation rate with few side effects (Durelli et al., 1994).

When IFN-β was first studied in MS, it was administered intrathecally in its native form in crude preparations from fibroblasts, to ensure access to the CNS (Jacobs et al., 1981). IFN-β inhibits the synthesis of IFN-γ, which has been shown to be present in
demyelinating lesions (Traugott et al., 1988), and can prevent its synergy with TNF, which has also been observed in MS lesions (Hofman et al., 1990). This inhibitory effect can prevent the upregulation of MHC class II molecules and activation of macrophages and therefore reduce inflammatory episodes. It was found that IFN-β treated patients had fewer exacerbations and this was confirmed in a double-blind placebo-controlled trial (Jacobs et al., 1987). However a similar study by Milanese et al., (1990) demonstrated an increase in relapse frequency in treated patients although this was using a preparation of natural IFN-β induced by synthetic or viral oligonucleotides (DeMaeyer and De Maeyer-Guignard, 1988).

There are currently 2 different recombinant IFN-β preparations in use for MS. IFN-β-1a is a glycosylated recombinant IFN-β produced in Chinese hamster ovary cells which is identical to human native IFN-β (Chernajovsky et al., 1984). IFN-β-1b is an unglycosylated recombinant protein produced in Escherichia Coli and modified from the native protein by substitution of L-cysteine by L-serine at position 17 to provide improved in vivo stability (Khosrovi 1984). Two placebo-controlled phase III clinical trials in 1993 of recombinant IFN-β-1b have produced the most promising therapy to date (The IFN-β MS study Group 1993; Paty et al., 1993). IFN-β-1b was administered subcutaneously every other day as low dose, 1.6 Million International Units (MIU) or high dose, 8.0 MIU. The high dose group had a significantly lower exacerbation rate per year, and increased the number of patients exacerbation free after 2 years of therapy. These studies were also the first to show decreased MRI activity in conjunction with positive treatment. There was also a 75% reduction in the formation of new lesions in the high dose group. IFN-β-1b was well tolerated but there were reports of injection site reactions and flu-like symptoms in treated groups.

A recent phase III clinical trial with IFN-β-1a showed that patients treated intramuscularly with 6.0 MIU of IFN-β-1a had a reduced exacerbation rate with a slower disability progression. In addition there was a reduction in the number of new gadolinium-enhanced lesions detected (Jacobs 1996). Unlike IFN-β-1b there were few injection site reactions. Over the two year period neutralising antibodies were seen in 45% of patients treated with IFN-β-1b and in 24% of patients treated with IFN-β-1a. This could potentially limit future administration of these drugs, and could account for the loss of treatment effect in some patients. The increase in neutralising antibodies to
IFN-β-1b may be due to its increased stability in vivo. Two recent phase III clinical trials have confirmed the therapeutic benefit of IFN-β. The use of recombinant IFN-β-1a in patients with remitting-relapsing MS has shown a reduction in the number of new MRI lesions as well as a reduction in the clinical relapse rate and an increased time to onset of sustained disease progression (PRISMS 1998). In patients with secondary-progressive MS, IFN-β-1b again reduced the incidence of clinical relapse, new MRI lesions and increased time to onset of sustained disease progression (European Study Group on Interferon β-1b in secondary progressive MS 1998).

The development and application of therapeutic agents has largely been pioneered by pre-clinical studies in animal models, which have provided an understanding of disease processes and have identified targets for treatment. Thus animal models of MS such as EAE are essential for testing and developing new therapeutic strategies.
### Table 1.2 Current therapy for MS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Outcome</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>↓ inflammation &amp; ↓ severity of relapse, does not alter frequency or limit progression of MS</td>
<td>no clinical studies</td>
<td>unknown with prolonged use due to psychosis, infections, bone necrosis, hypertension</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>↓ inflammation</td>
<td>no clinical studies</td>
<td>unknown with prolonged use due to psychosis, infections, bone necrosis, hypertension</td>
</tr>
<tr>
<td>Immunosuppressives</td>
<td>↓ inflammation</td>
<td>no clinical studies</td>
<td>unknown with prolonged use due to psychosis, infections, bone necrosis, hypertension</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>↓ immunosuppression</td>
<td>limited effect in early disease, ↓ Th cells</td>
<td>leukopenia, GI intolerance, nausea, sterility, ↑ risk of cancer, hair loss</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>↓ immunosuppression</td>
<td>slows progression of disability but not frequency of relapses</td>
<td>leukopenia, GI intolerance, nausea, sterility, ↑ risk of cancer, hair loss</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>↓ immunosuppression</td>
<td>↓ cytokine production, ↓ Th cells, no effect on frequency of relapse</td>
<td>allergic reactions</td>
</tr>
<tr>
<td>CAMPATH-1H</td>
<td>↓ immunosuppression</td>
<td>early results suggest no beneficial effect but shown modest effect on number of relapses</td>
<td>red blood cells and platelets</td>
</tr>
<tr>
<td>Immune Modulation</td>
<td>↓ immunosuppression</td>
<td>in conjunction with azathioprine had no effect compared to azathioprine alone, slight effect in conjunction with cyclophosphamide</td>
<td>mucosal irritation, chemical hepatitis</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>↓ immunosuppression</td>
<td>T cell deletion, increased cytokine secretion</td>
<td>significant exacerbation</td>
</tr>
<tr>
<td>Levamisole</td>
<td>↓ immunosuppression</td>
<td>no benefit in relapsing-remitting or chronic progressive MS</td>
<td>danger due to surgical procedure</td>
</tr>
</tbody>
</table>

ACTH: Adrenocorticotropic hormone; ↓ - reduction; ↑ - increase; NSAID - nonsteroidal antiinflammatory drugs; GI - gastrointestinal
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Outcome</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tolerance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP i.v.</td>
<td>specific immune tolerance</td>
<td>little benefit seen</td>
<td>local delayed hypersensitivity reactions at injection site, worsening of disease</td>
</tr>
<tr>
<td>Copolymer 1</td>
<td>desensitisation</td>
<td>lower exacerbation rate, some reduction in disability progression in early stage disease but not progressive disease</td>
<td>injection site irritation, anxiety, dizziness 15 min post injection</td>
</tr>
<tr>
<td>Oral Tolerance (MBP/PLP)</td>
<td>antigen-specific tolerance</td>
<td>possible reduction in relapse rate, large scale clinical trial still in progress</td>
<td></td>
</tr>
<tr>
<td><strong>Anti-Viral</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>anti-viral</td>
<td>exacerbation of disease, therapy discontinued</td>
<td>high rate of exacerbation</td>
</tr>
<tr>
<td>IFN-α</td>
<td>anti-viral</td>
<td>weak ↓ exacerbation rate in RR MS, or had little effect</td>
<td>fever, nausea, depression, leukopenia</td>
</tr>
<tr>
<td>IFN-β</td>
<td>anti-viral, down-regulates IFN-γ</td>
<td>↓ exacerbation rate, ↓ formation new lesions by MRI</td>
<td>injection site reactions, neutralising Ab</td>
</tr>
<tr>
<td><strong>Symptomatic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baclofen</td>
<td>↓ reflex activity in CNS</td>
<td>↓ spasticity, improves bladder &amp; bowel control</td>
<td>drowsiness, withdrawal may induce seizures &amp; hallucinations</td>
</tr>
<tr>
<td>Diazepam</td>
<td>↓ reflex activity in CNS</td>
<td>↓ spasticity but less effective than baclofen</td>
<td>drowsiness, dizziness, drug dependency</td>
</tr>
<tr>
<td>Cannabis</td>
<td>spasticity</td>
<td>anecdotal literature suggests ↓ spasticity &amp; improved emotional state, large scale clinical trials in progress</td>
<td>possible impaired memory &amp; personality changes</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>Fatty acids used to synthesise myelin</td>
<td>Possible ↓ in frequency, severity and duration of individual relapses, other studies showed no effect</td>
<td>no significant toxicity</td>
</tr>
<tr>
<td>IVIg</td>
<td>block autoantibodies in MS</td>
<td>possible benefit in early disease in slight reduction in relapse rate and clinical disability</td>
<td>eczema</td>
</tr>
<tr>
<td>Vitamins</td>
<td>replenish vitamin deficiency</td>
<td>basic studies have shown between 0% and 100% improvement</td>
<td>no risk</td>
</tr>
</tbody>
</table>

IVIg - intravenous immunoglobulin; i.v. intravenous; Ab - antibody; RR MS - relapsing-remitting multiple sclerosis
In order to investigate possible mechanisms involved in the pathogenesis of MS and new therapies for MS various animal models have been developed. There is no one universal model that displays all facets of MS but individual models can be used to study particular aspects of the disease, such as demyelination due to viral infection, the acute T cell mediated phase of inflammation, or mechanisms involved in relapse phases.

1.3.1. Viral Models of MS

Although the aetiology of MS is still unknown, epidemiological studies suggest that viral infection may play a role in the induction of MS. Therefore demyelinating viral models of MS may help investigate the role of virus in MS. The most common viral models are Theiler’s murine encephalomyelitis virus and Semliki Forest virus (SFV). TMEV is a single stranded RNA virus and is a natural pathogen of mice. It normally causes enteric infection, but in 1:2000 cases it may enter the CNS and cause disease. This may mimic the possible role of virus in MS where not all individuals infected with a particular virus will develop disease. The experimental model is efficient when TMEV is administered intracranially (i.c.). The virus infects neurons predominantly but can persist in oligodendrocytes and microglia. Persistence of virus causes demyelination, seen mainly in the spinal cord (Fazakerley et al., 1997). Paralysis of infected mice occurs in some models. SFV is also a single-stranded RNA virus but unlike TMEV it is neurotropic and neuroinvasive. It is administered intra-peritoneally (i.p.) and can cross the cerebral endothelium. The A774 strain enters the CNS and remains in small foci around the sites of entry (Fazakerley et al., 1997). The initiated immune response to SFV eliminates the virus and demyelinated lesions are observed. In this model CD8+ T cells are most likely to cause demyelination by their cytolytic activity on infected oligodendrocytes, as animals treated with depleting antibody to CD8+ T cells show no demyelination (Subak-Sharpe et al., 1993).

1.3.2. Experimental Allergic Encephalomyelitis (EAE)

Pasteur described a neuroparalytic disorder termed post vaccinal encephalomyelitis (PVE) in 1885, after rabies vaccine containing rabbit spinal cord was introduced into dogs. PVE was first described experimentally in primates sensitised to rabbit CNS
tissue (Rivers 1933). EAE is an autoimmune, demyelinating disease of the central nervous system exhibiting similar histopathology to MS. With the advent of Freund's-type adjuvants, EAE was described in a wide range of susceptible animal strains following injection of brain or spinal cord homogenates, or single myelin-associated proteins such as MBP and PLP emulsified in Freund's adjuvant. Adoptive transfer studies using T cells specific for CNS antigens can also induce EAE and highlights the likelihood that MS is immune-mediated. Using different induction protocols and routes of administration with various autoantigens and animal strains, a number of EAE models differing in disease course and pathology have been described. Acute EAE is a monophasic disease, which is a limited model for MS in that there is no relapse phase and disease is brief. The most common model has been demonstrated in Lewis rats which have been shown to be susceptible to chronic EAE after inoculation subcutaneously with CNS tissue, where they are then typically refractory to subsequent rechallenge (Raine and Traugott, 1984). This model exhibits acute inflammation in the brain stem and lumbar region of the spinal cord with perivascular cuffs containing mononuclear cell infiltrates but unlike MS there is little demyelination (Stepaniak et al., 1995). However, the injection of monoclonal antibodies specific for MOG could induce severe clinical relapses in Lewis rats, which were associated with significant demyelination in the spinal cord (Linington et al., 1992). In addition, recent studies have shown that injection of IL-12 i.p. can induce relapse in Lewis rat models of EAE and that DA rats immunised with whole myelin exhibit severe chronic relapsing and demyelinating EAE (Smith, et al., 1997; Lorentzen et al., 1995).

Chronic relapsing EAE (CREAE) is characterised by an acute phase of EAE followed by a period of spontaneous remission, then further relapses. This type of EAE is a more clinically relevant model for MS as it mimics the symptomology and temporal course of relapsing/remitting MS. CREAE has been described in many animal models including rat, guinea pig and mouse. CREAE in juvenile strain 13 guinea pigs exhibited lesions of a similar structure to MS where large foci of demyelination occurred in the cerebral and spinal cord white matter as well as periventricular lesions in the cerebral hemispheres although there was greater axonal sparing and peripheral nervous system involvement than with MS (Raine, C. S. 1997). The guinea pig strain 13 model develops primary demyelination over a protracted time course with relapses occurring between 3-5 months after the acute phase (Suckling, A. J. et al., 1984). However, the guinea pig model is limited as an immunological model due to limited knowledge of the
immune system and lack of reagents with which to probe this. In contrast the mouse EAE models have a much shorter time course and also a well-defined immune system making them amenable to study. CREAE was developed from acute EAE by immunising with 2 injections of spinal cord homogenate (SCH) in complete Freund’s adjuvant (CFA) one week apart (Brown and McFarlin, 1981). However some mice such as the SJL/J strain remain paralysed after the acute phase and the relapse phase can be highly unpredictable (Pettinelli and McFarline, 1981; Brown et al., 1982). Generally murine CREAE models display a similar topography to the guinea pig models but are less extensive. Often, small thin paraventricular lesions present in the distal regions of the spinal cord and primary demyelination are observed after the first relapse. Most murine models display mononuclear infiltrates in subpial regions and gliotic scarring with axonal loss (Raine, C. S. 1997). Although SCH has been shown to induce EAE in animal strains, individual myelin components have also been shown to induce EAE. MBP and PLP are the major myelin components and different strains respond to different encephalitogenic epitopes, although both MOG and myelin associated glycoprotein (MAG) proteins are also used. Generally murine CREAE models exhibit similar pathology although EAE induced by MOG exhibits different clinical and histopathological features to MBP or PLP induced EAE (Mendel et al., 1995). SJL/J (H-2^d^) mice are susceptible to MBP peptide 89-169 (Pettinelli et al., 1982). SJL/J mice are also susceptible to PLP 139-151 (Tuohy et al., 1989), SWR mice to PLP 103-116 (Tuohy et al., 1988) and PL/J mice to PLP 43-64 (Whitham et al., 1991). In addition EAE models have been described in primates. Rhesus monkeys exhibit a haemorrhaging necrotic disease but more recently marmosets have been shown to produce a chronic relapsing primary demyelinating disease with similar pathology to MS (van Lambalgen et al., 1987; Genain, et al., 1997). The nervous and immune systems are highly conserved between humans and non-human primates and therefore these models may be useful in assessing new therapies for MS.

1.3.3. Immunopathology of the Biozzi ABH CREAE model

This study has used the Biozzi ABH (antibody high) mouse strain (H-2^dql^), which is susceptible to a number of myelin epitopes (Amor et al., 1993) and produces a robust and reproducible relapsing model for MS (Baker et al., 1990). Both males and females are equally susceptible and lesions are present in perivascular cuffs. Meningeal infiltrate is observed in the cervical region of the spinal cord and infiltrate is also
present in the cerebellum (Baker et al., 1990). Infiltrate in the acute phase is composed of polymorphonuclear cells (PMN), monocytes and T cells in contrast to subsequent relapse phases where there are less PMN cells, although the infiltrate is more extensive (Baker et al., 1990). Lesion infiltrates are mainly composed of IL-2 receptor (IL-2R)$^+$ CD4$^+$ T cells and MHC class II$^+$ macrophages with GFAP$^+$ astrocytes present in and around the lesions (Baker et al., 1990). During remission there are fewer cells present, mainly IL-2R$^-$ CD4$^+$ T cells and few MHC class II$^+$ macrophages (Baker et al., 1990). At the height of disease ultrastructural analysis demonstrated white matter infiltration but little nerve damage. At post-acute remission however demyelination and remyelination were observed as well as nerve damage. During the relapse phase there was greater demyelination, axonal loss and extensive astrogliosis around lesions (Baker et al., 1990). A study by Allen demonstrated the cell types present in the spinal cord throughout the different episodes of CREAE in the Biozzi ABH mouse by immunoperoxidase staining (Allen et al., 1993). The acute phase of disease was associated with an increase in T cells and macrophages as well as a small number of B cells and peripheral mononuclear cells (PMN) cells. During remission there was an overall decrease in all infiltrating cell types. Similar to the acute phase, the relapse phase was associated with T cells and macrophages but also greater numbers of B cells, but fewer PMN cells (Allen et al., 1993). This model shares some of the architectural lesion structure seen in MS although lesions are confined to the spinal cord and are not as extensively distributed as in MS. However, it appears that the basic immunological processes involved in lesion formation in EAE maybe shared by MS which validates the use of EAE in the study of pathologic mechanisms and treatment regimes for MS. The study of the role of specific cell types or immunological molecules and their contribution to disease can be effectively studied in the well-defined, reproducible Biozzi ABH model of CREAE.

### 1.3.4. Immunotherapy of EAE

Currently novel therapies for EAE are moving away from conventional immunosuppressant pharmaceutical therapies and their associated toxicity, and towards biotechnological agents. These new advances have focused on the generation of a long-term state of “tolerance” to CNS antigens involved in disease, that does not require generalised immunosuppression. Studies of EAE pathogenesis have proved important in discerning the mechanisms involved in MS, such as the involvement of immune
modulatory molecules such as cytokines, MHC Ag and adhesion molecules and have suggested a putative pathogenic pathway of EAE (Figure 1.1). This pathway has provided many new targets for immunotherapeutic intervention and new therapies for EAE are being investigated, including blockade of signals through the trimolecular complex (TCR-MHC-peptide) by antibodies or synthetic peptides, immunomodulation or inhibition of pro-inflammatory responses by cytokine inhibitors or inhibitory cytokines and new strategies to facilitate the repair of the myelin sheath by oligodendrocyte grafting and growth factor delivery (reviewed by Brosnan, et al., 1997).

1.3.5. Genetic studies in EAE

From familial and twin studies it is thought that patients are genetically predisposed to MS, with a significant concordance rate between twins. The number and nature of these susceptibility genes are unknown but studies have suggested association with MHC molecules (Ebers et al., 1982; Sawcer et al., 1996; Kellar-Wood et al., 1995; The Multiple Sclerosis Genetics Group 1996). With the advent of identification of microsatellites in the mouse genome, susceptibility loci in EAE and physiologic pathways involved in the pathogenesis of EAE have started to be identified which may be of importance in EAE and of relevance to MS. Inbred mouse strains have a varying susceptibility to EAE, which indicates an important genetic influence on susceptibility. This has been linked to the MHC class II alloantigens (Fritz et al., 1985; Linthicum et al., 1994). However, there is a wide variety of susceptibility in H-2 congenic mice indicating that there are non-MHC genetic components, which are also necessary for EAE susceptibility (Linthicum et al., 1994; Amor et al., 1993). Studying crosses between strains of mice sharing a MHC haplotype but differing in susceptibility to EAE have elucidated important non-MHC susceptibility genes, and currently there are at least 10 putative Eae loci, which may be involved in modifying EAE (Baker et al., 1995; Sundvall et al., 1995; Encinas et al., 1996; Croxford et al., 1997; Butterfield et al., 1998). Interestingly, it was suggested that some of these putative loci may not be exclusive to EAE and have been demonstrated in insulin-dependent diabetes mellitus, systemic lupus erythmatosus and autoimmune orchitis and may reflect general
1. T cells (T) are primed against CNS antigens presented by dendritic cells (D) in peripheral lymph nodes and proliferate. 2. Primed T cells activate blood brain barrier endothelial cells and extravasate into CNS. 3. Primary recruitment of T cells are further activated by CNS antigen-presenting cells (APC), inducing the release of pro-inflammatory cytokines, which activate resident CNS cells such as astrocytes (A) and microglia (M). 4. Release of cytokines and chemokines induces secondary recruitment of macrophages (M) and B cells (B). 5. Demyelination of neurons (N) and destruction of oligodendrocytes (O) may be caused by immunological processes such as phagocytosis by macrophages/microglia binding to complement (C) and/or myelin specific antibodies (Ab) produced from infiltrating B cells, the direct toxic action of cytokines such as TNF and cytotoxic T cells (CT). Remission of disease may be partially due to release of anti-inflammatory cytokines such as TGF-β and IL-10 from regulatory cells (R).

autoimmune susceptibility loci (Todd et al., 1991; Kono et al., 1994; Meeker et al., 1995; Teuscher et al., 1998). Many of the putative loci in EAE are homologous to regions found in the human genome studies in MS patients (The Multiple Sclerosis Genetics Group 1996; Ebers et al., 1996; Sawcer et al., 1996; Croxford et al., 1997; Butterfield et al., 1998).
1.4. CYTOKINES

1.4.1. Cytokine Functions

Cytokines act as local messengers between cells and are produced by most cell types. They are important biological mediators of cellular communication and are involved in diverse processes such as inflammation, cell growth, development and repair. Cytokine production is upregulated by cellular activation and cytokines are effective at low concentrations, usually in the ng-pg range (Feldmann et al., 1996). Cytokines act via high affinity cytokine receptors on cell surfaces and most cells express multiple cytokine receptors. Soluble cytokine receptors are proteolytically shed from the cell surface or are the products of differentially spliced mRNA, and can be found in the CSF, serum and urine where some can act as natural cytokine inhibitors, such as tumour necrosis factor receptor (TNFR) whereas others act as agonists (i.e. IL-6-R). The cytokine network is complex with at least 100 known cytokines and growth factors identified at present. The elucidation of function of individual cytokines is hampered by the fact that they have different functions depending on which cell they interact with (pleiotropy) and the fact that different cytokines can produce the same outcome by sharing intra-cellular signalling pathways (cytokine redundancy). Cytokines can also induce the secretion of other cytokines by either “autocrine” (acting on self) or “paracrine” (acting on neighbouring cells) regulation and can act synergistically (Oswald, et al., 1992). Some cytokines have also been shown to block the production or secretion of other cytokines.

During an inflammatory episode, cells such as macrophages become activated and release pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1β and IL-6. These cytokines initiate the local inflammatory response by up-regulating adhesion molecules on endothelium close to the site of inflammation, as well as inducing chemokine release. Chemokines are small molecular weight proteins which are involved in the recruitment immune cells such as neutrophils, T cells and monocytes to the site of inflammation. These cells also release cytokines which amplify the response. The CD4+ T helper subset of cells in mice has been divided into T helper 1 (Th1) and Th2 type cells (Mosmann et al., 1989). Th1 cells typically produce pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ which favour the development of a type IV
hypersensitivity response, which is important for immunity against intracellular pathogens. Th2 cells are involved in allergic responses, important for immunity against extracellular pathogens, and produce anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 (Figure 1.2). Often T cells do not typically fall completely into one or the other category but populations are polarised towards one type. Indeed, in humans T cells do not belong to either category and are often termed Th0 as they may produce cytokines such as IL-4 and IFN-γ simultaneously (Prussin, 1997). However, the ratio between Th1 and Th2 cytokines may determine the type of immune response in particular situations. This can depend also on the MHC haplotype and the affinity of a particular antigen to MHC.

Cytokines secreted by antigen-presenting cells (APC) during antigen presentation to naïve CD4+ T cells can influence the cytokine profile of that cell. For naïve T cells to develop to Th1 cells, IL-12 produced by APC such as macrophages and dendritic cells, needs to be present during activation and is augmented by the presence of IFN-γ, which can maintain the expression of IL-12 receptors on CD4+ T cells (Guler, 1996). Differentiated Th2 cells do not respond to IL-12 as they lack the IL-12 β chain receptor, and therefore cannot activate Stat4, a nuclear DNA-binding complex involved in IL-12 signalling (Jacobsen et al., 1996). The development of CD4+ cells to the Th2 phenotype is mediated by IL-4. The source of IL-4 for this induction is thought to either come from naïve CD4+ T cells that produce small amounts which may accumulate at the site of antigen presentation and/or from a small population of CD4+ natural killer 1.1 cells identified in the mouse (Yoshimoto and Paul, 1994).

Th1 cytokines such as IFN-γ can inhibit the production of Th2 cytokines, and likewise Th2 cytokines such as IL-4 or IL-10 can inhibit the production of Th1 cytokines. It is thought that some disease states may be induced by a relative shift in the Th1/Th2 balance. In EAE, a disease where Th1 cells are more abundant than Th2, immunomodulation by Th2 cytokines may be useful in inhibiting disease. Other stimuli may play a role in determining polarity of cytokine expression from T cells. Co-stimulatory molecules present on the APC such as CD80 (B7.1) and CD86 (B7.2) can influence the cytokine phenotype induced (Kuchroo et al., 1995). Low affinity antigenic peptide-MHC complexes can serially trigger TCR inducing a sustained activating signal (Valitutti, et al., 1995), whereas peptide complexes with a TCR affinity
either higher or lower than the optimal can partially stimulate or antagonise T cells which may lead to different cytokine production (Valitutti et al., 1995).

**Figure 1.2 The influence of cytokines on the differentiation of CD4⁺ cells.**

Th0 cells produce a mixture of Th1 and Th2 cytokines, but in the presence of pro-inflammatory cytokines such as IFN-γ or IL-12 they can be influenced to a Th1-type profile. The presence of anti-inflammatory cytokines such as IL-4 and IL-10 can induce a Th2-type profile in Th0 cells. Th1 cytokines can inhibit expression of Th2 cytokines and likewise Th2 cytokines can inhibit Th1 cytokine production.

### 1.4.2. Cytokines in MS and EAE

Evidence from EAE studies has suggested that cytokines play an important role in the pathogenesis of disease. From these studies immunohistological examination of MS tissue revealed similar patterns of cytokine involvement to that in EAE. Many studies have shown the presence of cytokines within lesions in MS as well as EAE (Table 1.3).
Normally CNS endothelia express low levels of adhesion molecules. However, the action of pro-inflammatory cytokines and chemokines from activated leucocytes and resident CNS cells can induce adhesion molecules on the BBB and facilitate entry to the CNS. Adhesion molecules such as ICAM-1 (CD54) and VCAM-1 (CD105) are upregulated by TNF-α and IFN-γ and IL-1 in the case of ICAM-1 (CD54) (Cannella et al., 1995; Yednock et al., 1992). Once cells have entered the CNS they recognise antigen in the context of MHC class II Ag presented by microglial cells, and are further stimulated. This leads to a cascade of cytokine production, which induces a secondary wave of cellular recruitment as well as activating resident CNS cells.

1.4.3. Pro-inflammatory cytokines

IFN-γ

IFN-γ producing cells have been detected in the CSF of some MS patients, and a clinical trial using IFN-γ induced clinical relapse (Panitch et al., 1987). The activation of macrophages/microglia by IFN-γ induces the production of a variety of inflammatory mediators including TNF, nitric oxide, reactive oxygen species, proteases (MacMicking et al., 1992) and induces the upregulation of Fc receptors which increase the phagocytic capacity of macrophages. These factors may play a role in demyelination and the loss of oligodendrocytes. IFN-γ has been shown to upregulate MHC class II molecules in the CNS on astrocytes, oligodendrocytes, neurons and microglia (Wong et al., 1984) and during disease this could lead to increased antigen presentation and an expansion of the immune response. In EAE however, the role of IFN-γ is not so clear. In a study where the IFN-γ gene had been disrupted in a susceptible mouse strain, EAE was still inducible, suggesting that the initiation and severity of EAE do not depend on IFN-γ (Ferber et al., 1996). Another study using IFN-γ knockout mice on the “low-responder” BALB/c background, showed that without IFN-γ these mice were now susceptible to EAE, and that IFN-γ may have a regulatory role in EAE (Krakowski et al., 1996). The administration of anti-IFN-γ mAb also reversed a genetically-resistant strain of mice to an EAE-susceptible phenotype (Billiau et al., 1988; Duong et al., 1994).
### Table 1.3 The presence of cytokines in MS and EAE

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sources</th>
<th>Effects</th>
<th>Detection in disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>M, A, T, B, Mi, E</td>
<td>T cell and M activation</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IL-2</td>
<td>T</td>
<td>T cell proliferation</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IL-4</td>
<td>T</td>
<td>B cell activation, IgE switching</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IL-5</td>
<td>T</td>
<td>Eosinophil growth factor</td>
<td>EAE</td>
</tr>
<tr>
<td>IL-6</td>
<td>T, M, Mi, A, E</td>
<td>T and B cell growth; acute phase proteins</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IL-8</td>
<td>T, M</td>
<td>Chemotactic factor</td>
<td>EAE</td>
</tr>
<tr>
<td>IL-10</td>
<td>T, M, B, A</td>
<td>Negative regulator of M</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IL-12</td>
<td>M, B</td>
<td>Induces Th1 differentiation, activates NK cells</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>T, Mi, M, A, E, F</td>
<td>Inhibits cell growth, antiinflammatory</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>TGF-β2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>M, Mi, T, A</td>
<td>Local inflammation, E activation</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>TNF-β (LT)</td>
<td>T, Mi</td>
<td>Killing, E activation</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>T, NK</td>
<td>M activation, MHC I, II Ag expression</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>IFN-α</td>
<td>T, B, M</td>
<td>Anti-viral, MHC I Ag expression</td>
<td>?</td>
</tr>
<tr>
<td>IFN-β</td>
<td>F</td>
<td>Anti-viral, MHC I Ag expression</td>
<td>?</td>
</tr>
</tbody>
</table>

T = T cells; B = B cells; M = Macrophages; Mi = Microglia; A = Astrocytes; F = Fibroblasts; E = Endothelial cells; NK = Natural Killer cells; LT = lymphotoxin
TNF

TNF is an important mediator in MS and EAE. Elevated levels of TNF have been detected in the CSF of MS patients (Maimone et al., 1991; Spuler et al., 1996). TNF-α and TNF-β (lymphotoxin) have been identified in many studies investigating cytokines in MS lesions (Selmaj et al., 1991b; Woodroffe et al., 1993; Hofman et al., 1989). TNF-α is produced by neurons, astrocytes and microglia and the upregulation of its mRNA in PBMC is associated with disease activity in MS (Rieckmann et al., 1995). In addition, TNF has been identified in microglia and infiltrating leucocytes in the CNS of mice with EAE (Renno et al., 1995). TNF-β is produced by infiltrating leucocytes and microglia. TNF can synergise with IFN-γ, to induce the expression of both adhesion molecules and MHC class II molecules and the death of oligodendrocytes in vitro by apoptosis (Selmaj et al., 1988; Selmaj et al., 1991c). TNF mediates its effects via 2 receptors CD120a (TNFR-1, p55) and CD120b (TNFR-2, p75) both of which can be cleaved to produce soluble receptor. Increased levels of soluble TNFR have been detected in MS patients and is associated with disease activity (Matsuda et al., 1994). Specific polymorphisms of TNF-α and TNF-β alleles independent of the HLA-DR2 haplotype have been suggested as important factors in the susceptibility to MS (Kirk et al., 1997) whilst another study has shown that individually, TNF polymorphisms are not linked to a higher predisposition to MS but when present together they may contribute to the development of MS (Mycko et al., 1998a).

1.4.4. Anti-inflammatory cytokines

Although the exact mechanism of demyelination in MS and EAE is unknown, pathology may be due to myelin specific antibodies, either by fixing complement or by antibody dependent cellular cytotoxicity. Th2 cytokines IL-4 and IL-10 are involved in B cell proliferation and class switching of immunoglobulins and have been detected in both MS and EAE lesions (Table 1.3). IL-4 is involved in the class switching of IgM to IgE and has also been detected in the CSF of MS patients (Link et al., 1994). Some cytokines however, may also play a positive role in MS and EAE by inducing remission of disease. Both IL-10 and TGF-β messenger RNA (mRNA) have been implicated in the stabilisation of EAE (Kennedy et al., 1992; Issazadeh et al., 1995a) and MS (Rieckmann et al., 1994). Studies showing that Th1 cytokine production can be
modulated or inhibited by Th2 cytokines has led to the investigation of cytokine therapy in EAE and MS.

1.4.5. Chemokines in MS and EAE

Chemokines are small molecular weight proteins that are involved in chemotactic activity at sites of tissue damage. Two families of chemokines have been described. The C-X-C chemokines (a different nonconserved residue separates two cysteine residues) attract neutrophils whereas the C-C family (two cysteine residues together) attract monocytes, and leucocytes (reviewed by Hedrick, J. A., and Zlotnick, A. 1996). An important step in the pathology of EAE, and MS is the recruitment and perivascular migration into the CNS of T cells and monocytes, which is influenced by the action of chemokines (Haelens et al., 1996). Many studies have demonstrated the presence of both types of chemokine in lesions and CSF of MS tissue and in EAE tissue (Table 1.4).
Table 1.4 The presence of chemokines in MS and EAE.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Cellular Source</th>
<th>Effects</th>
<th>Detection in disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>A, M, E, F</td>
<td>recruit Mi, M, A, T, Eo</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>MCP-2</td>
<td>F, T</td>
<td>recruit Mi, M, Eo, T</td>
<td>MS</td>
</tr>
<tr>
<td>MCP-3</td>
<td>F, T</td>
<td>recruit Mi, M, Eo, T</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>RANTES</td>
<td>E, Pa, M, Mi, T</td>
<td>recruits Mi, N, L</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>A, M, CD8+ T</td>
<td>recruits Mi</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>M, Mi, CD8+ T</td>
<td>recruits Mi</td>
<td>MS, EAE</td>
</tr>
<tr>
<td>TCA3</td>
<td>T</td>
<td>recruit Mi, M, N, VC</td>
<td>EAE</td>
</tr>
<tr>
<td>GRO-α</td>
<td>E, A</td>
<td>recruit A, B, N, and OG proliferation</td>
<td>EAE</td>
</tr>
<tr>
<td>IL-8</td>
<td>T, M, E, A</td>
<td>recruit N, Mi, B</td>
<td>MS</td>
</tr>
<tr>
<td>IP-10</td>
<td>A, CD8+ T, E</td>
<td>recruits Mi, T, N</td>
<td>EAE</td>
</tr>
</tbody>
</table>

MCP – monocyte chemoattractant protein; RANTES – regulated upon activation, normal T cell expressed and secreted; MIP – macrophage inflammatory protein; TCA – T cell activation gene; IP – IFN-inducible protein; GRO-α - chemokine growth related oncogene alpha; T - T lymphocyte; M - macrophage; A - astrocyte; N - neutrophil; Mi - microglia; E - endothelial cell; Pa - perivascular astrocyte; CD8+ T- CD8+ T cell; VC – vascular smooth muscle cell; OG – oligodendrocyte; B – B lymphocyte; F- fibroblast; Eo - eosinophil
1.5. GENE THERAPY

1.5.1. Principles of Gene Therapy

Recent advances in the genetics of human disease and molecular biology have been applied to a new therapeutic concept, gene therapy. As the defective genes responsible for a multitude of single abnormal gene disorders are discovered, so new techniques have evolved whereby replacement genes can be used to correct the disease phenotype. This methodology could also be extended to provide genes whose products can have a therapeutic effect in damaged tissue, or factors which can modulate the disease processes in acquired genetic diseases, such as cancer or autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

Currently there is great interest in biological therapeutic agents as they are "natural" agents whose activities and mechanisms of action appear to be well understood. These can be produced in high quantities by recombinant DNA techniques and hopefully will be less toxic than chemical therapeutic agents. However, biological agents generally have a short half-life and therefore patients require frequent administration. Using gene therapy it is hoped this can be overcome and patients can receive equal benefit from a single injection of genetic material that will allow long-term production of the therapeutic agent. In addition localised expression in the target tissue may limit potentially harmful side-effects seen with systemic administration. Gene therapy can be defined as "the delivery of a functional gene for expression in somatic tissues with the intent to cure a disease". In order to achieve efficient transfer of genes to the target cells they are usually packaged into a vector, which aids delivery. Currently there are two main vector-types for the delivery of therapeutic genes, viral and non-viral vectors. Each type of vector has its advantages and disadvantages.

There are many variables which may influence which vector is the optimal one for a particular disease, such as tissue or cell type targeted, route of administration, size of genetic material to be transferred and required duration of expression of gene products. Currently viral vectors have shown to be more efficient at gene delivery than non-viral vectors. Vectors can be delivered either ex vivo into cells explanted from a patient, transduced with the therapeutic gene and implanted back to the patient, or in vivo where the vector is delivered directly to the target tissue of the patient (Gage, F. 1998).
1.5.2. Retroviral Vectors

Most recombinant retroviruses studied are based on the amphotropic murine leukæmia viruses (MLV) which have been well characterised and can infect both mouse and other cell types. Once a number of viral genes have been removed the retrovirus can accommodate 7 kb of exogenous DNA and as most viral genes are absent, they have low immunogenicity. Replication-deficient recombinant retroviral vectors are very efficient at infecting cells via natural MLV-specific amphotropic receptors, and have the advantage over other vectors in that they can integrate stably into the host genome, therefore permanently altering the cells genotype (Figure 1.3). However, retrovirus can only infect dividing cells and this is a disadvantage when the target tissue is postmitotic such as the CNS. Retrovirus is often used to infect cells *ex vivo*. Many cell types can be cultured *in vitro* and infected with retrovirus, cloned and then transplanted back into the target tissue, providing a much more efficient and localised gene expression (Gage, F. 1998). Retroviral vectors typically contain an antibiotic resistance gene so that explanted cells can be selected and cloned before transplantation. Cells such as fibroblasts have often been used in gene therapy protocols as they can be cultured easily from patients skin and can function as an autograft avoiding any immunological consequences of non-self cells being implanted (Kang, U 1995). Rat dermal fibroblasts have been shown to survive *in vivo* for up to 8.5 months in rat recipients (Palmer et al., 1991), although studies have shown that stable long-term gene expression is dependent upon the cell type, promoter class and target tissue (Roemer et al., 1991). Many vectors retain the long terminal repeats (LTR) of the original retroviral vector, but may contain other viral promoters such as the cytomegalovirus (CMV), Simian Virus 40 (SV40) and Rous Sarcoma Virus (RSV). Donor cells however, often recognise the viral promoter sequences and can silence them by methylation or other mechanisms, which reduces the longevity of transgene expression. Studies have shown that viral LTR in murine fibroblast cell lines are subject to methylation and that transgene expression is reduced regardless of the lifespan of the fibroblast (Hoeben et al., 1991). Similar data has also been shown using the CMV promoter, which does not provide long-term *in vivo* production of transgene compared to a housekeeping promoter, dihydrofolate reductase (DHFR) where transgene expression could be detected for at least up to 3 months (Scharffmann et al., 1991). The type of promoter can also confer specificity upon the cells expressing the transgene, for example the neuron-specific enolase (NSE) promoter is only active in neurons (Forss-
Petter, et al., 1990). Other cell types that have been used for gene therapy include astrocytes which may have an advantage in CNS gene therapy by being of CNS-origin, and lymphocytes which can be targeted to specific tissues from systemic administration to deliver transgene products locally. Currently the cellular vector approach to gene therapy is being investigated in a variety of disease models such as rheumatoid arthritis using immortalised fibroblasts or splenocytes (Triantaphyllopoulos et al., 1999; Mageed et al., 1998), a model of Parkinson’s disease using myoblasts, astrocytes or fibroblasts (Jiao et al., 1993; Yoshimoto et al., 1995; Fisher et al., 1991) and in treating experimental autoimmune neuritis with antigen specific T cells delivering nerve growth factor (NGF) (Kramer et al., 1995). Endothelial cells are potentially another useful vehicle for CNS gene delivery and have been shown to integrate into the brain parenchyma and vasculature (Quinonéro et al., 1997). The majority of currently approved clinical protocols using retroviruses are for cancer gene therapy, some inherited gene diseases, such as adenosine deaminase (ADA) deficiency, rheumatoid arthritis (Evans et al., 1998) and advanced cancer (Rosenberg et al., 1990). Potential disadvantages of this system include constitutive chronic expression of transgene, insertional mutagenesis or immunogenicity of transgene products or transplanted cells.
The therapeutic gene is cloned into plasmid DNA and packaged into infectious replication-deficient viral particles by a third generation packaging cell line containing 2 independent constructs. One construct expresses the env gene product and the other expresses the gag and pol gene products. This system reduces the chance of production of replication competent virus (RCV) as 3 separate recombination events are required to produce RCV. The plasmid DNA coding for therapeutic gene product contains the packaging signal $\psi^+$ allowing it to be packaged into the retroviral proteins. The packaging cell line is $\psi^-$ preventing packaging of replication competent virus. The infectious viral particles coding for the therapeutic gene can infect cells via specific cellular receptors and fuse with the cell membrane and become internalised. The viral core is shed and the proviral RNA is converted to proviral DNA and integrated into the hosts genome during cell division. Viral promoter long terminal repeats (LTR) drive expression of the therapeutic gene.
1.5.3. Adenoviral Vectors

Of the other viral vectors the adenoviral vector has been most studied. Adenoviruses are a frequent cause of upper respiratory tract infections and are also involved in pharyngitis, gastroenteritis, conjunctivitis and pneumonia (Taterka et al., 1992; Ginsberg et al., 1990; Trousdale et al., 1995; Engel 1995). This DNA virus has been used in in vivo studies as these viruses can infect a wide range of cell types both dividing and non-dividing, with a very high efficiency. They can hold up to 35 kb of exogenous DNA and can be produced at very high titres (10^{11} viral particles per ml). Although transferred genetic material remains epichromosomal and therefore transgene expression is limited, it produces a high level of expression of transgene (Figure 1.4). Many studies have investigated the duration of transgene expression of adenovirus in the CNS using marker proteins such as β-galactosidase. Results from these studies demonstrated that adenovirus could infect neuronal, glial, and ependymal cells as well as astrocytes and that this expression lasted between a few days to weeks (Bajocchi et al., 1993; Akli et al., 1993; Davidson et al., 1993). First generation adenoviral vectors have had the early (E)1 region genes removed to produce replication-deficient vector and the E3 region removed to allow the insertion of the therapeutic DNA construct. However, these vectors have been shown to produce immune responses (Worgall et al., 1997; Wood et al., 1996), probably due to viral protein expression as the viral genome is still intact. A recent study has shown that cells infected with an inactive adenovirus could still activate an immune response in the absence of viral replication or de novo protein synthesis (Kafri et al., 1998). This inflammatory response will limit the duration of expression of the transgene as well as potentially increasing severity of disease and preventing the readministration of adenovirus vector to the patient. In order to try to overcome this problem second-generation adenovirus vectors have had more viral genes deleted. Current approved therapies using adenovirus have been in cystic fibrosis and in some cancer studies (Schuler et al., 1998; Crystal et al., 1994).
Chapter 1 Introduction

Figure 1.4 Adenoviral infection of target cells.

The therapeutic gene is cloned into the E1 deleted replication-deficient adenovirus vector and packaged into infectious viral particles by wild type helper adenovirus containing the E1 gene for packaging. The infectious particles bind to specific cellular receptors and are internalised into an endosome. The adenovirus escapes the endosome by lowering the pH and exists epichromosomally in the target cells nucleus to express therapeutic gene.
1.5.4. Other Viral Vectors

The Adeno-associated virus (AAV) is a promising candidate for human gene therapy as it is non-pathogenic. AAV can infect non-replicating cells and in humans, integrates into a specific region of chromosome 19. Up to 96% of the viral genome can be deleted but even so it can only accommodate up to 5kb of exogenous DNA and their production requires co-infection with helper adenovirus, which may contaminate the AAV preparation. However, as the vector is almost free of viral genes this may reduce its immunogenicity. AAV has been tested in models of Parkinson’s disease expressing transgene for up to 3 months in neurons and glial cells (Kaplitt et al., 1994). AAV does not have a natural tropism for the CNS and therefore must be injected directly into the CNS. In contrast the Herpes simplex virus (HSV) type 1, which is neurotrophic, can accommodate transgenes of up to 30 kb. HSV have a natural tropism for neurons and would be of use in gene therapy of the CNS, however, at present vectors in use are replication-competent and are likely to be highly immunogenic. The HSV genome is more complicated than that of the adenovirus and therefore deletion of viral genes has been more difficult. The early HSV viral genes code for cytopathic products and these genes must be deleted to produce HSV vectors for gene therapy in the future. Another disadvantage is that at present transgene expression appears to be unstable and is transient. HSV delivers transgenes episomaly and therefore expression is limited.

It has also been shown that HSV vectors can become latent in the CNS and this is another limiting factor for their use although for treatment of brain tumor by a short burst of toxic agent they may prove a useful tool (Oligino et al., 1998). A further complication of HSV vectors at present is their immunogenicity. However, it is hoped that toxicity and cytotoxicity may be reduced by constructing HSV vectors containing the HSV origin of DNA replication, a packaging signal and the therapeutic gene, called an “amplicon”. However, these require helper virus for packaging and at present it has been difficult to produce high titres of virus free from helper virus.

Other viral vectors being considered for gene therapy include neurotropic viruses such as alphavirus, rhabdovirus, Theiler’s virus and pseudorabies virus. Theiler’s virus infects neuronal cells via specific receptors whereas the other viruses fuse with the cell membrane. The major advantage that these vectors may have is that they can be delivered systemically but will target the CNS. In conjunction with CNS cell-specific
promoters such as glial fibrillary acid protein GFAP and NSE it may be possible to target specific CNS cell subsets from systemic administration.

1.5.5. Non-Viral Vectors

The main advantage of non-viral vectors is that they are much safer to use and are non-immunogenic compared to viral vectors. Plasmid DNA exists epichromosomally so there is also no toxicity due to insertional mutagenesis as with retroviral vectors. They also can theoretically contain therapeutic genes of any size. However, at present targeting of DNA to specific cells is difficult and transduction efficiency is low and requires many copies of the plasmid to attain suitable expression. To address this problem studies are concentrating on producing more efficient vectors by producing cationic lipid-DNA complexes to aid entry to cells (Figure 1.5). A study using a cationic liposome-treated plasmid DNA complex coding for a β-galactosidase gene injected into mice brains showed that β-galactosidase positive cells could be detected up to 9 days post injection around the site of injection and also in the contra-lateral hemisphere (Ono et al., 1990). Another study using naked DNA injected into the epidermis of pigs showed β-galactosidase expression for up to 3 weeks and an IL-8 plasmid DNA was functionally active and induced neutrophil recruitment to the injected area (Hengge et al., 1995). A model of Parkinson's disease has also been treated by injection of a plasmid DNA-cationic liposome complex (CLC) into the CNS of rats producing tyrosine hydroxylase, where expression was detected for up to 15 days post injection (Cao et al., 1995). Despite these studies it appears that the duration of expression needs to be increased to avoid frequent repeat administration. Other methods of targeting DNA to cells include receptor-mediated methods and direct injection of the DNA to the target tissue. Wolff et al., (1990) was the first to show that muscle tissue is permissive to DNA uptake, probably due to its multi-nuclear and post-mitotic nature. Plasmid DNA expression has been shown to be present in mouse skeletal muscle for up to 19 months (Wolff et al., 1992). The longevity of expression is dependent on the injection vehicle, target tissue, age and species of recipient, and promoter used (Wolff et al., 1991; Wolff et al., 1992; Manthorpe et al., 1993; Wells et al., 1992; Danko et al., 1994). Viral promoters such as CMV, RSV and SV40 show a much greater level of expression than eukaryotic promoters. However each promoter has advantages and disadvantages. The CMV is a strong promoter but expression appears to decrease with
time (Manthorpe et al., 1993), whereas the RSV promoter has a lower expression level than the CMV but increases with time (Wolff et al., 1992; Davies et al., 1993). In contrast SV40 promoter expression peaks 3 days post administration but declines very rapidly (Davies et al., 1993). DNA encoding variable regions of T cell receptor Vβ8.2 as a suppressive vaccination has been successfully used to inhibit EAE in mice (Waisman et al., 1996). Cytokine genes have also been injected into mouse skeletal muscle and shown to regulate humoral or cellular immune responses \textit{in vivo} (Raz et al., 1993). Local delivery of therapeutic genes to other tissues such as the lungs via an aerosol could be an easy, non-invasive way to treat respiratory diseases such as asthma and cystic fibrosis. Currently however delivery is inefficient. Liposome-mediated gene therapy has been used clinically in cystic fibrosis trials (Rosenfeld et al., 1992; Crystal et al., 1994; Caplen et al., 1995).
The therapeutic gene is cloned into plasmid DNA and then binds to cationic liposomes which it is thought aid entry to the cell via fusion with the cell membrane. The majority of plasmid DNA-CLC enters the cell by endocytosis and the complex is broken down in the endosomes. However, a small proportion of complex escapes the endosome and exists epichromosomally in the target cell. The therapeutic gene can be driven by viral promoters or cell specific promoters to increase specificity of expression.
1.6. AIMS OF STUDY

Currently novel biological agents are being studied in EAE, a model of MS. However, a major limitation to this therapy is the very short half-life of these agents, requiring frequent administration, which can be costly and impractical. Therefore the aim of this study was to develop a means to produce therapeutic proteins \textit{in vivo} using gene therapy, which can modulate a chronic neuroinflammatory disease. Central to the concept of gene therapy is both the identification and construction of the optimal vector for the target agent, and the optimal vector for its delivery and expression. It is already established that inhibitory cytokines or cytokine inhibitors provide a potential method to modulate autoimmune CNS disease. Therefore this study will investigate the development of the optimal delivery system from vectors currently available (plasmid DNA, adenovirus and retrovirus) with which to treat EAE. This will include the different routes of administration, to determine whether efficacy can be achieved by local and/or systemic administration, and whether this strategy can provide a convenient treatment for EAE with increased benefit over conventional frequent protein administration. The use of gene therapy to deliver immunological agents may also prove to be a useful tool with which to dissect the pathogenesis of EAE as well for therapy.

1.6.1. Hypothesis

Gene delivery of cytokines or cytokine inhibitors either by plasmid DNA, adenovirus or retrovirally infected fibroblasts either systemically or locally have the potential to deliver therapeutic concentrations of immunological agents long-term to ameliorate the severity or inhibit CREAE in Biozzi ABH mice, and that this approach will have at least the same efficacy as repeat large dose bolus protein administration.
CHAPTER TWO

MATERIALS & METHODS
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2.1. Animals.

Biozzi ABH (H-2^dq1) mice were bred at The Institute of Ophthalmology, London, UK., and fed on a diet of RM1(E) pellets and water ad libitum. As males and females exhibited identical clinical and histological signs with equal frequency both sexes were used in the induction of CREAE (Baker et al., 1990).

2.2 Induction of CREAE.

Complete Freund's adjuvant (CFA) was prepared by mixing 16 \( \mu \)g Mycobacterium tuberculosis H37Ra (4 mg/ml) (Difco Laboratories, Detroit, Michigan, USA) and 4 \( \mu \)g Mycobacterium butyricum (0.5 mg/ml) (Difco Laboratories) in 4 ml of incomplete Freund's adjuvant (IFA) (Difco Laboratories). 1ml of this stock solution was added to 11.5 ml of IFA to make 12.5 ml of CFA for the inoculation emulsion. Spinal cord from Biozzi ABH mice was expelled by hydraulic pressure following insertion of a 20g needle (Becton Dickinson) and 20 ml syringe (Terumo, Luven, Belgium) filled with PBS (Sigma) into the lumbar region of the vertebral column. Spinal cord was homogenised with a glass homogeniser and freeze dried (Edwards Micro Modulyo, Sussex, UK). 33 mg lyophilised syngeneic spinal cord homogenate was reconstituted in 5 ml PBS and added to an equal volume of CFA. This mixture was sonicated (Bransonic Ultrasonic cleaner, Sigma, Poole, UK) for 10 minutes at room temperature and emulsified by drawing the mixture through a 1ml syringe to a consistency which did not disperse when added to water. This was loaded into 1 ml "luer lock" syringes (Becton Dickinson, Cowley, Oxford, UK) with 25g 16 mm needle (Becton Dickinson).

Biozzi ABH mice between the ages of 6-8 weeks were injected sub-cutaneously in the flank with 0.15 ml of the emulsion in 2 sites on day 0 and day 7 (Baker et al., 1990). Each animal received 1mg of lyophilised Biozzi ABH mouse SCH and 60 \( \mu \)g of mycobacteria at each time point.

2.3. Clinical Status of CREAE

Mice were evaluated neurologically according to the following grades: 0 = normal, 1 = loss of tail tone, 2 = loss of inverted righting reflex, 3= partial hind limb paralysis, 4 = total hind limb paralysis. Neurological signs observed of a lower severity than typically observed for a defined grade were reported as a grade in brackets, and scored 0.5 lower.
to aid in the statistical analysis (O’Neill et al., 1992).

2.4. Clinical Phases of CREAЕ

Following CREAЕ induction mice exhibited weight loss (WL) between days 13-15 post-inoculation (p.i.), which correlated to an influx of T cells and macrophages into the CNS (Allen et al., 1993). The onset (OS) of clinical signs (grade 1) manifested by loss of tail tone occurred between day 15-17 p.i. By day 18-20 p.i. animals exhibited acute phase paralysis (AP) (grade 4) where the inflammatory infiltrate was at its maximum level in the CNS (Allen et al., 1993). Animals gained weight and clinical signs abated over the next few days, day 20-24 p.i., the post acute phase (PA). Animals then entered a period of remission (RM) by day 24-28 p.i., where they experienced some residual loss of tail tone (grade 0.5). By day 35-40 p.i. animals relapsed (RL) and paralysis reoccurred. However, the development of paralysis time course is quicker in the RL phase compared to the AP. The RL phase correlated to an increase in T cells and macrophages infiltrating the CNS but also the presence of Ig+ cells, unlike the AP (Allen et al., 1993).

2.5. Sampling of Tissues

2.5.1. Tissue

Spinal cord (either dissected from the cervical region, or expelled from the cervical spinal column by applying hydraulic pressure through the lumbar spinal column from a 20g needle and 20 ml syringe filled with PBS), brain or anterior tibialis muscle tissue were sampled from normal mice, or mice at various stages during CREAЕ and following treatments of EAE mice. Tissue was mounted in Tissue-Tek mounting medium (Raymond A. Lamb, London UK) and snap frozen in liquid nitrogen cooled iso-pentane (BDH, Poole, UK) and specimens were stored at −70 °C. Cryostat sections were cut at a thickness of 8 μm, air dried overnight and fixed in acetone (BDH) for 10 min at room temperature, and stored at -20°C prior to analysis.
2.5.2. Serum

Animals were anaesthetised by CO\textsubscript{2} and were bled into the thoracic cavity by severing the right atrium of the heart. Blood was collected into 1.5 ml eppendorf tubes and allowed to clot at 4 °C for 1 hour. The tubes were centrifuged at 500g for 1 min using a bench top microfuge (Anderman Centrifuge 5415, Hamburg, Germany). Serum was removed from the tube and transferred to a clean eppendorf tube leaving the blood clot at the bottom of the old tube. Serum was centrifuged again to remove any erythrocytes still present in the serum. Serum was stored at -20 °C until used.

2.5.3. Peripheral blood mononuclear cells

Animals were killed by CO\textsubscript{2} overdose and animals were bled into the heart cavity as described above and blood was collected into heparinised tubes (Sigma). Blood was centrifuged at 500g for 5 min, then erythrocytes were lysed twice at 37 °C for 5 min by the addition of 0.83% ammonium chloride (Sigma) and washed in RPMI 1640 medium (Sigma) following each incubation and cytopsins prepared (Cytospin 2, Shandon Southern Products, Cheshire, UK). Alternatively, leucocytes were isolated by layering 1.5 ml heparinised blood above Lympholyte M (Cedarlane Labs, Peterborough, UK), and centrifuged at 400g for 10 min at room temperature. Cells were removed from the interface and washed in RPMI 1640 medium (Sigma) prior to use.

2.5.4. Splenocytes

Following euthanasia the spleen was removed and a single cell suspension prepared by teasing the spleen through a 200g wire mesh strainer. Erythrocytes were lysed by a single incubation with 0.83% NH\textsubscript{4}Cl as described above.

2.5.5. Isolation of spinal cord infiltrates

Animals terminally anaesthetised by CO\textsubscript{2} were perfused with 25 ml RPMI 1640 medium (Sigma) via the left ventricle. The spinal cord was then expelled by hydraulic pressure following insertion of a 20g needle (Becton Dickinson) and 20 ml syringe (Terumo, Luven, Belgium) filled with PBS (Sigma) into the lumbar region of the vertebral column. A previous study has shown histochemically that this procedure
strips the meninges from the cord (Allen et al., 1993). Cords from each group were pooled in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated foetal calf serum (FCS) and homogenised in a glass homogeniser. The resulting suspension was filtered through a 50 μm nylon mesh (R. Cadisch and Sons, London, UK) and centrifuged at 400g for 10 min at room temperature. The pellet was suspended in 1 ml of 100% Percoll (Pharmacia, Milton Keynes, UK) in a 17 x 120 mm Falcon conical tube (Becton Dickinson), above which 2 ml 80% Percoll, 3 ml 40% Percoll, and 2 ml 30% Percoll were layered successively. After centrifugation at 400g for 10 min at room temperature, the cell interface layer between the 80% and 40% Percoll layers was isolated and used for flow cytometry analysis (see below).

2.5.6. Urine
Urine was collected in eppendorf tubes and stored at -20°C until used.

2.5.7. CSF
CSF (1-15 μl) was withdrawn from the foramen magnum using a sharpened 2 μl pipette tip into a small tube from terminally CO₂ anaesthetised mice. Care was taken not to use samples contaminated with blood. Samples were centrifuged and the supernatant aliquoted in equal volumes and stored at -20 °C until use.

2.6. Therapeutic Agents

2.6.1. Recombinant Cytokines
Recombinant mouse IL-10 was a kind gift from Professor Marc Feldman (Kennedy Institute of Rheumatology, London, UK).

2.6.2. Cytokine Plasmids.
Plasmids and retroviral vectors expressing cytokines and cytokine inhibitors were as follows: Human p55 TNFR (extracellular domain) fused to a mouse IgG1 backbone was kindly provided by Prof. B. Beutler (Southwestern University, Dallas, Texas) and was driven by the CMV promoter (Peppel et al., 1991). The human p55 TNFR fusion protein was then cloned into pBabe-bleo, driven by the Moloney Murine Leukaemia
Virus (MMuLV) LTR promoter as described (Triantaphyllopoulos et al., 1998). The cDNA’s for murine IL-4 (mIL-4) and human TGF-β were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and cloned into the pBabe-neo retroviral vector (Morgenstern et al., 1990). mIL-4 (0.59 kb BamH1 fragment) was cloned into the BamH1 site of pBabe-neo, and the cloning of TGF-β has been described previously (Chernajovsky et al., 1997). The human p75 dimeric TNFR is a novel molecule consisting of 2 extracellular monomeric domains of human p75 TNFR linked by a flexible polyglycine-serine peptide linker. This molecule has a smaller molecular weight than the Ig fusion proteins, allowing greater penetration to tissues and therefore a greater biological activity. Human p75 TNFR extracellular domain (ECD) was digested from pVL1393-human p75 TNF-R ECD (In-Vitrogen, Leek, The Netherlands) with NcoI and Xba1 restriction enzymes and cloned into pCITE plasmid (Novagen, Madison, WI, USA) (Neve et al., 1996). This comprised the 5’ strand of the final dimer. The 3’ strand of human p75 TNF-R ECD was amplified by PCR from the pVL1393-human p75 TNF-R ECD and cloned downstream from a polyglycine linker sequence (Brigido et al., 1993; Neve et al., 1996). The 5’ ECD was digested from pCITE and together with the 3’ECD from pIg16 were cloned in tandem into pUC18 vector (Pharmacia) to produce the human p75 TNF-R ECD-dimer construct TRIP-4 (Neve et al., 1996). TRIP-4 was then digested with NcoI and BamH1 and ligated into the retroviral vector MFG (Dranoff et al., 1993; Neve et al., 1996) kindly provided by Dr. P.D. Robbins (University of Pittsburgh, Pennsylvania), as described previously (Chernajovsky et al., 1999). A genomic clone of mouse TNF (kindly provided by Dr. N. Sarvetnick, Scripps Institute, La Jolla, California) was cloned into pBabe-bleo (Mageed et al., 1998). Mouse IL-10 cDNA was obtained from Dr. K. Moore (DNAX, Palo Alto, California) and was cloned (as a 1.2 kb XhoI fragment) into the SalI site of pBabe-puro (Morgenstern et al., 1990). Mouse IFN-β, driven by the MMuLV LTR in pBabe-bleo or a plasmid with a neuron-specific enolase (NSE) promoter as were described previously (Triantaphyllopoulos et al., 1998). The plasmid pCH110 expressing the bacterial beta-galactosidase gene under the early SV40 promoter was purchased from Pharmacia Biotech (Uppsala, Sweden).
2.6.3. Expression of Cytokine Plasmids.

Plasmid DNA was prepared as described previously (Neve et al. 1996). Briefly, *Escherichia coli* cultures containing the plasmids of interest were grown at 37 °C. Following lysis of bacteria and addition of RNAase A (10 mg/ml), samples were centrifuged at 25,000g at 4 °C for 40 mins. and filtered through sterile gauze. DNA was precipitated with 60% propan-2-ol at -20 °C for 30 mins. Samples were centrifuged for 20 mins at 1,500g and pellets washed with ethanol and respun for 5 mins at 1,500g before being applied to QIAGEN columns (QIAGEN Inc., Chatsworth, CA) and eluted with wash buffer. After precipitation, plasmids were resuspended in normal saline prior to injection of 100 μg of the naked DNA. Plasmids were transfected into COS-7 cells for transient expression assays or permanently transfected using the GPenv-AM12 packaging cell lines (Markowitz et al., 1988) and were used to confirm the functional activity of the plasmid constructs using cytokine Enzyme Linked Immunoabsorbant Assay (ELISA) or biological assays and western blots. The IFNβ-NSE plasmid was assessed following transfection into neuroblastoma cells (Triantaphyllopoulos et al., 1998). ELISA was used to detect p75 dTNFR from supernatant collected from permanent transfections in the GPenvAM12 packaging cell line (Markowitz et al., 1988). p55 TNFR-Ig production from permanent transfections in GPenvAM12 cell lines was detected by western blotting and by protection from TNF cytotoxicity assay on WEHI 164 fibrosarcoma cells (clone 13) (Triantaphyllopoulos et al., 1998). Viral supernatants from permanent transfections in GPenvAM12 cells were collected and stored at -70 °C prior to their use.

2.6.4. Production of Plasmid DNA-Cationic Liposome Complexes

100 μg of plasmid DNA was dissolved in 30 μl saline for injection intra-muscularly (i.m.) or intra-cranially (i.c.). In some instances this was prepared by mixing plasmid DNA (100 μg) with 3 μg Lipofectin reagent (Gibco BRL, NY, USA) to form the cationic liposome complex (CLC) according to the manufacturers instructions. Briefly, plasmid DNA dissolved in saline was mixed with Lipofectin reagent in PBS (Sigma) and the resulting plasmid DNA-CLC mixture was incubated at room temperature for 15 min. prior to i.c. injection. Controls consisted of either untreated mice or injected with lipofectin dissolved in PBS.
2.6.5. Immunosuppressive Reagents for in vivo costimulatory blockade.

The 10.3.16 mouse IgG2a mAb specific for MHC class II (H-2A Ia.17) antigens (Oi et al., 1978) was supplied by Dr. A. Morgan (Celltech Slough, UK). A fusion protein of the extracellular domain of mouse CTLA4 and Fc regions of mouse IgG2a (CTLA4-mIg) (Finck et al., 1994) was supplied by Dr. G. Larsen (Genetics Institute, Cambridge MA), and L6 mouse IgG2a fusion protein a gift from Dr. J. Ledbetter (Bristol-Myers Squibb, Seattle, WA, USA) (Finck et al., 1994) served as a control. A similar fusion protein of mouse CTLA4 and the constant regions of human IgG1 (CTLA4-hIg) was protein-G (Hitrap Columns, Pharmacia Biotech, Uppsala, Sweden) purified from tissue culture supernatant of J337 cells transfected with CTLA4-hY1 (Lane et al., 1993). The rat 1G10 mAb specific for mouse CD80 (B7-1), (Nabavi et al., 1992) and 2D10 mAb specific for CD86 (B7-2), (Chen et al., 1994) were supplied by Dr. G. Powers (Hoffmann-La Roche, Nutley, NJ) or mAb was protein G purified (Hitrap Columns, Pharmacia Biotech, Uppsala, Sweden), from tissue culture hybridoma supernatant. Hamster Ig UC-4F10 hybridoma specific for mouse CTLA4 (Walunas et al., 1994) was supplied by Dr. J. Bluestone (University of Chicago, IL, USA) and protein G purified from tissue culture supernatant.

2.6.6. Adenoviral Gene Vectors.

Replication deficient E1 deletion mutants of type 5 human adenovirus (Ad) coding LacZ from Escherichia coli (AdRL) and the extracellular domain of mouse CTLA4-human IgG1 fusion protein (AdCTLA) driven by the Rous Sarcoma virus (RSV) promoter were generated as described previously (Oral et al., 1997). Adenovirus coding murine IL-10 (AdRLIL10) was similarly constructed. An adenoviral vector coding an immunoadhesin of the extracellular domain of human p55 TNF receptor and mouse IgG which has a cytomegalovirus promoter (TNFV), (Kolls et al., 1995) was provided by Dr. B. Beutler (Howard Hughes Medical Institute, Dallas, TX). The recombinant adenovirus vectors were generated and supplied by A. P. Byrnes (University of Oxford, UK), M. A. Wood (University of Oxford, UK), M. J. Dallman (Imperial College, London, UK), and K. Brown (Kennedy Institute of Rheumatology, London, UK). Adenovirus were grown in 293 cells until cells died from cytotoxicity. Virus particles were released from the cell pellet by four freeze-thaw cycles. The resulting viral particles were purified twice over
caesium chloride to remove soluble proteins and increase the concentration of virus as described previously (Green and Wold. 1979). Adenovirus was then dialysed against PBS buffer to remove caesium chloride and glycerol was added to a final volume of 10%. Finally, adenoviral preparations were passed through a 0.2 μm filter and stored at -70 °C prior to use. Viral titres and the absence of wild type virus were assessed by Dr. Robin Ali (Institute of Ophthalmology, London, UK).

2.6.7. Detection of Adenoviral Gene products.

Following injection of adenovirus, CNS tissues were snap frozen with liquid nitrogen and 8 μm cryostat sections were cut. Following AdRL injection, sections were stained to detect β-galactosidase activity as described later. To detect AdCTLA, serial sections were stained using an indirect avidin/biotin-immunoperoxidase technique described later (O’Neill et al., 1993), using UC-4F10 mAb to detect mouse CTLA4 and biotinylated anti-hamster Ig or biotinylated anti-human Ig diluted 1:100 in 5% NMS/PBS (Vector Labs. Peterborough, UK). Samples were then incubated with avidin/biotin peroxidase complex (Vectorstain Elite Kit, Vector Labs., Peterborough, UK). The substrate was developed using the diaminobenzidine chromagen (60 μg diaminobenzidine per 100 ml PBS plus 60 μl 30% H₂O₂), (O’Neill et al., 1993). Sections were incubated for 30s with haematoxylin and the counterstain was visualised by immersion in tap water. These were mounted with DPX (BDH).

Following administration of 5x10⁶ plaque forming units (PFU) of AdRIL10 to the CNS of mice CSF and serum were removed 3 days post i.e. injection and stored at -20°C. Samples were then analysed for the presence of murine IL-10 by ELISA as described later. Controls consisted of untreated mice or mice injected with AdCTLA.

Adenovirus coding for the human p55 TNFR-IgG (TNFV) was from the same batch as used by Dr. E. Quattrocchi to block the effects of TNF in a murine collagen-induced arthritis model (Quattrocchi, et al., 1999). This study examined the detection of the p55 TNFR-IgG delivered from the TNFV after systemic administration. They reported microgram levels of product in the serum of mice for at least 10 days.
2.6.8. In vivo detection of adenoviral delivered CTLA4-hIg

CTLA4-hIg was detected using a sandwich ELISA following coating of 96 well microtiter plates (MaxiSorp, Nunc, UK) plates with 50 ng of CTLA4-specific mAb (UC-4F10), cerebrospinal fluid (CSF) diluted 1:50 or serum samples diluted 1:10 and 1:100 at 4 °C overnight. Following blocking with 10% FCS in PBS samples were incubated 3 times with wash buffer (0.05% Tween-20 (Sigma), then 50 µl of peroxidase conjugated rabbit anti-human IgG (Sigma) diluted 1:2000 was incubated with the samples for 1 hour at room temperature. Following 3 washes in wash buffer the substrate was developed with the addition of 10 µl 30% H₂O₂ (Sigma) to 11 ml substrate buffer consisting of 150 mg ABTS (2,2’Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 0.1 M citric acid (Sigma) in double distilled (dd)H₂O (pH4.35). The optical density (OD) of duplicate samples were read at 405 nM. The CTLA4-hIg protein served as a standard with a detection limit of 0.5-1 ng/ml.

2.6.9. Detection of Immunogenicity of CTLA4-hIg in mouse serum

Immunogenicity of CTLA4-hIg was assessed using ELISA. 96 well microtiter plates (MaxiSorp, Nunc, UK) were coated overnight at 4 °C with 50 ng/well CTLA4-hIg protein diluted in 0.1M NaPO₄, pH 9. Following blocking with 10% FCS in PBS, serum samples diluted 1:10 in blocking buffer were incubated for 4h at room temperature. Following incubation 3 times with wash buffer (0.05% Tween-20 (Sigma), 50 µl of 1:2000 dilution of peroxidase conjugated rabbit anti-mouse Ig (Dako Ltd. Cambridge, UK) was incubated with the samples for 1 hour at room temperature. The substrate was developed as described above. The OD of duplicate samples were read at 405 nM.


Fibroblast cell lines were generated from the kidneys of day 18 foetal ABH mice. Kidneys were homogenized through a 200g wire mesh and maintained in an incubator at 35 °C, 5% CO₂, in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco BRL) containing 10% heat inactivated (56 °C for 30 min) FCS, 1 mM sodium pyruvate (Sigma), 10 mM non-essential amino acids (Sigma), 2 mM glutamine (Sigma), 0.2 mM 2-mercaptoethanol and gentamycin (1:1000) (Sigma). At 75% confluence fibroblasts
were immortalised using a retroviral vector, containing a neomycin resistance gene to G418 (Gibco), encoding a temperature-sensitive non-SV40 origin binding U19 mutant of the SV40 large-T antigen (Almazan et al., 1992). Briefly, prewarmed (37 °C) retroviral medium from SVU 19.5 cell lines was applied to the cells in the presence of 0.8 µg/ml polybrene (Sigma). Cells were incubated at 37 °C for 24 hours in the medium. Cells were then refed with DMEM supplemented with 10% FCS containing 0.5 mg/ml G418, and refed every 2 days, for 1-2 weeks. Viable colonies of immortalised fibroblasts were named temperature-sensitive fibroblasts (tsF) and were refed with G418-free medium. Cells were grown in plastic slide chambers and stained for the presence of large-T antigen. Briefly, cells were fixed in 4% formaldehyde for 10 mins. and then 50 mM Tris-HCl (pH 7.5) for 10 mins. Cells were then permeabilised with 0.2% Triton X-100 (Sigma) in PBS for 10 mins. Non-specific binding was blocked with 10% FCS in DMEM for 10 mins. Cells were incubated with mouse anti-SV40 large-T antigen antibody (clone number Pab100, a generous gift from Dr. P. Jat, Ludwig Institute, London, UK) (1:100) in 5% normal mouse serum (NMS)/PBS for 1 hour, and then goat anti-mouse Ig conjugated to fluorescein isothiocyanate (FITC) (1:100) (Vector Labs. Inc., CA, USA) in 5% NMS/PBS for 30 mins. Each step was preceded by 3 washes with PBS. Cells were visualised using a Zeiss Axiophot microscope. Controls were fibroblasts without primary antibody or non-transduced primary Biozzi ABH fibroblasts. Immortalised fibroblasts were cloned by limiting dilution to 96 well plates (Nunc) at 0.5 cells per well and clones expanded in the presence of 10% FCS DMEM.

2.6.11. Retroviral infection of tsF to produce cytokines and p75 TNF Receptor.

5x10⁴ Biozzi ABH tsF cells were seeded per 9mm diameter cell culture dish with replication-deficient retroviral supernatant from packaging cell lines coding for murine IL-10, murine IFN-β and soluble human p75 dTNFR, in the presence of 0.8 µg/ml polybrene (Sigma) for 24 h. This was repeated 2 days later. Cells were refed with DMEM 10% FCS for 48h and then expanded.
2.6.12. Selection and cloning of retrovirally infected tsF

Cells were removed from 175cm² flasks by first washing with serum-free media, Hanks Balanced Salt Solution (HBSS) (Sigma) without CaCl₂ or MgSO₄, and then incubated with 1x Trypsin-EDTA (Sigma) solution in HBSS at 37 °C. Cells were washed in 10% FCS DMEM and spun at 1500g for 5 mins. The MFG retroviral vector has no selectable marker so infected cells were expanded and cloned. p75 dTNFR-producing clones were generated by limiting dilution to 0.5 cells/well in 96 well plates. Wells containing one cell were allowed to grow and supernatant from these clones were tested by ELISA (see below) for the presence of human p75 TNFR. A tsF clone positive for human p75 dTNFR was named oc5. Positive selection of human p75 dTNFR tsF clones was also performed by immunohistochemical staining (see below) of tsF clones with an antibody specific for human p75 TNFR (clone no. 4D1B10(MR2-1) Caltag Labs., CA, USA). Cells infected with retrovirus coding for IL-10 were initially selected with the addition of 1.5 mg/ml puromycin (Sigma) respectively, for 2 weeks, changing medium every 2-4 days prior to cloning (see below). Supernatant from these cells was tested by ELISA for the presence of IL-10 (R&D Systems, MN, USA).

2.7. Administration of Therapeutic Agents

2.7.1. Central Nervous System Injection

Therapeutic agents were administered to the CNS in a 30 µl volume of PBS (Sigma) either before the anticipated onset of disease (day 12 p.i.), at onset of clinical signs (grade 1), or during remission (day 27 p.i.). Mice anaesthetized with halothane (May and Baker, Dagenham, UK), were injected intracranially (i.c.), “freehand”, into the right frontal cortex prior to or after onset of disease using 27g insulin syringes (Surgicon, Leeds, UK) (Baker et al., 1990; O’Neill et al., 1992). The needle was inserted as superficially as possible (3-5 mm), although the possibility of intra-ventricular injection could not be excluded. The needle was left in place for 15-20s to reduce “back-flow” of agents. Generally, i.c. injections were well-tolerated by the mice. Any mice showing evidence of brain damage as a result of the i.c. injection were excluded from the study. Controls consisted of injections of the appropriate vehicle, empty gene vectors or vectors coding for irrelevant genes.
2.7.2. Intra-muscular injection

Mice were anaesthetised by i.p. injection of 0.3 ml/kg of a mixture (1:1) of Hypnorm (Janssen, Wantage, U.K.) and Hypnovel (Roche, Welwyn Garden City, U.K.) diluted in H₂O. These received a single dose of 100 μg DNA in 50 μl PBS, injected i.m. in the right *tibialis anterior* muscle. In some instances 50 μl of a dose of 1.2% BaCl₂ (gift from Dominic Wells, Charing Cross and Westminster Hospital, London) in PBS was injected into the same muscle 5 days prior to plasmid injection, to induce myoproliferation, thus facilitating the uptake of plasmid into the regenerating muscle tissue (Wells et al., 1995). Cytokine plasmid DNA (100 μg in saline) was injected i.m. on day 0 of EAE induction. Controls consisted of injections of the same volume of PBS at the same time point and site of administration.

2.7.3. Systemic Injection

Mice received therapeutic agents in a volume of 100 μl per injection. Mice were injected intra-peritoneally (i.p.) at day 0 or day 7 p.i. using 25g needle (Microlance 3, Becton Dickinson, Oxford, UK) and 1 ml syringe (Becton Dickinson). Control groups consisted of untreated SCH mice or mice injected i.p. at the same time point with vehicle.

2.8. Linking of Fluorescent Marker to tsF.

Cells were linked to green fluorescent dye PKH2-GL (Sigma) according to manufacturer's protocol. Briefly, tsF cells were trypsinised into a single cell suspension, and diluted to 10⁷ cells/ml. Cells were washed in HBSS (Sigma) and centrifuged for 5 min (400g). Cells were resuspended to a concentration of 10⁷ cells/ml in provided diluent (diluent A) and then added to 4x10⁻⁶ M PKH2-GL dye dissolved in supplied diluent (Sigma). The sample was mixed and incubated at room temperature for 5 min. An equal volume of 10% FCS (Labtech) in DMEM (Sigma) was added to stop the binding reaction and incubated for 1 min at room temp. Cells were centrifuged for 5 min (400g) and then washed in 10% FCS 3 times.
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2.9. Immunohistochemistry

Immunohistochemistry was performed on frozen sections prepared as mentioned above. Endogenous peroxidase activity was blocked by incubating sections in 0.03% H$_2$O$_2$ in PBS for 10 min. Then non-specific protein binding was blocked by incubating sections in 5% NMS/PBS for 30 min. Primary antibodies (Table 2.1) were diluted 1:100 in 5% NMS/PBS and incubated for an hour. Then secondary antibodies diluted 1:50 in 5% NMS/PBS were incubated for 30 min. Secondary antibodies used were peroxidase conjugated anti-rat and anti-rabbit Ig (Vector Labs, Peterborough, UK). Peroxidase activity was developed by incubation in 60 µg dianamobenzidine (DAB) chromogen (Sigma) dissolved in 100 ml PBS with 60 µl 30% H$_2$O$_2$ (Sigma) for 20s. Sections were lightly counter stained with haematoxylin (BDH) and mounted in DPX (BDH). Three washes with PBS were performed between each step of the staining protocol (Baker et al., 1990). Positive controls for the Ab staining were cytokine-retrovirus transfected Biozzi ABH mouse fibroblast cytospins and human peripheral blood lymphocyte cytospins as described above. Cross reactivity of the human specific mAb were checked using mouse spleen cell cytospins as described above. Negative controls consisted of omission of primary antibody or irrelevant isotype matched control as described previously (Baker et al., 1990). Isotype matched controls did not induce a detectable signal (Baker et al., 1990). Controls also consisted of staining with mAb of CNS and muscle tissue injected with plasmid DNA of a different specificity to the primary antibody.

2.9.1. Primary antibodies

All primary antibodies (Table 2.1) were diluted in 5% normal mouse serum (NMS) dissolved in phosphate buffered saline (PBS) (Sigma).
Table 2.1

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Clone no.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat anti-mouse IL-10</td>
<td>JES5-2A5</td>
<td>Pharmingen, San Diego, CA, USA</td>
</tr>
<tr>
<td>Rabbit anti-mouse TGF-β1</td>
<td>SC-146</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>Rat anti-mouse CD120a</td>
<td>HM103</td>
<td>Dr. W. Buurman, Univ. Maastricht, Netherlands</td>
</tr>
<tr>
<td>Rat anti-mouse C120b</td>
<td>HM102</td>
<td>Dr. W. Buurman, Univ. Maastricht, Netherlands</td>
</tr>
<tr>
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<td>BVD4-1D11</td>
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<tr>
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<td>4D1B10(MR2-1)</td>
<td>Caltag Labs., CA, USA</td>
</tr>
<tr>
<td>Rat anti-mouse CD4</td>
<td>YTS 191</td>
<td>Prof. H. Waldmann, University of Oxford, UK</td>
</tr>
<tr>
<td>Rat anti-mouse CD8</td>
<td>YTS 169.4</td>
<td>Prof. H. Waldmann, University of Oxford, UK</td>
</tr>
<tr>
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<td>MI/70</td>
<td>Serotec</td>
</tr>
<tr>
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<td>OX-6</td>
<td>Serotec</td>
</tr>
<tr>
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<td>1G10</td>
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<td>2D10</td>
<td>Hoffman-LaRoche, Nutley, NJ, USA</td>
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<tr>
<td>Hamster anti-CD152</td>
<td>UC-4F10</td>
<td>Dr. J. Bluestone, Univ. Chicago, IL, USA</td>
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<tr>
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<td>A-8792</td>
<td>Sigma, Poole, UK</td>
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<td>B220, RA3-6B2</td>
<td>Caltag Labs, CA, USA</td>
</tr>
<tr>
<td>Mouse anti-SV40 large-T Ag</td>
<td>Pab 100</td>
<td>Dr. P. Jat, Ludwig Institute, London, UK</td>
</tr>
<tr>
<td>Rat anti-mouse IL-6</td>
<td>MP5-20F3</td>
<td>Pharmingen, San Diego, CA, USA</td>
</tr>
</tbody>
</table>

2.9.2. Secondary antibodies

Secondary antibodies were diluted 1:100 in 5% NMS/PBS and incubated for 30 min. Secondary antibodies used were biotinylated rabbit anti-rat Ig, biotinylated goat anti-hamster Ig, FITC conjugated goat anti-mouse Ig and biotinylated goat anti-rabbit Ig (Dako Ltd, Cambridge, UK). These were diluted 1:100 in 5% NMS/PBS. Negative control sections consisted of secondary antibodies only.

2.9.3. Histology of fixed tissues embedded in wax

Spinal cords were removed and fixed in 10% formal saline and embedded in paraffin wax and processed for routine histology. Serial longitudinal sections of the whole cord were cut and stained with haematoxylin and eosin (BDH). The number of distinct lesions were counted in 5 x 0.77 mm² fields at the 4 corners (white matter) and centre (grey matter) of each randomly selected area per section, and 12 sections at different levels from cord were examined and mean number of lesions/section/animal calculated.
2.10. Flow cytometry of isolated cells from EAE spinal cord

Cells isolated from mouse spinal cords were washed twice in HBSS medium (Gibco), and counted. 1-3x10^5 cells were then incubated with 5% normal mouse serum (NMS) in PBS (Sigma) with the primary antibody for 1 hour. Cells were then washed and resuspended in fixative (1% formaldehyde/PBS). Primary antibodies used were (Table 2.1): rat anti-mouse CD4 monoclonal antibody (mAb) –Tricolor (TC) conjugated (Caltag Labs, CA, USA, Clone no. CT-CD4), rat anti-mouse mAb CD8a-phycoerythrin (PE) conjugated (Clone KT15, Serotec, Oxford, UK), rat anti-mouse mAb CD11b (Mac-1) –PE conjugated (Clone no. MI70, Serotec) and mouse anti-rat/mouse mAb I-A-FITC conjugated (Clone no. OX-6, Serotec). Antibody concentrations were used according to manufacturers instructions. Cells were analysed using a FACScan flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson).

2.11. ELISA

2.11.1. Mouse p55 and p75 TNFR ELISA assay.

Serum samples at various time points following disease induction were collected and stored at -20 °C until used. Samples were tested for the presence of mouse p55 TNFR (CD120a) and mouse p75 TNFR (CD 120b). 96 well ELISA plates (Nunc Maxisorb, Life Technologies, Glasgow, UK) were coated with either 10 or 3 μg/ml anti-mouse CD120a, b TNFR antibodies respectively (HM102, HM103, a kind gift from Dr. W. Buurman, University of Maastricht, Netherlands) in 0.1 M Na_2HPO_4 (pH 9.0) (Sigma) (Bemelmans et al., 1993). The plate was sealed and incubated overnight at 4 °C. The plate was washed 3 times with wash buffer (0.05% Tween-20) (Sigma) in PBS (Sigma). Non-specific binding was blocked by incubation with blocking buffer (10% Foetal Calf Serum (FCS) in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Paisley, UK) for 30 min. at room temperature. The plate was washed 3 times with washing buffer, and mouse CD120a and CD120b standards (provided by Dr. W. Buurman) and samples diluted in blocking buffer were incubated overnight at 4 °C. Samples were diluted 1:10 and standards were in the range 1000 pg/ml to 0 pg/ml. The plate was washed 6 times and 1 μg/ml biotinylated polyclonal rabbit anti-mouse 55 and 75 kDa TNFR antibodies in blocking buffer were incubated for 1 hour at room temperature. The plate was
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washed 6 times and 1:2000 streptavidin-peroxidase (Dako Ltd., Cambridge, UK) solution was incubated at room temperature for 30 mins. Following 8 washes of the plate, colour development of the substrate was achieved by incubating 100 µl of the substrate to each well for 20 min. at room temperature. The substrate consisted of 10 µl 30% H₂O₂ (Sigma) added to 11 ml substrate buffer, 150 mg ABTS (Sigma) in 0.1 M citric acid (Sigma) in double distilled (dd)H₂O (pH 4.35). The optical density (O.D.) was read at 405 nm using a Titertek Multiskan ELISA reader (Flow Laboratories, Helsinki, Finland). Readings from wells with substrate solution but not coated with antibody were used to determine background readings, and controls included samples taken from mice inoculated with CFA alone that contained no SCH.

2.11.2. Human p75 TNFR, mouse IFN-γ and IL-10, and TGF-β ELISA assays.

Supernatants collected from tsF-transduced cells plated into 175 cm³ flasks (Marathon, UK) and grown in 10% FCS in 5% CO₂ or serum, urine and CSF from oc5 and tsF treated mice were stored at -20 °C until used. Human p75 ELISA was performed using Hbt Hycult Biotechnology kit (Uden, Netherlands). 96 well ELISA plates (Nunc Maxisorb, Life Technologies) were coated with 50 µl 2 µg/ml anti-human p75 TNFR or 2 µg/ml anti-human p55 TNFR (a gift from Dr. W. Buurman) antibody in 0.1 M Na₂HPO₄ (pH 9.0). The plate was sealed and incubated overnight at 4 °C. The plate was then washed 3 times with wash buffer (0.05% Tween-20 in PBS) (Sigma). Non-specific binding was blocked by incubation with blocking buffer (10% FCS in DMEM) for 30 mins. at room temperature. The plate was washed 3 times with washing buffer and standards and samples diluted in blocking buffer were incubated overnight at 4 °C. Samples were diluted 1:10 and 1:100 and standards were between 1000 pg/ml and 7.5 pg/ml. The plate was washed 6 times and 100 µl 1µg/ml biotinylated polyclonal anti-human p75 TNFR or anti-human p55 TNFR antibody in blocking buffer was added for 1 hour at room temperature. The plate was washed 6 times and 100 µl 1:2000 streptavidin-peroxidase (Dako Ltd.) solution was incubated at room temperature for 30 mins. Following 8 washes of the plate, 10 µl 30% H₂O₂ was added to 150 mg ABTS in 0.1 M citric acid (Sigma) in ddH₂O (pH 4.35), 200 µl of which was dispensed into each well, and incubated for 30 mins at room temperature. The O.D. was read at 405 nm. Readings from wells with substrate solution but not coated with antibody were used to determine background readings, and controls included samples taken from untreated
mice and tsF treated mice.

Mouse IFN-γ ELISA was performed using the Cytoscreen Immunoassay kit (Biosource International, CA, USA) according to the manufacturer’s instructions. The TGF-β ELISA was performed using a Promega kit (WI, USA). All TGF-β samples were acid-activated before ELISA according to the instructions. DMEM 10% FCS was used to measure background concentrations of TGF-β in growth media.

Mouse IL-10 ELISA kit (R&D Systems, MN, USA) was used according to manufacturer’s instructions. All CSF samples were diluted 1:50 and 1:100 in diluent provided, and serum samples were diluted 1:4 in provided diluent for all cytokine ELISA kits.

2.12. β-galactosidase Staining

Muscle and brain tissue injected with the LacZ construct (pCH110) or AdRL were removed between 7 and 10 days p.i. and fixed in a 0.005% glutaraldehyde/2% paraformaldehyde solution (Agar Scientific, Cambridge, UK), in sodium phosphate buffer (pH 7.3) for 30 mins. Tissue was then washed in PBS, and placed into a staining solution containing: 50 mM Tris-HCl (pH 7.5), 2.5 mM Ferro-ferricyanide, 15 mM NaCl, 1 mM MgCl₂ (Sigma) and 0.5 mg/ml X-Gal (Gibco BRL) in N,N-dimethyl formamide (Sigma) in PBS. The tissue block was incubated at 37 °C for 2 hours. Tissue was then washed in PBS and placed into formalin (Agar Scientific, Cambridge, UK). Expression of LacZ construct was seen by blue staining in the tissue block by light microscopy. Negative controls were either untreated tissue or tissue injected with cytokine plasmid DNA or AdCTLA.

2.13. ³[H]-thymidine incorporation into lymph node cells.

Following injection of gene construct into the brain, 50 µl of 2.5% oxazolone in [4:1] acetone:olive oil was applied to the inner thigh of one hindlimb. Three days later the draining inguinal lymph nodes from a minimum of 3 animals per group were removed, pooled and single cell suspensions prepared. 5 x 10⁵ cells/well were cultured in quadruplicate in supplemented RPMI-1640 medium and 10% FCS (as for DMEM 10%
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FCS, see above) overnight at 37 °C in the presence of 1 μCi 3H-thymidine as previously described (O’Neill et al., 1991; O’Neill et al., 1993; Baker et al., 1992; Baker et al., 1994), and the proliferative response assessed by β scintillation counting. Results are expressed as mean ± SD counts per minute.


The outer edge of the right and left cortex of neonatal mouse brain was removed and placed into 10% FCS in DMEM (Gibco) and the meninges stripped off the cortex pieces to reduce the contamination from smooth muscle cells and endothelial cells from blood vessels. These outer cortex segments were used to reduce contamination of the culture by ependymal cells. The tissue was passed through a 60 μm gauze filter into 10% FCS DMEM (Gibco). The tissue/medium mix was then triturated through a 5ml pipette 5-10 times and split into 125 cm³ flasks. Cells were then immortalised using a retrovirus coding for the temperature-sensitive SV40 large T-antigen as discussed above. Cells were then refed with DMEM supplemented with 10% FCS containing 0.5 mg/ml G418 every 2-3 days and were left to become confluent. Within each flask different cell types existed so patches of different cell types were removed from the flask and replated into 125 cm³ flasks. Cells were refed every 2-3 days until confluent. Cells were then cloned by limiting dilution in 96 well plates as described above. In this way 6 different clones were produced and named temperature-sensitive astrocytes.

2.14.1. Immunocytochemical analysis of astrocyte clones.

Cells were plated into 8 well chamber slides (Nunc) and grown to 50% confluency in 10% FCS DMEM. Cells were then washed 3 times in PBS (Sigma) following each of the incubations. Cells were then fixed in 4% paraformaldehyde for 20 min. Cells were permeabilised with 0.25% TritonX-100 (Sigma) in PBS for 10 min. Blocking of non-specific binding by incubation with 10% FCS in PBS for 15 min. was followed by incubation with primary antibodies for 2 hours. Primary antibodies used were: rabbit anti-glial fibrillary acidic protein (GFAP) mAb (1:100, DAKO), mouse anti-Galactocerebroside C (Gal C) (clone H8, 1:4), mouse anti-A2B5 ganglioside mAb (1:1), mouse anti-O4 (oligodendrocyte marker) mAb (1:1) and mouse anti-vimentin mAb (1:40) all gifts from Dr. Guus Wolswijk, Ludwig Institute for Cancer Research, London,
UK. All primary antibodies were diluted in 10% FCS DMEM. To block non-specific binding of the secondary antibodies cells were incubated with 10% goat serum in PBS for 15 min. Secondary antibody swine anti-rabbit Ig FITC (Dako) or sheep anti-mouse Ig FITC (Sigma) were diluted 1:50 in 10% goat serum and incubated for 2 hours. Cells were then washed in PBS 3 times and mounted with Vectashield (Vector Labs., Peterborough, UK). Staining was visualised using a Zeiss Axiophot 135M fluorescence microscope (Zeiss, Germany).

2.15. Statistical Analysis.

Results of treatment of EAE were presented as the mean clinical score, mean group score or mean day of onset ± standard error of the mean (SEM), and the statistical significance between groups determined using the Mann-Whitney U 2-tailed non-parametric ranking test, with MINITAB 10.51 Xtra software. Differences in antibody levels were assessed by Students $t$ test analysis using SigmaStat software, which incorporates analysis of normality and equality of variances between groups.
CHAPTER 3

IMMUNOHISTOCHEMICAL CHARACTERISATION OF Th2 CYTOKINES AND TNF RECEPTOR EXPRESSION IN THE CNS DURING CREAЕ
3.1. INTRODUCTION

EAE is mediated by CD4$^{+}$ T cells and the pro-inflammatory cytokines they produce initiate a cascade of events that lead to the pathology seen as described in chapter 1. Studies have demonstrated pro-inflammatory cytokines or their mRNA in the spinal cord of different models of EAE at different time points during disease and also in MS (Begolka et al., 1998; Okuda et al., 1998a; Issazadeh et al., 1996; Selmaj et al., 1991). Both IL-10 and TGF-β have been shown to be upregulated during recovery of EAE (Kennedy et al., 1992; Issazadeh et al., 1995a). The presence of anti-inflammatory Th2 cytokines in the CNS of mice during EAE has not been studied as extensively as the pro-inflammatory cytokines and the results observed appear to be dependent upon the species and strain used, whether the disease course is acute or relapsing and on the encephalitogenic peptide used to induce EAE (Begolka et al., 1998; Okuda et al., 1998a; Issazadeh et al., 1996; Diab et al., 1997).

3.2. AIM OF STUDY

The expression of anti-inflammatory cytokines in the CNS at different stages of disease may suggest potential mechanisms involved in the pathogenesis and importantly in the spontaneous recovery of EAE/MS and may be an indicator of potential benefit of anti-inflammatory cytokine therapy. Therefore this study has investigated the expression of Th2 cytokines by immunohistochemistry, both in normal CNS tissue and during the different stages of disease. TNF has been shown to play a major role in the pathogenesis of EAE and MS and mediates some of its effects via TNFR. Therefore the expression of both the 55 kDa and 75 kDa TNFR (p55 and p75 TNFR) in CNS tissue and their soluble forms in the serum will also be investigated.
3.3. RESULTS

Immunohistochemical analysis of normal mouse CNS tissue indicated that IL-6, IL-10, p55 and p75 TNFR could be detected (Table 3.1). IL-6 staining was localised to cells with an astrocyte-like morphology in brain and spinal cord sections (Figure 3.1a). IL-10 appeared to be present in a high proportion of parenchymal cells (Figure 3.1b). Both IL-4 and TGF-β could not be detected in normal tissue (Table 3.1). Low-level p55 TNFR staining could be detected on cells in the spinal cord (Figure 3.1c). However, p75 TNFR was only detected on very few cells (Table 3.1).

During the acute phase of disease perivascular lesions are seen throughout the spinal cord parenchyma consisting mainly of CD4+ T cells with low levels of CD8+ T cells and with macrophages and astrocytes present in and around the lesion (Baker et al., 1990). The expression of IL-4, IL-6, IL-10, TGF-β as well as p55 and p75 TNFR were upregulated during the acute phase and were mainly located either in the lesion or on cells around the lesion (Figure 3.2 a-f).

The remission phase correlates to a reduction in cell infiltrate in the spinal cord (Baker et al., 1990) and the presence of TGF-β, p55 and p75 TNFR were all greatly reduced consistent with a reduction of cellular activity (Table 3.1). However, IL-6 was still present on the border of resolving lesions and also in astrocyte-like cells surrounding the lesion (Figure 3.3a). IL-4 and IL-10 were both present on parenchymal cells and cells in and around lesions (Figure 3.3 b, c) to a greater degree than seen in the acute phase.

During relapse there is usually a more extensive inflammatory infiltrate than is seen in the acute phase and the disease progression is more rapid (Baker et al., 1990). IL-6 was detected around lesions but strikingly areas of astrogliosis could be seen, with many IL-6 positive astrocytes seen in localised areas of spinal cord parenchyma (Figure 3.4a). Low levels of TGF-β and IL-4 could be detected on parenchymal cells and around lesions (Table 3.1). Upregulation of both p55 (Figure 3.4b) and p75 TNFR could be detected during relapse on glial cells and around inflammatory cells in lesions (Table 3.1). IL-10 staining was present in and around the lesion infiltrate and glial cells (Table 3.1).
Chapter 3 Results

The presence of soluble mouse p55 and p75 TNFR was detected in serum from normal mice between 0.3-2.0 ng/ml by ELISA (Figure 3.5). Samples from mice injected with SCH at day 11 (D11) and at weight loss (WL) showed an increase of p55 TNFR with a peak of p75 TNFR (5.6 ng/ml) at WL (Figure 3.5). After this time point the p55 and p75 TNFR levels decreased back to normal levels except for a peak of p75 TNFR at the post-acute stage (PA). However, mice injected with complete Freund's adjuvant showed a similar increase in TNFR levels comparable to that seen in SCH treated EAE mice when sampled at day 11 (CFA11), day 18 acute phase (CFA18) and day 27 remission (CFA27) (Figure 3.5).
**Table 3.1** Anti-inflammatory cytokines and TNF receptors are present in the spinal cord of mice during the different phases of EAE.

<table>
<thead>
<tr>
<th>Cytokine or cytokine receptor</th>
<th>Normal</th>
<th>Acute EAE</th>
<th>Remission EAE</th>
<th>Relapse EAE</th>
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<tbody>
<tr>
<td>IL-4</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>p75 TNFR</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Detection of immunoregulatory cytokines immunohistochemically in the spinal cord of normal or CREAE mice during different time points of disease. + = positive staining, +/- = low level staining, - = no staining present.
Figure 3.1 Immunohistochemical staining of cytokines in normal CNS tissue.

(a) IL-6, (b) IL-10 and (c) p55 TNFR present in the normal spinal cord (➡️). Magnification is x200. Sections were lightly counter stained and isotype matched controls showed no positive staining. Three untreated mice were sampled for the presence of cytokines in the normal spinal cord.
Figure 3.2 Immunohistochemical staining in the spinal cord during the acute phase of CREA.E.

(a) IL-6 positive cells around lesions (►), and in the parenchyma (►), (b) IL-4 positive cells in the lesion (►), (c) IL-10 positive cells around the lesion (►), (d) TGF-β1 present at the lesion edge (►), (e) p55 TNFR positive cells around lesions (►) and in the parenchyma (►), and (f) p75 TNFR around a lesion (►). Magnification is x200 (Fig. 2a, b, d) and x400 (Fig. 2c, e, f). Sections were lightly counter stained and isotype matched controls showed no positive staining. Five mice were sampled for the presence of cytokines during the acute phase of EAE.
Figure 3.3 Immunohistochemical staining in spinal cord during the remission phase of CREAЕ.

(a) IL-6 positive cells around the lesion () and cells in the parenchyma (), (b) high level expression of IL-4 around the lesion () and on parenchymal cells (), and (c) IL-10 around lesions (). Magnification is x200. Sections were lightly counter stained and isotype matched controls showed no positive staining. Five mice were sampled for the presence of cytokines during the remission phase of EAE.
Figure 3.4 Immunohistochemical staining in spinal cord during the relapse phase of CREA E.

(a) IL-6 positive astrocytes indicating astrogliosis (※) x200, and (b) high level expression of p55 TNFR around lesion (●), x400. Sections were lightly counter stained and isotype matched controls showed no positive staining. Four mice were sampled for the presence of cytokines during the relapse phase of EAE.
Figure 3.5 Serum levels of soluble TNFR during CREAE by ELISA.

Time points analysed were (as described in more detail in chapter 2): N - normal, D11 - day 11 post disease-induction (p.i.), WL - weight loss, OS - onset, AP - acute phase, WG - weight gain, PA - post-acute, RM - remission, RL - relapse, CFA11 - CFA day11 p.i., CFA18 - CFA day18 p.i., CFA27 - CFA day27 p.i. Serum TNFR levels from mice with CREAE were not statistically significant from mice injected with CFA. The graph demonstrates the variance of soluble TNFR within groups and the median value is represented by a line. Groups consisted of 4-8 mice.
3.4. DISCUSSION

This study has demonstrated the presence of immunoregulatory cytokines IL-4, IL-6, IL-10 and TNFR within spinal cord lesions during the acute, remission and relapse phases of CREAE. Interestingly IL-6, IL-10 and both TNFR could also be detected in CNS tissue in normal mice and supports the expression of p75 TNFR but not p55 TNFR, in normal human CNS tissue (Raine, C. S. 1997). A previous study in Biozzi ABH mice has shown that pro-inflammatory cytokines such as IL-1, IFN-γ and TNF-α are absent in the CNS of normal mice (Baker et al., 1991). This suggests that immunoregulatory cytokines and cytokine receptors may be present in low quantities in the CNS to inhibit potentially unwanted non-specific inflammation. IL-6 and IL-10 appeared to be present in glial cells, which support in vitro studies which have shown the production of IL-6 from astrocytes (Aloisi et al., 1992) and IL-10 from microglia (Williams et al., 1996).

During the acute and relapse phase of CREAE pro-inflammatory cytokines such as IL-1, IFN-γ and TNF-α are expressed in lesions by infiltrating cells as well as resident CNS cells (Baker et al., 1990). Interestingly this study shows that immunoregulatory cytokines such as IL-4 and IL-10 are also present in lesions at these time points. Studies have shown the benefit of these cytokines in treating EAE (Racke et al., 1994; Rott et al., 1994; Mathiesen et al., 1997; Shaw et al., 1997), and that IL-10 can inhibit the production of pro-inflammatory cytokines (Fiorentino et al., 1991). Therefore the presence of these cytokines at this time point suggest that they may be present in quantities too low to modulate the inflammatory condition which supports a study in mouse EAE where levels of IL-4 and IL-10 mRNA were found to be 10-fold lower than those of pro-inflammatory cytokines (Begolka et al., 1998). Alternatively, they may be involved in the disease process such as the generation of antibody responses, although the majority of previous studies suggests that these cytokines are beneficial in reducing the severity of EAE.

In addition the target antigen may induce different forms of disease and this may shape the cytokine profile observed as reported in a study comparing IL-10 and IL-4 mRNA expression, in SJL/J mice inoculated with Theiler's murine encephalomyelitis virus (TMEV) or proteolipid protein (PLP) 139-151 peptide-induced disease (Begolka et al., 1998). Similarly in an MBP relapsing model of EAE IL-4, IL-10 and TGF-β mRNA
were absent in the CNS during the acute phase whereas a MOG non-relapsing EAE model showed the expression of all 3 cytokines mRNA (Okuda et al., 1998a).

It has been suggested that auto-antibodies may play a role in demyelination in MS (Genain et al., 1999). One feature of MS is the presence of oligoclonal immunoglobulin bands in the CSF of patients (Pirttila et al., 1995) and IL-4, which can induce immunoglobulin class switching from IgM to IgG, has also been detected in the CSF of MS patients (Link et al., 1994). This highlights the “pleiotropic” nature of cytokines and the potential for different outcomes of cytokine therapy in EAE depending on the time point of administration.

IL-6 is a pleiotropic cytokine and has both pro- and anti-inflammatory properties (Gijbels et al., 1992). Although IL-6 has not been observed in the CSF of MS patients it is produced by astrocytes in acute and chronic active plaques in MS CNS tissue, and elevated levels have been observed in EAE during onset of disease (Hauser et al., 1990; Maimone et al., 1997; Gijbels et al., 1990). Double-labelling of sections has confirmed that parenchymal cells around the lesions are GFAP+ astrocytes (Baker et al., 1996). The role of IL-6 in EAE has yet to be definitively elucidated although IL-6-deficient mice show resistance to EAE induced by myelin oligodendrocyte glycoprotein (MOG), and IL-6 specific antibodies also reduce EAE (Okuda et al., 1998b; Eugster et al., 1998; Gijbels et al., 1995). In this model of CREAE IL-6 was shown to be present during the acute and relapse phases of CREAE. This cytokine has pleiotropic functions and in early disease may induce pro-inflammatory cytokine release, BBB breakdown, B cell proliferation and antibody production (Campbell et al., 1993). However, IL-6 has also been implicated in the differentiation and survival of oligodendrocyte precursors, which may be beneficial in remyelination (Barres et al., 1993). IL-6 has also been shown to induce astrocyte proliferation (Selmaj et al., 1990). This would be consistent with this study which demonstrated IL-6 in gliotic astrocytes in the parenchyma and surrounding lesions during relapse. This may be involved in the scarring (sclerosis) seen in MS plaques (Kost-Mikucki et al., 1991).

TGF-β is a pleiotropic cytokine and has been shown to have inhibitory effects on EAE and can inhibit TNF production (Santambrogio et al., 1993). In this study TGF-β was present around the lesion at low levels during the acute and relapse phases and this
supports studies in other EAE models (Okuda et al., 1998a; Issazadeh et al., 1995b). TGF-β is normally secreted in a latent form and needs to be proteolytically cleaved in order to become activated (Gleizes et al., 1997). Therefore immunohistochemical detection of TGF-β in the CNS is not an indicator of bioactivity. It is often detected within the extracellular matrix of blood vessels (Falanga et al., 1992). However, in a study using transgenic mice overexpressing active TGF-β in the CNS, EAE was more severe, indicating that the level and/or activity of TGF-β expression maybe important in the microenvironment during EAE (Wyss-Corey et al., 1997).

Many studies have shown the importance of TNF in EAE pathology and it has been detected in MS lesions (Hofman et al., 1989; Selmaj et al., 1991). Soluble TNFR are thought to play a role in inflammation by acting as TNF inhibitors, and soluble TNFR have been detected in the serum or CSF from MS patients. While it has been suggested that sTNFR correlate to BBB permeability (Sharief and Hentges. 1991) there appears to be no consistent correlation of TNF or TNFR levels to disease episodes (Hofman et al., 1989; Selmaj et al., 1991; Chofflon et al., 1992; Spuler et al., 1996). In this study there was no correlation between soluble TNFR levels in the serum of CREAE mice and specific time points of CREAE although there was a non-significant increase in soluble CD120, probably as a consequence of immune activation induced by the adjuvant. The different findings between studies in MS may depend on whether samples came from the target organ, i.e. CNS, or from the periphery. Both p55 and p75 TNFR could be detected by immunohistochemistry in spinal cord lesions in the acute and relapse phases of CREAE. This supports the upregulation of both p55 and p75 TNFR demonstrated on oligodendrocytes and microglia in active MS lesions (Raine 1997). p55 TNFR is thought to play a role in cytotoxicity and its presence in the CNS during CREAE may reflect a role for TNF-mediated demyelination of neurons and/or the destruction of oligodendrocytes (Sipe et al., 1996), where cytotoxicity of membrane-bound TNF is more efficient than secreted TNF (Zajicek et al., 1992). Rat astrocytes, microglia and oligodendrocytes have been shown to constituently express the p55 TNFR mRNA which can be upregulated by IFN-γ, whereas TNF-α only upregulates TNFR mRNA in oligodendrocytes. In contrast p75 TNFR mRNA was only constituently expressed in microglia, where both TNF-α and IFN-γ upregulated its production (Dopp et al., 1997). This difference in sensitivity of oligodendrocytes to TNF-α may play an important role in demyelination. During the remission phase, levels of both TNFR were down-
regulated which may reflect the shedding of TNFR from cells in the CNS. Soluble TNFR binds TNF and may play a role in TNF neutralisation after the inflammatory episode in the CNS, and could be partly responsible for the spontaneous recovery of EAE. This correlates to studies using anti-TNF agents to successfully treat EAE (Baker et al., 1994; Selmaj et al., 1991; Ruddle et al., 1990) and suggests TNFR may be a useful therapeutic agent for gene therapy of EAE.

The overall observation of cytokine presence during EAE appears to be very contradictory with both pro- and anti-inflammatory cytokines being present simultaneously in EAE lesions and the surrounding parenchyma. However the level of expression may be an important factor in the outcome of cytokine release in the CNS rather than simply its presence in the lesion. From in vitro studies it is apparent that most cytokines have functions that may both help or hinder the disease course, possibly dependant upon the concentration of cytokine, other cytokines present in the microenvironment and the activation state of surrounding cells (Merrill et al., 1996). It is thought that MS is a “Th1-type” disorder mediated by CD4+ T cells producing pro-inflammatory cytokines (Krakowski et al., 1997). While this is very likely an important mechanism in the pathology of MS, other factors such as antibody production, immunoglobulin class switching and complement fixation may become apparent at later stages of disease and may be important in demyelination, where the “Th2-type” anti-inflammatory cytokines may actually enhance this process (Baker et al., 1996; Storch et al., 1998; Genain et al., 1996). The variation between EAE studies may reflect the different species, strains and induction protocols used and the complex cytokine interactions between infiltrating cells and resident CNS cells and may account for the diverse pathology and cytokine expression seen in individuals with MS. However, low levels of detectable TNFR during remission may correlate to endogenous TNF neutralisation and supports the use of TNFR to treat EAE.

Although present within the parenchyma, cytokines were often observed around lesions. Therefore their detection maybe reflective of the different cell types which may harbour these products rather than a specific role in disease pathogenesis. Further characterisation of the cytokine expression by individual cell types during EAE at different time points may further the understanding of the pathogenesis of EAE and give clues to further treatment strategies.
CHAPTER FOUR

CYTOKINE GENE THERAPY IN EAE BY LOCAL PLASMID DNA-CATIONIC LIPOSOME COMPLEX (CLC) ADMINISTRATION TO THE CNS
4.1. INTRODUCTION

In an attempt to modulate the pathogenic Th1 response occurring in during disease development to a non-pathogenic Th2 response, studies have shown that EAE can be suppressed by the systemic administration of inhibitory cytokines, such as IL-4, TGF-β and IL-10 (Rott et al., 1994; Racke et al., 1991; Racke et al., 1994). IFN-β has been shown to be an effective therapy for MS and is thus another candidate for immunomodulation of EAE (Paty et al., 1993). Soluble cytokine receptors for TNF (p55 and p75 TNFR), and anti-TNF antibodies have also been shown to inhibit EAE by antagonising pathogenic cytokine activity (Baker et al., 1994). Such immunotherapy administered systemically is however limited by the short biological half-life of cytokines, and disease returns following cessation of treatment.

Plasmid DNA is incorporated into cells epichromosomally and therefore can be used to transfect most cell types. It has also been reported to be non-immunogenic when delivered in vivo to non-human primates (Jiao et al., 1992). Studies have shown expression of plasmid DNA delivered gene products for up to 19 months in skeletal muscle (Wolff et al., 1992). Therefore, gene therapy by the direct injection of plasmid DNA coding for cytokine genes may provide a method to deliver long-term cytokine expression with which to treat EAE.

4.2. AIM

To investigate the use of gene therapy of EAE by inhibitory cytokines or cytokine inhibitors administered locally using plasmid DNA as a vector to provide a simple and safe method of extended expression of cytokine or cytokine inhibitor at physiological concentrations from a single injection.
4.3. RESULTS

4.3.1. Systemic Cytokine Gene Therapy Using Plasmid DNA

Mice were injected in the anterior tibialis muscle with either cytokine plasmids or saline as control. Successful injection into the target tissue caused muscle contraction leading to movement of the foot as shown in Figure 4.1. A single i.m. injection of 100 μg cytokine DNA in 50 μl saline administered on the day of immunization, to target the induction phase of EAE, failed to ameliorate disease severity or the onset of disease (Table 4.1). Immunohistochemical staining of the injected muscle, failed to show any increase in cytokine protein production beyond endogenous levels observed within the tissue. Injection of 1.2% BaCl₂ solution into the mouse tibialis anterior muscle, 5 days prior to DNA injection induces myoproliferation, and is reported to increase plasmid uptake (Wells et al., 1995), at the time of DNA injection. Figure 4.2 shows (a) normal muscle tissue, (b) 4 day post BaCl₂ injection and (c) 7 days post BaCl₂ injection. Figure 4.2b shows myoproliferation in the muscle tissue with more nuclei visible compared to normal muscle tissue (Figure 4.2a). After 7 days there is evidence of gross tissue remodelling (Figure 4.2c). Plasmid DNA injection after BaCl₂ treatment again failed to make an impact on disease when compared to untreated or BaCl₂ pre-treated animals, except for the p55 TNFR-Ig fusion protein DNA under the transcriptional control of a CMV promoter, which significantly reduced the severity of clinical disease (p < 0.05), and appeared to delay, although non-significantly, the onset of disease (Table 4.2). Although using a reporter gene LacZ construct (pCH110), expression of β-galactosidase could be seen in some muscle fibres following the route of injection (Figure 4.3), again immunohistochemistry could not demonstrate significant cytokine production above that seen endogenously.

4.3.2. CNS-directed protein therapy using recombinant IL-10 protein

To examine the potential impact of IL-10 in the effector phase of EAE local i.c. administration of recombinant IL-10 protein between 0.1 μg and 20 μg/ml was studied. This was injected into the CNS following the onset of clinical disease. Surprisingly local administration of up to 20 μg of mouse IL-10 protein had no inhibitory effect upon severity or disease course compared to the control group (Figure 4.4).
4.3.3. CNS-directed cytokine gene therapy with naked DNA.

Having established that systemic delivery of plasmid cytokine DNA during sensitisation did not significantly alter EAE disease course, but that local delivery of the cytokine protein could have a transient effect (Baker et al., 1994), the potential of local gene delivery to the CNS was studied. 100 μg cytokine DNA (volume 30 μl) was injected i.c. on day 12 p.i. shortly before the onset of disease (Table 4.3), in an attempt to modulate the effector phase of the disease course. The majority of the constructs failed to make any significant impact on the disease course (Table 4.3). Following i.c. injection of the IL-4 plasmid DNA construct there was a reduction in disease incidence, by 20%, compared with SCH control animals which all developed disease. However, the effect of the IL-4 construct upon disease severity within the whole group (Table 4.3) was just beyond statistical significance (p = 0.055), as the animals which developed disease exhibited comparable severity to that of control animals.

4.3.4. Immunostaining of CNS-directed gene expression from cytokine DNA-CLC.

Cationic liposomes facilitate the uptake of DNA into cells (Felgner et al., 1987), and injection of DNA-CLC has been shown to increase the CNS expression of reporter gene constructs (Ono et al., 1990). Using immunocytochemical techniques it was difficult to definitively detect gene delivered-protein expression following the injection of the DNA construct. The Ab reactive with human TGF-β also detected mouse TGF-β within the extracellular matrix and thus it was not possible to distinguish any expression above that normally expressed endogenously. Likewise IL-10 protein could be readily detected in normal CNS tissue. This was present in astrocytes, axons within the white matter and neural cell bodies within the grey matter as described in Chapter 3. This staining was inhibited by co-incubation of the mAb with rIL-10 protein. Interestingly, injection of two different batches of 100-500 μg IL-10 mAb (Clone no. JES5-2A5, Pharmingen, San Diego, CA, USA) directly into the CNS of mice either at the onset of EAE or in normal animals proved fatal in Biozzi ABH mice (n=5). IL-4 protein expression was below the level of detection. The biotynilated mouse mAb (Htr-9) specific for human p55 TNFR1 (CD120a) was found to cross-react with mouse CD120a, at all dilutions (1:50-1:16,000 of 1 mg/ml) tested, when used to stain mouse lymph node cell cytospins, and was therefore not used for CNS staining. Mouse Ig is
readily detected within normal mouse CNS tissue (Baker et al., 1990) and consequently could not be used to detect the Ig portion of the p55 fusion protein. In contrast it was possible to distinguish the expression of the human p75 dTNFR from the endogenously expressed mouse p75 TNFR using biotinylated anti-human CD120b. Human p75 positive resident cells were detected around the injection site within 3 days p.i. (Figure 4.5). Sparse expression could be detected by 6 days p.i. but by day 18 p.i no positive staining was detected (n=5). Sections of normal brain taken between 24 hours and 21 days post i.c. DNA-CLC injection failed to show any abnormality associated with CNS cytokine production, or any evidence of leucocyte infiltration.

4.3.5. Therapeutic effect of CNS-directed gene transfer using cytokine DNA-CLC.

Although protein expression following plasmid DNA-CLC was clearly at low levels these were examined as a means of ameliorating EAE. Again mice received a single i.c. injection of 100 μg cytokine DNA, but with 3 μg lipofectin (total volume 30 μl), on day 12 p.i. Mice injected i.c. with CLC alone showed no significant difference to untreated controls (p>0.05) in disease incidence, clinical severity or disease onset (Table 4.4). In contrast to that observed following injection of naked DNA alone (Table 4.3), cytokine DNA-CLC coding IFN-β, IL-4, TGF-β, p55 TNFR-Ig, and p75 dTNFR all significantly (p<0.001) reduced the severity of disease compared to either the untreated or CLC-treated controls (Table 4.4). CNS injection of IFN-β-NSE DNA-CLC also significantly (p<0.02) inhibited the severity of EAE compared with the CLC control group although this was to a lower degree than that observed following injection of IFN-β DNA-CLC, which was driven by the MMuLV-LTR (Table 4.4). Mice injected with p75 dTNFR DNA-CLC showed the greatest inhibition of disease course compared to untreated (p<0.0001) and CLC treated (p<0.005) animals (Figure 4.6), and reduced the disease incidence by 56% (Table 4.4). The disease frequency following injection of IL-4, TGF-β and IFN-β was similarly reduced by 30-40%. In contrast injection of LacZ or IL-10 DNA-CLC failed to affect the disease incidence or severity (Table 4.4).
Halothane anaesthetised Biozzi ABH mice were injected i.m. into the *anterior tibialis* hindlimb muscle with 50 μl saline. Normally the angle of the foot follows the line of the hindlimb (a). Immediately after successful i.m. injection in the direction demonstrated by the arrow (b), the *anterior tibialis* muscle contracts and the foot is perpendicular to the line of the hindlimb.
Figure 4.2 Histological Analysis of BaCl$_2$-induced myoproliferation in the anterior tibialis.

Halothane anaesthetised mice were injected i.m. with 50 µl 1.2% BaCl$_2$, a myoproliferative compound. Anterior tibialis muscle was removed from mice for histological analysis (n=3). Anterior tibialis muscle from (a) untreated mouse shows normal muscle fibre structure. 4 days post i.m. injection with 1.2% BaCl$_2$ myoproliferation of muscle fibres has occurred and there are more nuclei present (b). 7 days post i.m. injection of 1.2% BaCl$_2$ myoproliferation of muscle fibres is not present and gross remodelling of muscle fibres is evident (c). Sections were counterstained with haematoxylin. Magnification x100.
Figure 4.3 Detection of positive β-galactosidase muscle fibres following i.m. injection in anterior tibialis muscle of plasmid DNA coding for Lac Z

Halothane anaesthetised Biozzi ABH mice were injected i.m. into the anterior tibialis muscle with 100 μg plasmid DNA coding for β-Galactosidase (n=4). 24 hours post i.m. injection β-galactosidase positive muscle fibres could be detected along the route of injection (→).
Animals were injected with SCH in CFA on days 0 and 7. Mice were injected i.c. day 12 p.i. with either 0.1, 5 or 20 μg of recombinant mouse IL-10 protein, or were untreated (n=8-10). The results represent the mean group clinical disease score ± SEM. Mice injected with recombinant IL-10 showed equal severity of EAE to untreated mice at all dose ranges tested.
**Figure 4.5** Detection of human p75 dTNFR expression in the CNS following i.c. injection of plasmid DNA-CLC.

Arrows show human p75 dTNFR-positive cells around site of injection 3 days post-injection of 100 μg human p75 dTNFR DNA-CLC (30 μl), into the right cortex (n=5). Sections were not counterstained. Magnification x200.
### Table 4.1 Failure of intra-muscular injection of cytokine DNA construct to inhibit EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score ± S.E.M.</th>
<th>Mean Day of Onset ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>8/8</td>
<td>3.2 ± 0.6</td>
<td>15.6 ± 0.9</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>6/6</td>
<td>3.9 ± 0.1</td>
<td>15.2 ± 0.8</td>
</tr>
<tr>
<td>IL-10</td>
<td>6/6</td>
<td>3.8 ± 0.3</td>
<td>15.8 ± 1.1</td>
</tr>
<tr>
<td>TNF</td>
<td>6/6</td>
<td>4.0 ± 0.1</td>
<td>16.0 ± 0.6</td>
</tr>
<tr>
<td>IL-4</td>
<td>6/6</td>
<td>3.8 ± 0.2</td>
<td>16.5 ± 1.8</td>
</tr>
<tr>
<td>TGF-β</td>
<td>6/6</td>
<td>3.4 ± 0.3</td>
<td>16.2 ± 0.8</td>
</tr>
</tbody>
</table>

Animals were injected with SCH in Freund's adjuvant on day 0 & 7. Animals were injected i.m. into the anterior tibialis with 50μl 100 μg naked DNA coding for p55 TNFR-Ig under control of a CMV promoter, and IL-10, IL-4, TNF and TGF-β with a MMuLV LTR promoter, on day 0. The results represent the mean maximum clinical score ± SEM of all animals within the group and the mean day of onset of the animals which developed EAE.
Table 4.2 Partial inhibition of EAE by intra-muscular injection of cytokine DNA constructs into myoproliferating muscle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score ± S.E.M.</th>
<th>Mean Day of Onset ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>22/22</td>
<td>3.4 ± 0.2</td>
<td>16.4 ± 0.3</td>
</tr>
<tr>
<td>BaCl₂ alone</td>
<td>10/10</td>
<td>3.5 ± 0.2</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>p75 dTNFR</td>
<td>5/5</td>
<td>3.6 ± 0.3</td>
<td>17.6 ± 1.0</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>13/14</td>
<td>2.6 ± 0.3 *</td>
<td>16.0 ± 0.4</td>
</tr>
<tr>
<td>IL-10</td>
<td>9/9</td>
<td>3.1 ± 0.3</td>
<td>18.3 ± 0.8</td>
</tr>
<tr>
<td>TNF</td>
<td>9/9</td>
<td>3.6 ± 0.3</td>
<td>17.0 ± 0.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>6/6</td>
<td>2.8 ± 0.5</td>
<td>18.3 ± 1.0</td>
</tr>
<tr>
<td>TGF-β</td>
<td>15/15</td>
<td>3.0 ± 0.2</td>
<td>16.2 ± 0.4</td>
</tr>
</tbody>
</table>

Animals were injected with SCH in Freund's adjuvant on day 0 & 7. 1.2% barium chloride (BaCl₂) solution was injected i.m. into the anterior tibialis 5 days prior to 50 µl 100 µg naked DNA coding for p55TNFR-Ig with a CMV promoter, and human p75 dTNFR, IL-10, IL-4, TNF and TGF-β under MMuLV LTR promoter, on day 0. The results represent the mean maximum clinical score ± SEM of all animals within the group and the mean day of onset of the animals which developed EAE.

* p < 0.05 compared to untreated animals.
### Table 4.3 Failure of CNS injection of cytokine DNA construct to inhibit EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score ± S.E.M.</th>
<th>Mean day of Onset ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>21/21</td>
<td>3.5 ± 0.2</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>TGF-β</td>
<td>7/7</td>
<td>2.9 ± 0.4</td>
<td>15.6 ± 0.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>8/10</td>
<td>2.5 ± 0.5</td>
<td>16.1 ± 0.4</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>7/7</td>
<td>3.6 ± 0.2</td>
<td>16.4 ± 0.6</td>
</tr>
<tr>
<td>IFN-β</td>
<td>6/6</td>
<td>3.4 ± 0.3</td>
<td>15.9 ± 0.4</td>
</tr>
<tr>
<td>IFN-β [NSE]</td>
<td>5/5</td>
<td>3.1 ± 0.2</td>
<td>15.6 ± 0.5</td>
</tr>
</tbody>
</table>

Animals were injected with SCH in Freund's adjuvant on day 0 & 7. 30 µl of 100 µg of naked DNA coding for TGF-β, IL-4, IFN-β, and p55 TNFR-Ig under MMuLV LTR promoter, and IFN-β under neuron-specific enolase promoter, were injected i.c. on day 12. The results represent the mean maximum clinical score ± SEM of all animals within the group and the mean day of onset of the animals which developed EAE.
Table 4.4 Inhibition of EAE by local administration of cytokine DNA-CLC to the CNS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. EAE</th>
<th>Mean Group Score ± S.E.M.</th>
<th>Mean day of Onset ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLC</td>
<td>8/8</td>
<td>3.8 ± 0.1</td>
<td>15.0 ± 0.3</td>
</tr>
<tr>
<td>untreated</td>
<td>23/23</td>
<td>3.7 ± 0.1</td>
<td>15.1 ± 0.3</td>
</tr>
<tr>
<td>LacZ</td>
<td>7/7</td>
<td>3.2 ± 0.2</td>
<td>14.7 ± 0.3</td>
</tr>
<tr>
<td>TGF-β</td>
<td>10/15</td>
<td>2.0 ± 0.4 ***</td>
<td>16.4 ± 0.5</td>
</tr>
<tr>
<td>IL-4</td>
<td>9/13</td>
<td>2.3 ± 0.4 ***</td>
<td>16.2 ± 0.5 *</td>
</tr>
<tr>
<td>IL-10</td>
<td>11/11</td>
<td>3.6 ± 0.2</td>
<td>16.2 ± 0.3 *</td>
</tr>
<tr>
<td>p75 dTNFR</td>
<td>5/9</td>
<td>1.0 ± 0.3 ***</td>
<td>17.2 ± 0.6 **</td>
</tr>
<tr>
<td>p55 TNFR-Ig</td>
<td>11/11</td>
<td>3.0 ± 0.3 *</td>
<td>16.4 ± 0.2 ***</td>
</tr>
<tr>
<td>IFN-β</td>
<td>4/7</td>
<td>1.6 ± 0.6 **</td>
<td>14.8 ± 0.3</td>
</tr>
<tr>
<td>IFN-β [NSE]</td>
<td>16/18</td>
<td>2.7 ± 0.3 *</td>
<td>15.4 ± 0.3</td>
</tr>
</tbody>
</table>

Animals were injected with SCH in Freund's adjuvant on day 0 & 7. 30 μl of 100 μg of naked DNA coding for LacZ, TGF-β, IL-4, IFN-β, TNFR-Ig p55, and dTNFR p75 under MMuLV LTR promoter and IFN-β under neuron-specific enolase promoter were mixed with lipofectin and injected i.e. on day 12. The results represent the mean maximum clinical score ± SEM of all animals within the group and the mean day of onset of the animals which developed EAE.

* p < 0.05, ** p < 0.01, *** p < 0.005 compared to animals injected with CLC only.
**Figure 4.6** Inhibition of EAE following intra-cerebral injection of human p75 dTNFR plasmid DNA-CLC at effector stage of disease.

Animals were injected with SCH in CFA on days 0 and 7. Mice were injected i.c. day 12 p.i. with 100 μg of human p75 dTNFR plasmid DNA-CLC (■) (n=9) or CLC vehicle alone (O) (n=8), or were untreated (●) (n=23). The results represent the mean group clinical score ± SEM. Mice injected with p75 dTNFR plasmid DNA-CLC exhibited significantly reduced clinical disease score (p < 0.005) and a delayed mean day of onset (p < 0.01) of clinical signs compared to untreated mice or mice treated with CLC vehicle alone.
This study demonstrated that local gene delivery of therapeutic cytokine DNA-CLC to the CNS of mice just prior to the onset of EAE can significantly reduce the severity and onset of clinical disease.

Systemic cytokine gene therapy targeting the induction phase of EAE in lymphoid tissue by a single i.m. administration of cytokine plasmid DNA to the anterior tibialis muscle failed to influence the clinical progression of EAE, even following administration of a myoproliferative agent which has been shown to give a 5-40 fold increase in efficiency of gene transfer in the regenerating anterior tibialis and quadracep muscle fibres (Wells et al., 1995; Allen et al., 1993; Davis et al., 1993). This ineffectiveness in EAE treatment is probably due to inefficient gene transfer of the cytokine DNA, as immunohistochemical analysis of injected tissue did not show levels of cytokine above endogenous levels. Although immunohistochemistry failed to detect cytokine expression/production when DNA coding for p55 TNFR-Ig was delivered into myoproliferating tissue on day 0 p.i. there was a slight reduction in clinical severity of EAE, and may relate to a prolonged systemic half-life mediated by the Ig domains of the immunoadhesin. In addition the p55TNFR-Ig fusion protein was under the control of a CMV viral promoter, which may produce a greater gene expression than the MMuLV LTR promoter present in the other plasmids. However, this may be a statistical artifact (Type I error) and represent statistical significance but not biological significance. Injection of plasmid DNA i.m. has shown success in the lupus-prone mouse (MRL/lpr/lpr) model where RSV-driven TGF-β cytokine genes can be expressed for prolonged periods and mimic the beneficial characteristics of the cytokine itself injected in vivo (Danko et al., 1994). However DNA was administered in five separate injections to the quadriceps muscle of BALB/c mice (Danko et al., 1994), compared to a single injection in the anterior tibialis muscle in this ABH mouse CREAE model, thus targeting a larger area of muscle fibres. The difference observed in efficiency of uptake may also be related to the mouse strain used (Davis et al., 1993; Raz et al., 1993).

Previous studies have demonstrated the increased efficacy of therapeutic agents when delivered locally to the site of inflammation (Baker et al., 1994). Therefore cytokine DNA was delivered to the CNS during the effector phase of EAE. Such gross-tissue remodelling as in the i.m. injections (induced by the myoproliferative agent) and
repeated injection is not feasible in the CNS. As with i.m. administration, cytokine plasmid DNA injected into the CNS has no mechanism by which to target the nucleus of resident CNS cells unlike virus, and results in poor gene transfer, which may account for the relative lack of therapeutic effect. However, studies have shown that an increase in uptake efficiency of reporter gene constructs can be achieved by forming a DNA-CLC, producing a lipid complex which fuses with the plasma membrane of target cells and facilitates uptake of DNA (Ono et al., 1990; Waisman et al., 1996). Such reporter gene constructs have been shown to transfect neuronal cells, astrocytes, the myelin-forming oligodendrocytes and ependymal cells (Ono et al., 1990; Waisman et al., 1996), and was detected for at least 9 days following reporter gene DNA-CLC injection (Ono et al., 1990). However DNA-CLC can produce biologically significant expression as shown by the ability of tyrosine hydroxylase gene therapy to control a rat model of Parkinson's disease (Waisman et al., 1996).

In this study cytokine DNA-CLC had efficacy at inhibiting EAE compared with that following similar injection of naked DNA alone although it was still difficult to detect protein expression, indicating a low transfection efficacy or low levels of expression. Although immunohistochemistry could detect cytokine expression/production, the use of in situ hybridisation or quantitative polymerase chain reaction (PCR) to detect cytokine mRNA may have been a more sensitive technique.

Injection of plasmids expressing IL-4, IFN-β, TNFR and TGF-β DNA-CLC into the CNS inhibited the development of clinical EAE. This supports the inhibitory action seen in EAE when these proteins are administered systemically (Baker et al., 1990; Röcken et al., 1994; Ruuls et al., 1996; Racke et al., 1991; Racke et al., 1994; Baker et al., 1996). However, therapeutic effect may be observed with much lower doses when administered locally within the CNS (Baker et al., 1994). Although the efficacy of particular cytokines in different EAE models may be controversial, TGF-β and TNF-inhibitory molecules have most consistently shown inhibitory activity (Racke et al., 1991; Baker et al., 1996). Although both p55 TNFR-Ig and p75 dTNFR gene therapy exhibited a therapeutic effect this was particularly evident with the p75 dTNFR. The p75 dTNFR dimer, has a 100-fold greater affinity for TNF compared with the monomeric TNFR, and is effective in the treatment of collagen-induced arthritis (Neve et al., 1996; Chernajovsky et al., 1999). In addition, the smaller molecular weight of the
p75 dTNFR (59kDa) compared to the p55 TNFR-Ig (approximately 180kDa) may result in increased tissue penetrance and bioactivity of the p75 dTNFR (Neve et al., 1996). In vivo immunogenicity to the p75 dTNFR may also be decreased as the human Ig tail which is highly immunogenic in mice, is not present (Williams et al., 1995) (Chapter 5). Although the mechanism of action has to be elucidated, it has been shown using inhibitory proteins that TNF neutralisation during effector phase EAE inhibits leucocyte accumulation along the neuroaxis (Cross et al., 1993), and may also inhibit damage to myelin (Selmaj et al., 1988). Studies in TNF gene-targeted mice suggest that TNF-induced chemotactic factors may however be a more important target for TNF inhibition than leucocyte extravasation in EAE (Körner et al., 1997b).

TGF-β is involved in the natural resolution of clinical EAE in mice (Racke et al., 1991), and is thought to be a major mediator of oral tolerance, which can effectively inhibit clinical EAE (Santos et al., 1994). Although it has recently been reported that constitutive expression of active TGF-β by astrocytes in the CNS of a TGF-β transgenic mouse under control of the GFAP promoter can enhance the clinical expression of EAE (Wyss-Corey et al., 1997), this was not noted in this study following TGF-β DNA-CLC injection. While this may relate to different levels of production of the cytokine, importantly the TGF-β construct used here would be secreted in the inactive procytokine form. This would be activated at the site of inflammation to form an active compound. Local delivery of this construct using lymphocyte cell vectors has been shown to be effective at inhibiting collagen-induced arthritis (Chernajovsky et al., 1997), and indicates that local TGF-β immunotherapy may be effective in the control of organ-specific autoimmune disease.

The first cytokine to show significant positive benefit in the control of progression of MS is IFN-β. Although subcutaneous (s.c.) administration of IFN-β protein has shown a significant reduction in the frequency of relapse in some patients (Paty et al., 1993), initial studies also suggested possible efficacy following intrathecal IFN-β administration (Jacobs et al., 1986). This study indicated that mouse IFN-β gene vector delivered locally to the CNS also exhibited immunoinhibitory effects. While both treatment with IFN-β and IFN-β driven by the neuron-specific enolase (NSE) promoter reduced clinical severity, MMuLV LTR-driven IFN-β was more effective. This difference is probably due to the more selective expression of the NSE-promoter in
CNS neurons compared to the MMuLV LTR-driven IFN-β which could be expressed in other resident CNS cells, such as glia. While the mechanisms of action require elucidation, the efficacy of gene therapy shown here and protein therapy observed in Lewis rat and murine EAE following prolonged IFN-β protein administration indicate that this mechanism is in part mediated by immunomodulatory effects (Ruuls et al., 1996; Yu et al., 1996b). These may be independent of any potential anti-viral effects. Although efficacy in rat EAE was noted following continued s.c. treatment with rat IFN-β, disease severity was exacerbated once treatment was stopped (Ruuls et al., 1996).

EAE is transferred with neuroantigen-specific Th1 T cells to normal mice and MS is exacerbated by treatment with IFN-γ (Panitch et al., 1987). In mice at least, Th1 responses may be suppressed by immune deviation by Th2 cytokines such as IL-4 and IL-10. At present there is conflicting data using IL-10 to treat EAE and it has been reported that IL-10 mRNA is upregulated during the recovery phase of EAE in mice and may be a by-product of the recovery process (Kennedy et al., 1992). Although IL-10 protein has been shown to suppress EAE in Lewis rats (Rott et al., 1994) other studies in mice using human IL-10 protein suggest either no effect or disease exacerbation (Cannella et al., 1996).

Surprisingly this part of the study does not support a major role of CNS directed IL-10 therapy in the control of murine EAE using recombinant IL-10. However it was readily possible to detect high levels of endogenous IL-10 protein in normal CNS. It is therefore possible that the exogenously delivered IL-10 failed to exert any influence above that already present in the CNS, which may not be actively involved with the disease process. The significance of high levels of CNS IL-10 requires further elucidation. The rapid death of animals following local anti-IL-10 Ab treatment suggests that IL-10 may be physiologically important and play a role in neuronal function. This study suggests however that IL-4 is the major Th2 cytokine which can inhibit the development of EAE.

Direct injection of genetic material has some advantages over other methods of gene therapy in that it avoids the use of infectious virus, such as adenovirus vectors, which have been proved to be immunogenic in mice. This inherent immunogenicity may
reduce expression and limit repeat administration (Wood et al., 1996). Sections of brain removed from normal mice between 24 hours and 21 days post i.c. injection with plasmid DNA-CLC failed to show any evidence of leucocyte infiltrate. Plasmid DNA exists epichromosomally, and compared to other methods of delivery uptake is less efficient and expression of protein is transient, although delivery of naked DNA has been shown to persist for up to 19 months in muscle cells (Wolff et al., 1992; Leinwand et al., 1991; Leiden et al., 1994; Gal et al., 1993). With the current understanding of the cytokine network however, the appropriate levels of expression for optimal efficacy are currently unknown and will require controllable expression to identify this. The limited transfection efficiency by plasmid DNA constructs seen here, suggest that other gene targeting methods may be more suitable to resolve this issue.

Whilst this study indicates that plasmid DNA injections in this current protocol may be too inefficient for further clinical development in this animal system, this study highlights some potential gene targets for therapy, which probably influence different pathways within the progression of disease. It has also been revealed that cytokine DNA can produce therapeutic cytokines in vivo in the CNS, which can influence the clinical severity and course of EAE and which may be developed further with more suitable gene vectors for long-term gene expression.
CHAPTER FIVE

CNS ADENOVIRAL GENE THERAPY CODING FOR CTLA4-Ig FUSION PROTEIN IN EAE
5.1. INTRODUCTION

In the previous chapter it was demonstrated that plasmid DNA and even DNA-CLC fail to induce readily detectable levels of protein expression. Such detection was complicated further by the use of mouse gene therapy agents against a background of endogenous expression of native mouse proteins. Therefore to examine expression following gene transfer, therapeutic agents were examined to which reagents were available, to dissociate gene expression from endogenous protein production. In addition adenovirus-mediated gene delivery was investigated, although it is well established that adenoviral vectors induce T cell and antibody responses, which limit expression to weeks (Reichel et al., 1998; Byrnes et al., 1995; Wood et al., 1996). It is evident that immunosuppressive agents such as CTLA4-Ig delivered by gene therapy can self-protect themselves from immune-mediated removal (Ali et al., 1998), which may allow longer duration of expression. Adenoviral vectors are reported to deliver genes with high efficiency to post-mitotic cells and it is therefore widely used in in vivo gene transfer approaches. Adenoviral vectors may thus form useful tools to identify important therapeutic gene products for immunological control of disease.

T cell activation is initiated through the TCR-CD3 complex following recognition of specific antigen presented in the context of syngeneic MHC class I or class II antigens. In addition to TCR-derived signals, costimulatory signals derived from interactions between the CD28 molecule on the T cell and the B7 family of counter receptors [CD80 (B7-1) and CD86 (B7-2)] have been shown to be required for sustained T cell activation. CTLA4 (CD152), which is a cell surface molecule expressed by T cells following activation, has a much higher affinity for B7 molecules compared with CD28, and is a negative regulator of T cell activation (Boise et al., 1995). In contrast to some of the stimulating/agonist capabilities of mAb specific for CTLA4 (Karandikar et al., 1996; Perrin et al., 1996a), CTLA4-immunoglobulin fusion proteins (CTLA4-Ig) appear to act as CD28 antagonists and prevent costimulation in vitro and inhibit in vivo T cell priming in a variety of immunological diseases (Finck et al., 1994; Wallace et al., 1995; Steurer et al., 1995; Tang et al., 1996).

By targeting TCR stimulation and costimulation, EAE has been effectively inhibited using either mAb specific for MHC class II Ag (Steinman et al., 1981; Smith et al.,
1994) or CTLA4-Ig fusion proteins (Perrin et al., 1995; Cross et al., 1995; Arima et al., 1996; Khoury et al., 1995; Perrin et al., 1996b). However these therapies were most effective when administered during T cell priming and in most cases exhibited more limited or no effect when administered after the encephalitogenic T cell response had been generated (Steinman et al., 1981; Perrin et al., 1995; Arima et al., 1996). This suggested a limited requirement of costimulation for the activation of effector T cells (Perrin et al., 1995; Arima et al., 1996), and in vitro memory cells have been shown to be less dependent on accessory cell costimulation than naive T cells (Croft et al., 1994). However, the CNS is shielded from the periphery by the blood-brain barrier (BBB). Naive cells typically do not enter the CNS, but activated cells, irrespective of their specificity can cross the BBB (Wekerle et al., 1986; Hickey et al., 1991). These cells must be stimulated further by antigen, presented locally, for CNS inflammation to develop. These local APC probably represent the perivascular microglia, which can express MHC class II Ag, even in normal situations (Butter et al., 1991a). Large proteins, including immunoglobulin have limited access to the CNS even during the BBB dysfunction that occurs during clinical EAE (Butter et al., 1991b; O'Neill et al., 1993). Therefore the reagents targeting T cell stimulation may have had limited access to their target antigens, which may additionally account for their limited action in late stage EAE (Steinman et al., 1981; Perrin et al., 1995; Cross et al., 1995; Arima et al., 1996). Previously, it has been shown that local delivery of cytostatic inhibitors of T cell activation and anti-cytokine reagents can have significantly enhanced activity compared with that achievable following i.p. administration (O'Neill et al., 1991; Baker et al., 1992; Baker et al., 1994).

5.2. AIM

To elucidate adenoviral mediated inhibition of T cell activation and the role of local antigen presentation within the CNS, inhibitory reagents and adenoviral vectors expressing therapeutic agents specific for T cell stimulatory molecules were injected into the CNS, 1-2 days before the development of clinical signs. This study indicates the importance of costimulatory molecules in the effector stage of the neuroimmunological disease CREAE, the value of targeting cell surface molecules and again highlights the importance of CNS delivery.
5.3. RESULTS

5.3.1. Centrally active CTLA4-Ig fusion protein in effector phase EAE.

Following EAE induction mAb were administered on day 13 p.i., 2-3 days before the anticipated onset of clinical signs (Table 5.1). An i.p. injection of 100 μg H-2A specific mAb had no demonstrable effect on the course of clinical EAE (group score 3.6 ± 0.3) (Table 5.1). In contrast a similar injection of 80 μg of the Ig isotype-matched CTLA4-mIg fusion protein significantly inhibited the development of disease (group score 0.7 ± 0.3) (Table 5.1). The combined data from the different experiments (Table 5.1) strongly suggest a dose dependence in efficacy, shown by an increased frequency of disease expression. In order to demonstrate further the importance of local antigen presentation in the development of EAE these reagents were locally administered (i.c.) into the CNS. When doses of 5 μg of CTLA4-mIg were administered (Table 5.1) the severity of signs were significantly lower (group score 1.2 ± 0.1) than untreated controls (group score 3.6 ± 0.2) and animals receiving CTLA4-mIg i.p. (group score 2.8 ± 0.4). Likewise in contrast to the i.p. injection of H-2A-specific mAb, central administration of this mAb significantly inhibited disease (group score 0.3 ± 0.2), suggesting the importance of local antigen presentation in the development of clinical EAE (Table 5.1).

The efficacy of mouse CTLA4-mIg in effector mouse EAE function (Table 5.1) contrasts with previous studies using i.p. administration of a human CTLA4-hIg fusion protein (Perrin et al., 1995). Therefore different CTLA4-Ig fusion proteins, injected i.p., were examined. The administration of 60 μg of CTLA4-hIg on day 13 p.i. failed to inhibit the incidence, severity or onset of the disease. In contrast CTLA4-mIg inhibited both the day of onset and the severity of the disease (day of onset 17.0 ± 1.4; group score 1.8 ± 0.5) compared with control mIgG2a fusion protein (L6) (day of onset 14.7 ± 0.8; group score 3.4 ± 0.2) (Table 5.1). Furthermore when in the same experiment 60 μg of CTLA4-Ig was injected i.p. on day 11 p.i., CTLA4-mIg (5/7 affected; group score 1.3 ± 0.5; mean day of onset 17.4 ± 1.4) significantly (p<0.02) inhibited disease severity compared with similarly injected CTLA4-hIg animals (7/7 affected; group score 3.6 ± 0.2; mean day of onset 16.3 ± 1.0). When centrally (i.c.) administered on day 13 p.i. CTLA4-hIg again failed to prevent the animals from developing EAE but did significantly delay the onset of disease (day of onset 17.6 ± 1.4), compared with untreated controls (day of onset 15.5 ± 1.3) (Table 5.1). CTLA4-hIg i.c. was however
highly immunogenic and a significant (p<0.05) fusion protein-specific antibody response was detected within 4 days of administration (Figure 5.1) when the inhibitory effect of the protein was waning. In comparison to normal serum this anti-hIg was further elevated (p<0.01) by 8 days following CTLA4-hIg administration. This may account for the low efficacy of CTLA4-hIg in controlling EAE. This protein did however exhibit some immediate efficacy at controlling clinical signs when administered at disease onset (Figure 5.2a), where a biological effect is detectable within 1 day following administration of the immunoadhesin.

5.3.2. Post-onset inhibition of EAE following blockade of CD28/B7 costimulation.

There was no detectable expression of CD80 or CD86 antigens by immunohistochemistry in the CNS of normal animals. Following the development of clinical EAE however, CD80, and particularly CD86 were evident on infiltrating mononuclear cells and were upregulated by resident CNS-derived cells (Figure 5.3). The severity of disease in animals injected i.e. with 80 µg CTLA4-mIg following the onset of signs was significantly (p<0.01) less severe than in mice injected i.p. with 80 µg CTLA4-mIg (Figure 5.2a), which again pointed to an increased efficacy in controlling EAE when immunoinhibitory reagents are delivered to the CNS. Following i.c. injection both CTLA4-hIg (Figure 5.2a) and low dose CTLA4-mIg (Figure 5.2b) effectively stabilised the progression of clinical signs. To examine the influence of the putative CTLA4-Ig ligands, animals were similarly treated with a B7-specific mAb. The i.c. injection of animals with CD80-specific mAb exhibited minimal inhibitory effect and no apparent enhancement of disease, and did not differ significantly from the control group (Figure 5.4). The CD86-specific mAb induced a more pronounced amelioration of disease. For the first 48 hours following injection the clinical scores of animals receiving CD86-specific mAb were significantly reduced (p<0.05) compared with disease in control and CD80-specific mAb-treated animals (Figure 5.4). Although the predominant suppression of disease appeared to be due to the injection of the CD86-specific mAb, co-injection of both CD80 + CD86-specific mAb at disease onset inhibited the progression of clinical signs markedly, and significantly (p<0.005) inhibited the disease severity compared with control animals (Figure 5.4). The rate of increase in clinical disease within the first 24 hours of treatment was significantly reduced compared to that observed with either CD80 (p<0.001) or CD86 (p<0.01) specific mAb administered individually (Figure 5.4), and clinical scores of CD80 +
Chapter 5 Results

CD86 mAb-treated animals remained significantly lower (p<0.05) than observed in CD86-specific mAb-treated animals for 3 days following a single i.c. administration. This may suggest that both CD28 ligands are important in EAE effector cell function or that there is an additive effect.

5.3.3. Adenoviral CTLA4-Ig gene expression.

Chronic neuroinflammatory diseases may require repeated administration of immunoinhibitory molecules. Having established that CTLA4-Ig fusion proteins irrespective of isotype delivered into the CNS at the onset of clinical signs can ameliorate the effector phase of active EAE, the potential value of CTLA4-Ig for gene therapy was examined. Beta galactosidase activity from central adenoviral delivery of the reporter gene could readily be identified not only in neural tissue around the injection site (Figure 5.5), but was also demonstrable in ependymal cells lining the ventricles and astrocyte-like cells (Figure 5.5) and around the pial surfaces at sites distant from the injection site. This was evident within 24 hours following injection (n=3) and remained for at least 3 weeks (n=5), although protein expression was not evident by 6 weeks (n=3) post-injection. Similarly CTLA4-hIg protein could be detected within CNS tissue from animals injected with the AdCTLA vector (Figure 5.6a). This could also be distinguished from any possible endogenous expression of mouse CTLA4 by host cells, by the co-localisation of human IgG to the same areas (Figure 5.6b). That this protein was secreted was demonstrated by detection of CTLA4-hIg protein in the CSF on day 1 p.i. (40.2 ± 11.3 μg/ml detected in 3 of 5 animals), day 4 p.i. (492.4 ± 83.0 μg/ml n=6) and day 9 p.i. (493.3 ± 14.8 μg/ml n=5) following i.c. administration of 5 x 10^6 PFU AdCTLA. This contrasts with undetectable levels in the serum on day 1 (n=5), 72.7 ± 11.0μg/ml on day 4 and 66.2 ± 12.4 μg/ml on day 9 following i.c. injection of AdCTLA.

5.3.4. Adenoviral CTLA4-Ig gene therapy in EAE.

Animals were injected i.c. with either 1x10^7 or 5x10^6 PFU of AdCTLA on day 12 p.i. Although such injection failed to prevent animals from developing EAE at all doses tested (Table 5.2), there was a significant amelioration (p<0.02) of the severity of the disease induced compared to either untreated or AdRL-treated animals (Figure 5.7). Thus gene delivered CTLA4-hIg was more potent at inhibiting disease than delivery of
Chapter 5 Results

the protein alone, and interestingly failed to induce a marked fusion protein-specific antibody response within 5-8 days following administration, in contrast to that observed following CTLA4-hIg protein administration (Figure 5.1). As a positive control to show that the ELISA could detect an immune response to adenovirus-produced CTLA4-hIg, it was shown that BALB/c mice developed a significant (p<0.05) anti-Ig response by 3 weeks post-injection compared with AdRL injected animals (Figure 5.1). The severity of clinical EAE in AdCTLA-treated animals (group score 0.8 ± 0.4) was significantly decreased compared with animals injected with 5 x 10^6 PFU AdRL10 (group score 2.8 ± 0.4) (Table 5.2). This latter group did not differ significantly from AdRL treated animals (group score 3.5 ± 0.3), although there may have been some slight amelioration of signs (Table 5.2). In the same experiment a TNFR-Ig vector (TNFV) also inhibited disease (group score 1.5 ± 0.5) compared with AdRL recipient controls (group score 3.5 ± 0.3) (Table 5.2). To examine the effect of AdCTLA on histological lesions an additional experiment was performed but the animals were killed by day 18-19 p.i. (Table 5.2), at a time when controls were exhibiting clinical EAE. Disease in ABH is primarily a spinal cord disease with minimal involvement of the brain. In AdCTLA-treated animals yet to develop disease, spinal cords contained only a small number of perivascular lesions (4 ± 3 lesions/section. n=5) (Figure 5.8), which were more numerous with increased clinical activity (8 ± 4 lesions/section n=4), but in contrast to AdRL and other controls (14 ± 5 lesions/section. n=6) a marked subpial infiltration was not a major feature (Figure 5.8). This data indicates that the development and intensity of lesion load is delayed and reduced respectively, in mice receiving AdCTLA and thus correlates well with the clinical profile observed.

Local i.c. AdCTLA gene therapy can produce high local concentrations of CTLA4-hIg contained within the BBB which are capable of inhibiting CNS autoimmune disease (Table 5.2), but produce systemic levels which failed to substantially influence the generation of unrelated (oxazolone) primary immune responses in lymphoid tissues (Figure 5.9). Intra-peritoneal administration of 2 x 100 μg CTLA4-mIg protein inhibited blastogenesis of T cells within oxazolone-treated draining lymph nodes as observed by microscopy and thymidine incorporation (Figure 5.9). Similarly 3 x 400 μg i.p. CTLA4-hIg protein could also inhibit T cell proliferation in oxazolone stimulated lymph nodes (Figure 5.9). In contrast 3 x 200μg i.p. CTLA4-hIg protein was ineffective. This again indicates the more potent immunoinhibitory activity of the mCTLA4-mIg protein, compared with the mCTLA4-hIg protein. Gene delivery of potent
immunosuppressive agents to the target tissue, particularly the CNS, can achieve therapeutic concentrations while limiting potentially undesirable generalised peripheral immunosuppression.
### Table 5.1  
**Inhibition of EAE using MHC class II-specific mAb and CTLA4-Ig fusion protein**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg)</th>
<th>Route</th>
<th>No. EAE</th>
<th>Group Score</th>
<th>EAE Score</th>
<th>Day of onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>i.c.</td>
<td>9/9</td>
<td>3.3 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>14.6 ± 1.6</td>
</tr>
<tr>
<td>H-2A mAb</td>
<td>100</td>
<td>i.p.</td>
<td>5/5</td>
<td>3.6 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td>15.4 ± 0.9</td>
</tr>
<tr>
<td>H-2A mAb</td>
<td>100</td>
<td>i.c.</td>
<td>2/5</td>
<td>0.3 ± 0.2*</td>
<td>0.8 ± 0.2</td>
<td>15.5 ± 0.7</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>-</td>
<td>17/17</td>
<td>3.7 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>17.1 ± 1.9</td>
</tr>
<tr>
<td>CTLA4-mIg</td>
<td>80</td>
<td>i.p.</td>
<td>4/9</td>
<td>0.7 ± 0.3**</td>
<td>1.6 ± 0.3</td>
<td>17.0 ± 2.2</td>
</tr>
<tr>
<td>CTLA4-mIg</td>
<td>80</td>
<td>i.c.</td>
<td>2/8</td>
<td>0.1 ± 0.1**</td>
<td>0.5 ± 0.0</td>
<td>17.5 ± 0.7</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>-</td>
<td>14/14</td>
<td>3.1 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>15.5 ± 1.3</td>
</tr>
<tr>
<td>control mIg</td>
<td>60</td>
<td>i.p.</td>
<td>7/7</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>14.7 ± 0.8</td>
</tr>
<tr>
<td>CTLA4-mIg</td>
<td>60</td>
<td>i.p.</td>
<td>6/7</td>
<td>1.8 ± 0.5*</td>
<td>2.1 ± 0.6</td>
<td>17.0 ± 1.4*</td>
</tr>
<tr>
<td>CTLA4-hIg</td>
<td>60</td>
<td>i.p.</td>
<td>7/7</td>
<td>2.9 ± 0.4</td>
<td>2.9 ± 0.4</td>
<td>15.9 ± 0.7</td>
</tr>
<tr>
<td>CTLA4-hIg</td>
<td>60</td>
<td>i.c.</td>
<td>7/8</td>
<td>3.0 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>17.6 ± 1.4*</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>-</td>
<td>27/27</td>
<td>3.6 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>15.2 ± 1.1</td>
</tr>
<tr>
<td>CTLA4-mIg</td>
<td>5</td>
<td>i.p.</td>
<td>6/6</td>
<td>2.8 ± 0.4*</td>
<td>2.8 ± 0.4</td>
<td>16.5 ± 1.5</td>
</tr>
<tr>
<td>CTLA4-mIg</td>
<td>5</td>
<td>i.c.</td>
<td>7/7</td>
<td>1.2 ± 0.1**#</td>
<td>1.2 ± 0.1</td>
<td>16.5 ± 1.5</td>
</tr>
</tbody>
</table>

ABH mice were injected with SCH in CFA on day 0 and 7. Animals received a single i.p. or i.c. injection of MHC class II antigen-specific mAb (10.3.6), mouse CTLA4-mouse IgG2a fusion protein (CTLA4-mIg), control mouse IgG2a fusion protein (L6), mouse CTLA4-human IgG1(CTLA4-hIg) fusion protein dissolved in saline, on day 13 p.i. The results represent the number of animals which developed clinical EAE within a group; the mean ± SEM maximum clinical score of all animals in the group; and the mean ± SEM maximum clinical score and mean ± SD day of onset of signs of the animals within the group which developed EAE. Mice were observed up to day 24 p.i. The data presented was derived from 4 separate experiments.

* P<0.05 compared with the vehicle/untreated control group.

**P<0.002 compared with the vehicle/untreated control group.

# P<0.05 compared with group of animals injected i.p. with CTLA4-mIg
Table 5.2 Gene delivered CTLA4-Ig injected locally into the CNS inhibits EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (PFU)</th>
<th>No. EAE</th>
<th>Group score</th>
<th>EAE score</th>
<th>Day of onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>10/11</td>
<td>3.2 ± 0.3</td>
<td>3.6 ± 0.1</td>
<td>15.5 ± 1.0</td>
</tr>
<tr>
<td>AdRL</td>
<td>1 x 10⁷</td>
<td>8/8</td>
<td>2.6 ± 0.4</td>
<td>2.6 ± 0.4</td>
<td>15.5 ± 1.3</td>
</tr>
<tr>
<td>AdCTLA</td>
<td>1 x 10⁷</td>
<td>5/7</td>
<td>1.2 ± 0.5**</td>
<td>1.7 ± 0.2**</td>
<td>16.8 ± 1.1*</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>7/7</td>
<td>3.7 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>15.1 ± 1.8</td>
</tr>
<tr>
<td>AdRL</td>
<td>5 x 10⁶</td>
<td>8/8</td>
<td>3.3 ± 0.4</td>
<td>3.3 ± 0.4</td>
<td>15.3 ± 0.9</td>
</tr>
<tr>
<td>AdCTLA</td>
<td>5 x 10⁶</td>
<td>4/7</td>
<td>0.9 ± 0.4**,#</td>
<td>1.9 ± 0.7*</td>
<td>17.3 ± 1.0⁷</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>11/13</td>
<td>2.8 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>16.2 ± 1.0</td>
</tr>
<tr>
<td>AdRL</td>
<td>5 x 10⁶</td>
<td>8/8</td>
<td>3.5 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>16.8 ± 1.4</td>
</tr>
<tr>
<td>AdRIL10</td>
<td>5 x 10⁶</td>
<td>7/7</td>
<td>2.8 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>16.3 ± 1.3</td>
</tr>
<tr>
<td>TNFV</td>
<td>5 x 10⁶</td>
<td>6/8</td>
<td>1.5 ± 0.5#</td>
<td>2.0 ± 0.6*</td>
<td>18.8 ± 2.1**,#</td>
</tr>
<tr>
<td>AdCTLA</td>
<td>5 x 10⁶</td>
<td>4/8</td>
<td>0.8 ± 0.4**,#</td>
<td>1.6 ± 0.4**,#</td>
<td>17.3 ± 2.1</td>
</tr>
</tbody>
</table>

ABH mice were injected i.c. with various doses (PFU) of viral particles encoding β-galactosidase (AdRL) or mouse CTLA4-human IgG1 fusion protein (AdCTLA), interleukin 10 (AdRIL10) or p55 TNFR-Ig (TNFV) on day 12 p.i. following EAE induction using SCH in CFA. The results represent the number of animals which developed clinical EAE within a group; the mean ± SEM maximum clinical score of all animals in the group; and the mean ± SEM maximum clinical score and mean ± SD day of onset of signs of the animals within the group which developed EAE. Animals were observed to day 24 p.i. The table is a compilation of 3 separate experiments.

* P< 0.05 compared with untreated controls.
** P< 0.02 compared with untreated controls.
# P< 0.02 compared with the AdRL treated groups.
ABH mice (hatched boxes) were injected i.c. with 60 μg CTLA4-hlg protein or $5 \times 10^6$ PFU. $AdCTLA$ (n=8). Serum was collected 4 (n=4) or 8 days (n=7) post-injection and CTLA4-hlg specific antibody response assessed by ELISA, which detected antibody responses to human immunoglobulin. Serum from BALB/c mice (blank boxes) which were injected into the CNS with $AdCTLA$ (n=7) or $AdRL$ (n=4) 3 weeks earlier were used as positive and negative controls, respectively, for the detection capacity of the ELISA for adenovirus-produced CTLA4-hlg. The results represent the group means + SD optical densities (arbitrary units) at 405nm. * $p<0.05$ **$p<0.01$ compared with normal ABH mouse serum or * $p<0.05$ compared with serum from BALB/c mice injected with $AdRL$. 

Figure 5.1. Immunogenicity of CTLA4-hlg Protein.
Figure 5.2 Inhibition of clinical EAE using locally applied CTLA4-Ig fusion protein

ABH mice were injected with SCH in CFA on day 0 and 7. Graph A shows the differences between CNS and i.p. administration of CTLA4-Ig fusion proteins. Animals received 80 μg i.e. of control (L6) mouse Ig (■), 80 μg i.p. CTLA4-mIg (●), 60 μg i.c. of CTLA4-hIg (▲) or 80 μg i.c.CTLA4-mIg (▼) dissolved in saline. These were administered following the onset (†) of clinical signs. Graph B shows the dose-response of i.e. injected CTLA4-mIg. Animals were untreated (□) or received a single injection of either 80 μg i.c. CTLA4-mIg (▼), 5 μg i.c. CTLA4-mIg (▽), 0.5 μg CTLA4-mIg (△), 0.05 μg CTLA4-mIg (○) dissolved in saline. These were administered following the onset (†) of clinical signs. The results represent the mean + SEM clinical score of all animals within the group, which contained a minimum of 6, typically 8 animals per group.
Chapter 5 Results

Figure 5.3 Upregulation of CD80 and CD86 in spinal cords of mice with severe EAE.

ABH mice were injected with SCH in CFA on day 0 and 7. Spinal cords were removed from mice exhibiting severe disease (grade 4) and snap frozen (n=5). Serial sections were stained using indirect peroxidase with mAb to either (A) CD80 or (B) CD86. Sections were not counterstained and show the co-localisation of CD80 and CD86 to infiltrating mononuclear cells and resident CNS cells (arrows). (x 100)
ABH mice were injected with SCH in CFA on day 0 and 7. Animals were untreated (■) or received a single injection i.c. of either: 150 μg CD80 (▼), 150 μg CD86 (△) -specific mAb alone or a combination of 150 μg of each of CD80 and CD86 (●) specific mAb, dissolved in saline. These were administered following the onset (†) of clinical signs. The results represent the mean + SEM clinical score of all animals within each group from 2 separate experiments, which contained a final number of between 15-17 animals per group.
Figure 5.5 Adenoviral delivered β-galactosidase activity in the CNS

ABH mice were injected i.c. with $1 \times 10^7$ PFU AdRL. Frozen sections were stained with X-gal for 2 hours after which β-galactosidase activity could be detected in (a) neural tissue at the injection site (insert) and ependymal cells lining the ventricles 3 days following i.c. injection (n=3). (Magnification x25, x200 insert). (b) shows β-galactosidase activity in ependymal cells lining the fourth ventricle (n=5) (magnification x100), and (c) demonstrates β-galactosidase activity in a glial cell 10 days post i.c injection (n=3) (magnification x200). Control sections of i.c. AdCTLA injected brain did not show any β-galactosidase activity. Sections were not counterstained.
ABH mice were injected i.c. with $1 \times 10^7$ PFU AdCTLA on 12 p.i. following EAE induction (n=3). On day 21 p.i. brain tissue was removed and serial sections stained using indirect peroxidase to detect (a) CTLA4 and (b) human IgG1. Cells showing co-localisation of both mouse CTLA4 and human Ig were detected (arrow) in and surrounding the injection site. Sections were counterstained with haematoxylin. Magnification x125.
Figure 5.7 Inhibition of clinical EAE using gene delivered mouse CTLA4-human IgG1 fusion protein.

ABH mice were injected with SCH in CFA on day 0 and 7. Animals were untreated (●) or received a single injection i.e. of either $5 \times 10^6$ PFU AdRL (▼) or $5 \times 10^6$ PFU AdCTLA (▲). These were administered following the onset of clinical signs. The results represent the mean ± SEM clinical score of all animals within the group, which contained between 7-8 animals per group.
Figure 5.8 Adenoviral delivery of CTLA-hlg reduces the lesion load within the CNS.

Spinal cord from (a) SCH mice, (b) AdRL treated mice and (c) AdCTLA treated mice were removed at the timepoint when mice with the normal disease course were at maximum clinical severity (grade 4). Paraffin sections were counterstained with haematoxylin and eosin. AdCTLA treated mice showed very few perivascular cuffs compared with SCH or AdRL treated mice. Groups consisted of 5 animals. Magnification x100.
Figure 5.9 Adenovirus delivered CTLA4-Ig fusion protein in the CNS does not inhibit systemic primary proliferative responses

ABH mice were injected i.c. with 5 x 10^6 PFU AdCTLA or 30 μl PBS on either day -1 or day -7. 50 μl of 2.5% oxazolone (OX) or vehicle (acetone:olive oil. AOO) was applied epicutaneously to the hindlimb on day 0. Animals were injected i.p. with either 100 μl PBS or 100 μg CTLA4-mIg protein on day 0 and 1. In a separate experiment a group of animals were also injected i.p. with 400 ng CTLA4-hIg fusion protein or human serum IgG (hlg) on day 0, 1 and 2. Inguinal lymph nodes were removed on day 3 and 5 x 10^6 lymph node cells cultured overnight in the presence of 1 μCi ^3H-thymidine. The results represent the mean + SD thymidine incorporation from quadruplicate samples. Groups consisted of 3-5 mice.
5.4. DISCUSSION

This study indicated that effector T cell function could be downregulated more effectively by central administration of inhibitors of T cell stimulation, compared with their i.p. administration which showed a more limited efficacy. Antibodies specific for the MHC class II antigens (10.3.6) were the first mAb used to inhibit clinical EAE (Steinman et al., 1981), and can inhibit primary in vivo proliferative responses in lymphoid tissues (Steinman et al., 1981). These were highly effective inhibitors of EAE when administered during T cell priming but the majority of animals developed EAE when administered shortly before the onset of disease (Steinman et al., 1981). The organ-specificity of such autoimmune diseases indicates that primed T cells are activated by their specific antigens expressed within the target tissue, via local antigen presentation. This is supported by the demonstration that central injection of MHC class II-specific mAb inhibits disease even when administered late after EAE induction, and was active at doses which had failed to inhibit disease when administered i.p. (Steinman et al., 1981). Additionally this suggests that mAb administered i.p. either cannot penetrate the BBB or do so but at a level that is not high enough to be therapeutic.

Although studies on diabetes and active EAE using human CTLA4-hIgG1 fusion proteins have both shown an inhibitory effect when administered early in the disease course or during disease induction respectively, in both cases late phase diabetes and adoptive EAE were not inhibited. It was thus suggested that T cell priming was more sensitive to CD28 costimulation than effector cell function (Perrin et al., 1995; Lenschow et al., 1995). This current study however clearly indicates that mouse CTLA4-Ig fusion proteins can inhibit effector T cell function, but in keeping with previous studies using human CTLA4-hIg (Perrin et al., 1995; Arima et al., 1996), mouse CTLA4-hIg administered i.p., at the doses examined, exhibited a minimal inhibitory action. However in rat active EAE multiple i.p. administrations of CTLA4-hIg at the onset of EAE is effective at inhibiting late stage disease (Khoury et al., 1995). This was thought to be due to a deviation from an encephalitogenic Th1 response towards a suppressive Th2 function (Khoury et al., 1995), and has been supported in other studies (Sayegh et al., 1995). Studies in murine collagen induced arthritis and contact dermatitis however indicate that mCTLA4-mIg can inhibit both Th1 and Th2 antibody and T cell responses (Tang et al., 1996; Webb et al., 1996).
Human Ig fusion proteins are highly immunogenic in mice and rapidly induce an anti-Ig Ab response which may limit their pharmacokinetic and inhibitory profiles in vivo (Williams et al., 1995; Srinivas et al., 1996). This may be a major factor in the low efficacy of CTLA4-hIg protein in this study. Mouse CTLA4 may also have greater functional activity on binding to its mouse ligands than human CTLA4 (Wallace et al., 1994) and may account for the more potent inhibitory activity of mouse CTLA4 fusion proteins than observed previously with human CTLA4-Ig constructs (Perrin et al., 1995). The differences however between the fusion proteins observed here suggest that ligand binding, and blockage of CD80/86 interactions by CTLA4 may not be the only mechanism of action. This also appears to be dependent on the isotype of the Ig fusion protein, which is compatible with the limited action of human CTLA4-hIg fusion proteins on adoptive EAE (Perrin et al., 1995). Whereas CTLA4-mIg (1-100μg/ml) could inhibit concanavalin A (stimulated with 10μg/ml Con A for 48h) induced splenocyte proliferation in vitro, 10-100μg/ml of this CTLA4-hIg fusion protein (Lane et al., 1993) was not inhibitory. Similarly the CTLA4-mIg fusion protein was more efficient than CTLA4-hIg at inhibiting systemic oxazolone-induced T cell proliferation, as a lower amount of CTLA4-mIg was required to induce comparable inhibition to CTLA4-hIg. The increased efficacy of the CTLA4-mIg may reflect more limited interactions between human Fc regions and mouse cells of this particular construct. The mouse IgG2a isotype of the fusion protein is capable of complement fixation and cell depletion through antibody-dependent cellular cytotoxicity, although in some instances these interactions are not necessary for function as CTLA4 non-complement and non-Fc receptor binding Ig mutants are still active in vivo (Steurer et al., 1995).

Macrophages are much less sensitive to the action of potentially depleting mAb than lymphocytes, but significant T cell and B220+ B cell (splenocyte) depletion following a single i.p. administration of CTLA4-Ig was not evident by microscopy and correlates to other studies (Finck et al., 1994). It is possible however that activated cells which upregulate B7 antigens are susceptible to deletion. The action of these reagents may therefore not only be through inhibition of CD28 dependent signals by blocking B7 interactions but could also deliver regulatory signals to stimulated antigen-specific T cells or APC (Boise et al., 1995; Vanderlugt et al., 1997). Although this study demonstrates the activity of CTLA4-mIg during the effector stage of EAE, importantly there is a significantly enhanced effect of centrally applied CTLA4-Ig over i.p. administration and suggests that the important stimulatory event occurs within the CNS.
Antigen-specific T cells initiating the cascade of events leading to clinical EAE are localised to the perivascular space (Cross et al., 1990), and the major cells expressing MHC class II antigens in vivo are microglia and mainly the perivascular microglia (Butter et al., 1991a). This suggests that the major antigen presenting cell in EAE is the perivascular microglial cells, whose stimulatory and costimulatory activities can be effectively prevented by central administration of inhibitory agents into the CNS. These cells express both CD80 and CD86 antigens (Windhagen et al., 1995), which can bind to CD28 and transmit positive costimulatory signals to T cells, but these may be qualitatively different (Perrin et al., 1996b; Lenschow et al., 1995; Vanderlugt et al., 1997; Kuchroo et al., 1995; Miller et al., 1995). Recent reports in EAE have proposed that CD80 signals prime for a Th1 dominated response which may exacerbate clinical disease (Vanderlugt et al., 1997; Kuchroo et al., 1995; Miller et al., 1995) whereas CD86 induces a Th2 response which can ameliorate disease (Kuchroo et al., 1995).

In contrast the data in EAE reported here are similar to those reported in murine diabetes, contact dermatitis and collagen induced arthritis, which demonstrate a lack of, or minimal, inhibitory effect of CD80-specific mAb and a (modest) beneficial effect of CD86-specific mAb (Lenschow et al., 1995; Webb et al., 1996; Reiser et al., 1996). This may relate to the expression of CD86 on lymphocytes in addition to that found on APC. An enhanced effect in disease amelioration was evident following combination of both CD80 and CD86-specific mAb as seen in heart allograft rejection, and collagen-induced arthritis (Webb et al., 1996; Pearson et al., 1997). In another study in adoptive EAE where the donor encephalitogenic cells were incubated in vitro with B7-specific mAb, the combination of both CD80 and CD86-specific mAb most effectively inhibited disease transfer (Racke et al., 1995). This suggests that both CD80 and CD86 deliver signals necessary for the effector encephalitogenic T cell response. However when injected into animals shortly after active EAE induction, an anti-CD80 mAb inhibited disease whereas an anti-CD86 mAb induced a mild exacerbation, but a combination of both was not as proficient as CD80-specific mAb alone at inhibiting disease (Perrin et al., 1996b). Furthermore it appears that the effects observed with B7-specific antibodies may not be a consequence of antigen blockade, but may induce signalling via the B7 molecules. Treatment of SJL/J mice during EAE remission with CD80 F(ab) inhibited the relapse of disease, whereas intact CD80-specific mAb had the opposite effect of enhancing disease (Vanderlugt et al., 1997; Miller et al., 1995). Therefore the effect of B7-reactive agents appears to be dependent on the form of the immunoregulatory...
molecule administered, and the timing and route of administration relative to the development of effector function, and possibly the animal strain analysed. In this ABH mouse EAE model the data suggest that primed cells are more sensitive to inhibition of both B7 ligands, although it is not possible here to dissociate whether the \textit{in vivo} effect was due to alterations in function of T cells, APC or both cell types.

This study also investigated gene therapy approaches and demonstrated that local (intracranial) gene therapy can make a significant impact on CNS autoimmune disease, while limiting suppression of unrelated peripheral immune responses, and that CTLA4-Ig may make a suitable tool for gene therapy. The lack of endogenous CNS-cell proliferation make retroviral vectors impractical and plasmid DNA induces minimal gene product expression as discussed in chapter 4. In contrast, adenoviral vectors produce readily detectable levels of protein which can be sustained within the CNS for at least a number of weeks (Wood et al., 1996; Byrnes et al., 1995). Ad\textit{CTLA} delivered gene products appeared to be less immunogenic than a bolus of high-dose CTLA4-hIg protein and produced a high, sustained local concentration of protein which may account for the increased efficacy observed. With constant production by Ad\textit{CTLA} there was no evidence of disease exacerbation shown here, compared with the increase in disease severity found in some studies using repeated i.p. CTLA4-Ig protein administration (Racke et al., 1995). These first generation viral vectors however, will have a low clinical value due to their inherent immunogenicity, which limits the length of transgene expression (Wood et al., 1996; Byrnes et al., 1995; Kay et al., 1995), although such vector immunogenicity is reduced by co-expression of CTLA4-Ig (Kay et al., 1995). These adenoviral vectors do however provide a means to evaluate therapeutic gene candidates. Central administration of p55 TNFR-Ig protein (Baker et al., 1994) and TNF\textit{V} shown here can both inhibit disease, whereas Ad\textit{RIL10} exhibited little or no effect. This is consistent with the results following i.c. injection of 100 µg plasmid IL-10 DNA or recombinant IL-10 protein (Chapter 4) and is consistent with the high endogenous levels of immunocytochemically-detectable IL-10 in normal ABH mouse CNS discussed in chapters 3 and 4. Further studies with the next generation of non-immunogenic vectors such as AAV may promote long-term gene expression for treatment of chronic autoimmune disease.

Local delivery of inhibitory agents may exhibit a greater impact than can be achieved following systemic administration, and requires lower doses for effect (O’Neill et al.,
Furthermore this can limit, potentially undesirable, systemic immunosuppression (Baker et al., 1992). Targeting of costimulatory pathways using CTLA4-Ig can inhibit effector cell function and may be of value in treating chronic human autoimmune diseases, such as multiple sclerosis. However, recently it has been reported that myelin-specific activated T cells in multiple sclerosis patients have a decreased \textit{in vitro} dependence of CD28-mediated costimulation compared with myelin-specific naive T cells from healthy individuals (Scholz et al., 1998; Lovett-Racke et al., 1998), although incubation with CTLA4-hIg protein did induce some inhibition of proliferative capacity in one study (Lovett-Racke et al., 1998). These studies are consistent with the reduced requirement of accessory cell costimulation of memory versus naive T cells \textit{in vitro} (Croft et al., 1994), however the action of these reagents may not solely induce blockade of Ag and could induce additional effects \textit{in vivo}.

This study highlights the value of adenovirus in high efficiency gene delivery for the identification of target genes with which to treat EAE, although expression is transient. This is further compounded by the use of potentially immunogenic products. However, following the development of a more suitable vector for gene delivery, agents targeting costimulation will be of value in modulating EAE.
CHAPTER SIX

GENE THERAPY FOR CREAЕ USING RETROVIRALLY ENGINEERED FIBROBLASTS EXPRESSING A SOLUBLE TNF RECEPTOR
6.1. INTRODUCTION

Studies have shown TNF to be a potent mediator in several inflammatory disorders such as rheumatoid arthritis, septic shock, and MS (Tracey et al., 1988; Brosnan et al., 1988). Correlations have also been made between levels of TNF-α in the CSF of MS patients and the severity and progress of MS (Sharief et al., 1991; Tsukuda et al., 1991). There is now strong evidence to support a role for TNF in the pathogenesis of EAE as discussed in Chapter 1. Many studies have investigated the use of anti-TNF agents in EAE. However, treatment is not curative and on cessation of anti-TNF therapy relapse of disease is observed.

The study discussed in Chapter 4 showed that a DNA-cationic liposome complex coding for human p75 dTNFR injected directly into the CNS of mice with EAE ameliorated disease and delayed disease onset. However, gene delivery by DNA plasmid was shown to be inefficient, and delivery of product was limited to days. Adenoviral vectors have a high efficiency of infection to both replicating and non-replicating cells in vivo but expression is often limited to weeks or months as they evoke inflammatory responses as discussed in Chapter 5. Retroviral vectors however, can integrate into the host cell genome of replicating cells and have been used almost exclusively ex vivo to infect various cell types for use as biological pumps as discussed in Chapter 1.

6.2. AIM

Therefore this study will investigate the use of immortalised Biozzi ABH mouse kidney fibroblasts as a suitable cellular vehicle for the retroviral delivery of soluble human p75 dTNFR in vivo with which to treat EAE. Stable long-term production of human dTNFR from the retrovirally engineered fibroblasts will allow the study of anti-TNF agents to inhibit the acute phase as well as the relapse phase of CREAE. Long-term inhibition of TNF may also provide a useful tool with which to investigate the role of TNF in the pathogenesis of EAE.
6.3. RESULTS

6.3.1. *In vitro Cytokine Profile of temperature-sensitive fibroblasts.*

To produce a stable cell line for gene delivery, Biozzi ABH mouse foetal kidney fibroblasts were first immortalised using a temperature-sensitive SV40 large T antigen, expanded and named temperature-sensitive fibroblasts (tsF). These were then infected with an MFG retroviral vector, with expression driven by MMuLV LTR, coding for soluble human p75 dTNFR prior to cloning by limiting dilution. These cells could be grown in bulk culture (Figure 6.1). Supernatants from 13 clones were analysed by ELISA for the production of human p75 dTNFR. One clone of the dTNFR-tsF (oc5) produced 1.7 ng human p75 dTNFR/ml/1x10^6 cells/24 h, compared to tsF alone, which produced undetectable levels of human p75 dTNFR. Cytospins of dTNFR-tsF, tsF and tsF infected with a retrovirus coding for a murine IL-12 p40 homodimer were stained with an antibody to human p75. dTNFR-tsF cells stained positive for human p75 at an antibody dilution of 1:400 but tsF and tsF/IL-12 did not stain (Figure 6.2). Control mouse specific IL-12 mAb stained cells expressing the IL-12 p40. In addition, tsF could be shown to express SV40 large T antigen by immunohistochemistry (Figure 6.3). Although the dTNFR-tsF cells produced undetectable levels of IFN-β by anti-viral assay (Triantaphyllopoulos et al., 1998), or IFN-γ, IL-10 and IL-4 compared to positive controls as assessed by ELISA a TGF-β ELISA demonstrated that dTNFR-tsF and tsF cells produced 0.66 and 2.10 ng/ml TGF-β/1x10^6 cells respectively.

6.3.2. Detection of dTNFR-tsF cells and dTNFR production *in vivo* after CNS implantation

Fluorescently (PKH2)-labeled dTNFR-tsF (2 x10^5) when injected into the right cortex were detected in the parenchyma surrounding the injection site and also in the meninges close to the injection site (Figure 6.4). Cells could be detected in this way up to three months post i.c. injection but due to the half-life of the fluorescent marker could not be positively identified after this time point. SV40 large T antigen was rapidly shut down, as assessed by immunohistochemistry, following *in vivo* transplantation and consistent with this, cells were not found to be tumorigenic. Following i.c. injection of 2 x10^5 dTNFR-tsF cells into the right cerebral cortex, brains were removed 2 days post injection and immunostained for human p75 dTNFR on cryostat sections to demonstrate
that cells could express their transgene product \textit{in situ}. The use of human p75 dTNFR allowed the TNFR produced from the dTNFR-tsF to be distinguished from endogenous mouse p75 TNFR. Human p75 dTNFR peroxidase staining was detected in the right cerebral cortex on a population of cells, which were detected close to the injection site (Figure 6.5).

\textbf{6.3.3. Time course of the production of human p75 dTNFR from dTNFR-tsF cells in vivo.}

Serum, urine and CSF were collected from mice injected i.c. with $2 \times 10^6$ dTNFR-tsF or tsF at various time points and stored at $-20^\circ\text{C}$ prior to p75 TNFR ELISA. Human p75 dTNFR could be detected in the urine, serum and CSF of mice injected i.c. with dTNFR-tsF for at least 5 weeks post-injection (longest time point examined) (Figure 6.6). p75 dTNFR was detected in the serum and urine of mice 24 hours after i.c. injection at similar concentrations ($80-90$ pg/ml) but decreased to lower levels during weeks 1-3 ($21-30$ pg/ml). Urinary concentrations of human p75 dTNFR showed a low level throughout the time course ($17-26$ pg/ml). CSF concentration was maintained at a high level ($\sim 600-700$ pg/ml) for at least 3 weeks. Human p75 dTNFR was not detected in urine, serum or CSF samples from untreated control mice or mice injected with the same number of non-transduced tsF cells.

\textbf{6.3.4. Therapeutic control of acute-phase EAE with dTNFR expressing cells.}

Having established that dTNFR production was maintained \textit{in vivo} for at least 5 weeks the therapeutic potential of injecting dTNFR-tsF cells at different time points both during disease induction and once disease was induced were investigated. Following disease induction dTNFR-tsF cells were administered either i.p., to target peripheral lymphoid priming of encephalitogenic T cells or locally to the CNS to inhibit the effector T cell response. Systemic administration of $1 \times 10^6$ dTNFR-tsF cells administered i.p. had no effect upon disease severity (Table 6.1). However, increasing the dose to $5 \times 10^6$ dTNFR-tsF cells, significantly inhibited the development of disease and delayed the onset of clinical signs, compared to tsF treated mice (Table 6.1). There was no significant increase in disease inhibition by increasing the dose of cells to $1 \times 10^7$ on day 7 p.i. compared to that seen with $5 \times 10^6$ cells, suggesting that the optimal response had been achieved (Table 6.1). Comparison of $5 \times 10^6$ dTNFR-tsF cells
administered i.p. at day 0 or 7 p.i. suggest that systemic TNF neutralisation during early or late time points of the priming stage of disease can inhibit EAE to a similar degree (Tables 6.1, 6.2). In groups of mice treated with dTNFR-tsF the mean percentage incidence of mice exhibiting EAE was significantly reduced (69.6%) compared to tsF (90.5%) and untreated controls (96.8%) (p < 0.03). A further control consisted of tsF retrovirally engineered to produce mouse IFN-β (Triantaphyllopoulos et al., 1998). When 2x10^6 IFNβ-tsF cells were injected i.c. day 12 p.i. there was no effect upon disease severity (mean clinical score = 3.5 ± 0.1; n=6) or mean day of onset (18.2 ± 0.4) compared to tsF treated mice (mean clinical score = 3.8 ± 0.2; mean day of onset = 17.5 ± 0.5; n=6). The lack of efficacy seen with the IFNβ-tsF may be due to the low level of cytokine produced (Triantaphyllopoulos et al., 1998).

The efficacy of dTNFR-tsF cells in EAE inhibition could be further increased when implanted into the CNS. A lower dose of dTNFR-tsF (2x10^6) cells implanted into the CNS day 7 p.i. or just prior to disease onset (day 12 p.i.) inhibited disease severity to a equal or greater degree compared to i.p. administration of more than double the number of cells (Table 6.2). To investigate the potential of neutralising TNF during established disease, dTNFR-tsF cells were administered i.c. at the onset of clinical signs (clinical grade 1). The continuation of disease severity was moderately inhibited by the administration of dTNFR-tsF cells (EAE score 2.0 ± 0.4) compared to tsF treated mice (EAE score 3.3 ± 0.2) (Table 6.2).

Mice injected with non-transduced tsF at all time points and by all routes showed no significant difference in mean disease score or onset of disease when compared with the untreated SCH inoculated mice (Tables 6.1, 6.2, 6.3).

6.3.5. Therapeutic control of relapse phase EAE with dTNFR expressing cells.

EAE in ABH mice follows a relapsing-remitting disease profile, which allows investigation of the relapse phase, which may be more clinically relevant to MS than acute disease models of EAE (Baker et al., 1990). The dTNFR-tsF cells have been shown to express their transgene product for at least 5 weeks in vivo. Therefore, mice injected with dTNFR-tsF cells in the CNS day 7 p.i. were allowed to pass through the acute phase of disease to study the long-term inhibition of TNF on the relapse phase of
Chapter 6 Results

disease. As seen in previous experiments, dTNFR-tsF treated mice had a significantly ameliorated mean clinical score compared to tsF treated mice (p < 0.0005) during the acute phase, with an EAE incidence of 53% compared to 100% for tsF-treated and untreated mice (Table 6.3). In addition, the dTNFR-tsF treated group exhibited a lower incidence rate of relapse (41%) compared to tsF-treated group (69%) and the mean clinical score was significantly reduced compared to tsF treated mice (p < 0.05) (Table 6.3; Figure 6.7).

This study also investigated the inhibition of the relapse phase in mice which had been allowed to pass through the acute phase of disease without treatment. Following acute phase disease (day 15-25 p.i.) mice were injected i.c. with 2 x 10^6 dTNFR-tsF or non-transduced tsF cells during the remission phase (day 28 p.i.). After transplantation mice injected with dTNFR-tsF cells showed a significantly lower clinical score during the relapse phase (day 30 onwards) compared to control tsF treated mice (p < 0.05) (Table 6.3, Figure 6.8). Notably the incidence of relapse was reduced (33%) compared to either tsF treated group (100%) or untreated group (78%) (Table 6.3).

6.3.6. CNS inflammation is reduced in dTNFR-tsF treated mice.

Lumbar regions of spinal cord were taken from mice day 18-19 p.i. when there was a maximal difference in clinical disease between dTNFR-tsF treated and other groups and were embedded in wax, and counterstained with haematoxylin and eosin for histological analysis. Spinal cords from dTNFR-tsF treated mice (n=5) that had no clinical disease and no weight loss showed no perivascular infiltrate, or lesions in the parenchyma, although there was a slight infiltrate in the meninges of one animal. This contrasts to mice from tsF treated (n=5) or control SCH (n=5) groups which were at maximum clinical disease (grade 4) and showed perivascular lesions and substantial cellular infiltrate in the parenchyma and meninges (Figure 6.9). Mice from the dTNFR-tsF group that experienced EAE had a similar inflammatory profile to that of mice from tsF treated or untreated SCH mice.
Table 6.1 Therapeutic effects of systemic gene-delivered dTNF during the priming stage of EAE.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Dose</th>
<th>Route</th>
<th>No.</th>
<th>Mean Clinical Score ± SEM</th>
<th>Mean EAE Score ± SEM</th>
<th>Mean Day of Onset ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>6/6</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>17.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>tsF</td>
<td>1x10^6</td>
<td>i.p.</td>
<td>7/8</td>
<td>2.9 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>dTNFR-tsF</td>
<td>1x10^6</td>
<td>i.p.</td>
<td>5/7</td>
<td>2.7 ± 0.7</td>
<td>3.8 ± 0.1</td>
<td>18.4 ± 0.7</td>
</tr>
<tr>
<td>0</td>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>13/14</td>
<td>3.5 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td>18.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>tsF</td>
<td>5x10^6</td>
<td>i.p.</td>
<td>7/8</td>
<td>3.3 ± 0.5</td>
<td>3.8 ± 0.1</td>
<td>19.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>dTNFR-tsF</td>
<td>5x10^6</td>
<td>i.p.</td>
<td>5/8</td>
<td>1.9 ± 0.6^*</td>
<td>3.0 ± 0.3</td>
<td>21.4 ± 0.6^*</td>
</tr>
<tr>
<td>7</td>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>11/11</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>16.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>tsF</td>
<td>1x10^7</td>
<td>i.p.</td>
<td>8/8</td>
<td>3.2 ± 0.4</td>
<td>3.2 ± 0.4</td>
<td>18.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>dTNFR-tsF</td>
<td>1x10^7</td>
<td>i.p.</td>
<td>6/8</td>
<td>1.3 ± 0.5^*</td>
<td>1.7 ± 0.5</td>
<td>19.3 ± 0.8</td>
</tr>
</tbody>
</table>

ABH mice were inoculated with SCH in CFA on day 0 and 7. Animals were untreated (SCH) or received either dTNFR-tsF or tsF cells alone on day 0 or 7 post disease induction. The results represent the number of mice which developed EAE, the mean clinical score ± SEM of each group, the mean EAE score of each group and the mean day of onset of EAE. The mean clinical score includes all mice in the group whereas the mean EAE score was determined by assessing only mice with EAE.

* p < 0.05 compared to tsF treated mice.
Table 6.2. Local administration to the CNS increases the efficacy of gene-delivered dTNFR.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose Route</th>
<th>No.</th>
<th>Mean Clinical Score ± SEM</th>
<th>Mean EAE Score ± SEM</th>
<th>Mean Day of Onset ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 7 SCH</td>
<td>- -</td>
<td>34/36</td>
<td>3.5 ± 0.2</td>
<td>3.7 ± 0.1</td>
<td>17.3 ± 0.4</td>
</tr>
<tr>
<td>tsF 5x10⁶</td>
<td>i.p.</td>
<td>20/23</td>
<td>3.1 ± 0.3</td>
<td>3.5 ± 0.2</td>
<td>18.0 ± 0.5</td>
</tr>
<tr>
<td>dTNFR-tsF 5x10⁶</td>
<td>i.p.</td>
<td>16/23</td>
<td>1.9 ± 0.3***</td>
<td>2.6 ± 0.3**</td>
<td>18.8 ± 0.6</td>
</tr>
<tr>
<td>day 7 SCH</td>
<td>- -</td>
<td>18/18</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>tsF 2x10⁶</td>
<td>i.c.</td>
<td>13/13</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>16.5 ± 0.5</td>
</tr>
<tr>
<td>dTNFR-tsF 2x10⁶</td>
<td>i.c.</td>
<td>10/17</td>
<td>1.4 ± 0.3***</td>
<td>2.5 ± 0.3**</td>
<td>17.1 ± 0.9</td>
</tr>
<tr>
<td>day 12 SCH</td>
<td>- -</td>
<td>15/15</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>15.2 ± 0.5</td>
</tr>
<tr>
<td>tsF 5x10⁶</td>
<td>i.p.</td>
<td>14/14</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>16.9 ± 0.5</td>
</tr>
<tr>
<td>dTNFR-tsF 5x10⁶</td>
<td>i.p.</td>
<td>10/15</td>
<td>2.1 ± 0.4**</td>
<td>3.2 ± 0.2*</td>
<td>16.8 ± 0.5</td>
</tr>
<tr>
<td>day 12 SCH</td>
<td>- -</td>
<td>16/16</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>14.4 ± 0.5</td>
</tr>
<tr>
<td>tsF 2x10⁶</td>
<td>i.c.</td>
<td>16/16</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>14.8 ± 0.3</td>
</tr>
<tr>
<td>dTNFR-tsF 2x10⁶</td>
<td>i.c.</td>
<td>11/13</td>
<td>2.2 ± 0.4***</td>
<td>2.6 ± 0.4***</td>
<td>16.7 ± 0.5*</td>
</tr>
<tr>
<td>onset SCH</td>
<td>- -</td>
<td>14/14</td>
<td>-</td>
<td>3.3 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>tsF 2x10⁶</td>
<td>i.c.</td>
<td>6/6</td>
<td>-</td>
<td>3.3 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>dTNFR-tsF 2x10⁶</td>
<td>i.c.</td>
<td>10/10</td>
<td>-</td>
<td>2.0 ± 0.4*</td>
<td>-</td>
</tr>
</tbody>
</table>

ABH mice were inoculated with SCH in CFA on day 0 and 7. Animals were untreated (SCH) or received either dTNFR-tsF or tsF cells alone on day 7 or 12 post disease induction or at onset of disease (grade 1). The results represent the number of mice which developed EAE, the mean clinical score ± SEM of each group, the mean EAE score of each group and the mean day of onset of EAE. The mean clinical score includes all mice in the group whereas the mean EAE score was determined by assessing only mice with EAE.

* p < 0.05, ** p < 0.005, *** p < 0.0002 compared to tsF treated mice.
**Table 6.3 Therapeutic effect of anti-TNF therapy on the relapse phase of EAE**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Route</th>
<th>Day of treatment p.i.</th>
<th>No. EAE</th>
<th>Mean Clinical Score ± SEM</th>
<th>No. Relapse</th>
<th>Mean Relapse Score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9/9</td>
<td>3.7 ± 0.1</td>
<td>7/9</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>tsF</td>
<td>2x10^6</td>
<td>i.c.</td>
<td>7</td>
<td>13/13</td>
<td>3.4 ± 0.1</td>
<td>9/13</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>dTNFR-tsF</td>
<td>2x10^6</td>
<td>i.c.</td>
<td>7</td>
<td>9/17</td>
<td>1.4 ± 0.4 **</td>
<td>7/17</td>
<td>1.9 ± 0.4 *</td>
</tr>
<tr>
<td>tsF</td>
<td>2x10^6</td>
<td>i.c.</td>
<td>28</td>
<td>9/9</td>
<td>3.6 ± 0.2</td>
<td>9/9</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>dTNFR-tsF</td>
<td>2x10^6</td>
<td>i.c.</td>
<td>28</td>
<td>12/12</td>
<td>3.5 ± 0.2</td>
<td>4/12</td>
<td>2.1 ± 0.4 *</td>
</tr>
</tbody>
</table>

ABH mice were inoculated with SCH in CFA on day 0 and 7. Animals were untreated (SCH) or received either dTNFR-tsF or tsF cells alone on day 7 or 28 post disease induction. The results represent the number of mice which developed EAE, the mean clinical score ± SEM of each group, the mean relapse score of each group and the mean day of onset of EAE.

* p < 0.05 when compared to tsF treated mice
** p < 0.0005 when compared to tsF treated mice
Figure 6.1 Temperature-sensitive immortalised fibroblasts can be grown in bulk culture at 37°C.

Biozzi ABH foetal fibroblasts were immortalised with a retrovirus coding for SV40 large T antigen. These cells could be grown in bulk culture at 35°C more efficiently than primary fibroblast cultures. (a) Immortalised fibroblasts could be grown to confluency in culture (x10) and (b) exhibited an appearance typical of immortalised cells at low cell numbers (x32).
Figure 6.2 Cytospins of immortalised fibroblasts express CD120b after infection with retrovirus coding for human soluble p75 dTNFR.

Immortalised tsF were infected with retrovirus coding for human p75 dTNFR (dTNFR-tsF) or murine IL-12 p40 homodimer (p40 IL-12-tsF). (a) cytospins of dTNFR-tsF cells showed production of human p75 TNFR when stained with anti-human p75 TNFR mAb, in contrast to (b) p40 IL-12-tsF cells. p40 IL-12-tsF (c) did show production of murine p40 IL-12. Cytospins were counterstained with haematoxylin. Magnification x100.
Figure 6.3 Temperature-sensitive immortalised fibroblasts (tsF) express the SV40 large T antigen at 35°C in vitro.

Immortalised fibroblasts (tsF) were grown in vitro at 35°C on chamber slides. (a) shows tsF cells under light phase microscopy (magnification x200), and (b) shows the same tsF cells after fluorescent staining of the SV40 large T antigen in the nucleus (magnification x200).
Chapter 6 Results

Figure 6.4 Fluorescently linked dTNFR-tsF cells are present around the injection site after i.c. injection.

2 x 10^6 dTNFR-tsF were linked to the fluorescent marker, PKH2, and injected i.c. into the right cerebral cortex of anaesthetised mice (n=5). 24 hours post i.c. injection brains were removed and cryostat sectioned. (a) shows a large mass of dTNFR-tsF cells in the meninges and surrounding parenchyma close to the site of injection. Magnification x100.
Figure 6.5 CNS localisation and production of human p75 dTNFR after i.c. injection of dTNFR-tsF to the cerebral cortex.

2 x 10^5 dTNFR-tsF cells were injected into the right cerebral cortex of anaesthetised mice and the brains removed 2 days post injection and stained for human p75 dTNFR to show the distribution of dTNFR-tsF within the CNS (n=4). Positive human p75 dTNFR peroxidase staining could be detected on a population of cells, which appeared close to the injection site (→). Cryostat sections were not counterstained. Magnification x 200.
Figure 6.6 Time course of production of human soluble p75 dTNFR from dTNFR-tsF in vivo after CNS administration.

Time course of in vivo production of human soluble p75 TNFR was measured in urine (•), serum (○) and cerebrospinal fluid (▼) from ABH mice injected with 2x10^6 dTNFR-tsF cells i.c. Samples from untreated mice or tsF treated mice showed no positivity for human p75 TNFR. Sensitivity of ELISA < 5pg/ml. Groups consisted of 3-6 mice.
Figure 6.7 Successful inhibition of the acute and relapse phase of EAE by a single injection of dTNFR-tsF locally to the CNS prior to the acute phase.

Mice were injected with spinal cord homogenate in CFA day 0 and day 7. The data shows that the acute phase of EAE can be ameliorated with local delivery of human p75 dTNFR from cloned immortalized fibroblasts (p< 0.0005) compared to mice injected at the same time point and route with immortalized fibroblasts only (tsF). Mice were injected i.c. with 2x10^6 dTNFR-tsF (O) or tsF (•) day 7 p.i. (↓). Although the single injection of dTNFR-tsF did not prevent onset of the relapse phase of EAE it could still ameliorate the severity of disease 5 weeks later (p < 0.05) to a similar degree to that seen when dTNFR-tsF were administered during remission (day 28 p.i.) (Figure 6.8). The results represent the mean clinical score of all animals in the group (n=13-17 mice).
Figure 6.8 Successful inhibition of the relapse phase of EAE by a single injection of dTNFR-tsF locally to the CNS during the remission phase.

Mice were injected with spinal cord homogenate in CFA day 0 and day 7. Mice were allowed to proceed through the acute phase untreated. Mice were injected i.c. with $2 \times 10^6$ dTNFR-tsF (O) or tsF (●) during remission of EAE day 28 p.i. (↓). The data shows that although the single injection of dTNFR-tsF did not prevent onset of the relapse phase of EAE it could still ameliorate the severity of relapse ($p < 0.05$) compared to mice treated with the same quantity of tsF at the same time point. The results represent the mean clinical score of all animals in the group (n=9-12 mice).
Figure 6.9 Immortalised fibroblasts retrovirally engineered to express human p75 dTNFR implanted in the CNS of mice with EAE reduces the lesion load in the lumbar spinal cord.

Lumbar regions of spinal cord were removed day 18-19 p.i. embedded in wax, sectioned and counterstained with haematoxylin and eosin for histology. (a) and (b) show spinal cord sections from tsF and untreated SCH mice respectively at maximal paralysis. Perivascular lesions can be seen in both cases with some inflammatory cell infiltrate present in the meninges (magnification x100). (c) shows a lack of perivascular lesions and inflammatory cell infiltrate in the spinal cord of a dTNFR-tsF treated mouse at an equivalent time point. Groups contained 5 mice each.
6.4. DISCUSSION

This study demonstrates that genetically engineered cell vectors can survive long term in the CNS and produce a therapeutic concentration of protein capable of inhibiting CNS inflammatory disease over two disease episodes, both the acute phase and relapse phase of EAE.

Chapter 4 demonstrated that a DNA construct coding for dTNFR complexed with cationic liposome can ameliorate acute EAE, when injected into the CNS. However, production was limited to between 3-6 days, in contrast to using a retroviral/cell vector system as described here where transgene production could be detected for weeks. Following CNS delivery of dTNFR-tsF cells, high levels of dTNFR in the CSF could be detected compared with that detected in peripheral compartments. The capacity to maintain a concentration gradient of immunosuppressive agents within the CNS may be important to limit non-specific effects in the periphery, and will be aided by the relatively short half-life of dTNFR. However this study suggests that the dTNFR is active when administered both centrally and systemically. The dTNFR-tsF cells were at least as effective at inhibiting EAE as previous reports using multiple-dose protein therapy, despite producing 1000-fold lower protein concentrations than bolus protein injections (Ruddle et al., 1990; Selmaj et al., 1991; Baker et al., 1994). Furthermore the neutralisation of TNF in EAE by dTNFR-expressing cells produced an effect comparable to a delay in onset of EAE seen in Tnf gene-deleted mice (Körner et al., 1997a), and supports the inhibition seen with plasmid DNA coding for human p75 dTNFR in Chapter 4 and studies using anti-TNF mAb or TNF fusion proteins to inhibit TNF-mediated pathology (Ruddle et al., 1990; Baker et al., 1994).

The time course of production of human p75 dTNFR from dTNFR-tsF cells was investigated in order to predict suitable time points for administration during EAE. Production of the human p75 dTNFR was observed for at least 5 weeks in normal mice injected i.c.

The p75 dTNFR gene product used here inhibited disease development, particularly when administered locally to the CNS. This would be consistent with an important TNF-dependent mechanism in the CNS, which is involved in the pathogenesis of EAE. TNF is a pleiotropic cytokine and has many important actions as described in Chapter 1,
and is thought to have a major role in the recruitment of inflammatory cells, possibly through the regulation of vascular adhesion molecules or the induction of other proinflammatory cytokines (Selmaj et al., 1995; Selmaj et al., 1998; Barton et al., 1994). However it has also been suggested that TNF-specific (p55 TNFR-Ig) treatment may decrease the activation state of encephalitogenic T cells rather than inhibiting T cell infiltration into the CNS (Körner et al., 1997b). The onset of weight loss correlates with blood:brain barrier dysfunction and cellular recruitment (Allen et al., 1993), and in some cases of TNF-specific protein therapy, animals showed weight loss even though disease was reduced (Körner et al., 1997b). Whilst some dTNFR-tsF treated mice in this study did develop EAE there was a number of animals that were completely protected from EAE, showing stable weight progression, and no CNS infiltrate. This may indicate a higher degree of TNF neutralisation by the dTNFR-tsF cells, inhibiting TNF-induced cachexia, compared to the bolus protein administration in some studies (Körner et al., 1997b). This study also demonstrates that both the development of acute phase EAE and the development of relapse may also be inhibited by dTNFR.

Although many studies have shown correlation of TNF and soluble TNFR levels in serum and CSF to severity of MS, there remains much controversy over the results (Hofman et al., 1989; Selmaj et al., 1991; Chofflon et al., 1992; Spuler et al., 1996). However as described in Chapter 3, analysis of serum samples for the presence of mouse CD120a and CD120b throughout the relapsing course described by Allen et al., 1993 failed to demonstrate any correlation with disease activity. The effect of TNF-neutralisation in vivo in this study however further indicates the importance of TNF in the pathogenesis of CREAE and may possibly suggest that TNF may be a useful target in the control of MS in humans if access to the CNS can be addressed.

Interestingly, either mice injected i.c. with dTNFR-tsF cells prior to the acute phase and allowed to relapse or mice injected i.c. during remission showed a significant amelioration of clinical signs in the relapse phase compared to tsF treated mice (p < 0.05), as well as reducing the incidence of EAE. However, the acute phase showed a greater tendency for inhibition than the relapse phase, and this difference in the ability of p75 dTNFR to block the acute phase compared to the relapse phase suggests that different requirements for TNF are involved in the two processes. Studies with TNF gene-targeted mice have shown that TNF is important in the initial stages of EAE but that disease onset is only delayed and can proceed to its normal course (Riminton et al.,
1998). TNF is undoubtedly important in the upregulation of adhesion molecules on CNS endothelia, however, macrophages/microglia produce both IL-1 and IL-6 and can induce IFN-γ production, which can also act on endothelia to upregulate adhesion molecules. Therefore functional "cytokine redundancy" may explain the access to the CNS of a new wave of T cells in the absence of TNF (Wong et al., 1992; Des Vries et al., 1994) where other cytokines can compensate for the absence or neutralisation of TNF and its effect.

The neutralisation of TNF in the acute phase may block the secondary recruitment of monocytes to the CNS and be sufficient to inhibit EAE. In contrast, the relapse phase includes other cell types such as microglia/macrophages, which have been activated previously in the CNS and may control disease progression independently of TNF and therefore be refractory to TNF neutralisation. Alternatively, expression of chemokines may be present, with different temporal TNF-dependencies. It has been shown that TNF can induce production of murine monocyte chemoattractant protein-1 (MCP-1α) from astrocytes but not from microglial cells which could produce macrophage inflammatory protein-1α (MIP-1α) when stimulated with LPS (Hayashi et al., 1995). Alternatively, a greater number of B cells have been observed in the relapse phase of EAE in Biozzi ABH mice compared with the acute phase (Allen et al., 1993). Therefore B cells may have different requirements to T cells independent of TNF.

However while TNF-specific mAb have reduced the severity of rheumatoid arthritis and Crohn's disease (Elliot et al., 1994; Moreland et al., 1997) the same antibody and a p55 TNFR-IgG1 fusion protein administered to septic shock patients had no survival benefit, and in the case of the high dose TNFR-IgG1 group the high dose was associated with a higher mortality rate amongst the patients (Fisher et al., 1996; Abraham et al., 1998). Similarly it has been reported that a p55 TNFR-Ig fusion protein increased exacerbations in 2 MS patients (Martin et al., 1998). Thus it appears that different TNF antagonists have different immunogenic and pharmacological properties. Endogenous TNF production is controlled by natural feedback mechanisms (Carballo et al., 1998; Di Santo et al., 1995). It is therefore likely that the degree of neutralisation may have important consequences on the development of either pro- or anti-inflammatory events depending on the timing and concentration administered. Therefore care must be taken the extrapolation of animal data into the clinic.
Although many cell types are currently being studied for vehicles in gene therapy, fibroblasts are an attractive vector for human therapy as they can be easily cultured from skin biopsies of any individual, and thus eliminate any potential allogeneic immune response. These can readily be stably infected by retrovirus *ex vivo*. At present fibroblasts are being used as vehicles to deliver therapeutic products in a variety of disease models, such as Parkinson's disease, leukaemia, Alzheimer's disease, CNS glioma and melanoma, and arthritis (Levivier et al., 1995; Wang et al., 1996; Fisher et al., 1993; Glick et al., 1997; Triantaphyllopoulos et al., 1999; Pelletier et al., 1997; Bakker et al., 1997). As cells delivered to the CNS are viable, and are capable of producing endogenous growth factors, an added benefit in immunosuppression, neuroprotection and graft survival may come from the use of CNS-derived cells. For example oligodendrocytes which could be used to promote remyelination in damaged tissue (O'Leary et al., 1997) or astrocytes which may provide growth factors (see Appendix A).

Retrovirally-engineered fibroblasts can effectively deliver therapeutic concentrations of dTNFR to mice either systemically or locally in the CNS. This *ex vivo* gene therapy procedure administered locally to the CNS could also be used to deliver a variety of other immunosuppressive products, or neuroprotection/regeneration agents either alone or in combination. While efficacy has been shown here in an inflammatory CNS model, this approach will be of relevance to a variety of other conditions where proteins need to be delivered long term to the CNS. This strategy provides the opportunity to deliver proteins long term, without frequent repeat administration unlike antibody or fusion protein therapy. This offers a useful tool to dissect neurological disease pathways experimentally and could be developed towards the control of neurological disorders such as MS.
CHAPTER SEVEN

DIFFERENT THERAPEUTIC OUTCOMES IN EAE USING MURINE IL-10 AS PROTEIN OR EXPRESSED BY ADENOVIRUS OR RETROVIRALLY TRANSDUCED SYNGENEIC FIBROBLASTS
Chapter 7 Introduction

7.1. INTRODUCTION

IL-10 is an anti-inflammatory cytokine produced by Th2 cells and macrophages and it has been shown to suppress pro-inflammatory cytokines from activated Th1 cells (Fiorentino et al., 1989) probably indirectly, by down-regulating IFN-γ-induced MHC class II antigens on APC such as macrophages and microglial cells (Frei et al., 1994). IL-10 also down regulates B7 molecules on APC, costimulatory molecules required for T cell activation (Ding et al., 1993) and inhibits the production of reactive oxygen and nitrogen intermediates from microglia (Bogdan et al., 1991; Cunha et al., 1992; Gazzinelli et al., 1992), which may cause damage to myelin and oligodendrocytes (Merrill et al., 1993).

IL-10 has been shown to be present in perivascular astrocytic endfeet not only from MS brains but also from normal brains suggesting a regulatory role at the BBB (Cannella et al., 1995). In addition IL-10 has been shown to stimulate the production of IL-1ra and soluble tumour necrosis factor receptor p75 and p55 (TNFR p75, p55) from mononuclear cells and fibroblasts respectively (Seitz et al., 1995). This data suggests an immunoregulatory role for IL-10 and it has been shown to inhibit EAE (Rott et al., 1994; Willenborg et al., 1995; Xiao et al., 1998) although one study showed human IL-10 to have no effect or to worsen severity of EAE in mice (Cannella et al., 1996). IL-10 has also been reported to be elevated in the serum and CSF of MS patients receiving IFN-β therapy (Martin et al., 1998) and IL-10 mRNA has been shown to be upregulated in the CNS during the remission phase of EAE (Kennedy et al., 1992). Gene therapy can be used to overcome the large daily administration of IL-10 needed for therapy of EAE. Two recent studies have used T cells transfected with either cDNA or retrovirus coding for IL-10 with which to treat EAE (Mathisen et al., 1997; Shaw et al., 1997). Interestingly the study using IL-10 transfected hybridoma cells showed no effect upon EAE, although IL-4 transfected hybridoma cells did inhibit EAE. However, animals were reported to have eventually died from tumours due to hybridoma overgrowth (Shaw et al., 1997). In contrast, the study using transfected memory T cells showed an amelioration of disease (Mathisen et al., 1997).

Chapters 4 and 5 showed that an IL-10 DNA-cationic liposome complex (CLC), adenovirus coding for IL-10 and a bolus dose of recombinant IL-10 had no effect upon
EAE when administered locally into the CNS. Whilst DNA-CLC has a low duration of expression and adenovirus is highly immunogenic, the study in Chapter 6 has shown that syngeneic immortalised fibroblasts infected with a retrovirus coding human soluble p75 dTNFR can be used as biological minipumps with which to treat EAE. These provide long-term expression of therapeutic protein and exhibit no apparent immunogenicity in this model.

7.2. AIM

Therefore this study will investigate the use of retroviral/fibroblast vector with which to deliver IL-10 locally to the CNS to treat EAE. Chapters 4 and 5 demonstrated delivery of IL-10 to the CNS as recombinant protein, or by expression from plasmid DNA-CLC or adenovirus. Therefore a direct comparison can be made on the different vector systems when administered by the same route and in the same model of disease. In addition the long-term expression of IL-10 in the CNS from the retroviral/fibroblast vector allows the study of the mechanisms by which IL-10 exerts its influence on EAE.
Chapter 7 Results

7.3. RESULTS

7.3.1. In vitro and in vivo detection of gene delivered IL-10

Chapter 6 demonstrated that tsF cells can exist and produce their transgene product for months following CNS transplantation, whereas adenoviral transgenes are expressed transiently, but at high levels (Chapter 5). To determine that the IL-10.tsF cells could express IL-10 in vivo, CSF and serum was sampled from mice injected i.c. with 2x10^6 IL-10.tsF cells, and analysed by ELISA. Samples were collected 3 days post i.c. injection of IL-10.tsF or AdRlLlO or control groups. CSF samples from 5x10^6 PFU ADRILl0 (n=5) contained 110.3 ± 22.2 ng/ml IL-10 compared to mice injected i.c. with 5x10^5 PFU AdRlLlO (n=4) which contained 5 ± 1.6 ng/ml IL-10. CSF from mice injected i.c. with IL-10.tsF cells (n=3) contained 19.0 ± 1.0 ng/ml IL-10. However, CSF samples from mice injected with 5x10^4 PFU AdRlLl0, 5x10^6 PFU AdRL, 2x10^6 non-transduced tsF or 100 μg plasmid DNA-CLC coding for IL-10 did not contain levels of IL-10 above the threshold of detection by ELISA (15.0 pg/ml). Serum IL-10 concentration from all groups was below the detection limit. In addition the in vivo production of IL-10 from AdRlLl0 has previously been shown to have efficacy in a model of murine arthritis (Quattrocchi et al., 1999). ELISA of tissue culture supernatants from confluent immortalised mouse fibroblasts infected with retrovirus coding for murine IL-10 (IL-10.tsF) produced 3.3 ng IL-10 /ml/1x10^6 cells/ 24h in contrast to non-infected tsF or tsF cells infected with a retrovirus coding for murine IFN-β where murine IL-10 production was undetectable.

Chapter 6 demonstrated that the p75 dTNFR-tsF cells produced TGF-β which has also been shown to ameliorate the clinical signs of EAE. Therefore the levels of TGF-β produced from IL-10.tsF were tested by ELISA. IL-10.tsF produced 904 pg TGF-β /ml/5 x 10^6 cells/24 h compared to tsF cells which produced 2130 pg TGF-β /ml/5 x 10^6 cells/24 h. The level of TGF-β present in the tissue culture medium was subtracted from the values obtained for cell supernatants. ELISA sensitivity was less than 7pg/ml.

At the dose of tsF (2x10^6, Chapter 6) where there was no amelioration of EAE when injected i.c., it was assumed that TGF-β was having no effect upon the disease course. Therefore as the IL-10.tsF cells produced less than half the TGF-β than the non-
transduced tsF cells, a dose of 2x10^6 was used to treat EAE where TGF-β would have no immediate role in disease amelioration. However, its presence in the CNS during disease could have other effects as discussed later.

7.3.2. IL-10 CNS Gene Therapy in EAE.

In 3 separate experiments a total of 30 mice were injected i.c. on day 12 p.i. before the onset of clinical signs, with either 2x10^6 IL-10.tsF or tsF cells. The inhibition of EAE using IL-10.tsF was reproducible and the results from one typical experiment are shown in Table 7.1. IL-10 delivered by IL-10.tsF cells significantly reduced the mean clinical score of EAE (2.2 ± 0.2; p < 0.05) compared to tsF treated mice (3.3 ± 0.3) although it had no effect on the mean onset of disease (Table 7.1). Most IL-10.tsF treated mice did not progress above grade 2 where they stabilised for 3-4 days before returning to mild clinical disability of a slight loss of tail tone, grade 0.5, typical in post-acute remission animals. In contrast, control SCH and tsF treated mice progressed to maximum clinical severity, complete hindlimb paralysis (grade 4) before returning to mild clinical disease. Figure 7.1 represents three experiments pooled together and demonstrates the stabilised course of EAE after CNS administration of IL-10.tsF compared to tsF treated and untreated SCH mice.

Previously we have demonstrated the lack of efficacy on EAE inhibition of IL-10 delivered as murine recombinant protein; adenovirus (5x10^6 PFU); or plasmid DNA-cationic liposome complex (Chapters 4 and 5). The difference in efficacy of vector expressed IL-10 in EAE may be due to an unknown factor secreted by the tsF. Therefore non-transduced tsF (2x10^6) were co-injected with 5x10^6 PFU AdRIL10 into the CNS, in a total volume of 30 µl PBS day 12 p.i. as in the other treatment protocols. The tsF/AdRIL10 treatment had no effect upon the severity compared to tsF treated mice (tsF/AdRIL10 mean clinical score 3.1 ± 0.5; tsF mean clinical score 3.8 ± 0.1).

To investigate the importance of the level of IL-10 delivered to the CNS the adenovirus titre was reduced so to deliver approximately an equivalent concentration of mouse IL-10 in vivo as the IL-10.tsF. In addition the concentration of recombinant murine IL-10 was also reduced. Adenoviral delivery of IL-10 however, from 5x10^6, 5x10^5, 5x10^4 PFU AdRIL10 and direct injection of 2x10^5, 5x10^3, 100, 10 and 1 ng/ml IL-10 all had no
significant inhibitory effect on EAE (Table 7.1).

7.3.3. **IL-10 gene therapy reduces the perivascular lesion load in the CNS but increases CNS inflammatory cell infiltrate.**

In untreated EAE the number of infiltrating cells closely correlates with the clinical score (Allen et al., 1993). Wax embedded sections from the lumbar region of spinal cords from untreated SCH, tsF or IL-10.tsF treated groups were divided according to their EAE clinical score and the number of perivascular cuffs containing inflammatory cell infiltrate (perivascular lesions) assessed microscopically from 3 sections from each mouse per group. Each group contained 6 mice. Sections from untreated SCH mice at grade 4 had a mean lesion number of 14.3 ± 2.8 per section, where discrete perivascular cuffs were present in parenchyma of the spinal cord, with infiltrate both in the parenchyma and in the meninges. Spinal cord from tsF treated mice at grade 4 had a mean lesion count of 11.5 ± 1.3 per section, and displayed similar pathology to the SCH group. Sections from IL-10.tsF treated mice at grade 4 had a lower mean lesion number of 4.7 ± 1.3.

The mean lesion number from untreated SCH and tsF treated mice grade 2 were 6.8 ± 1.0 and 13.3 ± 2.9 per section respectively. In contrast the IL-10.tsF treated mice at grade 2 had a significantly lower mean lesion number of 2.2 ± 0.5 per section (p < 0.01). The untreated SCH and tsF treated spinal cords shared similar pathology with perivascular lesions in the parenchyma and with inflammatory infiltrate widespread throughout the parenchyma and meninges (Figure 7.2a, b). However, despite a lower lesion count in the IL-10.tsF treated mice there appeared to be higher levels of infiltrate in the parenchyma surrounding the meninges and the meninges itself, which was not localised to perivascular tissue and did not form discrete lesions (Figure 7.2c). In some instances large numbers of infiltrating cells were seen in the meninges but not infiltrating the spinal cord parenchyma (Figure 7.2d). IL-10.tsF treated mice, which had stabilised disease for at least 3 days resulting only in loss of tail tone (grade 1), had a mean lesion number of 0.6 ± 0.2. In contrast, tsF treated mice at grade 1, which typically had been observed to continue the disease course to maximum disability (total hind limb paralysis, grade 4) had a mean lesion number of 6.6 ± 2.6. However, as with the IL-10.tsF treated mice at grade 2, the spinal cord of IL-10.tsF treated mice contained large numbers of cells in the meninges but not invading the perivascular space or
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7.3.4. IL-10 gene therapy down-regulates MHC class II antigen on resident CNS cells but not on infiltrating cells.

Immunofluorescent detection of the MHC class II antigen in cryostat sections from the spinal cord from untreated SCH and tsF treated mice at grade 2 and grade 4 showed MHC class II⁺ cells in lesions and notably within the surrounding parenchyma, which corresponded to CD11b⁺ (i.e. macrophages/microglia) cells (tsF treated mouse at grade 2 shown, Figure 7.3a, b). However, there was little or no positive MHC class II Ag staining in the IL-10.tsF-treated group at grade 2 on resident cells within the parenchyma compared to CD11b⁺ cells, although MHC class II⁺ cells were observed on infiltrating cells in perivascular lesions (Figure 7.3c, d).

7.3.5. IL-10 gene therapy induces a shift in the CD4⁺:CD8⁺ T cell ratio and induces B cell infiltration.

A previous immunocytochemical study in the ABH mouse during the evolution and resolution of acute EAE showed a constant ratio of CD4⁺ to CD8⁺ cells in the spinal cord at approximately 8-9:1 (Allen et al., 1993). Likewise flow cytometry demonstrated an ~8:1 ratio of CD4⁺:CD8⁺ of CNS inflammatory cells in untreated mice (Figure 7.4). In contrast this ratio was reduced to ~4:1 in IL-10.tsF disease stabilised mice (Figure 7.4). In tsF treated mice there appeared to be an apparent increase in the ratio of CD4⁺:CD8⁺ cells (Figure 7.4). However, this was not observed in wax histology sections from tsF treated mice (Figures 7.6, 7.7). This was also supported by immunocytochemistry in frozen spinal cord sections (Figure 7.5). The numbers of CD8⁺ T cells were low in sections from untreated and tsF treated mice yet there was a significant (p < 0.002) increase in the number of CD8⁺ cells present in the parenchyma and especially the meningeal infiltrate of spinal cords taken from IL-10.tsF treated mice stabilised at grade 2 compared with all other groups (Figure 7.6).

CD4⁺ T cells could be detected by immunohistochemistry in cryostat sections of spinal cord in the meninges, parenchyma and perivascular lesions in the spinal cord of SCH and tsF treated mice at grade 4. In both groups the mean numbers of cells per section of CD4⁺ cells were similar, around 200+ cells (Figure 7.5). Spinal cords from untreated
SCH mice and tsF treated mice at clinical grade 2 had comparable numbers of CD4+ cells and less than observed in the same groups at grade 4 (Figure 7.7a, b). In contrast, in IL-10.tsF treated mice at grade 2 showing inhibition of EAE there was a significant increase in the number of CD4+ cells compared to the tsF treated or untreated SCH mice at grade 2 (p < 0.01) (Figure 7.7c). However, these mice had stabilised at grade 2 for three days compared with tsF treated and control SCH mice, which were sacrificed at first signs of grade 2 clinical disease. Therefore they were sacrificed at a timepoint equivalent to that of grade 4 in tsF treated and control SCH mice, which is supported by a similar quantity of CD4+ cells in the spinal cord of grade 4 mice (Figure 7.5).

Spinal cord sections analysed at maximum clinical severity from untreated SCH and tsF treated mice had low levels of B220+ cells (Figure 7.5). IL-10.tsF treated mice stabilised at grade 2 exhibited a significantly greater number of B220+ cells present in the meninges and surrounding parenchyma compared to untreated SCH or tsF treated mice at grade 2 and 4 (p < 0.0005) (Figure 7.8).
Table 7.1 Successful CNS-directed IL-10 gene therapy of EAE by IL-10.tsF but not by adenoviral delivery or recombinant IL-10 injection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose</th>
<th>No. EAE</th>
<th>Mean clinical score ± SEM</th>
<th>Mean EAE score ± SEM</th>
<th>Mean day of onset ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>10/10</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>16.5 ± 0.5</td>
</tr>
<tr>
<td>tsF</td>
<td>i.c.</td>
<td>2x10^6</td>
<td>10/10</td>
<td>3.3 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>16.4 ± 0.5</td>
</tr>
<tr>
<td>IL-10.tsF</td>
<td>i.c.</td>
<td>2x10^6</td>
<td>8/10</td>
<td>1.8 ± 0.4**</td>
<td>2.2 ± 0.2*</td>
<td>17.0 ± 0.5</td>
</tr>
<tr>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>7/7</td>
<td>3.1 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>saline</td>
<td>i.c.</td>
<td>-</td>
<td>5/5</td>
<td>3.2 ± 0.4</td>
<td>3.2 ± 0.4</td>
<td>15.6 ± 0.4</td>
</tr>
<tr>
<td>rIL-10</td>
<td>i.c.</td>
<td>2x10^3 ng</td>
<td>7/7</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.4</td>
<td>15.0 ± 0.0</td>
</tr>
<tr>
<td>rIL-10</td>
<td>i.c.</td>
<td>5x10^3 ng</td>
<td>9/9</td>
<td>2.9 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>15.8 ± 0.2</td>
</tr>
<tr>
<td>rIL-10</td>
<td>i.c.</td>
<td>100 ng</td>
<td>8/8</td>
<td>3.0 ± 0.4</td>
<td>3.0 ± 0.4</td>
<td>15.5 ± 0.4</td>
</tr>
<tr>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>7/7</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>14.1 ± 0.3</td>
</tr>
<tr>
<td>rIL-10</td>
<td>i.c.</td>
<td>10ng</td>
<td>6/6</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>13.5 ± 0.2</td>
</tr>
<tr>
<td>rIL-10</td>
<td>i.c.</td>
<td>1ng</td>
<td>5/5</td>
<td>4.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>SCH</td>
<td>-</td>
<td>-</td>
<td>6/6</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>13.7 ± 0.7</td>
</tr>
<tr>
<td>AdRl</td>
<td>i.c.</td>
<td>5x10^6 pfu</td>
<td>7/7</td>
<td>3.7 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>14.6 ± 0.6</td>
</tr>
<tr>
<td>AdRl10</td>
<td>i.c.</td>
<td>5x10^6 pfu</td>
<td>7/7</td>
<td>4.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>14.6 ± 0.3</td>
</tr>
<tr>
<td>AdRl10</td>
<td>i.c.</td>
<td>5x10^5 pfu</td>
<td>6/6</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>13.8 ± 0.2</td>
</tr>
<tr>
<td>AdRl10</td>
<td>i.c.</td>
<td>5x10^4 pfu</td>
<td>6/6</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>13.8 ± 0.3</td>
</tr>
</tbody>
</table>

Mice were inoculated with SCH in CFA on day 0 and day 7. Mice were either untreated (SCH) or injected i.c. day 12 p.i. with either 2x10^6 immortalised fibroblasts (tsF), tsF producing IL-10 (IL-10.tsF), adenovirus coding for IL-10 (AdRl10) or Lac Z (AdRL), recombinant murine IL-10 (rIL-10) or saline. The table shows the results of four separate experiments. The results represent the number of mice which developed EAE, the mean clinical score ± SEM of each group, the mean EAE score of each group ± SEM and the mean day of onset of EAE ± SEM.

* p < 0.05 compared to tsF treated or SCH mice

** p < 0.005 compared to tsF treated or SCH mice
Figure 7.1 Delivery of IL-10 from IL-10.tsF implanted into the CNS prior to disease onset inhibits EAE.

Mice were inoculated with SCH on day 0 and day 7. Mice were either untreated (SCH) (○) or injected i.c. day 12 p.i. (✓) with either 2x10⁶ immortalised fibroblasts (tsF) (●) or tsF producing IL-10 (IL-10.tsF) (▼). The data shows the inhibition of EAE in mice treated with IL-10.tsF cells (mean clinical score = 1.8 ± 0.4) compared to tsF treated (mean clinical score = 3.3 ± 0.3) or untreated SCH mice (mean clinical score = 3.9 ± 0.1). The results represent the mean clinical score ± SEM of all animals from three experiments pooled together (n=20-30).
Figure 7.2 Treatment of EAE with IL-10.tsF reduces the number of perivascular lesions in the lumbar region of the spinal cord

Mice were inoculated with SCH on day 0 and day 7. Mice were injected i.c. with $2 \times 10^6$ tsF or IL-10.tsF or were untreated (SCH). Lumbar region spinal cord was removed from untreated SCH mice and tsF treated mice at maximum clinical disease and from mice treated with IL-10.tsF at an equivalent time-point where they experienced grade 2 clinical disease. Spinal cord was wax processed for histological assessment of mean lesion number/section ± for each group. Groups consisted of 6 mice. Sections were fixed in 10% formal saline and serially sectioned. (a) and (b) show the accumulation of perivascular inflammatory infiltrate typically seen in spinal cord of untreated mice or tsF treated mice with maximum clinical disease. In contrast, (c) shows the increased inflammatory infiltrate seen in spinal cord from IL-10.tsF treated mice at an equivalent time-point to those of untreated and tsF treated mice. Although there is increased infiltrate present in the IL-10 treated spinal cords, it is distributed randomly in the parenchyma, is not associated to perivascular regions and does not form discrete lesions. (d) demonstrates a large number of inflammatory infiltrate contained within the meninges from IL-10.tsF treated animal. (Magnification x100).
Figure 7.3 Inhibition of EAE by IL-10.tsF implanted into the CNS is associated with a decrease in MHC class II antigen expression on resident CNS cells but not on infiltrating immune cells.

Mice were inoculated with SCH on day 0 and day 7. Mice were injected i.c. with $2 \times 10^6$ tsF or IL-10.tsF. Lumbar region spinal cord was removed from tsF treated mice and IL-10.tsF treated mice at grade 2 and was frozen for cryostat sectioning. Spinal cord sections were analysed by immunohistochemistry for the presence of the H2-A antigen (OX-6) using a FITC conjugated antibody and CD11b using a peroxidase conjugated secondary antibody developed with DAB chromogen. Figure (a) shows OX-6 positive cells both on parenchymal (→) and infiltrating cells (↗) from tsF-treated mice. Figure (b) demonstrates CD11b⁺ cells present on infiltrating cells (↗) and on parenchymal cells (→) in a similar distribution to the H2-A positive cells in (a). Figures (c) and (d) show spinal cord from IL-10.tsF treated mice. Although CD11b⁺ cells are present both in perivascular lesions (↗) and on parenchymal cells (→) (Figure d), IL-10 treatment of EAE is associated with a decrease in MHC class II activity on parenchymal cells but not on infiltrating cells (↗) (Figure c). Groups consisted of 4 mice. Magnification x100.
Figure 7.4 IL-10.tsF therapy shifts the ratio of infiltrating CD4⁺:CD8⁺ T cells in the spinal cord of mice with EAE

Biozzi ABH mice were inoculated with SCH day 0 and 7. Mice were either untreated (SCH), or injected i.c. with 2x10⁶ tsF or IL-10.tsF day 12 p.i. At clinical grade 2 spinal cords were removed from untreated SCH mice, tsF treated mice and IL-10.tsF treated mice and T cells were isolated by Percoll gradient centrifugation. Isolated T cells were stained with antibodies to CD4 (TC conjugated) or CD8 (PE conjugated) and analysed by FACS. EAE mice at grade 2 had a CD4:CD8 ratio of 8:1 as seen previously (Allen et al., 1993). However, IL-10.tsF treated mice had a lower CD4:CD8 ratio, which corresponded to the increase in CD8 cells by wax histology (Figure 7.5). Groups consisted of 3-5 mice.
Figure 7.5 Inhibition of EAE by CNS IL-10-tsF gene therapy is associated with a significant increase in CD8+ and B220+ cell types

Mice were inoculated with SCH on day 0 and day 7. Mice were injected i.c. with 2x10^6 tsF or IL-10.tsF. Lumbar region spinal cord was removed from untreated SCH mice, tsF and IL-10.tsF treated mice and was frozen for cryostat sectioning (n=4-5). Sections were analysed for the presence of CD4+, CD8+ and B220+ cells by immunohistochemistry. Figure 4 shows the mean number of each cell type per section from untreated SCH, tsF treated or IL-10.tsF treated mice at grade 2 (SCH2, tsF2, IL-10.tsF2) or control SCH or tsF treated mice at grade 4 (SCH4, tsF4). In the spinal cord of IL-10.tsF treated mice at grade 2, there were significantly greater numbers of CD8+ and B220+ cells, compared to untreated and tsF treated mice both at grade 2 and grade 4.

* p < 0.002 compared to control SCH and tsF treated mice.

** p < 0.0005 compared to control SCH and tsF treated mice.
Figure 7.6 IL-10.tsF implanted in the CNS of mice induce an increase in CD8⁺ T cell numbers

Biozzi ABH mice were inoculated with SCH day 0 and day 7. Mice were untreated (SCH), or injected i.c. with 2x10⁶ tsF or IL-10.tsF day 12 p.i. (n=3-4) Mice were sacrificed at clinical grade 2 and cryostat sections cut from the spinal cord. Sections were analysed for the presence of CD8⁺ cells by immunohistochemistry. Figure 7.6 shows CD8⁺ cells in the spinal cords from IL-10.tsF treated mice present in the parenchyma (a) (→) and also in the meninges (b). In contrast, in spinal cord taken from untreated (c) or tsF treated (d) mice fewer CD8⁺ T cells were observed. Sections were not counterstained. Magnification x125.
Biozzi ABH mice were inoculated with SCH day 0 and day 7. Mice were untreated (SCH), or injected i.c. with 2x10^6 tsF or IL-10.tsF day 12 p.i. (n=3-4). Mice were sacrificed at clinical grade 2 and cryostat sections cut from the spinal cord. Sections were analysed for the presence of CD4^+ cells by immunohistochemistry. (a) and (b) show CD4^+ cells in the spinal cords from SCH and tsF treated mice present in the parenchyma (→) and also in perivascular lesions (⇒). (c) demonstrates an equal number of CD4^+ cells throughout the parenchyma (→→) of spinal cords of IL-10.tsF treated mice but not associated to perivascular regions. Sections were not counterstained. Magnification x125.
Biozzi ABH mice were inoculated with SCH day 0 and day 7. Mice were untreated (SCH), or injected i.e. with 2x10^6 tsF or IL-10.tsF day 12 p.i. (n=3-4). Mice were sacrificed at clinical grade 2 and cryostat sections cut from the spinal cord. Sections were analysed for the presence of B220⁺ cells by immunohistochemistry. (a) and (b) show very few B220⁺ cells in the spinal cords from SCH and tsF treated mice. (c) however shows a greater number of B220⁺ cells in the spinal cords of IL-10.tsF treated mice. Sections were not counterstained. Magnification x125.
7.4. DISCUSSION

This study has shown that IL-10 is a potent immunoregulatory cytokine, which can inhibit EAE when expressed constitutively from syngeneic fibroblasts injected intracranially. IL-10 has been shown to have a half-life of around 2 h \textit{in vivo} (Li et al., 1994) and a previous study administering large quantities of IL-10 frequently, showed that EAE could be inhibited when IL-10 was administered systemically during the priming phase of EAE (Rott et al., 1994). Therefore, gene therapy can be used to administer IL-10 long-term from a single injection to inhibit EAE. This study compared the delivery of IL-10 to the CNS by direct i.e. injection of protein, adenovirus or retrovirally-engineered fibroblasts to inhibit EAE using consistent methods in the same model. Using these approaches, disease was only modulated using the retrovirally delivered product.

Injection of a wide dose range (2x10^5 – 1 ng/ml) of recombinant IL-10 protein into the CNS prior to disease onset had no effect upon EAE disease course or severity, possibly due to lack of sustained effect, as it was not feasible to perform repeated i.c. injections, and the rapid clearance of the protein. In Chapters 4 and 5, IL-10 was delivered into the CNS either by plasmid DNA-CLC or by adenovirus in order to treat EAE under the same conditions as this study. In both cases IL-10 treatment was unsuccessful. Chapter 4 demonstrated that plasmid DNA-CLC gene therapy induces limited expression of transgene over a short period of time and has no effect upon EAE. The short half-life of IL-10 and low level expression may not be enough to inhibit the onset of EAE. Likewise, a bolus i.c. dose of recombinant IL-10 protein had no effect upon EAE disease course or severity, possibly due to either too large a dose or lack of sustained effect due to a short half-life of the protein. In contrast, Chapter 5 demonstrated that adenovirus can produce a large quantity (high ng-μg/ml) of transgene over a two week period prior to its elimination by the immune response (Reichel et al., 1998). The sudden increase of IL-10 on such a scale may induce endogenous feedback mechanisms in order to limit the unphysiological levels of IL-10 in the CNS. IL-10-expressing tsF have been shown to produce levels of IL-10 in the low ng/ml range \textit{in vivo} and the study in Chapter 6 demonstrated effective concentrations of transgene expression for at least 5 weeks post-implantation. This suggests that the level of IL-10 produced by the gene vector may be critical in determining whether IL-10 gene therapy
is successful. Therefore the adenoviral titre was reduced to produce similar levels of IL-10 in the CNS in vivo as the IL-10.tsF. However EAE was still not inhibited. Adenoviral delivery of viral IL-10 has been effective in models of collagen-induced arthritis, but in this circumstance was delivered systemically, where the environment may not be as sensitively regulated as the CNS and where the volume of body compartments will reduce the level of IL-10 reaching the target organ (Apparailly et al., 1998; Ma et al., 1998). Therefore the success of the retroviral expressed IL-10 may lie in the duration and level of viral promoter expression and the physiological concentrations of IL-10 produced over a long time period rather than a sudden increase of high levels of IL-10 as seen with adenovirus (Chapter 5) and large bolus protein administration (Chapter 4).

The difference in efficacy of IL-10 delivered by immortalised fibroblasts (tsF) in EAE compared to other vectors may be due to synergy between IL-10 and an unknown factor secreted by the tsF. Although we have not detected IL-4, IL-10 or IFN-β in cultured tsF these cells produce 2.1ng TGF-β/ml/5 x 10^6 cells/24h. TGF-β has been shown to inhibit EAE (Racke et al., 1991). The IL-10.tsF likewise produced 0.9 ng TGF-β/ml/5 x 10^6 cells/24h. It was not possible to examine the effect of neutralising IL-10 from IL-10.tsF with IL-10-specific mAb, as these can induce mortality in ABH mice when injected i.c. (Chapter 4). Similarly the use of antibodies to TGF-β would not only neutralise the TGF-β from the tsF but also endogenous TGF-β in the CNS. Therefore we investigated the co-administration of non-transduced tsF and AdRIL10 injected i.c. day 12 p.i. to study the possible synergy, yet this failed to affect the disease course. While this may indicate that synergy of factors such as TGF-β produced by tsF with IL-10 may not be the cause of the increased efficacy of tsF delivered IL-10, it is also possible that the effect depends on the nature of how IL-10 affects the intracellular function of the cell vector itself. In addition, the distribution of adenoviral infection of the CNS (Figure 5.5) may lead to the expression of IL-10 in a different region to the endogenous substances released by the tsF cells which remain relatively stationary around the site of injection and surrounding parenchyma and meninges (Figure 6.4). Further studies using IL-10.tsF produced from TGF-β knockout mice may help resolve this issue.

A recent study using transgenic mice constitutively producing human IL-10 (400-700
Chapter 7 Discussion

pg/ml in serum) demonstrated protection from induction of EAE (Cua et al., 1999). Mice failed to show histological evidence of CNS infiltration, which was associated with suppression of auto-reactive Th1 cell function (Cua et al., 1999). In contrast to the transgenic model, this study used a retroviral system to deliver murine IL-10 when T cells had already been primed. Unlike the transgenic model, production was restricted to the CNS compartment, and clinical disease was initiated but ameliorated to a less severe form.

Long-term delivery of IL-10 to the CNS also allows the study of the mechanism by which IL-10 may inhibit EAE at the target organ rather than the effects of IL-10 on peripheral lymphoid organs seen by systemic IL-10 administration. This was reflected by a different histological profile in the CNS, although a large number of inflammatory cells accumulated within the meninges and underlying tissue. It was evident that microglial activation had not been initiated, as represented by the upregulation of MHC class II antigens, in animals injected with IL-10.tsf. This may reflect a direct action of cytokine inhibition of parenchymal microglia/macrophage function, or that the release of pro-inflammatory T cell products such as IFN-γ, which is a potent up regulator of adhesion, costimulatory molecules and MHC class II antigens was prevented thus limiting further expansion of the lesion. Furthermore IL-10 may also regulate other molecules such as metalloproteases, which may be required for extravasation across the vessel basement membranes and into the parenchyma (Cuzner et al., 1999). In addition, IL-10 has also been shown to inhibit inflammatory cytokines (Fiorentino et al., 1989), inhibit costimulatory molecules on APC and IL-1 and TNF-α induced ICAM-1 expression on glial cells (Shrikant et al., 1995) as well as down regulating IL-2 transcription factors c-fos and c-jun in CD4+ cells which directly inhibits proliferation of T cells (Perrin et al., 1999).

There was a significant increase in frequency of CD8+ T cells and B cells in IL-10.tsf treated mice. This supports previous work which has shown that IL-10 can promote the growth of activated CD8+ cells (Groux et al., 1998; Chen et al., 1991) and that increased pancreatic infiltration of CD4+, CD8+ T and B cells were observed in an IL-10 transgenic model in NOD mice which led to an earlier onset of diabetes (Wogensen et al., 1993; Wogensen et al., 1994). The relative increase in CD8+ cells compared to CD4+ cells after IL-10 treatment was demonstrated by the reduction in the ratio of
CD4\(^+\):CD8\(^+\) cells in mice treated with IL-10.tsF as determined by FACS analysis. IL-10 is known to inhibit Ag-specific proliferation and cytokine production from CD4\(^+\) cells and this suggests that IL-10 may differentially regulate CD4\(^+\) and CD8\(^+\) cells. IL-10 has also been shown to down-regulate CD80 and CD86 on monocytes, therefore the greater increase of CD8\(^+\) cells in the CNS may reflect different requirements for costimulation between the two cell types (Ding et al., 1993). Further study of the IL-10-induced infiltration of immune cells into the CNS may suggest the involvement of "regulatory cells" in the inhibition of EAE.

Studies have shown that IL-10 can inhibit the in vitro chemotactic response of chemokines such as RANTES and IL-8 (Tan et al., 1995), as well as mRNA expression of IFN-\(\gamma\)-inducible protein (IP)-10 in mouse peritoneal macrophages (Tebo et al., 1998), and monocyte chemoattractant protein (MCP) -1, macrophage inflammatory protein (MIP) -1\(\alpha\) and MIP-1\(\beta\) mRNA from rat astrocytes (Guo et al., 1998) which are responsible for migration of neutrophils, T cells and monocytes (Merrill et al., 1996). However, IL-10 has also been shown to upregulate expression of the human \(\beta\) (CC) chemokine (HCC-4), which has chemoattractant properties for monocytes (Hedrick et al., 1998). The possibility of IL-10 stimulating the release of other chemoattractants for cells not usually observed in high numbers in EAE infiltrates, such as B cells and CD8\(^+\) T cells, may partially account for the increase in cellular infiltrate seen in IL-10.tsF treated mice. In addition, IL-10 may also regulate other molecules such as metalloproteases, which maybe required for extravasation across the basement membranes and into the parenchyma. Taken together, the effects of IL-10 on metalloproteases and chemokines suggests that IL-10 is an important regulatory factor in cellular extravasation and migration through the CNS. In addition, IL-10 has been shown to enhance endothelial cell VCAM-1 (CD105) expression (Fiehn et al., 1997) which binds to ligand \(\alpha 4\beta 1\) integrin, VLA-4 (CD49d), and is involved in cell adhesion to endothelial cells in microvessels during inflammatory episodes.

Despite greater numbers of infiltrating cells in the spinal cord IL-10.tsF treated mice had a lower mean lesion number compared with tsF treated and untreated mice at clinical grade 2 (impaired righting reflex). Interestingly, mice from IL-10.tsF and tsF treated groups at grade 1 exhibited lesions and demonstrated the presence of perivascular lesions at an early stage of EAE supporting the theory that the
inflammatory process is an important initial stage in the pathology of EAE and MS.

These results contrast with other studies of IL-10 treatment for EAE where little or no inflammatory infiltrate was observed in the CNS of treated mice after IL-10 treatment (Rott et al., 1994; Xiao et al., 1998). However, as IL-10 has a short half-life, the protein used in these studies would only be effective in the region of hours and this may account for the difference between studies. In addition, these studies have investigated the effect of IL-10 on peripheral lymphoid tissue during priming and may represent the reduced antigen-specific proliferation of T cells via the down-regulation of MHC class II molecules on APC (de Waal Malefyt et al., 1991; Rott et al., 1994; Frei et al., 1994). However, in this study, T cell priming in the peripheral lymphoid tissue has already taken place and activated T cells have entered the CNS. This conclusion is supported by the observation that in IL-10-tsF treated spinal cords MHC class II+ infiltrating cells from the periphery are unaffected, whereas resident CD11b+ CNS cells are MHC class II- in contrast to the resident cells in SCH and tsF treated mice. This suggests that the action of IL-10 serves to prevent the local activation of resident microglia.

This study has shown the benefit of local implantation of IL-10 producing immortalised fibroblasts to the CNS to consistently and significantly reduce EAE from a single administration. In addition this study has highlighted the importance of selecting the correct vector for the therapeutic gene to be administered. In the case of cytokine gene therapy little is known about the relative levels of cytokines in vivo and the feedback loops which control production and inhibition, especially in the CNS. Therefore, although in vitro experiments play an important role in determining cytokine function this study highlights the importance for in vivo experiments in animal models to determine the role of cytokines in their local environment.

The increased inflammatory infiltrate observed in IL-10 treated spinal cord highlights one potential limitation associated with cytokine therapy in the CNS. Cytokines often have "pleiotropic" properties and chronic expression may result in many different responses. Although severity of disease is significantly reduced by IL-10 gene delivery, chronic expression of "beneficial" cytokines may result in exacerbation of disease or other pathologies. Through the use of inducible promoters to regulate expression of proteins, it may be possible to investigate this further.
CHAPTER EIGHT

FINAL CONCLUSIONS

&

FUTURE WORK
This study has investigated the use of gene therapy to deliver immunomodulatory agents to inhibit EAE in Biozzi ABH mice, a mouse model for the human disease MS. Little research has been conducted in gene therapy for EAE. The Biozzi ABH model of EAE is very reproducible and well characterised with a predictable time course including acute, remission and relapse phases (Baker et al., 1990). This allows the accurate study of immunosuppressive agents on the pathogenesis of ongoing acute disease as well as the relapse phase, which is more clinically relevant to MS patients with relapsing/remitting MS.

As has been described, one route to study the action of cytokines in EAE has been systemic delivery of bolus proteins or cytokine inhibitors, yet these agents may require long-term administration to be effective. An alternative route has been the generation of transgenic or gene “knockout” mice. However, these mice may have serious defects rendering them physiologically compromised, e.g. TNF-β gene knockout mice do not have normal lymph node architecture (Korner et al., 1997). There is redundancy in the immune system, particularly in relation to cytokines. Therefore for the knockout animal to survive it may compensate for the missing proteins using alternative mechanisms. This may lead to immunological effects unrelated to the EAE process. Furthermore, if the action of the “knockout” affects the general development of T cell responses then animals fail to become primed and therefore show no disease expression (Steinman, L. 1997). This again is a poor representative of the human system where disease is established. Therefore, gene therapy can be utilised to deliver cytokines or cytokine inhibitors to modulate or neutralise cytokines long-term, to treat EAE and dissect possible functions of cytokines in the pathogenesis of EAE.

This study has demonstrated a variety of methods for the delivery of genes coding for Th2-type cytokines or cytokine inhibitors by non-viral plasmid DNA-CLC or by viral vectors administered either in vivo or ex vivo, which can successfully express their therapeutic gene in vivo at therapeutic concentrations to inhibit the severity and time course of EAE.

8.1. Gene Therapy vs Protein Therapy

Generally it was observed in this study that therapeutic agents can be delivered to the CNS by gene vectors at 1000-fold lower concentrations (ng-pg/ml range) than other
studies using systemic bolus protein administration (µg/ml range). Although adenoviral vectors appeared to express therapeutic proteins in the µg/ml range the leakage to the periphery was low and systemic concentrations were considerably lower than seen in the CNS. The greater expression levels of the adenovirus may make it suitable for delivery of neutralising agents rather than cytokines. The study of low dose/long-term delivery of therapeutic agents to the CNS may prove beneficial in long-term immunosuppressive therapies for treatment of human CNS disorders. This strategy may reduce unwanted peripheral immunosuppression and other side effects often associated with large dose systemic administration protein therapy.

8.2. Route of Gene Vector Delivery

The route of administration of therapeutic agents can affect their efficacy in treating CNS disorders. The delivery of 5 µg/ml CTLA4-mIg protein administered to the CNS had the same inhibitory effect on EAE as 80 µg/ml delivered systemically. This principle is important to gene therapy as therapeutic agents will be expressed long-term and therefore there is potential for unwanted side effects due to systemic toxicity. The studies described here using plasmid DNA-CLC, adenovirus or retrovirally infected immortalised fibroblasts (tsF) all clearly showed that local administration of vectors to the CNS greatly increased the efficacy of the therapeutic agents expressed in inhibiting EAE. The study in Chapter 5 using CTLA4-hIg demonstrated that direct injection to the CNS of the protein was less effective in inhibiting EAE compared to adenoviral delivery. The long-term expression of the adenoviral delivered CTLA4-hIg may be self-protective and reduce the immune response to the hIg backbone of the fusion protein compared to the single protein injection where the fusion protein will be metabolised and will no longer inhibit the immune response to itself.

In addition, this study has demonstrated that adenoviral delivery of a therapeutic concentration of CTLA4-hIg locally to the CNS does not induce peripheral immunosuppression and that the peripheral lymphoid organs can still mount immune responses. Studies of the relative CSF and serum levels following CNS injection of AdCTLA or immortalised fibroblasts expressing dTNFR or IL-10, demonstrate a significantly higher CNS concentration of gene product than in the serum. The ineffectiveness of CNS adenoviral-delivered CTLA4-hIg to inhibit general systemic
immune responses should be a requirement for human gene therapy to treat disorders of the CNS so as not to leave patients severely immunocompromised and to reduce systemic toxicity.

Blockade of costimulation has proved of little therapeutic use in the effector phase of EAE (Arima et al., 1996; Perrin et al., 1995; Steinman et al., 1981). This may be due to a reduced requirement of costimulation by the effector cells. A study has shown that myelin specific T cells from MS patients have a decreased CD28-dependance for costimulation \textit{in vitro} (Scholz et al., 1998). However, the large molecular weights of therapeutic agents such as CTLA4-Ig fusion proteins administered systemically may limit entry to the CNS across the BBB. Therefore in late stage disease where T cells have entered the CNS these fusion proteins may have a reduced efficacy in disease inhibition. Blockade of costimulation in the CNS by CNS adenoviral delivery of CTLA4-hIg (Chapter 5) demonstrates that primed effector cells require further costimulation once they enter the CNS to induce EAE. This highlights the importance of directing the therapy to the target organ of disease and provides a more accurate analysis of the CNS mechanisms involved in EAE.

8.3. Timepoint of Gene Vector Delivery

Many studies of therapeutic agents which successfully inhibit EAE, have proven of limited value in clinical situations. Generally previous EAE studies have concentrated on treatment of the induction phase of disease, whereas treatment during the effector phase or remission may have more value in predicting the outcome of therapies in the clinic, particularly as in MS patients disease is ongoing and advanced.

This study has demonstrated that retrovirally infected fibroblasts producing p75 dTNFR, administered to the CNS during remission (day 28 p.i.), can inhibit the relapse phase of mice which have already experienced an acute attack of EAE. In addition when p75 dTNFR producing fibroblasts were administered to the CNS at onset of clinical signs both the acute phase and subsequent relapse phase were significantly inhibited. Furthermore, a number of treated mice did not relapse within the period observed (up to 45 days p.i.). Prior to disease onset the timepoint of administration did not affect the efficacy of p75 dTNFR producing fibroblasts to inhibit EAE.
8.4. Comparison in the Kinetics of Vector Expression

Chapter 4 demonstrated that plasmid DNA coding for Th2 cytokines and the TNF antagonist especially, p75 dTNFR, complexed to cationic liposomes can express sufficient gene product to inhibit EAE. However, expression was limited to 6 days and few p75 dTNFR-transduced cells were observed in the CNS following i.c. injection. Systemic i.m. injection in contrast had no effect upon the severity of EAE and a plasmid coding for β–galactosidase showed that only a few muscle fibres along the route of injection were transduced. The use of a myoproliferative agent 5 days prior to DNA injection had no effect on the efficiency of DNA transduction in muscle. In comparison to plasmid DNA-CLC, adenoviral delivery had a greater transfection efficiency as well as increased production of transgene for a greater duration, making this a more suitable vector for gene delivery to the CNS. However, adenovirus has been shown to be highly immunogenic and this raises the question of its suitability in treatment of human diseases. Repeated administration of adenovirus may be necessary as the transgene exists epichromosomally and therefore production will be reduced with time due to immunological memory neutralising the viral vector. Repeat administration may increase potential immune responses in the CNS which may exacerbate disease. Second generation adenoviral vectors with further deletion of viral genes, may overcome the disadvantage of adenoviral vectors.

However, a recent report studying the use of “E1-deleted, E2A temperature-sensitive” adenovirus delivery of ornithine transcarbamylase (OCT) to the liver of patients with OCT deficiency resulted in the death of the patient (Lehrman, S. 1999). A huge dose (3.8 x 10^{13} PFU) of adenovirus was delivered to the liver in a condition where an inflammatory response would cause severe complications, as adenovirus infected cells would become the target of cytotoxic T cells and would be destroyed. It has been reported in the literature that replication-deficient adenovirus can induce inflammatory responses (Ferry and Heard. 1998; Juillard et al., 1995; Yang et al., 1994), therefore this disregard for previous studies may hinder the further use of in vivo viral gene therapy, especially adenovirus, in the clinic.

The use of retroviral vectors in Chapters 6 and 7, to infect a cellular “vehicle”, provided a more stable gene delivery which could express the gene product for at least up to 5
weeks *in vivo*. Using this vector system, immortalised fibroblasts genetically engineered to produce dTNFR, when implanted into the CNS could significantly inhibit EAE in both the acute phase and more clinically relevant relapse phase. Cells implanted into the CNS prior to the acute phase inhibited EAE to the same degree as cells implanted during remission suggesting that production of dTNFR remains at therapeutic levels *in vivo* throughout the 5 weeks observed. In addition, immortalised fibroblasts retrovirally engineered to express murine IL-10 also had a stabilising effect upon EAE severity when implanted in the CNS during active disease. Interestingly, the injection of recombinant IL-10, plasmid DNA-CLC or adenovirus expressing IL-10 into the CNS at the same time point had no effect on disease severity. It is likely that the tsF cells produce many other substances and that these may synergise with the retroviral-coded IL-10 to increase its efficacy. This however, is also a possibility when cells are infected with any of the other gene therapy agents. ELISA of tissue culture supernatant from tsF cells for the presence of cytokines demonstrated a lack of detectable levels of IL-4, IL-10, IFN-γ and IFN-β, with only TGF-β present. However, co-injection of adenovirus coding for IL-10 and non-transduced tsF demonstrated no activity in EAE suppression suggesting that synergy between IL-10 and an endogenous factor from the tsF was not responsible for the disparity between studies. The quantity and duration of expression of IL-10 was different between the vectors used in this study and may be critical in determining the efficacy of IL-10 in EAE inhibition as discussed in Chapter 7.

8.5. Cytokine Gene Therapy For EAE

The main limitation of cytokine therapy at present is that it is difficult to predict the effect of a cytokine or cytokine inhibitor *in vivo* as cytokine interactions are local, temporally-regulated and their pleiotropic nature can produce conflicting data in animal studies. The balance of cytokines present, location and concentration produced as well as the cell types present, are all variables in how cytokines exert their effects. Another potential problem for cytokine therapy is the over-expression of a particular cytokine inducing negative feedback loops. The IL-10 study demonstrated the importance understanding the mode of action of the therapeutic agent and of selecting the correct vector for its delivery. Many previous studies of cytokine therapy in EAE have resulted in contradictory reports, which are probably due to different species and strains of
animals, different IL-10 preparations and delivery methods. Therefore, chapter 7 studied four different methods of IL-10 delivery in the same model using identical protocols for an accurate comparison of vector delivery. Only retroviral delivery of IL-10 from immortalised fibroblasts inhibited EAE. Kinetics of delivery from this vector were characterised by low-level expression over 5 weeks. In contrast, adenoviral vectors and direct injection of bolus protein resulted in high CSF concentrations of IL-10 over a shorter period. Plasmid DNA-CLC expressed very low transfection efficiency in vivo and the few cells transfected expressed transgene for 3-6 days. Therefore, with cytokine therapy the concentration and duration administered may be vital in determining its success. Currently, the most significant approach to cytokine therapy in MS has been the long-term use of IFN-β (Paty, D. W. and Li, D. K. 1993).

Therefore the optimal vector for gene therapy depends upon the product to be delivered. In the case of neutralising agents such as anti-TNF molecules or CTLA4-Ig adenoviral-type delivery may be the vector of choice as these agents work in a dose-dependent way. However, until the function and control of cytokines is better understood the delivery of cytokines to the CNS may be unpredictable. Gene delivery of cytokines and cytokine inhibitors can be used to study the involvement of the cytokine network in the pathogenesis of EAE. The use of regulatable promoters may provide a more controlled system to study the effect of cytokines in vivo.

It may be that the inhibition observed by cytokine therapy in these studies represents the greatest amount of inhibition achievable with cytokine therapy in EAE. Few studies investigating cytokine therapy of EAE have shown complete inhibition of disease and certainly not when administered after the acute phase of disease. The down-regulation or neutralisation of some pro-inflammatory cytokines does not exclude other cytokines with over-lapping functions to partially correct for this loss, so-called "cytokine redundancy". To overcome this, multiple cytokines and cytokine inhibitors could be employed. For example, retrovirally-engineered fibroblasts producing IL-10 and p75 dTNFR could be combined easily for example and together may cause greater inhibition of disease.

We have shown that fibroblasts produce endogenous factors such as TGF-β, and whilst this study did not demonstrate synergy between transgene products and endogenous
products this may be a possibility and may increase or decrease the efficacy of transgene products. Therefore the characterisation of other cell types, such as astrocytes, for gene therapy may allow not only delivery of transgene but also growth and repair factors which may aid repair of damaged neurons in EAE and trophic factors to aid migration of oligodendrocytes to areas of inflammation. In addition this gene therapy strategy could be used to deliver other agents into the CNS to inhibit EAE, such as anti-CD4 antibodies, which have been shown to completely abrogate EAE (O’Neill et al., 1993).

Recent reports have described the use of gene therapy to treat EAE using hybridoma cells and myelin antigen-specific T cells to deliver IL-10, IL-4 or TGF-β (Mathisen et al., 1997; Shaw et al., 1997; Chen et al., 1998). This method has the potential to target delivery of cytokines to the CNS by less invasive systemic administration as it uses the inherent trafficking potential of the lymphocytes. However, numbers of circulating cells entering the CNS after i.v administration have been shown to be low and therefore the concentration of cytokine delivered to the CNS cannot be predicted (Brennan et al., 1999). Activated CNS-antigen specific T cells are not limited to the CNS and could express their transgene throughout the systemic compartments if the cells are activated in vitro prior to injection. These cells require long-term expansion in vitro and may be pathogenic. In addition, hybridoma cells will continue to proliferate in vivo and therefore may deliver increasing quantities of transgene product as well as initiating potential tumour formation. A more sophisticated targeting system has recently been described. It is unlikely that large quantities of self-reactive T lymphocytes specific for myelin antigens will be isolated from patients for use to deliver therapeutic genes into the CNS. However, autologous T cells could be isolated and engineered to confer an antibody–type specificity to CNS myelin antigens. These “T-bodies” are described as the fusion between variable domains of a specific antibody to the TCR signalling molecules of the CD3 complex of a T lymphocyte (Annenkov et al., 1998; Chernajovsky, Y. 1999). A T cell chimaera specific for a CNS antigen will only become activated upon presentation of that antigen but does not have an MHC restriction. In addition, the engineered T cell has a reduced response to its cognate antigen, which may reduce potential activation of pathogenic T cells. Such methods could be used to engineer any T cell and confer CNS specificity thus reducing the requirement for in vitro stimulation. Importantly, these cells could be deleted by co-expressed suicide
genes such as HSV-thymidine kinase, where activation of the T-body will cause proliferation which is necessary for ganciclovir-mediated apoptosis.

8.6. Gene Therapy for MS?

The future of gene therapy for MS may be the use of genetically modified cells producing therapeutic agents encapsulated in a semi-permeable membrane, which allows the implantation of cells to the lumbar intra-thecal space, by a minor operation (Aebischer et al., 1996) and removes many of the potential problems inherent in most other gene delivery protocols. This method allows the outward diffusion of therapeutic transgene products, the inward diffusion of growth factors for cells and prevents potential limiting immune responses to implanted cells. Through use of selective pore sizes these membranes can exclude antibodies and immune cells. Using this protocol ciliary neurotrophic factor has been detected in the CSF of amyotrophic lateral sclerosis patients up to 17 weeks (Aebischer et al., 1996). Therefore, the first generation gene therapy studies described in this study could provide data for trials using this encapsulated delivery vehicle to treat a variety CNS inflammatory disorders. In cases of adverse events encapsulated cells can be removed easily, and where therapy is successful, cells can be used to reduce inflammation in the CNS and during remission, cells can be switched to deliver nerve growth and repair factors. It would also be possible to deliver a variety of different therapeutic agents by mixing cells within the polymer capsules. Using this method to treat MS patients would seem advantageous rather than direct delivery of genetically engineered cells or virus to the CNS. In addition, different therapeutic genes delivered could be administered according to the patients' individual requirements, as MS symptoms may be due to different arms of the immune system (Lucchinetti et al., 1998). In addition, immunomodulatory agents could be delivered during relapse phases of disease and nerve repair factors delivered during the remission phases.

This study has developed first generation gene therapy techniques, which in their present form would not be used therapeutically. Hopefully it will provide the basis upon which further, more advanced techniques can be built upon with the emphasis on suitability to the patients to be treated. As it has been established that local gene therapy can be beneficial a further study investigating a variety of other logical potential therapeutic candidates, different cell types relevant to human therapy, promoter
regulation, suicide genes, and specific cell targeting can be undertaken. Once the same level of benefit can be achieved with these regulatory and safety factors in place then gene therapy may be used to treat not only MS, but other human CNS disorders.
8.7. Future prospects for gene therapy of EAE

Currently constructs are being produced coding for CD4 antibodies as well as anti-CD4 F(ab) and F(ab)² antibodies. These will be cloned into retroviral plasmids and used to infect both immortalised astrocytes and fibroblasts. Previous treatments using systemic administration of CD4 antibodies have shown complete abrogation of EAE. It is hoped that using these new constructs delivered locally to the CNS that the same degree of inhibition can be achieved. In addition, astrocyte and fibroblast delivery will be compared to determine if there is any added benefit to transgene delivery using cells endogenous to the CNS. Preliminary protein therapy using CD4 agents administered to the CNS has shown inhibition of EAE. In addition, plasmid DNA-CLC coding for anti-CD4 Fab fragments injected day 12 p.i. has shown a delay in the onset of EAE.

A CTLA4-mouseIg construct with a mutant complement fixing and Fc receptor binding site is currently being cloned into a retroviral vector for infection of astrocytes and fibroblasts. This construct will not opsonise for macrophages or induce complement lysis. The use of this construct in studies similar to those in Chapter 5 may clarify the earlier work on EAE using 2 different isotypes of CTLA4-Ig. Any inhibition of disease will be due to specific blockade of the CD28:B7 pathway and not due to potential cell deletion.

Splenocytes from an MBP transgenic mouse will be infected with retrovirus coding for p75 dTNFR, IL-4, TGF-β or IL-12 p40 homodimer. These will then be administered systemically to treat passive EAE but should target the CNS due to their T cell receptor specificity. Preliminary studies have demonstrated the efficacy of IL-4 and TGF-β retrovirally-engineered splenocytes administered i.v. to inhibit EAE.

The production of immortalised fibroblasts from TGF-β knockout mice may allow optimal dosing of cells to the CNS to deliver the transgene, without the possibility of TGF-β having an effect on disease.

IL-10 producing fibroblasts will be administered during remission to inhibit subsequent relapses and determine the effect of long term IL-10 therapy on EAE disease course. Analysis of inflammatory cells isolated from the spinal cords of mice treated with IL-10.tsf and p75 dTNFR producing cells may allow further analysis of the mechanisms
by which these therapies work, and insights to the pathogenesis of EAE.

IFN-β producing fibroblasts have been cloned and will be studied to investigate their anti-inflammatory properties in passive EAE as well as their anti-viral properties in Theiler's virus induced EAE.

Fibroblasts producing IL-12 p40 homodimer, IL-4, TGF-β, and soluble complement receptor 1 under MMuLV LTR and CMV promoters have been produced and will also be studied in inhibiting EAE.
Appendix A

Production of immortalised CNS glial cells for CNS gene delivery.

Chapters 6 and 7 have demonstrated the efficacy of retrovirally-infected immortalised fibroblasts to deliver therapeutic proteins to treat EAE. This system is a stable vector system capable of long-term delivery of transgene product and can successfully ameliorate EAE over 2 disease episodes. Chapters 4, 5 and 6 have also shown that local delivery of the therapeutic gene to the CNS is more efficient at treating EAE than systemic delivery, even at lower doses, and can avoid unwanted non-specific peripheral immunosuppression. Previous studies have developed cellular vectors such as fibroblasts and myoblasts for delivery of gene products to the CNS (Fisher et al., 1991; Jiao et al., 1992). In an attempt to optimise the use of cellular vehicles for retroviral infection for CNS gene therapy endogenous CNS cells such as astrocytes may prove to be a more efficient and beneficial vector.

In a primary demyelinating disease such as MS lesions are evident at different time points and with different activities as described in Chapter 1. However, astrocytes are seen in most lesions whether acute, chronic active or even silent (Raine, C.S. 1997). Their role in MS is not clear, however reactive astrocytes are present at the lesion edge as well as in the centre of the lesion and in surrounding white matter parenchyma (Lee and Brosnan, 1997). It is thought that in the early stage of lesion formation astrocytes provide soluble factors, which may facilitate repair and that the lesion centre in chronic lesions consist mainly of astrocytes (Lee and Brosnan 1997). This astrocytic scar may be responsible for the eventual lack of repair and remyelination in the CNS of MS patients and may be a cause of the permanent disability associated with MS.

Astrocytes originate from the CNS and therefore may persist longer in their endogenous micro-environment after transplantation into the CNS, than other cell types such as fibroblasts. In EAE and MS, neuronal dysfunction and eventual loss leads to many of the permanent disabilities seen in patients. Currently astrocytes have been isolated and genetically engineered to produce a variety of soluble factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and tyrosine hydroxylase (TH) for gene therapy of Parkinson’s disease (Castillo et al., 1994; Cunningham et al., 1991; Lundberg et al., 1996).
Six astrocyte clones were derived from Biozzi ABH mouse cerebral cortex which were immortalised with the retrovirus coding for temperature-sensitive SV40 large T antigen allowing astrocytes to be maintained in bulk culture in vitro and were named temperature-sensitive astrocytes (tsA). The different clones were characterised by immunocytochemistry and could be shown to retain their astrocyte markers after immortalisation. Immortalised astrocytes were positive for glial fibrillary acidic protein (GFAP), galactocerebroside (GalC), and the structural protein vimentin, but not A2B5 ganglioside or oligodendrocyte marker O4. GFAP and vimentin staining was present throughout the cytoplasm of the tsA whereas GalC staining was present in discrete vesicles throughout the cell and around the nucleus but not expressed on the cell surface. Vimentin is a structural protein and usually associated with immature astrocytes or reactive astrocytes in vivo (Lee and Brosnan. 1997). Cells were also positive for GalC, a marker usually associated with oligodendrocytes (Raff et al., 1979) and normally not present in type I or type II astrocytes.

This immunohistochemical detection of markers present on astrocytes or oligodendrocytes suggests that the cells isolated in this study may reflect astrocyte progenitors and provide preliminary evidence that astrocytes and oligodendrocytes are derived from the same stem cells or are capable of differentiating along either pathway at an early stage of development. The different morphology of the tsA clones isolated here may reflect astrocyte progenitor cells at different stages of differentiation.

Whilst under constant conditions in culture (35°C) these immortalised cells remain in their immortalised phenotype. However, once implanted into the CNS, where murine body temperature (39°C) will switch off the temperature-sensitive immortalising element of the large T antigen. These cells may be responsive to growth and differentiation factors secreted in the micro-environment of the CNS and develop along specific pathways. Astrocytes have been shown to produce a wide variety of growth and differentiation factors, which act on oligodendrocytes, microglia and neurons (Eddlestone and Mucke. 1993), which may be beneficial during EAE inducing survival and repair of neurones and differentiation of oligodendrocytes from their progenitors. A previous study has shown that insulin-like growth factor (IGF)-1 produced by astrocytes correlates to an increase in remyelination in EAE (Yao et al., 1995). If the astrocyte progenitors can be modulated to develop into oligodendrocyte type cells either in vitro by adding different growth factors to the culture medium or in vivo, by factors
secreted in the CNS, then these cells may be useful in repair of damaged neurons during MS and EAE. Previous studies have shown benefit of cultures oligodendrocytes in remyelinating neurons (Groves et al., 1993).

Astrocytes have been shown to stably integrate into the CNS and can migrate throughout the brain (Booss et al., 1991; Jacque et al., 1992) although other studies have shown little migration from the area of transplant (Yoshimoto et al., 1995; Lundberg et al., 1996). Chapter 2 described astrogliosis in the CNS during EAE and may reflect the recruitment of astrocytes from adjacent parenchyma by soluble factors released in the CNS as part of the inflammatory response in EAE. Whilst in some studies this might be seen as a disadvantage in the treatment of EAE this could be beneficial as it would allow genetically engineered astrocytes to deliver therapeutic products directly to the sites of lesions.

Cloned immortalised astrocytes have now been developed with the potential to provide an improved cellular vehicle for delivery of retrovirus coded therapeutic proteins to the CNS with which to treat CNS disorders. Currently these cells are being engineered to express anti-CD4 immunoglobulin fragments for CNS gene therapy of EAE.
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LIST OF PUBLICATIONS
PUBLICATIONS


**ABSTRACTS**


Figure 8.1. Maps of vectors used in this study
### Table 8.1. VECTOR PRODUCTION, CLONING AND EXPRESSION

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<td>IFN-β [NSE]</td>
<td>Prof. Y. Chernajovsky</td>
<td>Dr. K.A. Triantaphyllopoulos</td>
<td>Prof. Y. Chernajovsky</td>
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<td>TNF</td>
<td>Dr. N. Sarvetnick</td>
<td>Dr. R. A. Mageed</td>
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<td>β-galactosidase</td>
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<td>human p75 dTNFR</td>
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<td>Dr. K.A.</td>
<td>Dr. K.A.</td>
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<td>mouse CTLA4-human IgG1</td>
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<td>J. L. Croxford</td>
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<td>AdRIL-10</td>
<td>K. Browne</td>
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<td>Dr. E. Quattrocchi</td>
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<td>AdCTLA</td>
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<td>Dr. A. Byrnes/Dr. M. Dallman</td>
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Dr. K. Moore  
Prof. Y. Chernajovsky  
ATCC (American Type Culture Collection)  
Dr. N. Sarvetnick  
Pharmacia Biotech  
In-Vitrogen  
Prof. B. Beutler  
Dr. G. Larson  
K. Browne  
Prof. M. A. Wood  
Dr. K.A. Triantaphyllopoulos  
Dr. R. A. Mageed  
Dr. R. Neve  
Dr. A. Byrnes  
Dr. M. Dallman  
Dr. E. Quattrocchi  
J. L. Croxford

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Leek, Netherlands  
Southwestern University, Dallas, Texas, USA  
Genetics Institute, Cambridge, MA, USA  
Kennedy Institute of Rheumatology, London, UK  
University of Oxford, Oxford, UK  
Kennedy Institute of Rheumatology, London, UK  
Kennedy Institute of Rheumatology, London, UK  
University of Oxford, Oxford, UK  
Imperial College, London, UK  
Kennedy Institute of Rheumatology, London, UK  
Institute of Ophthalmology, London, UK