Variability in the Immune Response to Theiler’s Murine Encephalomyelitis Virus in Different Strains of Mice

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Declaration
I hereby declare that the dissertation entitled "Variability in the Immune Response to Theiler's Murine Encephalomyelitis Virus in Different Strains of Mice" is not substantially the same as any I have submitted for a degree, diploma or qualification. The dissertation is a result of my own work, and includes nothing which is the outcome of work done in collaboration, except where duly acknowledged.

Acknowledgements
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Finally, I would like to express my deepest gratitude to my wife Alison, my parents and my granny Ina MacLean, without whom none of this would have been possible. I dedicate this thesis to you all.
Summary

Theiler's Murine Encephalomyelitis Virus (TMEV) has been a favoured model for Multiple Sclerosis (MS) since 1975 when Lipton first reported that infection with TMEV caused a biphasic Central Nervous System (CNS) disease leading to demyelination. TMEV is a picornavirus belonging to the cardiovirus genus and is a natural enteric pathogen of mice which can occasionally initiate a chronic persistent infection of the CNS. This depends on the strain and dose of virus and the strain, age and sex of the mouse. Intracerebral infection of all mouse strains with the avirulent BeAn strain of TMEV results in an acute encephalomyelitis which in susceptible mouse strains, is followed by a persistent CNS infection with lesions of inflammatory demyelination or in resistant mouse strains eradication of the virus. On the other hand i.c. infection with the neurovirulent GDVII strain of Theiler's virus results in a fulminant encephalitis in mice of all genetic backgrounds.

The main aim of this study was to determine the cytokine and immunoglobulin profiles elicited in different mouse strains during the acute phase of infection. mRNA transcript levels for numerous cytokines were studied in the brains and spinal cords in Balb/c (resistant), CBA (intermediately susceptibility) and SJL/J (susceptible) mice, during the acute phase of disease, using the technique of RNase protection assay (RPA). The RPA included analysis of transcripts for TNFβ, TNFα, TGFβ, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-12p40 and IL-12p35. There were similarities between the strains in the levels of pro-inflammatory cytokines expressed including TNFα, TNFβ and IL1α. However, there were several fundamental differences between the strains including the inability of susceptible SJL/J mice to express IL-1β in the brain and the spinal cord when compared to Balb/c and CBA mice. SJL/J mice had an increase in expression of IL-4 and IL-10 and a decrease in expression of IL-2 and IFNγ when
compared to Balb/c and CBA mice. Expression of pro-inflammatory, anti-inflammatory, Th1 and Th2 type cytokines correlated with the increase in levels of cellular infiltrates (CD3+, CD4+, CD8+ and F4/80+) in the CNS. Anti-viral immunoglobulin isotypes were also different in the three mouse strains studied. All strains produced similar levels of IgM however, Balb/c mice had significantly increased levels of IgG1 and IgG2a compared to CBA and SJL/J mice during the acute phase of disease.

This study also investigated TMEV persistence in CBA (intermediately susceptible) mice and the cytokine and anti-viral immunoglobulin isotypes associated with persistence. Virus persisted for >60 days in 50% of infected CBA mice, as determined by RT-PCR. Animals in which virus persisted had significantly increased RNA transcripts in the CNS for TNFα, IL-12p35 and IL-12p40. Persistently infected animals also had increased levels of anti-viral IgG1, IgG2a and IgG2b when compared to animals which had cleared the virus.

The importance of interferons α/β and γ were investigated. Virus spread extensively throughout the white matter regions of the brains in IFNα/βR0/0 mice (constructed on a genetically resistant background (H-2b)), during the acute phase of infection, indicating the importance of IFNα/β in preventing infection of oligodendrocytes. Infection of IFNγR0/0 mice (also on a genetically resistant background) resulted in viral persistence and increased levels of anti-viral IgM, IgG1, IgG2a and IgG2b, demonstrating IFNγ is essential for viral clearance. Perforin is a functional effector molecule in CTL killing, therefore, its role(s) during the acute and chronic phase of Theiler's virus infection was investigated to ascertain its importance. Studies in perforin knockout mice (also on a resistant genetic background) demonstrated that perforin is essential to control viral infection during the acute phase of infection, and is an absolute requirement for viral clearance.
Infection with GDVII resulted in high levels of virus replication in the brains and spinal cords of infected mice. Levels of TNFα, IL-1α, IL-2, IFNγ and IL-12p40 increase throughout infection in the brains of infected animals, and TNFα, IL-2 and IL-12p40 increase in the spinal cords. High virus titres, and an increase in the above pro-inflammatory cytokines correlated with an increase in levels of programmed cell death in CNS tissues.

Infection of neonatal mice with the BeAn strain results in 100% mortality, with increased virus titres in the CNS. Expression of TNFβ, TNFα, IL-4, IL-1α and IL-6 increased throughout the course of infection of neonatal mice. TNFα has been implicated in the phenomenon of death by shock. Therefore, TNFα may have important implications in the pathogenesis of Theiler’s virus infection in neonates.
### Abbreviations

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<td>adenosine triphosphate</td>
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<td>BDV</td>
<td>Borna disease virus</td>
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<td>BHK</td>
<td>Baby hamster kidney cells</td>
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<td>B2M</td>
<td>beta two microglobulin</td>
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<td>BS</td>
<td>blocking serum</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cm</td>
<td>centimetre</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CPE</td>
<td>cytopathic effect</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
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<tr>
<td>DAB</td>
<td>3,3' -diaminobenzidine tetrahydrochloride</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dUTP</td>
<td>2'-deoxyribo uridine triphosphate</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EMCV</td>
<td>encephalomyocarditis virus</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>fg</td>
<td>fentigram</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>gram</td>
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<td>GMEM</td>
<td>Glasgow’s modified Eagles medium</td>
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GTP  guanosine triphosphate
HCl  hydrochloric acid
HIV  human immunodeficiency virus
HRPO horseradish peroxidase
HSV  herpes simplex virus
HTLV human T-cell lymphotrophic virus
ic  intracerebral
im  intramuscular
in  intranasal
ip  intraperitoneal
iv  intravenous
Ig  immunoglobulin
IFN interferon
IL  interleukin
ISH in situ hybridisation
kb  kilobase
kDa kilo Dalton
L litre
LCMV lymphocytic choriomeningitis virus
LSB low salt buffer
µg microgram
µl microlitre
µM micromolar
mg milligram
min minute
ml millilitre
mM millimolar
mRNA messenger RNA
M molar
MHC major histocompatibility complex
MHV mouse hepatitis virus
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<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>Mr</td>
<td>molecular weight</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>nanomolar</td>
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<tr>
<td>nu/nu</td>
<td>mice homozygous for the nude mutation (athymic)</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<tr>
<td>NCS</td>
<td>newborn calf serum</td>
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<tr>
<td>NGS</td>
<td>newborn goat serum</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>pH</td>
<td>potential hydrogen</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBSA</td>
<td>PBS with 0.75% bovine serum albumin</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS with 0.05% Tween</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFU</td>
<td>plaque forming unit</td>
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<tr>
<td>PI</td>
<td>post-infection</td>
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<td>PID</td>
<td>post infection day</td>
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<tr>
<td>PIPES</td>
<td>1,4-piperazine diethane sulfonic acid</td>
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<tr>
<td>PRV</td>
<td>pseudorabies virus</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RPA</td>
<td>RNase protection assay</td>
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<tr>
<td>RPM</td>
<td>revolutions per minute</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>RV</td>
<td>rabies virus</td>
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<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>ssDNA</td>
<td>salmon sperm DNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
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<tr>
<td>SSC</td>
<td>standard saline citrate</td>
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SSPE  subacute sclerosing panencephalitis
trRNA  transfer RNA
TMEV  Theiler's murine encephalomyelitis virus
TO    Theiler's original
Tris   2-amino-2-(hydroxymethyl)-1,3-propandiol
UTP   uridine triphosphate
UTR   untranslated regions
UV    ultra violet
VP    viral protein
vRNA  viral RNA
VSV   Vesicular stomatitis virus
VV    Vaccinia virus
VZV   Varicella zoster virus
w/v   weight per volume
WNV   West Nile virus
YFV   Yellow fever virus
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1.0 Introduction

Over the past two decades there has been much progress made in the field of Central Nervous System (CNS) viral pathogenesis. This is due to a combination of factors which include:-

- The increasing clinical relevance of viral infections since bacterial and parasitic infections are becoming preventable either by vaccination or treatment.

- Advances in technology, such as the recombinant DNA revolution have made it possible to construct, clone and genetically manipulate viruses. This biological revolution has allowed scientists to decipher the key functional viral genes which are important in viral pathogenesis. The greater availability of reagents such as monoclonal antibodies, riboprobes, and the construction of genetically manipulated animals (which do or do not contain important genes) have all contributed to enable researchers to further characterise the cellular and molecular events of viral disease pathogenesis.

- The suitability of animal models to be used for studying immunological and cellular interactions of viruses with the CNS, which can be compared and contrasted to human diseases.

- CNS viral infections were also brought to the forefront of researchers minds with the emergence of HIV in the early 1980’s. HIV brought worldwide concern with the thought of a lethal viral infection spreading in pandemic proportions. In the early 1980’s the implications and pathology of HIV infection were largely unknown. However, HIV research has allowed scientists to characterise previously unknown information about the CNS, and also has brought with it, due to immunosuppression, the
emergence of other CNS virus manifestations which otherwise would have been asymptomatic for example progressive multifocal leukoencephalopathy (PML).

1.1 Determinants affecting the outcome of viral infection

Viral infections range from acute to chronic, asymptomatic to symptomatic, and non-life threatening to lethal. The ability of the infected species to clear a viral infection depends upon a variety of different viral and host factors, these include:

*Age of the host is an important factor in various human and animal viral infections. For example children produce a less severe infection when infected with Varicella zoster (VZV), mumps and Epstein-Barr virus (EBV), compared to the adult. The opposite is the case with other viral infections which are more severe in children compared to adults. These include rotaviruses and respiratory syncitial viral infections (Weinstein, 1957; Morgenson, 1979). There are other examples where this is the case for animal viruses (Fazakerley et al., 1993).

*Host genetics play an important role in determining whether the host develops pathology following viral infection. The importance of host genetics has been documented in many viral infections which include, picornaviruses (Lipton and Dal Canto, 1979; Bureau et al., 1992; Patick et al., 1991), myxoviruses (Haller et al., 1979, 1980; Staeheli et al., 1986a, b), retroviruses (Purchase et al., 1977), coronaviruses (Bang and Warwick, 1960; Knobler et al., 1984), rhabdoviruses (Lodmell, 1983), papovaviruses (Brinton and Nathanson, 1981), arenaviruses (Oldstone et al., 1984), poxviruses (O’Neil and Blanden, 1983) and herpesviruses (Chalmer et al., 1977; Lopez, 1980; Bancroft et al., 1981; Grundy et al., 1981; Allan and Shellam, 1984; Quinnan, 1987; Sprecher and Becker, 1987). The susceptibility or resistance to a particular virus is
frequently associated with genes associated with the immune response (Bureau et al., 1992). However, other non-immune genes have also been shown to also be important. These may include for example, genes for receptors which are necessary for viral uptake by cells (Boyle et al., 1987).

*The genotype of virus also determines the outcome of infection. Intranasal infection of mice with the BeAn strain of TMEV results in asymptomatic infection, however, when mice are inoculated with the GDVII strain of TMEV this results in fatal encephalitis in all mice (Lui et al., 1967). Viruses which exhibit a strain dependant virulence are reviewed by Tyler and Friedmann (1990).

*Several other factors are also important in determining the outcome of viral infection and these include dose and route of infection (Theiler and Gard, 1940 a, b; O'Leary et al., 1942; Sigel, 1952; Lui et al., 1967; Lipton et al., 1980), sex of the animal (Lodmell, 1983, Kappel et al., 1990), hormonal, emotional, nutritional and immunological status (Sheridan et al., 1998), stress (Hermann et al., 1993) and concomitant infections.

In the case of the CNS, clearly a major factor is the ability of the virus to gain access. This is usually via the blood or via the peripheral nerves and viruses and their different genotypes vary in their ability to cross the blood brain barrier (BBB) and to track along nerves. Many neurological diseases associated with viruses are considered to result from the presumably rare infection of the CNS by an otherwise non-neuroinvasive virus. Visna virus is an example of this.
Table 1.1 Virus infections of the CNS of medical and veterinary importance.

<table>
<thead>
<tr>
<th>Species</th>
<th>Virus Family</th>
<th>Virus</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Paramyxoviridae</td>
<td>Measles virus</td>
<td>Subacute-sclerosing panecephalitis</td>
</tr>
<tr>
<td></td>
<td>Papoviridae</td>
<td>JC papovavirus</td>
<td>Progressive</td>
</tr>
<tr>
<td></td>
<td>Togaviridae</td>
<td>Rubella virus</td>
<td>Multifocal leukoencephalopathy</td>
</tr>
<tr>
<td></td>
<td>Picornaviridae</td>
<td>Poliovirus</td>
<td>Chronic-progressive panecephalitis</td>
</tr>
<tr>
<td></td>
<td>Rhabdoviridae</td>
<td>Rabies virus</td>
<td>Poliomyelitis</td>
</tr>
<tr>
<td></td>
<td>Flaviviridae</td>
<td>JapaneseB encephalitis virus</td>
<td>Encephalitis</td>
</tr>
<tr>
<td></td>
<td>Herpesviruses</td>
<td>Herpes simplex virus</td>
<td>Encephalitis</td>
</tr>
<tr>
<td></td>
<td>Retroviruses</td>
<td>Human immunodeficiency virus</td>
<td>Encephalitis dementia in AIDS patients</td>
</tr>
<tr>
<td>Sheep</td>
<td>Retrovirus</td>
<td>Visna virus</td>
<td>Demyelinating leukoencephalopathy</td>
</tr>
<tr>
<td></td>
<td>Togaviridae</td>
<td>Border disease virus</td>
<td>Demyelination</td>
</tr>
<tr>
<td>Horses</td>
<td>Bornaviridae</td>
<td>Borna disease virus</td>
<td>Polioencephalitis</td>
</tr>
<tr>
<td>Goat</td>
<td>Retroviridae</td>
<td>Caprine Arthritis Encephalitis virus</td>
<td>Leukoencephalomyelitis of kids</td>
</tr>
<tr>
<td>Dog</td>
<td>Paramyxoviridae</td>
<td>Canine distemper virus</td>
<td>Post-infectious encephalitis and old dog encephalitis</td>
</tr>
</tbody>
</table>
1.2 Virus can infect cells with a variety of sequelae

The relationship between viruses and the CNS is complex and diverse. The unique structural and immunological components of the CNS coupled with the blood brain barrier (BBB), lack of lymphatic tissue and the long-life of many cells make it an ideal environment for an opportunistic viral infection to persist, cause damage and/or disease. There are various types of virus infection which include:

* Lytic or acute infection- virus infects the cell and produces progeny, often killing the host cell. The virus may go on to be cleared by an active immune response.

* Abortive infection- this is when virus enters the cell and produces some early gene products but is unable to complete a full round of replication. Therefore, the infection halts at this stage and is aborted.

* Latency- virus infects cells and lies dormant with minimal gene expression until a specific signal activates gene expression, producing infectious virus.

* Persistent infection- virus persists in a host cell longer than would be expected in the acute infection, viruses are thought to persist by a variety of mechanisms (see Section 1.11).

1.3 Cellular tropisms of viruses in the CNS

Viruses target many cell types in the CNS and these include neurones, oligodendrocytes, astrocytes and microglia. Neurones are the site for lytic acute infections eg. Japanese encephalitis virus (Ogata et al., 1997) and poliovirus (Abe et al., 1995), latent infection eg. Herpes simplex viruses (Garber et al., 1997; Sawtell, 1997), VZV (Mahalingam et al., 1996) and
persistent infections eg. measles virus (Isaacson et al., 1996; Rall et al., 1997), rubella, bornaviruses and Lymphocytic choriomeningitis virus (Cao et al., 1997). Persistent and latent viral infections are established in neurones for several reasons. Firstly, neurones are long-lived and irreplaceable and are relatively resistant to apoptosis and immune mediated destruction. Neurones normally lack the expression of MHC molecules for T cell recognition therefore viral antigen within these cells is not recognised by the immune system. A number of viruses infect oligodendrocytes and astrocytes and often cause lesions of demyelination. These include mouse hepatitis virus (Baraclatas et al., 1997), Theiler’s virus (Aubert et al., 1987), JC papovavirus (Divireddy, et al., 1996; Mesquita et al., 1996), Canine distemper virus (Graber et al 1995; Summers, et al., 1994), Herpes simplex virus (Kastrukoff, et al., 1994) and Semiliki Forest virus (Fazakerley et al., 1991). Microglia the intrinsic brain macrophage are the cell type in which Visna virus (Diguardo, et al., 1994), HIV (Nottet, et al., 1997) and canine distemper virus (Alldinger, et al., 1996) persist. Demyelination is also associated with persistent infection with these viruses. CNS viruses and cell types which they infect are summarised in Table 1.2.
Table 1.2 CNS viruses and the cell types they infect.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td></td>
<td>Poliovirus</td>
</tr>
<tr>
<td></td>
<td>Theiler's virus</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td></td>
<td>Varicella zoster virus</td>
</tr>
<tr>
<td></td>
<td>Visna virus</td>
</tr>
<tr>
<td></td>
<td>Rabies virus</td>
</tr>
<tr>
<td></td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td></td>
<td>Mouse Hepatitis virus ,MHV-4</td>
</tr>
<tr>
<td></td>
<td>Reovirus 3</td>
</tr>
<tr>
<td></td>
<td>Measles virus</td>
</tr>
<tr>
<td></td>
<td>Semiliki Forest virus</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td>Measles virus</td>
</tr>
<tr>
<td></td>
<td>JC papovavirus</td>
</tr>
<tr>
<td></td>
<td>Mouse hepatitis virus , JHM, ts8</td>
</tr>
<tr>
<td></td>
<td>Theiler's virus</td>
</tr>
<tr>
<td></td>
<td>Semiliki Forest virus</td>
</tr>
<tr>
<td></td>
<td>Ross River virus</td>
</tr>
<tr>
<td></td>
<td>Eastern Equine encephalitis virus</td>
</tr>
<tr>
<td></td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td></td>
<td>Visna virus</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Canine distemper virus</td>
</tr>
<tr>
<td></td>
<td>Visna virus</td>
</tr>
<tr>
<td></td>
<td>JC papovavirus</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td></td>
<td>Mouse hepatitis virus , JHM</td>
</tr>
<tr>
<td></td>
<td>Theiler's virus</td>
</tr>
<tr>
<td>Microglia</td>
<td>Canine distemper virus</td>
</tr>
<tr>
<td></td>
<td>Visna virus</td>
</tr>
<tr>
<td></td>
<td>HIV</td>
</tr>
<tr>
<td></td>
<td>Theiler's virus</td>
</tr>
</tbody>
</table>
1.4 Viruses which induce demyelination

As mentioned above one of the consequences of viral persistence in the CNS is demyelination. Demyelination is the loss of the insulating myelin sheath which surrounds neuronal axons. There are two forms of demyelination, primary demyelination which is the direct loss of or destruction to the myelin and secondary demyelination which is loss of myelin due to neuronal damage. Lesions of demyelination are associated with several viral diseases in animals and in man (Table 1.3). Almost all demyelinating diseases in man and animals of known aetiology are viral. Two well known diseases that cause demyelination are SSPE of children, which is caused by measles virus persisting in the CNS (Riadl and Linington, 1996; Allen, et al., 1996; Nagano et al., 1994), and PML which is produced in adults and children by JC virus (Armand, et al., 1997; Devireddy, et al., 1996).

Table 1.3 CNS viruses which can cause demyelination.

<table>
<thead>
<tr>
<th>Family</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Togaviridae</td>
<td>Semiliki Forest virus</td>
</tr>
<tr>
<td></td>
<td>Ross River virus</td>
</tr>
<tr>
<td></td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>Coronaviridae</td>
<td>Mouse Hepatitis virus</td>
</tr>
<tr>
<td>Herpesviridae</td>
<td>Herpes simplex I virus</td>
</tr>
<tr>
<td></td>
<td>Marek’s disease virus</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td>Measles virus</td>
</tr>
<tr>
<td></td>
<td>Canine distemper virus</td>
</tr>
<tr>
<td>Papovaviridae</td>
<td>JC virus</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Theiler’s virus</td>
</tr>
<tr>
<td>Retroviridae</td>
<td>Visna virus</td>
</tr>
<tr>
<td></td>
<td>HTLV-1</td>
</tr>
<tr>
<td></td>
<td>HIV</td>
</tr>
<tr>
<td>Rhabdoviridae</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td></td>
<td>Chandipura virus</td>
</tr>
</tbody>
</table>
In experimental animals there are several viruses which lead to demyelination. Theiler's virus is one of the best known models of viral induced demyelination. Although much studied the exact mechanism of demyelination caused by Theiler's virus is as yet unknown.

1.5 Discovery and Pathology of Theiler's Virus

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus belonging to the genus cardiovirus. The virus was originally isolated in 1934 by Max Theiler from the brains and spinal cords of a mouse with hind limb paralysis (Theiler 1934, 1937).

From Theiler's evidence, it was deduced that TMEV is a naturally occurring murine virus, whose route of transmission is faeco-oral. Infection of young adult mice usually leads to asymptomatic enteric infection (Olitsky 1939, 1940, Theiler and Gard, 1940b), however, in rare instances (0.05%) virus may spread to the CNS and cause clinical disease (Theiler 1937, Olitsky 1939). The clinical symptoms which may accompany CNS infection are acute encephalitis and paralysis.

Strains other than Theiler's original isolates (TO) have been isolated from around the world, and have been experimentally investigated (Daniels et al., 1952; Lipton et al., 1975; Fazakerley and Buchmeier, 1993). It was soon discovered that the TMEV family could be subdivided into two subgroups depending on their biological behaviour *in vivo* and *in vitro* (Table 1.4).

The GDVII and FA strains are highly neurovirulent *in vivo*, causing a rapid fatal encephalitis in infected mice (Theiler and Gard, 1940a). These strains predominantly infect neurones and astrocytes although oligodendrocytes may also be infected (Simas et al., 1995). In cell culture these two strains produce large plaques (Lipton, 1980).
Other TMEV isolates include TO, BeAn 8386, DA, Yale, TO4 and WW (Lipton et al., 1979, 1980). These isolates constitute the avirulent subgroup and are approximately one thousand times less virulent than the neurovirulent subgroup. In cell culture they form smaller plaques (Lipton, 1980). The avirulent isolates are capable of producing a biphasic disease in mice (Lipton et al., 1975). During the acute phase some animals develop hind-limb paralysis and a waddling gate, occurring 2-4 weeks post-inoculation. During this phase virus is predominantly in neurones of the grey matter. Animals that survive the acute phase can go on to develop a persistent CNS infection which results in a chronic inflammatory demyelinating disease (Lipton et al., 1984 a, b). During this phase the number of virally infected cells is greatly reduced, however, virus can be observed in oligodendrocytes, astrocytes and microglia (Brahic et al., 1981; Aubert et al., 1987; Lipton et al., 1995).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>GDVII</th>
<th>TO strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathology</td>
<td>Acute poliomyelitis</td>
<td>Early grey matter disease, persistence, demyelination.</td>
</tr>
<tr>
<td>Target cells (adults)</td>
<td>Neurones</td>
<td>Neurones, oligodendrocytes, astrocytes, macrophage.</td>
</tr>
<tr>
<td>Virulence LD$_{50}$</td>
<td>1-10</td>
<td>$10^4$-$10^5$</td>
</tr>
<tr>
<td>Plaque size (mm in vitro)</td>
<td>Large (2.5)</td>
<td>Small (0.5-1)</td>
</tr>
<tr>
<td>Temperature sensitivity</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

Table 1.4  Comparison of GDVII and TO subgroup of Theiler's virus. The biological characteristics of the two subgroups are shown. No differences are seen in the physical properties of the subgroups other than those related to the protein size.
1.6 Molecular structure of TMEV

TMEV is a single stranded, positive sense RNA virus of 8100 nucleotides. The organisation of the TMEV genome is typical of other picornaviruses as shown in Figure 1.1 (Stanway, 1990). The 5' untranslated region (UTR) of TMEV is longer than that seen in other picornaviruses. It also lacks the poly C tract preceding the open reading frame (ORF), seen in other picornaviruses. The single ORF encodes a polyprotein of 2303 amino acids. The polyprotein begins with a short leader sequence followed by 11 other genes that are typical of the picornavirus 4:3:4 genomic arrangement. The coding region is followed by another UTR at the 3' end which is followed by a poly (A) tail. The structural proteins are encoded towards the 5' end of the ORF designated the P1 region. This P1 area consists of 4 genes, VP1 (37 kDa), VP2 (34 kDa), VP3 (18 kDa) and VP4 (6 kDa), these are the capsid proteins. The P2 and P3 regions are the areas that code for the non-structural proteins which are downstream from the P1 region. P2 consists of 3 genes, encoding the 2A, 2B and 2C proteins, and P3 encodes 4 proteins 3A, 3B, 3C and 3D. These non-structural proteins are involved in viral replication and protein processing, and much is known of their activity (Roos et al., 1989; Palmenburg et al., 1990; Reuer et al., 1990; Hellen et al., 1991; Johnson and Sarnow, 1991; Hambidge and Sarnow et al., 1992; Kean et al., 1993; Johnson et al., 1995).

TMEV virions are small icosahedral particles approximately 20-30 nm in diameter with a sedimentation coefficient of 150 S and are stable at pH 3 or lower (Lipton and Friedmann, 1980). Inside the icosahedral protein capsid is a single stranded, positive sense RNA, the 5'-terminal UpUp of which is linked by a phosphodiester bond to the phenolic (O4) hydroxyl group of a tyrosine residue of a small virus encoded protein, Vpg, which is approximately 20 amino acids in length.
Recently the 3 dimensional structures of the BeAn, GDVII and DA strains of TMEV have been elucidated by X-ray crystallography (Lou et al., 1992; Grant et al., 1992; Toth, 1993 and Lou et al., 1996), see Figure 1.2.

The virion consists of 60 copies of each capsid protein VP1, VP2, VP3, and VP4. The arrangement of these capsid proteins forms a classical iscosohedral structure (Figure 1.2). VP1, VP2, and VP3 are exposed on the surface of the virion whereas VP4, which is the smallest protein, is completely internal. Picornaviruses have a five, three, and two fold axis of symmetry, and these are used to orientate the surface features.

VP1, VP2 and VP3 all have similar wedge shaped eight stranded anti-parallel β barrel sheets that package together to form the continuous protein shell of the virion. Along with VP4 the amino termini of VP1, VP2 and VP3 interact to form a detailed network on the inner surface of the capsid, whereas the carboxy termini of the three major capsid proteins, and most of the loops on the β strands (BC, CD, EF loops of VP1 and EF of VP2) are exposed on the outer surface. None of the capsid proteins are glycosylated, however, VP4 has a molecule of myristic acid linked to the amino terminal glycine.

A broad protrusion is present at the five fold axis where the VP1 molecules are clustered, forming a star-shaped plateau which slopes downwards across the surface of VP2 to the three fold axis. On the two fold axis there is a deep depression corresponding to the surface of VP3, which has been described as a “canyon” or “pit” on other picornaviruses, and has been proven to be of functional significance (Hogle et al., 1985; Rossmann et al., 1985; Luo et al., 1992 and Rossmann and Palmenberg, 1988). There are subtle structural differences between the neurovirulent GDVII and the avirulent BeAn and DA strains of TMEV. These structural differences are in small local areas and involve the interactions of the side chains of the amino acids involved. However small the differences may be they appear to have important
implications in determining the difference between neurovirulence and avirulence (Luo et al., 1987; Kim et al., 1990).

1.7 Viral Entry Into The Target cell

In in vitro cultures poliovirus (a picornavirus) RNA can be detected two hours after infection, and 30 minutes later infectious virus is detectable. In this time the virus replicates the positive sense genome, packages it into virions and effectively releases it from the cell. It is then recognised by a receptor on a neighbouring cell. A putative receptor for TMEV has been proposed (Kilpatrick and Lipton, 1991). Radiolabelled virus binds to a 34 kDa membrane protein on susceptible tissue culture cells, and more weakly to two other proteins which are 18 kDa and 100 kDa. The 34 kDa receptor is a sialoglycoprotein and the same receptor appears to be utilised by both BeAn and GDVII (Fontiadis et al., 1991). Interestingly however, if cultures of BHK-21 cells are pre-treated with neuraminidase before infection, the binding of BeAn infection is markedly inhibited (ie up to 90%), and GDVII binding is unaffected. This suggests that sialic acid residues are required for BeAn binding but not for GDVII. It has been hypothesised that the canyon which is present on the poliovirus and human rhinovirus (HRV) surface is the putative receptor binding site (Hogle et al., 1985; Rossman et al., 1985). In Mengo virus the canyon is replaced by a pit and the receptor binding site (which has been identified for Mengo virus) is in this pit. By analogy with Mengo virus the TMEV pit residues have been identified (Kim et al., 1990). Of the 30 pit residues in GDVII and BeAn there are only two differences, both of which occur in the VP1 region at positions 88 and 219 (Pevear et al., 1987). These differences may account for the differential binding to the cellular receptor observed between BeAn and GDVII.

The molecular events which include viral entry into the cell, translocation to the cytosol and initiation of transcription/translation are still poorly
understood for picornaviruses. Subsequent to attachment of ligand to receptor it has been proposed that the mechanism of viral entry into the cell is via receptor mediated endocytosis (Madshus et al., 1984a, 1984b). After attachment of the ligand to the receptor(s) (which cluster in clathrin coated pits, which are depressions in the cellular membrane) the complex is internalised by an invagination process, which leads to the formation of clathrin coated vesicles. Subsequently, a drop in pH inside these vesicles initiates changes in the capsid proteins, which in turn causes the RNA to be transferred from the virion to the cytosol of the infected cell. It is still unknown by what mechanism the viral RNA is released from the virion, however it has been suggested that the release occurs through the centre of a pentamer (Rossmann et al., 1985). However, it has been argued that since TMEV is insensitive to changes in pH, uncoating may be induced by virion binding to the host receptor (Luo et al., 1992).

1.8 Translation and Replication Strategies

After entry of viral RNA into the cytosol is completed the next steps to occur are the binding of viral RNA to ribosomes, formation of polyribosomes, transcription and translation (Figure 1.3). The positive sense viral RNA is translated into a single polyprotein using the host cell translational apparatus. This polyprotein is then proteolytically cleaved to yield components which are essential for viral replication. The cell does not contain all the necessary components for complete replication. These include specific proteases for cleavage of the polyprotein and an RNA-dependant RNA polymerase.

Following the production of the polyprotein and its functional protein products, the next stage in viral replication is the production of negative stranded RNA, which is subsequently used as a template to produce message sense viral RNA, which in turn will produce the capsid and other essential proteinaceous components for complete viral production (Figure 1.3).
The precise mechanisms of picornavirus RNA replication are not yet fully understood, however there is evidence that 3 forms of RNA are found in the cytoplasm of infected cells. These are 1) positive sense, single stranded RNA that is either viral RNA or mRNA (Hewlett et al., 1977; Nomoto et al., 1977), 2) double stranded replicative form RNA, ( Baltimore 1966; Richards and Ehrenfeld, 1989) and lastly 3) partially double stranded and partially single stranded replicative intermediate RNA ( Flanegan and Baltimore, 1977, 1979). These differences in RNA type may be accounted for by the way in which picornaviruses replicate. However, their exact role has yet to be elucidated.

There are two main models for replication of the positive stranded RNA into the complementary negative stranded RNA. In the first of these models VPg, or VPg linked to uridine monophosphate in the form of VPg-pU-pU is postulated to prime RNA synthesis ( Flanegan and Baltimore, 1977; Lee et al., 1977 and Nomoto, 1977). Baltimore suggested that uridinylated VPg hybridises to the 3' poly A tail of the positive stranded vRNA and this primes complementary strand synthesis with the assistance of the viral RNA dependent RNA polymerase (3Dpol).

The alternative theory of picornavirus replication is that a hair pin structure forms at the 3' end of the picornavirus RNA (Semler et al., 1989), possibly as a result of uridinylation of the poly A by terminal uridylyl transferase. The hair pin primes the elongation of the complementary strand. In this model VPg is thought to function in the trans-esterification required to break off the phosphodiesterase bond within the hair-pin loop (Tobin et al., 1989). The positive template strand and the complementary negative strand then separate meaning that the next stage of replication can occur. The transcription from positive sense to negative sense RNA by 3Dpol occurs on the smooth endoplasmic reticulum in the cell cytosol. Smooth membranes are essential for complete viral replication (Dales et al., 1965; Calguiri and Tamm, 1970; Freidmann and Lipton, 1980 and Lorch et al., 1981).
One of the most intensively researched areas of picornavirus replication is how the viral RNA can be translated when it lacks the 5' methylated GpppN (N being any nucleotide) cap structure generally present on eukaryotic mRNA's (Pilipenko et al., 1994, 1995). Cap independent initiation of translation is replaced by the encoding of an internal ribosome binding site in the 5' UTR (Bandyopadhyay et al., 1992). All picornaviruses have an unusual 5' UTR, they are comparatively long. In TMEV the length of the 5' UTR is 1064 nt. As stated previously, these mRNA's lack the methylated cap, but have a small viral polypeptide VPg, which is 20 amino acids in length. Viral RNA molecules which are to be translated lose their VPg proteins which results in the formation of elaborate secondary structures which form an IRES (Internal Ribosome Entry Site) or RLP (Ribosome Landing Pad), which the ribosomes bind to. The IRES has been mapped for TMEV between nt 500 and 1043-45 in the 5' UTR (Bandyopadhyay et al., 1992). Deletions in the IRES drastically reduce translation in BHK-21 cells.

Once the initiation complex is set, the ribosome scans the 5'UTR until it finds a starting window. The starting window is defined as the area where the ribosome begins translation, the starting window for TMEV was mapped to be 16-17 nt downstream of the IRES and was approximately 12 nt in length. Once an appropriate AUG initiation codon is recognised, translation occurs, providing that essential cellular factors are also present.

Translation of the large single ORF occurs in the absence of viral gene products and yields a single polypeptide. The polypeptide precursor is processed by a series of proteolytic cleavages into the mature gene products which are essential for further replication. The final products of a series of cleavages include structural proteins (VP1, VP2, VP3 and VP4), which assemble into monomers, pentamers, provirions and virions in that order, and the 8 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, 3D) which are involved in viral replication. Briefly, the mature proteins 2A (Roos et al.,
1989; Batson and Rundell, 1991) and 3C (Cheah et al., 1990; Jia et al., 1991; Kean et al., 1993) are the main proteases involved in cleavage of the polypeptide, although autocatalytic events are also involved in cleavage. The leader or L protein has been shown in in vitro studies to occur in two distinct forms termed L and L* (Roos et al., 1989; Kong and Roos, 1991). L* is synthesised from an alternative AUG codon 13 nt upstream of L’s initiation codon. The significance of L* remains uncertain; however mutational studies within the L region, which disrupt L* have shown a decrease in demyelination. This data is consistent with results from a previous study that showed that in recombinant virus which substituted GDVII for DA sequences in the 5' terminus (therefore lacking L* initiation codon) exhibited a decrease in demyelination. Interestingly, mutagenesis of the L protein has shown that it is completely dispensable in the infection of BHK 21 cells (Kong, 1994), however, in foot and mouth disease virus the L protein is a protease and is involved in proteolytic cleavages. It may have a similar role in TMEV infection (Palmenberg et al., 1990, Medina et al., 1993). The 3B (VPg) protein is covalently linked to the 5' end of the RNA and has been shown to play a role in the initiation of viral RNA synthesis (see above) (Barron and Baltimore, 1993, Ward and Flanagan, 1992; and Reur et al., 1990). The most conserved protein among picornaviruses is 3D. This is the RNA-dependant RNA polymerase which is vital for replication. It has conserved sequences with every other known DNA and RNA polymerase (Kuhn and Wimmer, 1987; Sankar and Porter, 1992). Very little is known about the involvement and interaction of these non-structural proteins, and it is still an area of research.

The morphogenesis of TMEV is a three step process which (1) involves the capsid proteins assembling from monomers into pentamers, (2) these pentamers assemble into provirions, which consist of the positive sense RNA genome packaged inside a 12 platted capsid, and finally (3) maturation of the provirion into the virion which involves cleavage of VP0. This occurs as infectious virions are released from the infected cell. Virus replication may
cause programmed cell death (apoptosis) or necrosis of the infected cell, which subsequently leads to the release of active virus which can then go on to infect other cells. Apoptosis has been a reported mechanism of cell death after infection with many different viruses, and recent in vitro studies have indicated that TMEV can also cause cell death by apoptosis (Jelachich and Lipton, 1996; Tsunoda et al., 1996). Interestingly the neurovirulent GDVII strain is at least 50 fold more efficient at inducing apoptosis relative to the avirulent BeAn strain. It has also been shown that in BHK-21 cells infected with BeAn virus the virions are associated with smooth membranes and it has been suggested that virions may exit the cell via channels or pores in the smooth membrane. However, very little evidence exists to substantiate this finding (Friedmann and Lipton, 1980). Cell necrosis or apoptosis is dependent on the cell type infected and the mechanism by which TMEV exits the cell may also depend on the infected cell type. Cell lysis has been observed in vitro in various cell types after TMEV infection i.e. BHK-21 (McCright and Fujinami, 1997), and in vivo in neurones (Lui et al., 1967; Stroop et al., 1981; Lipton, 1975; Rosethal et al., 1986; Wada and Fujinami, 1992). However, in other cell types TMEV is able to establish a persistent infection such as oligodendrocytes in vivo where viral RNA is said to be restricted at the negative strand stage (Cash et al., 1985, 1986, 1988). Also in vitro persistent infections have been established in a number of cell types including macrophage/microglia CNS cultures (Levy et al., 1992), glioma cell cultures (Patick, 1991), mixed astrocyte cell cultures (Rodriguez et al., 1988 and O'Hara et al., 1992), pure astrocyte cultures (Usherwood Thesis, 1994) and mouse fibroblast cell lines (Roos et al., 1982).

1.9 Differences Between Strains of TMEV at the Molecular Level

As stated previously, TMEV can be divided into 2 subgroups. The TO strains are avirulent whereas the neurovirulent strains FA and GDVII are lethal in mice inoculated i.c. within days after infection. The cloning and sequencing of
these 2 serologically related subgroups of TMEV strains has proven to be an invaluable tool in determining which regions in the neurovirulent and avirulent strains cause virulence and pathogenesis (Aubert and Brahic, 1995). The virulent and persistent strains did not evolve as two separate groups but rather the neurovirulent strains arose from a subgroup of persistent strains (Michaels and Brahic, 1996).

Following i.c. inoculation the neurovirulent strains of TMEV, GDVII and FA cause an explosion of viral replication and rapidly spread throughout the brain causing a fulminant encephalitis which results in death (Theiler and Gard, 1940b; Olistky, 1945; Simas et al., 1995). This occurs in mice of all genetic backgrounds and affected mice show breathing difficulties, fur which is stark and ruffled, circling behaviour, weight loss and flaccid paralysis culminating in death. The neurovirulence of GDVII is dependent on route of entry and dose of virus, with i.c. inoculation causing the highest mortality rates, with a LD50 of 0.1 PFU (Theiler and Gard, 1940b; Lui et al., 1967; Lipton, 1980). Intraperitoneal, intravenous, intranasal and oral inoculations are less effective, but they also give rise to abnormalities in the CNS, however demonstrating the neuroinvasiveness of the virus.

GDVII and FA strains of the virus contrast dramatically with the avirulent strains which constitute the TO subgroup and include TO (Theiler, 1934, 1937; Olitsy, 1939, 1945; Daniels et al., 1952), BeAn, DA, Yale and WW. The TO subgroup of viruses after i.c. inoculation can produce a chronic CNS infection in mice that survive acute infection (Daniels et al., 1952; Lipton, 1975; Dal Canto and Lipton, 1975; Lehrich et al., 1976). Chronic infection results in a persistent CNS infection with lesions of CNS demyelination, however this is dependent on mouse strain. All mouse strains undergo the acute phase of the disease, however susceptibility to chronic infection is dependent on genetic background (Bureau et al., 1992), age, sex of the animal (Kappel et al., 1990), dose, route and strain of virus. Penney and colleagues observed that neonates
or suckling mice are highly susceptible to the acute phase of the disease exhibiting 100% mortality (Penney et al., 1979; Rodriguez et al., 1983b). But, as mice increase in age they become increasingly resistant to infection (Steiner et al., 1984). Mouse strains can be divided on the basis of the incidence of chronic demyelination after TMEV infection. C57Bl/6 and one sub-strain of Balb/c mice are resistant, CBA, AKR and Swiss are intermediate and SJL/J and PLJ and SWR are highly susceptible (Lipton and Dal Canto, 1976; Clatch et al., 1987b; Bureau et al., 1992). This is discussed in Section 1.10.

The clinical signs associated with the acute disease (which occur 7-30 days post infection) are hunched posture, flaccid paralysis (most commonly associated with the hind limbs but forelimbs may also be affected), unsteady gait which usually results in waddling, weight loss, incontinency, shivering and stark ruffled fur (Theiler, 1934, 1937; Daniels et al., 1952; Lipton, 1975; Lipton and Dal Canto, 1976; Lehrich et al., 1976). Mortality during the acute phase of the disease is dependent on mouse strain. Mice surviving the acute phase of the disease, whether they show clinical signs or not, may, depending on mouse strain go on to develop chronic infection. Clinical signs associated with chronic infection are; ruffled fur, severe hind limb and/or forelimb paralysis, tremors, hunched posture and severe gait disturbance. This usually occurs around 60 days after infection, but can occur after this time (Daniels et al., 1952; Lipton, 1975; Lipton and Dal Canto, 1976).

Nucleotide sequences have been established for the DA (O’Hara et al., 1988), BeAn (Pevear et al., 1987) and GDVII (Pevear et al., 1987) strains of TMEV. When the nucleotide and amino acid sequences are compared it can be noted that there is a large degree of identity between the three strains, although obvious differences do exist. BeAn and GDVII are 90.4% identical at the nucleotide level and 95.7% identical at the amino acid level, whereas BeAn and DA (both TO subgroup) are 92% identical at the nucleotide level and 94.2% identical at the amino acid level. The 5’ and 3’ UTRs are relatively well
conserved between strains with 94.5% and 99.2% identity, between GDVII and BeAn and BeAn and DA. This implies that there are important functional elements located in these areas, therefore only a limited amount of variability is permitted to ensure the virus remains viable. On the other hand the largest sequence differences between the TMEV strains exists in the PI region of the viral genome. The VP1 region shows the most divergence with 92% amino acid identity between BeAn and DA and 92.75% identity between GDVII and BeAn. This is an area of particular interest. Construction and phenotyping of chimeric viruses suggests that the VP1 region is involved in determining persistence and demyelination.

Isolation and characterisation of two subgroups of TMEV strains with distinctive phenotypes has made it possible to construct chimeric recombinant viruses to elucidate the molecular components responsible for virulence, persistence and demyelination. It is known that persisting virus is an absolute prerequisite for demyelination to occur. However, is persistence the result of attenuated virulence or not? Full length cDNA clones for GDVII (Tangy et al., 1989; Calenoff et al., 1990), DA (McAllister et al., 1989; Roos et al., 1989), and BeAn (Calenoff et al., 1990) were constructed.

It has been discovered using chimeras derived from the above infectious viral cDNAs that the PI region coding for the capsid proteins is the most important region in determining neurovirulence. Neurovirulence is the result of complex interactions involving at least two genetic determinants one in the PI region and the other in the 5' UTR. Both these regions may contain other elements which confer virulence but how they interact with each other is as yet still unknown.

It is also clear that the determinants of demyelination and persistence are also multigenic. Mutations in the VP1 region in TO strains are able to affect their ability to persist and cause demyelination (Jarousse et al., 1994b), suggesting
that the VP1 region may be involved. Studies have also shown that amino acid 101 in the VP1 region can affect the viruses ability to persist (Zurbriggen and Fujinami, 1989; Zurbriggen et al., 1991); and a more recent study also showed that a mutation in amino acid 100 completely attenuates the ability of the virus to persist (Lui et al., 1998). The 5' UTR and L regions may also be involved in persistence and influence demyelination (Stein et al., 1992).

The VP2 area is also important in determining viral persistence, more specifically a change in a single amino acid at position 141 in VP2 from lysine to asparagine affects the virus's ability to persist (Jarousse et al., 1996), and also a mutation at amino acid 173 in VP2 from threonine to phenylalanine results in failure to persist and induce demyelination (Sato et al., 1996).

Further evidence supporting McAlister's hypothesis that the determinants of persistence lie in P1 (McAlister et al., 1990), is that recombinant viruses in which BeAn sequences are gradually replaced by GDVII sequences starting at the NH2 terminal suggest that confirmational determinants requiring homologous sequences in both the VP2 puff and VP1 loop regions which are in close contact at the virion surface, may underlie persistence (Adami et al., 1998).

All the above evidence indicates the viral capsid proteins contain determinants for persistence and demyelination. Although many important areas have been mapped in the TMEV genome and several amino acids have been identified as being important in virulence, demyelination and persistence, it is probable that even more exist and ongoing research will identify them.

It should be noted that work involving chimeras should be treated with caution, because chimeras are complex and may involve constructs from
different laboratories, whereby the infectious parental clones may differ. Contamination is also a major consideration.

1.10 Mouse Strain Susceptibility

It was first reported by Lipton and colleagues that there was a variation in demyelinating disease depending on which mouse strain was infected with TMEV (Lipton and Dal Canto, 1979). According to these authors, all strains of mice were susceptible to the acute phase of the disease, however, whether the mice went on to show signs of chronic infection depended upon mouse strain. Further work has shown that mouse strains can be classified into 3 groups. SJL/J and PL/J mice have a high susceptibility to chronic infection. An intermediate group consists of CBA, AKR and Swiss mice. Mice which are resistant to chronic infection include BALB/c and C57Bl/6. Differences in susceptibility were reported in the BALB/c strain of mice. There are two strains of BALB/c mice known as BALB/cAnNG and BALB/cByJ (Nicholson et al., 1994). The differences in susceptibility are due to unknown genetic differences within these strains.

It is of particular interest to note that CBA mice are intermediate in susceptibility to chronic infection. In a study particularly pertinent to this thesis, RNA and sections from the brains and spinal cords of CBA mice, sampled at various time points during the chronic phase of disease were investigated for viral persistence by using RT-PCR and in situ hybridisation. The results showed that 50% of mice had virus in both tissues, however, the remaining 50% were negative. Persistence of virus in the CNS thus varies between individual CBA mice (Simas & Fazakerley, 1996). This leaves the intriguing question of why individuals in an inbred strain of mouse with identical genetics should differ in susceptibility?
Lipton and Melvold (1984b) further investigated the genetic basis of susceptible and resistant mice. They crossed susceptible SJL/J (H-2$^s$) mice with resistant (H-2$^b$) mice and found that resistance was dominant, with only 15% of heterozygotes having chronic infection compared to 80-100% of the homozygous SJL/J mice. Subsequent studies have demonstrated that the H-2D locus has a role to play in susceptibility (Clatch et al., 1985; Rodriguez and David, 1985; Patrick et al., 1991). Studies in congeneric mice on a resistant C57Bl/6 background showed the haplotypes conferring susceptibility to demyelination were s, f, p, r, v and q and resistance were b, k and d. To define and map the MHC gene(s) involved in susceptibility, recombinant inbred strains of mice were used. Within the H-2 complex susceptibility was mapped to the H-2D region between genes D and L (Clatch et al., 1985, 1987b; Rodriguez et al., 1986). The role of MHC class I molecules was confirmed by later studies demonstrating that β-2-m -/- mice on a resistant background develop demyelinating lesions as compared to the wild type controls which did not (Fiette et al., 1995). Also congruent with this hypothesis is the observation that Lindsley et al. (1991) made which was that resistant C57Bl/6 mice had high numbers of virus-specific CD8+ T cells at day 7 post infection, but susceptible SJL/J mice had low levels of CD8+ T cells that were not detectable until day 13 post infection. These data illustrate the importance in developing a good CTL response early on in a viral infection.

Other non H-2 genes are also linked to susceptibility. These include T cell receptor β chain (Melvold et al., 1987), IFNγ gene on chromosome 10 (Bureau et al., 1993, Fiette et al., 1995), myelin basic protein on chromosome 18 (Bureau et al., 1993) and a gene located near to the carbonic anhydrase gene on chromosome 3 (Melvold et al., 1990). Sex of the animal may also be important when considering susceptibility with the general trend of males being more susceptible than females (Kappel et al., 1990). The reasons for this are largely unknown.
The above paragraph shows that the susceptibility to TMEV induced
demyelination is a complex process involving many genes. It should be noted
that many of these genes are linked to the immune response ie TCR, MHC,
IFNγ. It also should be stressed that in some cases these genes have only
been linked to susceptibility and are not necessarily the genes involved, only
further research will discover the precise locations.

1.11 Sites and Mechanisms of Persistence

The sites of viral persistence and the mechanisms by which the virus evades
an active neutralising antibody and cellular DTH responses have been areas
of much research over the last ten to fifteen years.

It is still the subject of much controversy as to which is the dominant cell type
where the virus establishes a persistent infection. It has been debated as to
whether macrophages or oligodendrocytes are the predominant cell type
involved.

In the acute phase of TMEV infection, depending on mouse strain, age, virus
strain and dose, neurones are the predominantly infected cell type and in
particular copies of viral RNA and antigen were discovered in the thalamic
nuclei, hypothalamus, brain stem and spinal cord motor neurones (Lipton,
1975; Wroblewska et al., 1977; Brahic, 1981; Dal Canto and Lipton, 1982).
These are mainly grey matter areas and no virus is detectable in the white
matter (Simas et al., 1995).

The cellular tropism is very different in the chronic infection. There have
been conflicting arguments as to what the dominant cell type is during
chronic infection. Lipton and co-workers suggest that astrocytes and
macrophages are the sites of viral persistence, in particular the macrophages
(Dal Canto and Lipton, 1982; Lipton, 1995). In contrast to this, Rodriguez
reported that oligodendrocytes were the main sites of virus persistence, and not macrophages, with a small proportion of astrocytes infected (Rodriguez et al., 1983a). Aubert and colleagues estimated only 10% of cells infected during persistence were macrophages/microglia, 25-40% were oligodendrocytes and 5-10% were astrocytes (50% of infected cell types were uncharacterisable due to limitations in the technique used) (Aubert et al., 1987). Infection of oligodendrocytes during the chronic infection was also observed by Simas & Fazakerely, 1996.

The mechanisms by which virus persists despite an active immune response is an area of tremendous interest. Several theories have been proposed as to why this is the case.

One hypothesis is that viral variants exist. The theory is that mutations in genes of the outer capsid proteins cause an antigenic drift and virus evades antibody. Rodriguez and co-workers isolated several viral variants from the CNS of an infected mouse, however when the isolated strains were challenged with anti-serum, it was discovered that all variants could be neutralised (Liebowitz and Rodriguez, 1983). This only accounts for antibody variants, CD4+ or CD 8+T cell variants may exist and evade an active immune response.

An alternative hypothesis is that once the infected cell is lysed the infected TMEV virions remain associated with the cellular membranes, and these cellular membranes occlude B cell epitopes, therefore denying access to the neutralising antibody and allowing virus to persist (Friedmann and Lorch, 1984; Frankell et al., 1987).

It has also been suggested that proteases contained in infected macrophages digest the B cell epitopes (Nitayaphan et al., 1985), rendering the virus undetectable by the neutralising antibody. This work has been reiterated in
*vitro* showing that there are trypsin sensitive epitopes in VP1. Neutralisation epitopes also exist at protease cleavage sites in other picornaviruses such as FMDV and PV.

Restricted replication has also been implicated in viral persistence. It has been postulated that TMEV exists in a state of restricted replication in glial cells (Cash et al., 1985, 1986, 1988), with a decrease in viral proteins expressed by the cells. This could result in the virus avoiding confrontation with the immune response and hence persistence. The restricted replication hypothesis was supported by findings by Johnston et al. (1995). They discovered that the levels of 3D polymerase were decreased in the spinal cords of mice with a persistent infection, indicating that a decreased amount of viral replication was occurring.

Blakemore also suggested the "Trojan Horse" hypothesis, whereby macrophages are persistently infected in the periphery, and recruitment of persistently infected macrophages into the CNS causes disease (Blakemore et al., 1988). It still remains an objective to recognise the mechanism by which virus persists. Perhaps more than one of the mechanisms suggested occur.

### 1.12 Mechanisms of Demyelination

Demyelination can occur either by direct damage to the myelinating cell the oligodendrocyte. This is primary demyelination. Alternatively, secondary demyelination can occur, which is the result of damage to the neurone leading to axonal loss and then loss of myelin. Infection with TMEV usually results in primary demyelination however, the mechanism of this demyelination is still unknown and several postulates exist as to how demyelination occurs.

There have been several mechanisms suggested for the pathogenesis of demyelination which occurs in susceptible mice after Theiler's virus infection.
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The first mechanism is that demyelination is directly due to viral infection and that there is no involvement of the immune response. The evidence supporting this is that nude mice (mice with no functional T cells) and mice depleted of CD4+ T cells do have areas of demyelination (Rosenthal et al., 1986; Zurbriggen et al., 1991). Other evidence which supports this hypothesis is that within the infected oligodendrocytes the inner glial loops swell prior to demyelination (Rodriguez et al., 1985), and also paracrystalline arrays of virus can be found in oligodendrocytes (Blakemore et al., 1988). However, it is unlikely that oligodendrocyte lysis is the major mechanism of demyelination because there is overwhelming evidence to substantiate the “non-specific bystander effect” theory.

A second hypothesis suggests that demyelination is immune-mediated. Immunosuppressive treatments result in a decrease or no demyelination (Lipton and Dal Canto, 1976, 1977). Other evidence that implicates the involvement of the immune system is that mononuclear cells have been observed stripping myelinated axons of their myelin while the oligodendrocyte cell bodies appeared normal (Lipton, 1975). The model proposed by Clatch and colleagues suggests an effector role for TMEV-specific DTH, wherein proinflammatory cytokines released by virus specific DTH T cell leads to recruitment, accumulation and activation of macrophages in the CNS. This subsequently leads to non-specific bystander demyelination (Figure 1.4). Evidence supporting this theory includes; the lack of a demonstrable peripheral or CNS activated T cell response to the immunodominant myelin epitopes within the first 40 days post infection (Barbano and Dal Canto, 1984; Miller et al., 1987b); the correlation between disease and high levels of MHC class II induced DTH (Clatch et al., 1985, 1986, 1987b); the disease acceleration after inoculation with CD4+ Th1 cell lines (Gerety et al., 1994b); demonstration of activated CD4+ T cells in the CNS post infection (Pope et al., 1996) and finally the tolerisation of animals to
developing disease after treatment with UV inactivated virions (Karpus et al., 1995) but not with myelin proteins (Miller et al., 1989).

Mice which have demyelinating lesions induced by TMEV have similarities in their histopathological features when compared to animals which have EAE (Experimental Autoimmune Encephalitis) (Dal Canto et al., 1996; Tsundoa and Fujinami, 1996; Dal Canto, 1995). EAE is a CNS inflammatory reaction induced by inoculating mice with myelin proteins in CFA. EAE is caused by antibody and T cell recognition of myelin basic protein, proteolipid protein or myelin oligodendrocyte protein (these proteins are all components of myelin). It has been speculated that TMEV induced demyelination may also be a result of the autoreactivity of T and B cells to myelin constituents.

It is possible that TMEV infection generates neoantigens in a number of ways, which include lysis of oligodendrocytes or DTH bystander damage resulting in the potential to generate autospecific T and B cells. There is also the possibility that TMEV may contain similar B and T cell epitopes as the CNS components (molecular mimicry) priming the immune system to attack self and cause demyelination. Until recently previous studies had failed to recognise any specific autoimmune responses after TMEV infection. However, there is now significant evidence to suggest that autoimmunity may be an important component in TMEV induced demyelination. Miller et al. (1997) recently showed that TMEV infected mice develop T cell reactivity (T cell proliferation and DTH) to an immunodominant epitope on PLP139-151. This was first detectable 3-4 weeks after the first signs of chronic disease were noticed. As myelin damage progressed 5-6 months post infection T cell and antibody responses to multiple myelin epitopes PLP178-190, PLP56-70 and MOG 92-106 were evident. Examination of the epitopes indicated that the responses did not arise by cross reactivity (molecular mimicry) with TMEV specific T cell epitopes (Figure 1.4.), but instead "Epitope spreading" was postulated to be involved.
These data suggest that autoimmune responses to myelin epitopes arise in the chronic phase of TMEV induced demyelination and these responses appear to be induced by de novo priming of self reactive T cells to autoantigens released secondary to virus specific T cell mediated demyelination. Miller’s current model for demyelination (Figure 1.4) is a continuation of Clatch’s, basically it suggests that persistent infection of CNS cells provides a continuous supply of viral epitopes which are presented via MHC class II and recognised by primed CD4+ Th1 cells of the Th1 cell subtype. This reaction releases pro inflammatory cytokines which cause recruitment of activated macrophage into the CNS. The products of these activated macrophages and T cells (ie TNFα, NO, IFNγ and O2 radicals) mediate the effector phase of demyelination but consequently cause the release of myelin proteins which causes the generation of autoreactive T and B cells which further contribute to the demyelination.

1.13 TMEV as a model for Multiple Sclerosis

In the 1940’s it was recognised that mouse TMEV infection had similarities to human polio virus infection causing flaccid paralysis, therefore TMEV was initially used as an animal model for polio. However, it was soon recognised that TMEV had the potential to be used as a model for MS which is a relatively prevalent disease in Westernised societies, affecting one in 2000 people. It is important on a human, social and economic scale that a cure/treatment for this disease is discovered. MS is one of the most common CNS diseases among young adults and yet its etiology remains largely unknown. Although inflammatory demyelinating lesions are a common pathological feature of the disease, the clinical course is very variable. Epidemiological evidence indicates that MS may be acquired environmentally, possibly by a virus. Several viruses have been implicated as the agent which causes MS, including recently HHV6 (Liedtke et al., 1995). There are numerous comparisons that can be made between TMEV-induced
disease and MS (Tsunoda and Fujinami, 1996; Dal Canto, 1996).
Fundamentally, both are natural endemic CNS diseases of a chronic nature.
Both MS and TMEV-induced disease are genetically influenced by several loci
including MHC and TcR. Like MS TMEV’s disease process is primarily
immune mediated with disease associated with CNS mononuclear
inflammatory infiltrates. The triggering agent of TMEV is a virus and the
triggering nature of MS may also be viral. TMEV is an excellent model for MS
for several reasons. Firstly, it has the advantage of being a naturally
occurring murine virus and therefore infection is studied in its natural host.
Another major advantage is the ever increasing number of laboratory
reagents available to investigate the molecular events and the disease
pathogenesis. In nature only a small percentage of mice develop
demyelinating disease after infection with TMEV, and it can also be noted that
in a human population only a small percentage of people develop clinical
signs of MS. This point must be highlighted as it is consistent with MS
having a common viral aetiology. Almost all of the naturally occurring CNS
demyelinating diseases of animals and man of known aetiology are viral, MS
may be no exception.

1.14 Immune Response to TMEV
The immune response to TMEV is a complex and multifactorial interaction
between both the cell mediated and humoral arms of the immune response.
There are quantitative and qualitative differences between these two arms of
the immune response and this may determine the difference between viral
clearance and persistence which is observed between mouse strains. Also the
difference in the immune response in mice resistant to and mice susceptible to
viral persistence is not only important in viral clearance but it also has
implications for the neuropathology observed in susceptible strains, although
this as yet is still poorly characterised.
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1.14.1 B Cell Immunity to TMEV

The role of antibodies in controlling and clearing picornaviruses has been well documented previously (Lea et al., 1994; Mateu et al., 1995; Smith et al., 1995). TMEV is no different from any of the other picornaviruses in this respect and it has been shown that B cells play an important role in the immune response to TMEV. However, although they play a crucial role in immunity to TMEV, they appear only to form part of the protective response.

The neutralising antibody response to TMEV was first shown by Lipton and Gonzalez-Scarano, (1978). They demonstrated that antibody able to neutralise virus was first detectable one week following inoculation but was slow to develop and did not peak until four to eight weeks post inoculation. Although the increase in neutralising antibody is slow to develop it does correlate with the decrease in infectious virus titre, in the acute phase of the disease. However, antibody clearly lacks the ability to clear virus, because TMEV can still persist in a susceptible mouse which has high antibody levels (Rabinowitz and Lipton, 1976).

Since Lipton’s initial studies there have been many others who have tried to further characterise the antibody response to TMEV. Pena Rossi and co-workers elucidated that IgM was the major isotype produced one and two weeks post inoculation in both resistant and susceptible strains (Pena Rossi et al., 1991). However, the levels of IgM were much higher in resistant C57Bl/6 mice than they were in susceptible SJL/J mice. Serum taken from C57Bl/6 mice at 2 weeks post infection was then transferred into susceptible SJL/J mice during the acute phase of Theiler’s virus disease. This dramatically decreased the number of virus antigen positive cells in the spinal cord, whereas serum taken from infected SJL/J mice at 2 weeks P.I. had no such effect. These data suggest that IgM is the major isotype involved in clearing
the virus during the acute phase of the disease. Decreased levels of IgM in SJL/J mice may contribute to their inability to clear virus.

SJL/J mice were protected from TMEV induced demyelinating disease after being pretreated with an attenuated virus. This protection was due to the presence of B cells and CD4+ T cells but was not connected with CD8+ T cells (Kurtz et al., 1995). While protection could not be transferred by neutralising antibody or serum from previously inoculated mice it did correlate with the number of plasma cells in the CNS during the first week post-infection.

A number of studies have observed intrathecal antibody synthesis in the CNS during Theiler's virus infection (Lipton and Gonzalez-Scarano, 1978; Roos et al., 1987; Cash et al., 1989). Antibody levels are increased in the CSF when compared to the serum of animals infected with TMEV (Lipton and Gonzalez-Scarano, 1978). It was discovered by isoelectric focusing that the antibodies in the CSF were oligoclonal IgM (Rodriguez et al., 1988). The antibodies in the CSF had a restricted repertoire, (predominantly for neutralisation) with some of the clones not present in the serum (Roos et al., 1987).

The antibody response during the chronic phase of the disease was the focus of a study carried out by Karpus and colleagues (Karpus et al., 1994). They discovered that C57Bl/6 mice without virus persistence had a significantly lower anti-viral antibody response at 7 weeks P.I. compared to SJL/J mice with a persistent infection, and that C57Bl/6 mice predominantly produced antibodies of the subclass IgG1, whereas SJL/J mice predominantly produced antibodies of the subclass IgG2a. These IgG's are considered to be indicative of the T helper cell response (Mossman et al., 1986, 1991; Huber et al., 1994, Kelso et al., 1995). The dogma suggests that IgG1 is associated with T helper cells of subclass 2 (Th2), whereas, IgG2a is associated with the T helper subclass 1 (Th1). Th1 responses are associated with production of proinflammatory cytokines involved in DTH which increase macrophage
activity. Differences in Ig class or subclass may directly influence the progression of the disease and/or be a marker of T-helper cell activity.

Xid mutant mice are unable to mount an antibody response. Intermediately susceptible CBA mice which have the Xid mutation have increased demyelination as compared to wild type CBA's (Rodriguez et al., 1990). The explanation proposed for this is that Xid mutant CBA's are unable to clear the virus effectively resulting in an increased virus titre and as a result increased demyelination. This confirms the importance of the antibody response in prevention of the pathology. However, mice on a resistant genetic background with the Xid mutation did not develop demyelination suggesting another mechanism of viral clearance is sufficient to eradicate the infection in these animals (perhaps anti-viral cytotoxic lymphocytes).

Other evidence supporting the importance of B cells in providing protection from disease includes the inability of B cell deficient mice to clear TMEV resulting in demyelinating disease; unlike the control group (Kurtz et al., 1995). Rodriguez et al. (1993) reported that anti-μ treatment from birth, aimed at suppressing B cells and antibody production, resulted in aggravation of inflammatory lesions in SJL/J mice infected with TMEV. This indicated that antibodies play a role in limiting spread in susceptible strains. No accurate conclusive results could be given as the μ depletions were incomplete.

Antibody epitopes which are involved in neutralisation of viral infectivity have been investigated (Inoue et al., 1994,; Kurtz et al., 1995, Usherwood et al., 1995). The antibody response is directed against the capsid proteins VP1 and VP2. However, serum from individuals immunised with purified VP1 showed a greater neutralisation capacity when compared to those individual immunised with purified VP2. This indicates that the viral capsid protein VP1 contained immunodominant epitopes (Clatch, 1987a; Scheu et al., 1989, Cash et al., 1989). This is consistent with other Picornaviruses such as
poliovirus and foot and mouth disease virus, where vaccination with VP1 prior to infection can lead to eradication of virus.

The use of synthetic peptides has defined the major linear antibody epitopes on BeAn virus which are recognised by SJL/J, BALB/c and C57Bl/6 mice. These were recognised to be VP1 12-25, VP1 146-160, VP1 262-276, VP2 2-16, VP2 165-179 and VP3 24-37 (Inoue et al., 1994). Only antibodies to VP1 262-276, VP2 2-16 and VP2 165-179 were able to neutralise plaque formation in vitro. Interestingly, the epitopes recognised by SJL/J mice are different from those recognised by BALB/c and C57Bl/6 mice. This may have important implications in viral pathogenesis. It has recently been found that preimmunisation with fusion proteins for VP1 and VP2 (but not VP3) results in the protection of susceptible mice from demyelination. These mice displayed high levels of neutralising antibody to the capsid proteins of the immunogen, indicating that immunisation with VP1 and/or VP2 results in complete protection from TMEV induced demyelinating disease.

In a separate study also using synthetically generated peptides Usherwood et al., (1995) determined B cell epitopes in the VP1 region, using serum from CBA mice. The immunodominant epitope was in the C terminal region and consisted of 52 amino acids VP1 225-276. Other peptides also strongly recognised included, VP1 25-39, VP1 81-95 and VP1 121-135. However, one must remember that in the assays used the peptides may not assume the same conformation as the corresponding areas on the intact virion therefore generally only linear epitopes can be determined.

In addition to virus specific antibodies it has been suggested by several authors that autoantibodies are generated during TMEV persistent infection (Rauch et al., 1987, Welsh et al., 1987). Antibodies against whole myelin and myelin basic protein have been shown to parallel chronic demyelinating disease. Whether autoantibodies promote disease or whether their
production is induced by the release of myelin proteins following damage, and is non-pathogenic, remains unclear.

The generation of antibodies is partially dependant on T cells, as was shown by Borrow and colleagues who depleted CBA mice of CD4+ lymphocytes before inoculation with TMEV. This resulted in an inability to generate neutralising antibody and death of the animals (Borrow et al., 1992). However, in another study, nude mice (which were on a C57BI/6 background) were able to generate an antibody response, although it was 3 fold less that the control group inoculated in parallel, suggesting that T cells were not absolutely required for the generation of B cell responses during the acute phase of the disease (Rodriguez et al., 1986, Zurbriggen et al., 1991).

1.14.2 T cell response

The cell mediated arm of the immune response is equally as important as the humoral arm. It is imperative to understand that both arms do not act independently of each other, but instead communicate to constitute a fully functional and competent immune response.

The cell mediated arm of the immune response to TMEV is very complex and has been the centre of much research carried out over the last ten to fifteen years. Lipton and co-workers first detected a splenic TMEV specific T cell proliferative response in SJL/J mice that had been inoculated with virus i.c (Lipton and Dal Canto, 1976). This splenic T cell proliferation response was first detectable 78 days post infection, and persisted for one year. To prove that the delayed detection of both neutralising antibody levels and T cell proliferation were not a result of virus-induced immunosuppression, sheep red blood cells (SRBC) were also inoculated. The immune response to these SRBC was normal, eliminating the possibility that neutralising antibodies and
T cell proliferation were delayed due to generalised immunosuppression (Lipton and Dal Canto, 1976).

Clatch and colleagues tried to characterise further the cell mediated immune response by comparing the responses in resistant and susceptible mouse strains (Clatch et al., 1985, 1986, 1987b). Both resistant and susceptible mice developed high anti-viral antibody responses during the chronic phase which rose with the same kinetics, however, the splenic TMEV specific proliferation response varied greatly between mouse strains. It was observed that susceptible SJL/J mice developed a specific DTH and T cell proliferation response within 10-14 days post infection, preceding the onset of clinical disease, and this remained elevated for 180 days. In contrast, resistant BALB/c mice developed low levels of T cell proliferation response and no measurable DTH (Clatch et al., 1987b). The results from this set of experiments led to the hypothesis that TMEV infected susceptible mice develop a virus specific DTH reaction which is absent in resistant strains, and that this subsequently causes the recruitment of T cells and macrophages into the CNS which causes non-specific bystander damage to the myelin sheaths and hence demyelination (Clatch et al., 1986; Miller et al., 1987, 1990; Peterson et al., 1993; Gerety et al., 1994a, 1994b). A pathological DTH is supported by the observation that demyelinating lesions contain many mononuclear cells and macrophages engaged in myelin stripping from nerve axons (Dal Canto and Lipton, 1975).

Further evidence supporting this hypothesis came from lymphocyte depletion studies. Susceptible mouse strains depleted of CD4+ lymphocytes before the onset of demyelinating disease showed a marked decrease in the incidence of disease. 77% of immunocompetent animals developed paralysis as compared to only 36% of animals depleted of CD4+ cells before the demyelination phase (Borrow et al., 1992; Welsh et al., 1987).
The depletion studies also showed that if animals were CD4+ depleted before infection, then they died approximately 1 month after inoculation. This was probably due to the fact that in the absence of CD4+ cells animals were unable to produce anti-viral antibodies and consequently were unable to clear the virus from the CNS resulting in a fatal infection (Borrow et al., 1992, Welsh et al., 1987). However, during the acute phase of the disease CD4+ cells probably play an important role in providing help to produce a good antiviral antibody response, and therefore protect the animal from fulminant encephalitis. However, antibody alone is incapable of completely clearing the virus from the host. Transfer of hyper-immune serum was able to prevent death in CD4+ depleted animals, but virus persisted and was able to induce demyelination (Welsh et al., 1987).

In conclusion, these studies have shown that although CD4+ cells are important, and play a protective role in clearing the virus in the acute phase of the disease, probably via antibody production, they conversely play a role in the pathogenesis of TMEV induced demyelination during the chronic phase.

The role of antigen specific CD4+ T cells was extended by Karpus and colleagues in some classical immunotolerance experiments (Karpus et al., 1994). A susceptible mouse strain was intravenously inoculated with UV inactivated TMEV which was coupled to syngeneic splenocytes via a carbodiimide bond, prior to inoculation with active TMEV. This resulted in decreased DTH and T cell response. There was also a decrease in the levels of splenic mRNA for IL-2, IFNγ and TNFβ, however there was no decrease in the levels of mRNA for IL-4, IL-6 and IL-10. Surprisingly anti-TMEV antibody levels in tolerised mice were 20-100 fold greater with a decrease in IgG2a and a dramatic increase in the amount of IgG1 when compared to the sham tolerised control group. These data suggest that Th1 cells (ie cells associated with IL-2, IFNγ and TNFβ and antibodies of the IgG2a isotype)
were specifically anergized with concomitant stimulation of Th2 cells (cells associated with IL-4, IL-6 and IL-10 cytokines and also antibodies of the IgG1 isotype). These data implicate Th2 cells in protection from developing chronic demyelinating disease and Th1 cells as effectors of disease. The induction of antigen specific in vivo anergy has important implications in the design of therapeutic strategies for immunopathological diseases mediated by Th1 cytokines especially T cell mediated autoimmune disorders.

Several other studies have been undertaken which have demonstrated the importance of T cells and these have all involved modulating the immune response, either by drugs or by monoclonal antibodies (Welsh et al., 1987; Borrow et al., 1993; Gatrill unpublished observations). Cyclophosphamide and/or anti-thymocyte serum cause a general immunosuppression (Lipton and Dal Canto, 1976, 1977; Roos et al., 1981). Treated mice show a decrease in the degree of mononuclear cell inflammation and demyelination. However, more specifically, animals given monoclonal antibodies to Ia MHC class II molecules after acute disease show a decrease in demyelination indicating that the expression of I-A antigen in the CNS between different strains may contribute to susceptibility (Rodriguez et al., 1986; Friedmann et al., 1987). MHC Class II molecules are expressed on antigen presenting cells and present foreign antigen to CD4+ T cells. RHAβ0/0 mice were constructed on a resistant background (H-2b) and are deficient in their ability to express MHC class II molecules, when these mice were infected with TMEV they were unable to clear the virus and developed lesions of demyelination (Fiette et al., 1996). However, the foci of demyelination were much smaller and fewer when compared to susceptible SJL/J mice. These studies indicate that demyelination can occur without CD4+ T cells, however the levels are not as large as they would be if CD4+ T cells were present. This implies that CD4+ T cells as important effector cells in the demyelinating process, but also demonstrates that demyelination can occur by a non-CD 4+ T cell dependent mechanism.
The importance of T cells in preventing demyelination is also observed in nude mice which are congenially deficient in T cells and which provide a useful model in which infection can be elucidated in the absence of mature T cells. TMEV infected nude mice had increased levels of CNS virus load and mortality when compared to a control group (Rosenthal et al., 1986; Zurbriggen et al., 1991). They did not generate any anti-viral antibodies and rapidly developed small lesions of demyelination. These studies illustrate that T cells provide an important contribution to controlling infection during the acute phase of the disease, but as with the RHAB0/0 mice it also illustrates that demyelination can occur in the absence of T cells. In these nude mice CNS virus persisted at high levels and was unable to be cleared. It is thought that in these mice demyelination results from lytic infection of oligodendrocytes. This is suggested by electron microscopy of the spinal cords of these animals which showed dying oligodendrocytes containing viral material (Blakemore et al., 1988). These data suggest that in resistant strains T cells prevent demyelination during the acute phase. Conversely, in susceptible and intermediately susceptible strains during the chronic phase, T cells cause demyelination.

T cell responses against whole virus have been widely reported (Lipton and Dal Canto, 1976) however, only recently has there been data to suggest the fine specificity of the T cell response to TMEV. As with other picornaviruses TMEV is composed of 4 structural proteins VP1, VP2, VP3 and VP4. It has been well documented in other epitope mapping studies of picornaviruses such as Mengo virus, Coxsackie B4 and FMDV (McPhee et al., 1994; Zamorano et al., 1994, Duque and Palemberg; 1996) that T cell epitopes are present in the VP1 capsid protein. This is also true of Theiler’s virus. A study using fusion proteins and synthetic peptides reported that for H-2s mice a CD4+ T cell epitope was present in VP1 between amino acids 233-244 (Yauch et al 1994). Other CD4+ T cell epitopes were elucidated by Gerety et al. (1994a, 1994b) in SJL/J (H-2s) mice VP2 274-284. Usherwood et al. (1995)
determined CD4+ T cell epitopes in CBA (H2-k) mice were located at VP1 33-47, 153-167, 166-180, 225-239, 233-244. Yauch also discovered a CD4+ T cell epitope at VP3 24-37 (Yauch et al., 1995). All of the above epitopes were located in capsid proteins. However, it is not to say that more epitopes do not exist as no studies have been carried out on the P2 and P3 regions of the genome. The further identification of determinants recognised by the virus specific T lymphocytes will help elucidate the mechanisms involved in cell-mediated immunopathogenesis and may have important implications in peptide therapy for virally induced demyelinating diseases.

Immunostaining of CNS material during the acute phase showed CD8+, CD4+ and MAC-1+ cells were all present by day 7 in resistant C57Bl/10 mice whereas only CD4+ and MAC-1+ cells were present in susceptible SJL/J mice. The authors suggested that CD8+ cells were involved in clearing the virus from C57Bl/10 mice, however, the markedly slower response in the SJL/J mice allowed the virus to persist and hence cause demyelination (Lindsley and Rodriguez, 1989).

The role of CD8+ T cells in the chronic infection is still unclear (Lindsley et al., 1991; Pena Rossi et al., 1991). As mentioned previously resistance to Theiler’s virus induced demyelination genetically maps to the MHC class I D region and is associated with up-regulation of class I products and the presence of class I restricted virus specific cytotoxic CD8+ T cells in the CNS. Haplotypes associated with susceptibility are s, f, p, r, v, q and with resistance are b, k, d.

Pena Rossi et al. (1991) showed for the first time that TMEV was able to elicit a CTL response. This was unusual because the only other known picornavirus to generate a CTL response was coxsackie B3 (Seko et al., 1996). Lindsley et al. (1991) also demonstrated that TMEV infection produced a specific CTL response which peaked at day 7 post infection, and noted no difference in response between resistant and susceptible strains. These experiments also
demonstrated that CTL's were present during the acute and chronic phases of the disease. As well as being detected in the CNS as early as day 7 post infection, virus specific CTL’s were isolated from the CNS of SJL/J mice with a chronic infection on day 226 post-infection. The above data argue against the hypothesis that CD8+ T cells confer resistance, however; although both resistant and susceptible strains generate specific CTL’s they may recognise different epitopes and this may explain the difference between viral clearance and persistence.

Capsid proteins VP1 and VP2 of TMEV serve as targets for T cell mediated cytotoxicity in resistant H-2b mice but not susceptible H-2s mice. This difference may be related to their differences in ability to clear virus from the CNS. Analysis is continuing to elucidate CD 8+ T cell epitopes (Lin et al., 1995).

Rodriguez and colleagues (1993) reported that the CD8+ depleted animals had a reduced severity of demyelination and the results were the same whether the animals were depleted at the time of infection or 15-17 days after. However, contradictory to Rodriguez, Borrow and co-workers reported that animals depleted of CD8+ T cells had an increase in severity of clinical disease and viral clearance was slower (Borrow et al., 1992). Borrow et al., 1992 also reported that depletion of animals at 4 weeks post-inoculation had no effect on the onset of clinical disease. Gerety and colleagues confirmed similar results to Borrow (Gerety et al., 1994a, 1994b). Obviously, these latter reports suggest opposite roles for CD8+ T-cells to the Rodriguez study. Rodriguez’s study suggests that CD8+ T cells contribute to demyelination perhaps by lysing virally infected oligodendrocytes, whereas Borrow suggests that CD8+ cells are restricting pathology and preventing demyelination presumably by clearing virus and thereby removing the stimulus for a continued DTH response.
β-2-microglobulin is a constituent of the MHC class I molecule, therefore β-2-microglobulin deficient mice have no functional CD8+ T cells. These mice were bred on a resistant background (C57Bl/6 x SV129). The results obtained when wild type and knockout mice were inoculated with virus were that the knock out mice were not able to clear virus and established a persistent infection and consequently demyelination (Rodriguez et al., 1993; Fiette et al., 1993). Encouragingly, the converse was true of wild type mice with no virus antigen or demyelination detectable. Also the anti-viral antibody response was unaffected by the absence of functional MHC class I.

Recent studies by Pope et al., (1996) have also illustrated by flow cytometry that CD8+ T cells exist in the CNS from day 20 to day 120 post infection in SJL/J mice however, the level of activated CD8+ T cells is low. This was measured by determining the amount of IL-2R on the surface of the CD8+ T cells. These results argue against a major role for CD8+ T cell mediated cytotoxicity mechanisms in the pathogenesis of chronic demyelinating disease in the SJL/J mouse strain inoculated with the BeAn strain of TMEV. Whereas CD8+ T cells infiltrating the CNS of SJL/J mice do not appear to function directly to cause demyelination they might have as yet an undefined role in the pathogenesis of the disease.

Further evidence demonstrating the importance of CTLs during the acute phase of infection was provided by Larsson-Sciard et al., (1997). It was discovered that in vivo administration of IL-2 by inoculation of transfected tumour cells resulted in complete protection from chronic demyelinating disease in the usually susceptible DBA/2 mice. IL-2 treatment resulted in a 3-4 fold increase in TMEV specific CTL precursors (Larsson-Sciard et al., 1997). As mentioned earlier CTL’s have a critical role in viral clearance therefore Larsson-Sciard’s results suggest that induction and recruitment of TMEV specific CTLs are necessary for clearing the virus, perhaps before it reaches immunopriviliged sites allowing chronic infection. The observation that
CD8+ T cells are detected earlier in the CNS of resistant mice supports the idea that CTLs are important in clearing the virus from the CNS during the acute phase of infection. Therefore the levels of IL-2 appear to be important in generating CTL precursors and CTLs necessary for clearing the viral infection.

The picture is not entirely clear but it seems that antibody is important in limiting the spread of the infection but appears to be incapable of clearing the infection whereas CD8+ T cells may have a role in eliminating remaining virus in the acute stage of the disease, but they are not absolutely required to prevent death from overwhelming viral replication. Demyelinating lesions are present in the absence of CD8+ cells, suggesting that these cells do not play a direct role in the pathology of demyelination. On the other hand mice depleted of CD4+ cells previous to virus inoculation all die due to lack of anti-viral antibodies, and animals CD4+ depleted after the acute phase of the disease have a decrease in demyelinating lesions. This implies that CD4+ cells have a protective role during the acute phase of the disease by facilitating production of anti-viral antibodies, but alternatively have a pathogenic role in the formation of demyelinating lesions during chronic infection. Further, there is some indication that Th2 responses may be protective and Th1 pathogenic.

1.14.3 Cytokines in TMEV infection

As with other CNS viral infections the cytokine response elicited during TMEV infection appears to be complicated.

There is evidence to suggest that TNFα is important in the demyelinating process. Levels of TNFα in cells isolated from spinal cords of SJL/J mice were investigated by ELISPOT assay, and it was demonstrated at day 40 post infection that relative to uninfected control mice, SJL/J mice had elevated
levels of TNFα. Levels of this cytokine correlated with disease severity and levels of inflammatory infiltrates in the CNS (Inoue et al., 1996). The number of TNFα producing cells in spinal cords of SJL/J were comparatively higher than the levels in resistant BALB/c and C57Bl/6 mice. Also the levels of TNFα in supernatants from cultured cells isolated from the CNS or spleens were much higher in clinically affected animals compared to resistant control animals. SJL/J mice treated with a monoclonal antibody to TNFα before the onset of clinical signs disease had decreased disease severity. The identity of the TNFα producing cells was not characterised. However it is known that TNFα is secreted by a variety of different cells of both immune and CNS origin. There is evidence that shows that TMEV infected astrocytes from both resistant and susceptible mouse strains produce IL-1α, IL-6 TNFα and TNFα receptor in vitro (Rubio and Torres, 1991; Rubio, 1994; Rubio and Seirra, 1993).

TNFα has been identified in acute and chronic MS lesions (Navickas and Link, 1996; Spuler et al., 1996; Brown et al., 1996; Philippe et al., 1996) and TNFα was discovered to have a profoundly cytotoxic effect on in vitro cultures of oligodendrocytes (Soliven et al., 1995; Qi and Dal Canto, 1996). TNFα also decreases mRNA expression of myelin proteins in primary oligodendrocyte cultures. These data all support a role for TNFα in the pathogenesis of Theiler’s virus infection.

Contradicting the above data, treatment of TMEV infected animals with TNFα ameliorated demyelination (Paya et al., 1990). There was no effect on grey matter disease but white matter disease was dramatically reduced. A possible explanation for this, as it contradicts the other results could be that it down regulates destructive T cells and macrophages, or it may be due to desensitisation of oligodendrocytes due to the large initial dose (tachphylaxis).
The interferon systems are extremely important in many viral infections. This is also true for TMEV. IFNa/β is an absolute requirement to prevent mortality from TMEV infection as described by Fiette et al., (1995). TMEV infection of mice with dysfunctional IFNa/β receptors died as early as day three post infection with extensive dissemination of virus throughout the CNS. IFNγ is also important in TMEV infection. Its functional role was elucidated by inoculating genetically engineered IFNγ receptor knockout mice which were on a resistant genetic background (Fiette et al., 1995). These mice had an inability to clear virus from the CNS and had extensive white matter demyelination, unlike the resistant WT control group. It has also been reported that treatment of susceptible mice with a monoclonal antibody to IFNγ increases viral persistence (Kohanawa et al., 1994). It could be speculated that IFNγ is necessary for generating an activated and fully functional cytotoxic T cell response which is necessary for viral clearance.

With regards to other viral infections, LCMV infected IFNγR°/° mice have normal antibody levels but greatly reduced levels of CTLs which results in persistence of LCMV in the CNS, this could also be true for TMEV (Moskophidis et al., 1994; Orange et al., 1996; Planz et al., 1997).

Evidence of the importance of IL-1β in TMEV infection was illustrated by experiments with LPS (Pullen et al., 1995). LPS is produced by gram negative bacteria and is known to potentiate immune responses and to trigger resident CNS cells to produce various inflammatory cytokines. The ability of LPS to augment demyelination after TMEV infection was investigated in resistant C57Bl/6 mice. When treated with LPS these mice developed clinical disease as compared to the control group which did not display any clinical signs. This indicated that the type and levels of cytokines produced are instrumental in determining whether or not the animal develops clinical signs. The effects of LPS could be mimicked in C57Bl/6 mice by administering IL-1β, suggesting that the increase in susceptibility to TMEV induced demyelination following LPS stimulation may function via IL-1β. These results have
important implications because they indicate how altering the balance of inflammatory cytokines may determine the difference in susceptible and resistant strains.

Therefore, it can be seen from the above data that many different cytokines have many different roles in TMEV infection, and the specific role(s) of individual cytokines is an area of active research.

1.14.4 Natural Killer Cells

Natural killer (NK) cells are important mediators of the innate immune response and are particularly important in some viral infections (Kagi et al., 1995). NK cells function by lysing infected cells in a non-MHC restricted manner, this is mediated by the perforin molecule. The importance of NK cells in TMEV infection was first investigated by Paya and colleagues (1989). They reported that susceptible SJL/J mice had increased levels of virus when compared to the resistant C57Bl/10 strain and suggested that this could be attributed to the SJL/J mice having a 50% reduction in NK cells compared to C57Bl/10 mice. Resistant C57Bl/10 mice when rendered NK cell deficient either by depletion using mAb NK1.1 or polyclonal anti asialo-GM1, had diffuse encephalitis in the grey matter during the acute phase of the disease, and mice treated with the anti asialo-GM1 developed lesions of demyelination. This data suggests that NK cells help to protect resistant mice from encephalitis.
1.15 Aim of thesis

The main aim of this thesis was to investigate the temporal changes in cytokine and immunoglobulin levels during the acute phase of disease. The rational for this was that different mouse strains have different susceptibilities, therefore it was of interest to determine whether different mouse strains elicit different immune responses during the acute phase of disease, which in turn may account for the differences between viral clearance and viral persistence. The powerful new technique of RNase Protection Assay was used to determine the expression of numerous cytokine mRNAs. Therefore from the cytokine environment it was possible to determine whether there was a pro or anti inflammatory, Th1 or Th2 immune response present. The cytokine and immunoglobulin profiles were determined during the chronic phase of Theiler's virus infection in intermediately susceptible CBA mice. During the chronic phase of Theiler's virus disease 50% of CBA mice clear the infection. Therefore comparing the cytokines expressed in animals in which virus was cleared and in which virus persisted would determine whether a particular type of cytokine environment i.e. type 1 or type 2 was present during the demyelinating phase of the disease.

Microsatellite DNA mapping has isolated a region near the IFN gene in determining susceptibility. Therefore the roles of type I and II interferon were investigated during the acute and chronic phase of Theiler's virus disease in IFNα/βR and IFNγR knockout mice. The importance of CTLs in clearing Theiler's virus was discussed in the introduction, however, the mechanism by which CTLs function during Theiler's virus was not known. Therefore, Theiler's virus infection in perforin deficient mice was investigated to ascertain its role during the acute and chronic phases of Theiler's virus infection.
Very little research has been carried out on the immunology of the neurovirulent GDVII strain of Theiler's virus therefore the levels of infectious virus, cytokine levels and amount of programmed cell death was investigated in detail. It has been recently reported that Sindbis virus induces a TNFα associated shock phenomenon in neonatal mice, therefore it was of interest to determine whether neonatal animals infected with Theiler's virus were undergoing the same process of TNFα shock.
Figure 1.1 The arrangement and function of genes in the Theiler’s virus genome. Showing the typical 4:3:4 arrangement seen in picornaviruses.
Figure 1.2 X-ray crystallography of a picornavirus (Human Rhinovirus 14).
Figure 1.3 The picornaviral infection cycle (adapted from Reukert, 1990) The first step in infection is the attachment of the virion to a cellular receptor (1). Transfer of the VPg-RNA genome occurs by a poorly understood mechanism involving a conformational change and loss of VP4. The viral genome is then translated by the normal host cell translation factors (2) and the resulting polyprotein is cleaved by viral proteases to liberate mature viral proteins. These are involved in viral replication (3 and 4) and in assembly of virions (6). The first stage of RNA replication is to copy the genomic RNA into negative strand RNA which involves the formation of double stranded (RF) replicative form (RF) RNA (3). Replicative intermediate (RI) forms of RNA are present when more positive stand RNA is synthesised for further translation (5) and for packaging into assembling virions (7). The packaging of the VPg-RNA is accompanied by a maturation cleavage of VP0 to form VP4 and VP2. Cell necrosis and/or apoptosis are the main routes of virion exit from the cell (8).
**Figure 1.4** Immune mediated theory of demyelination (adapted from Miller et al., 1997).

1) Viral antigen is recognised in the periphery and T cell is stimulated to proliferate and differentiate. 
2) Activated T cell penetrates the blood brain barrier and gains access to the CNS. 
3) Activated Th1 cells recognise viral antigen which is presented by MHC class II on the surface of a tissue antigen presenting cell. 
4) Proinflammatory cytokines and chemokines are released which attract monocytes from the periphery, stimulate macrophage activation, and cause tissue destruction. 
5) Activated macrophages produce proinflammatory cytokines as well as nitric oxide and O₂ radicals leading to further tissue destruction. 
6) Myelin is released into the periphery where it is presented by an APC, and recognised by a Th1 cell, 8) which then proliferates and differentiates and then crosses the BBB leading to further autoimmune destruction.
Chapter 2

Materials and Methods
## Chapter 2 - Materials and Methods

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2.1 Chemicals and reagents

Unless otherwise stated the chemicals used in this study were obtained from BDH Chemicals Ltd. (Dagenham, England), Sigma Chemical Company (Poole, England), GIBCO BRL (Paisley, Scotland) or Fisons Ltd. (Loughborough, England). All radiolabelled isotopes were obtained from Amersham International Plc (Amersham, England). The composition of buffers and reagents used in this thesis are listed in Appendix 1.

2.2 Virus

The BeAn 8386 and GDVII strains of TMEV used in this study were a gift from Prof. H. L. Lipton (Mount Sinai Medical Centre, New York, USA). Both BeAn 8386 and GDVII were grown in confluent BHK-21 cells maintained in Glasgow’s modified Eagles Medium (GMEM), supplemented with 10% foetal calf serum, 200 mM L-Glutamine, penicillin and streptomycin at 37°C, 4% CO₂. Infectious supernatant from these cultured cells was collected at day 3 post infection and clarified by centrifuging at 1000 g for 15 min, and stored at -70°C. The same batches of BeAn and GDVII were used throughout this study.

2.3 Animals

3-4 week old female CBA, SJL/J, BALB/c and C57Bl/6 mice were obtained from Bantin and Kingman. Female CBA and BALB/c mice older than 4 weeks old, Sv 129, interferon α/β receptor knock-out, interferon γ receptor knock-out, C57Bl/6 and perforin knock-out mice of various ages were all obtained from the Department of Veterinary Pathology's animal unit (University of Edinburgh, Scotland). Mice were maintained on a dry diet and lighting was set on the standard 12 hr light and 12 hr dark cycle.

2.4 Inoculation and clinical scoring of mice

2.4.1 Intracranial inoculations

Mice were inoculated intracranially with $10^4$ PFU of TMEV BeAn or GDVII in 20 μl of sterile phosphate buffered saline, under halothane anaesthetic.
2.4.2 Clinical grading of mice during the acute and chronic phases of Theiler’s virus.

Mice suffering from acute and chronic Theiler’s virus induced disease were graded from 1-5. A score of 1 indicated a gait impairment and increasing scores to 5 indicated a more severe paralysis, with 5 indicating 2 or more limbs been paralysed, leading to wasting and death.

2.5 Sampling and preparation of tissues for histology

Mice were bled by cardiac puncture and transcardially perfused with PBS, and tissues removed, 100 U of heparin was administered around the cardiac area to prevent clotting. Removed tissues were either fixed in isopentane or 4% phosphate buffered formal saline and subsequently processed for histology, or snap frozen in liquid nitrogen and stored at -70 °C until use in a virus titration assay or an RNA extraction assay. Serum was obtained from all sacrificed animals by centrifuging blood samples at 10,000 g for 5 min, and retaining the serum, this was then stored at -20°C.

2.6 Tissue Culture

Unless otherwise stated all tissue culture plasticware was obtained from Falcon Laboratories (Becton Dickinson, UK). All tissue culture procedures were carried out under standard aseptic conditions. New born calf serum was supplied by GIBCO BRL and Glasgows modified Eagles medium (GMEM), tryptose phosphate broth, Hank’s buffered balanced salts (HBBS) and PBS were all prepared at the Department of Veterinary Pathology, University of Edinburgh (see Appendix 1).

2.7 Virus Plaque Assay

Brains and spinal cords were removed from infected mice and homogenised in GMEM supplemented with 20 mM HEPES. Brains were homogenised 1:5 w:v and spinal cords 1:10 w:v. A polytron homogeniser was used to homogenise the samples. Ten-fold serial dilutions were made of the
Chapter 2

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Homogenised tissues and 200 μl of the appropriate dilution was added to HBBS (Hank’s buffered balanced saline) washed subconfluent monolayers of BHK cells in 6-well plates and incubated for 45 min at room temperature. Monolayers were then overlaid with 1% agar in GMEM and once the agar had set the cultures were overlaid with 2 ml GMEM to prevent dehydration. The samples were then incubated for 3 days at 37°C, 4% CO₂. They were subsequently fixed in 4% formal saline for 30 min and stained with 0.1% toluidine blue before counting.

2.8 Virus neutralisation assay

Virus neutralisation assays were carried out to determine the amount of neutralising antibody. Serial dilutions of serum samples from infected mice were assayed for neutralising antibody as described before (Rabinowitz and Lipton, 1976).

2.9 Total RNA Extraction

2.9.1 GTC Method

Total brain RNA was extracted from brains of infected mice using the GTC method (Chomczynski et al., 1987).

Half brains were homogenised in 12 ml of ice cold guanidinium thiocyanate (GTC) solution (4.0 M GTC, 25 mM Na Citrate pH 7.0, 0.5% Sarkosyl, 0.1 M β-Mercaptoethanol, 1-2 drops of Sigma anti-foam) using a glass dounce homogeniser that had been specifically prepared for RNA extraction. The dounce homogenisers were steeped in 10% DECON, rinsed thoroughly, stood in 1 M NaOH for 1 hr, rinsed in sterile distilled water, and steeped for a further hour in chromic acid 1 M, rinsed 6X in sterile water, dried and autoclaved. Homogenised tissue suspensions were laid onto a 2 ml cushion of CsCl (5.7 M) and EDTA (0.1 M, pH 7.0), which was DEPC treated; in Beckman polyallomer ultracentrifuge tubes (Beckman, UK) and centrifuged at 35 K for >16hr at 15°C, in a SW40 rotor (Beckman, UK). After centrifugation
was complete the GTC layer was removed and the interface washed with ice cold GTC solution, the GTC solution and CsCl, EDTA cushion was subsequently removed, leaving a gelatineous pellet which was washed with 70% ethanol and 1X TNE (0.1 M NaCl, Tris 10 mM, 1 mM EDTA). The pellet was spun down, resuspended in sterile water and ethanol precipitated at -20°C. The resulting pellet was lyophilised and resuspended in 1X TE, analysed (see Section 2.10) and stored at -20°C.

2.9.2 Extraction of RNA from cellular cultures
GMEM medium was discarded from the cells from which RNA was to be purified from, the monolayers were washed twice with PBS. 10 ml of GTC solution was added (5 min/RT) to instantaneously lyse the cellular monolayer. The thick viscous solution was removed and transferred to a screw capped tube. The tube was vigorously shaken to shear any DNA that was present. The preparation was then added onto a CsCl cushion and RNA extraction was continued and completed as for the mouse brain samples (see Section 2.9.1).

2.9.3 Trizol Method
RNA was extracted from spinal cords and baby mouse brains using the Trizol extraction method (GIBCO BRL, UK). Trizol is a ready to use RNA extraction buffer. Tissues were thoroughly homogenised in 1 ml of trizol using a tight fitting glass dounce homogeniser (which had been previously treated for RNA use only). Once a smooth suspension was achieved by homogenisation the preparation was allowed to stand for 10 min to enable the complete dissociation of nucleoprotein complexes. Next 0.2 ml of chloroform was added and samples were vortexed vigorously for 15 secs and incubated (15 min/RT). The samples were then centrifuged at 10,000 g for 15 min/4°C. Two phases were apparent and the RNA remained exclusively in the upper phase. The upper phase was extracted and transferred to a fresh eppendorf, 0.6 ml of isopropanol was added and samples were incubated (15 min/RT).
Samples were then centrifuged at 10,000 g 10min/RT. The precipitated RNA was then lyophilized, resuspended in 1X TE buffer, quantitated and qualitated (see Section 2.10).

2.10 Analysis of RNA
The integrity of RNA samples was analysed by running them in a 1% or 2% agarose gel in 40 mM Tris, 40 mM acetic acid and 1 mM EDTA, pH 7.2. Gels were run at 90 V, stained with ethidium bromide 0.5 mg/ml, and products were visualised under long wave uv radiation. The concentration and purity of the RNA was determined by measuring the absorbance at 260 nm and 280 nm on the spectrophotometer (Phillips, Scientific Instruments, UK).

2.11 RNase Protection Assay
The cDNA clones which were used to generate subclones for the RNase protection assay have been previously described (Hobbs et al., 1993). The brains and spinal cords were removed from infected animals, which had been perfused with sterile PBS, and total RNA was prepared using the GTC method (brains) and Trizol method (spinal cords) as described above. Cytokine transcripts were quantified by using a method previously described (Melton et al., 1984).

Probe synthesis
Radiolabelled RNA probe sets were synthesised as described by Hobbs et al., (1993). Briefly, UTP (10 μM), GTP (2.5 mM), ATP (2.5 mM), CTP (2.5 mM) and $^{32}$P UTP 300 Ci/mmol were lyophilised for 45 min. To the dried nucleotides DTT (100 mM), transcription buffer (5X), RNasin (40 U/μl) and linearised templates (50-100 μg/ml) were added, with the initiation of transcription occuring by the addition of T7 Polymerase (10 U/μl). This reaction was stopped after 1 hr by the addition of RQ1 DNase (2 U/μl) for 30 min at 37°C. The probes were then purified by phenol/chloroform, chloroform and isoamyl alcohol extractions and precipitated with ethanol and
ammonium acetate. The probes were then lyophilised and resuspended in hybridization buffer (80% formamide, 1 mM EDTA, 400 mM NaCl, 40 mM PIPES, pH 6.7).

Hybridizations
1.9 µl of probe was added to 14 µg of lyophilized total RNA and dissolved in 8 µl of hybridization buffer. Uninfected total brain RNA was used as a control and yeast transfer RNA was used as a control for the digestions. All the samples were overlaid with mineral oil, heated to 90 ºC and incubated overnight (12-16 hr) at 56ºC.

Digestions
After the hybridization step unhybridized single stranded RNA was digested by the addition of RNase A (100 µg/ml) and RNase T (1500 U/µl), in Tris (1 M, pH 7.5), NaCl (5 M), EDTA (0.5 M, pH 8.0). After incubation for 45 min at 30 ºC samples were treated with 18 µl of a mixture of Proteinase K (0.5 mg/ml), SDS (10%), yeast tRNA (2 mg/ml). The duplexes of RNA were isolated by extraction/precipitation as before, air dried and dissolved in loading buffer (80% formamide, 1 mM EDTA, 50 mM Tris Borate pH 8.3, 0.05 w/v xylene cyanole and bromophenol blue) and electrophorised in a standard 5% acrylamide/8 M urea sequencing gel. Dried gels were placed on film with intensifying screens and were developed after a 2 week exposure at -70ºC. Densitometry was used to quantitate the autoradiographic images. The length (nt) of the mRNA transcripts for the different probes were known, therefore to ensure the correct band was identified, the distance travelled by the different bands was plotted against known length, so that accurate identification of the bands occurred. Once the bands were identified the autoradiographic image was sent to Dr Monte Hobbs (Scripps Research Institute) to double check that the correct bands had been identified.
2.12 RNA Dot Blot Hybridization

Various concentrations of brain RNA were added to 100 µl of blotting buffer (50% formamide, 7% formaldehyde, 1X SSC and 10 µg/ml of tRNA). Samples were heated to 68°C for 15 min and cooled on ice. 200 µl of 20X SSC was added to each sample, giving a final concentration of 14X SSC. The membrane used for Northern blotting was a positively charged nylon membrane (Boehringer-Mannheim, Germany). Prior to use the membrane was soaked in sterile distilled water. The dot blot apparatus was set up according to the manufactures instructions (Biorad, UK). Wells in the dot blot apparatus were washed with 200 µl of 20X SSC under vacuum. Samples were applied with no vacuum, then once samples were loaded in the wells a vacuum was gently applied. The wells were washed with 15X SSC and the membrane was allowed to air dry. RNA was crosslinked to the membrane using uv radiation (12,000 joules/3 min). The membrane was prehybridized in prehybridization solution (see Appendix 1) >1 hr at 65°C. Unhydrolysed digoxigenin labelled riboprobe specific for TMEV was used at a concentration of 1/1000 and boiled in hybridization buffer before being added to the membrane. Hybridization occurred for 12-16 hr at 65°C. After hybridization the membrane was washed twice in 2X SSC, 0.1% SDS (15 min/37°C) once in 2X SSC, 0.1%SDS (15 min/65°C) and finally washed twice in 0.2X SSC, 0.1% SDS (8 min/65°C).

Detection of Digoxigenin

After hybridization, hybridized digoxigenin probe was detected by incubating with a sheep anti-digoxigenin antibody and by using an enhanced chemiluminescence kit according to the manufacturers instructions (Boehringer-Mannheim, Germany). Luiminescence was determined by using autoradiography.
2.13 PCR

2.13.1 Oligonucleotide primers

Oligonucleotide primers and their genomic location are shown in Table 2.1. All primers were derived from the area spanning nucleotides 3078 to 4000 which are in the VP1/VP2 region.

Primers were selected based on the sequences published by Pevear et al. (1987), and the primers used for nested PCR were selected internally from the original first round primers.

Table 2.1.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Primer</th>
<th>Nucleotide</th>
<th>Tm°C</th>
<th>Mwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGTAATCAAGGATGAACTG</td>
<td>2B'</td>
<td>3879/3901</td>
<td>60.6</td>
<td>5213</td>
</tr>
<tr>
<td>ACCCAGCCTTGTTGAGGA</td>
<td>2A</td>
<td>3494/3510</td>
<td>59.8</td>
<td>6214</td>
</tr>
<tr>
<td>CACTAAGGACTTTGGAGTCG</td>
<td>N1'</td>
<td>3635/3654</td>
<td>62.5</td>
<td>6205</td>
</tr>
<tr>
<td>GTTGTACAGCATGATCTTGG</td>
<td>N2</td>
<td>3787/3806</td>
<td>67.7</td>
<td>6154</td>
</tr>
</tbody>
</table>

^c Tm (melting temperature) at 100 pM oligo and 1M salt.

' indicates complementary to the published sequence.

d calculated molecular weight of the primer.

2.13.2 One Step Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction of viral RNA was performed using a GeneAmp Thermostable rTth Reverse Transcription RNA PCR kit (Perkin Elmer, UK). Viral RNA was reverse transcribed in a 20 μl reaction mixture containing 1 μg of total brain RNA, 1X reverse transcription buffer (1 mM MnCl₂, 200 mM of GTP, CTP, ATP, TTP) 5 U of rTth polymerase and primer 2B “the down stream primer” (at 0.75 mM). Each sample was overlaid with mineral oil and incubated for 15 min at 58 °C. After incubation and
cDNA synthesis the 20 µl was transferred to 80 µl of PCR cocktail (0.8X chelating buffer, 2.5 mM MgCl₂, 0.15 µM of primer 2A the “upstream primer” then reactions were subjected to 35 cycles of amplification.

94°C for 30 secs
55°C for 30 secs
60°C for 60 secs

The cycles of replication were carried out in a Techne PH3 thermal cycler (Techne Ltd., UK). One tenth of the RT-PCR product was run on a 1% or 2% agarose gel in 1X TAE. The gel was then stained with ethidium bromide (0.5 mg/ml), and viewed under a UV light. To obtain maximum sensitivity gels were Southern blotted.

2.13.3 Nested PCR
To obtain maximum sensitivity without Southern blotting a nested PCR was developed in which products from the first round of RT-PCR were further amplified by nested PCR (Hofner et al., 1994; Tilson et al., 1995).

The nested PCR cocktail contained 2 µl of the first round RT-PCR product, 46 µl of the PCR cocktail as described above and 0.15 µM of primer N1 the “upstream primer” internal to the first round PCR primers and 0.15 µM of primer N2 the “downstream primer” internal to the first round PCR primers, and 5 U of rTth polymerase. The samples were subjected to 35 cycles of amplification as described above (Section 2.13.2) and the products of nested PCR were run as described previously on a 1% agarose gel (Section 2.13.2).

2.13.4 Analysis of Nucleic Acids by Southern Blot
10 µl of RT-PCR products were run on a 1% agarose gel. The DNA was transferred to a Hybond N+ membrane (Boeheringer-Mannheim, Germany)
by Southern blotting (Maniatis, 1989), and cross-linked to the membrane by uv radiation 1200 joules/30 secs.

The blotted membrane was then prehybridized at 65°C, >1 hr in 50% formamide, 5X SSC, 2% blocking solution (Boehringer-Mannheim, Germany) 0.1% SDS, and 0.002% tRNA. Hybridization occurred overnight with a non-hydrolysed digoxigenin-labelled riboprobe used at a concentration of 1:1000. After hybridization a series of washes were undertaken:

2, 2X SSC, 0.1% SDS, 15 min RT
1, 2X SSC, 0.1% SDS, 15 min 65°C
2, 0.2 XSSC, 0.1% SDS, 8 min at 65°C

After the stringency washes the DNA was detected using an anti-digoxigenin antibody and CSPD enhanced chemiluminescence kit according to the manufacturers instructions (Boehringer-Mannheim, Germany).

2.14 Enzyme-linked immunosorbent assay (ELISA)

2.14.1 Antigen Purification

Thirty T175 flasks of BHK clone 2 cells were grown at 37°C and 4% CO₂ until 80% confluent. Monolayers were washed three times with PBS and infected with TMEV at an M.O.I. of 0.1 PFU and incubated for three days. Tissue culture supernatants were collected and centrifuged at 1,000 g (15 min/RT). 10% SDS was added to give a final concentration of 0.8% SDS (92 ml of supernatant and 8 ml of 10% SDS). Supernatants were centrifuged in an ultracentrifuge (Beckman, UK) at 35K (2 hr/20°C) in a 45 rotor. Once pelleted the virus was soaked in 10 mM Tris-HCl (pH7.5) (0°C/1-2 hr). The pellets were pooled in 3 ml and added to 6.6 ml of CsCl in 10 mM Tris-HCl (pH 7.5) with a density of 1.51 g/ml. The preparation was cooled (20 min/0°C), and centrifuged (10,000 g/10 min/4°C). This step was included to precipitate the SDS. The solutions density change was readjusted with CsCl to give a final weight of 1.35 g/ml. The preparation was centrifuged for 48 hr, 40 K, 4°C in a
SW 55 rotor (Beckman, UK). After high speed centrifugation the virus banded in the centre of the tube, this band was removed and dialysed in 10 mM Tris-HCl (pH7.5).

2.14.2 Protein Assay of TMEV BeAn Virus yield

A standard Bradford protein assay was carried out to determine the concentration of TMEV (BeAn) virus obtained after the rigorous purification described above (Section 2.14.1). 1 ml of Biorad Bradford protein dye reagent (1/5 dilution, in water) was added to variety of standards, and to purified virus (see Table 2.2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30.0 µl standard (2 mg/ml Bovine Serum Albumin) + 20.0 µl</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>15.0 µl</td>
<td>+ 35.0 µl</td>
</tr>
<tr>
<td>C</td>
<td>7.5 µl</td>
<td>+ 42.5 µl</td>
</tr>
<tr>
<td>D</td>
<td>2.5 µl</td>
<td>+ 47.5 µl</td>
</tr>
<tr>
<td>E</td>
<td>1.5 µl</td>
<td>+ 48.5 µl</td>
</tr>
<tr>
<td>F</td>
<td>0.5 µl</td>
<td>+ 49.5 µl</td>
</tr>
<tr>
<td>G</td>
<td>50.0 µl</td>
<td>+ 0.0 µl</td>
</tr>
</tbody>
</table>

The above were vortexed and the absorbance was measured at 595 nm in a 1 ml quartz cuvette using a spectrophotometer (Phillips Scientific Instruments, UK). Optical densities are displayed (Figure 2.1). Virus yield was obtained by extrapolation on the standard curve obtained from the BSA standards measured. The concentration of purified virus obtained was around 600 µg/ml.
2.14.3 Purified TMEV (BeAn) quantification by plaque assay.

Purified TMEV (BeAn) was also quantified by viral plaque assay as described in Section 2.7. The titre obtained was $6.5 \times 10^8$ PFU/ml. Therefore 1 mg of viral protein is equal to approximately $1.1 \times 10^9$ PFU/ml.

2.14.3 ELISA Procedure

A variety of 96 well plates were tested, but one, Immulon 4 (Dynatech, USA) had a significantly lower background with a higher specific absorbance compared to the rest. This absorbance decreased with serial dilution of antigen. These plates were chosen to carry out the assay. All wells were coated with 3 µg/ml of purified virus diluted in 50 µl of ELISA coating buffer (see appendix 1). Plates were wrapped in clingfilm and incubated O/N at 4°C, plates could be stored like this for several days. Serum samples that were to be analysed were diluted (1/100) in 0.05% Tween in PBS (PBST) containing 5% rabbit serum, 50 µl of each sample was added per well and samples were always analysed in duplicate. Positive and negative controls were always included in every assay, these were hyperimmune serum and serum from a known positive, also 5% rabbit serum and serum from mice inoculated with an unrelated virus were included as the negative controls. The wells were washed 3X PBST prior to the addition of the diluted serum samples, once the serum samples were added they were allowed to incubate for 2 hr at RT. Samples were then removed and the plates washed 3X PBST. 50 µl of secondary antibody (either rabbit anti mouse IgM, IgG1, IgG2a or IgG2b, which were all conjugated with horseradish peroxidase) diluted in 5% rabbit serum in PBST were added per well and the plates were incubated at RT/1 hr. Wells were again washed 3X with PBST prior to the addition of substrate. The substrate solution used was 5-aminosalicylic acid (Sigma, UK). 100 mg of 5-salicylic acid was dissolved in 100 ml 0.02 M sodium phosphate buffer (see appendix 1). 1 ml of 1% hydrogen peroxide was added to the mixture, 50 µl of the mixed solution was then immediately added to the wells and the colour was allowed to develop 5 min-1 hr. The reaction was stopped.
by the addition of 3 M NaOH, and the plates were then read on an ELISA
plate reader (Dynatech, USA) at 570 nm. Samples were considered positive
when the mean of the duplicates was greater than the mean plus two
standard deviations of the negative controls.

The ELISA experiments were not carried out as accurately as they might
have been, therefore the value of these experiments is questionable. In
these ELISA experiments for convenience a standard 1/100 dilution of
serum was used for all samples. However, if ELISA experiments were to be
carried out more thorough, the exact total immunoglobulin levels in each
individual sample should be determined by a separate ELISA. Therefore
when reading the ELISA data this point should be remembered.

2.15 Histology

2.15.1 Preparation of Slides
Slides used to mount the tissues on were biobond (British Biocell
International, UK) treated. Biobond produces a very strong adhesive between
glass and tissue and also minimises the amount of background.

Slides were soaked in 2% DECON for 1 hr and thoroughly washed with
distilled water to remove any residual DECON, slides were then dried at RT
and were covered to protect from dust. The slides were then treated for 4 min
at RT with 2% biobond solution in acetone. The slides were then rinsed for 5
min in distilled water and allowed to air dry, and stored until use in a dust
free environment.

2.15.2 Tissue Sectioning
Tissue section was undertaken by Neil McIntyre at the Histology Department,
Department of Veterinary Pathology, University of Edinburgh.
2.15.3 Immunocytochemistry

There were two methods of immunostaining used throughout this study depending on the type of tissue fixation method used. Method 1 was used for paraffin sections and method 2 was used on cryostat sections.

2.15.3.1 Method 1

Dewax 7 micron thick paraffin embedded tissues in xylene (15 min). Leave sections in 100% ethanol (5 min/RT). Place sections in 100% methanol, 0.3% H₂O₂ (30 min/RT). Hydrate sections through graded alcohols (1 min in each 100%, 95%, 70%, 50%, 30%), and wash in H₂O twice (1 min/RT). Sequentially treat sections with 1% Triton-X100 (1.5 min/RT), wash twice in H₂O (5 min/RT), wash in PBS (5 min/RT), digest the tissues in 10 µg/ml proteinase K in 20 mM Tris, 2 mM CaCl₂ (pH 7.0). Stop reaction by washing slides in PBS, glycine 2 g/L and 5 mM EDTA.

Sections were placed in a humidity chamber and blocked with 10% blocking solution. The blocking solution was made up as 5% animal serum which is the same species as the secondary antibody in PBS. Primary antibody was added at the appropriate dilution (see Table 2.3) in 2% blocking serum/ PBS, (> 1 hr/RT) Biotinylated secondary antibody was added at 200 µl/slide (see Table 2.3) and incubated >1hr at room temperature. Sections were then incubated with ABC peroxidase reagent according to the manufacturers instructions (Vector Laboratories, UK), sections were washed between each step three times in PBS. Diaminobenzidine (DAB) was used as a detection substrate according to the manufacturers instructions (Sigma, UK), sections were washed in PBS (5 min/RT), counterstained with haemotoxylin, dehydrated in graded alcohols (1 min in each), left in xylene (10 min) and mounted using Surgipath micromount medium (Surgipath, UK).
2.15.3.2 Method 2

10 micron thick cryostat sections were treated with 0.03% H$_2$O$_2$ in PBS for 10 minutes, again this was to block any endogenous peroxidase activity. Sections were placed in a humidity chamber and incubated sequentially with primary antibody (Table 2.4) diluted in PBS containing 5% blocking serum for 1 hr. The blocking serum used was the same as the species of secondary antibody. The sections were then treated with biotinylated secondary antibody for 1 hr, and then with ABC peroxidase (see Section 2.15.3.1) for 30 min. To enhance reactivity the sections were further incubated with swine anti-rabbit or a rabbit anti-goat peroxidase conjugate for 30 min. Between each stage sections were washed three times with PBS containing 0.01% Brij. The immunostaining was visualised using DAB (see Section 2.15.3.1) and the sections were counterstained with haemotoxylin and mounted using glycerol.

The working dilutions of primary antibodies were determined using concanavalin A stimulated splenocytes and CNS tissue from mice with clinical signs of chronic relapsing experimental autoimmune encephalitis.

The degree of immunoreactivity was determined on a 4-point scale based on the intensity of the staining and the number of positive cells or immunopositive areas in sections from three mice sampled at each time point.

2.15.4 Double staining with Terminal deoxynucleotide transferase mediated UTP Nick End Labelling (TUNEL) and Immunostaining

Sections were dewaxed in xylene (15 min/RT) and sequentially hydrated through graded alcohols (1 minute in 100%, 95%, 70%, 50%, 35%), treated with 1% Triton-X100 (1.5 min/RT), washed in PBS (5 min/RT) and then dipped in water and treated with 10 µg/ml of proteinase K in 20 mM Tris-HCl, 2 mM CaCl$_2$ pH 7.0. This reaction was stopped with PBS, 2 g/L glycine
and 5 mM EDTA (5 min/RT). These treatments were to permeabilise the tissue, after these treatments the sections washed in PBS (5 min/RT).

Positive control
As positive control for TUNEL, one section was equilibrated with DNase buffer (30 mM Tris-HCl pH 7.2, 140 mM sodium cacodylate, 4 mM MgCl₂ and 0.1 mM DTT) for 20 minutes at RT, then 5 µg/ml of DNase 1 in DNase buffer was added to the section (20 min/37°C). The slides were then washed three times in PBS (5 min/RT).

Next all tissues were equilibrated in TUNEL buffer (30 mM Tris-HCl pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), sections were then incubated with 0.3 U/µl of TdT (Terminal deoxynucleotide Transferase) and 20 µM digoxigenin labelled UTP in TUNEL buffer (2 hr/37°C). To prevent dehydration sections were covered with Nescofilm and incubated in a humidity chamber. The TUNEL reaction was terminated by removing the Nescofilm and leaving the section in 2X SSC (5 min/RT).

Immunostaining and Alkaline Phosphatase Detection
Sections were blocked with 3% normal goat serum (NGS, Sigma, UK) in PBS (20 min/RT), then incubated for > 1 hr at room temperature with sheep anti-digoxigenin (1/100) which was alkaline phosphate conjugated (Boehringer Mannheim, Germany), then washed three times with PBS (5 min/RT).

BCIP/NBT Detection
The BCIP/NBT detection was undertaken following the manufacturers instructions (Boehringer-Mannheim, Germany). The BCIP/NBT solution was incubated on the tissue sections (20 min/RT), until a blue/black colour appeared. Sections were then washed three times in PBS.
**Immunostaining for viral protein**

Tissue sections were incubated in 100% methanol with 0.3% H₂O₂ (> 30 min/RT), this was to quench endogenous peroxidase activity. Sections were blocked with 10% goat serum in PBS (20 min/RT) The primary antibody, which was rabbit anti BeAn in 2% goat serum and PBS was incubated (> 1 hr/RT). Sections were then incubated with the secondary antibody, goat anti-rabbit which was biotinylated and diluted in 2% NGS in PBS (> 1 hr/RT). Then washed three times in PBS.

**ABC/DAB Detection**

ABC/DAB detection was undertaken according to the manufacturers instructions (see Section 2.15.3.1). Sections were rinsed after this detection step and dehydrated through the graded alcohols and counterstained with methyl green, then mounted using Surgipath mountant.

**2.15.5 Restriction Endonuclease Digestion of DNA**

The starting plasmid contained the B2 fragment of TMEV which had been subcloned into pGem3 as described before ((Pevear et al., 1988). The construct contained the VP4, 3, 2, and 1 regions of the TMEV genome. The TMEV viral cDNA which was used for riboprobe synthesis was isolated by a standard restriction digest with Hind III yielding a cDNA fragment, of approximately 3 kb in length. This was purified by chloroform/phenol extraction. In all of the restriction digest reactions all the restriction endonucleases and reaction buffers were supplied by GIBCO BRL and were used according to the manufacturers instructions.

**2.15.6 In vitro Synthesis of Riboprobes**

Riboprobes were synthesised from a recombinant pGEM3 vector harbouring the 3.4 kb SalI, Xba1 cDNA restriction fragment of BeAn corresponding to nucleotides 1729-4733. Probes were labelled according to the manufacturers instructions. ³⁵S probes were synthesised using the riboprobe Gemini kit,
according to the manufacturer's instructions (Promega). A Boehringer RNA labelling kit was used to produce biotin and digoxigenin labelled riboprobe. These riboprobes were synthesised according to the manufacturer's specifications (Boehringer-Mannheim). The different transcription reactions are listed below for each probe. The purification and alkaline hydrolysis steps for all probes were the same, as recorded below.

2.15.6.1 $^{35}$S Labelled Riboprobe Synthesis

Transcription Reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water (DEPC treated)</td>
<td>1.1 μl</td>
</tr>
<tr>
<td>Transcription buffer (5X)</td>
<td>4.0 μl</td>
</tr>
<tr>
<td>DTT (100 mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>RNasin (25 U/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>GTP (10 mM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>CTP (10 mM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>UTP (10 mM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>ATP (300 μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>DNA linearized plasmid template</td>
<td>1.0 μl (1 μg)</td>
</tr>
<tr>
<td>$^{35}$S-ATP (1350 Ci/mM)</td>
<td>4.0 μl</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>1.0 μl</td>
</tr>
</tbody>
</table>

This mixture was thoroughly mixed by vortexing, pulse centrifuged and incubated at 37°C overnight in a heat block.

2.15.6.2 Digoxigenin labelled riboprobes

Transcription Reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearized plasmid template DNA</td>
<td>1.0 μl (1 μg)</td>
</tr>
<tr>
<td>NTP labelling mix containing digoxigenin</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Transcription buffer (10X)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>11.0 μl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1.0 μl</td>
</tr>
</tbody>
</table>
Chapter 2 Materials and Methods

T7 RNA polymerase (20 U/μl) 2.0 μl

This mixture was thoroughly mixed by vortexing, pulse centrifuged and incubated at 37°C overnight in a heat block.

2.15.6.3 Biotin labelled riboprobe

Transcription Reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>Linearized plasmid DNA</td>
<td>1.0 μl (1 μg)</td>
</tr>
<tr>
<td>Transcription buffer (10X)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>NTP labelling mixture containing biotin</td>
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</tr>
<tr>
<td>Sterile water</td>
<td>6.0 μl</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>2.0 μl</td>
</tr>
</tbody>
</table>

This mixture was thoroughly mixed by vortexing, pulse centrifuged and incubated at 37°C overnight in a heat block.

2.15.7 Purification of 35S, Biotin and Digoxigenin labelled riboprobes

After transcription samples were taken out of the heat block and pulse centrifuged to spin down any condensation which may have occurred. To terminate the reaction 1 μl of RQ1 DNase (1U) was added and incubated for 15 min/37°C. The reaction mixture was then made up to 100 μl with sterile water and 1 μl of 1 mg/ml of tRNA was added. An equal volume of phenol/chloroform was added and centrifuged for 5 min/10000g the upper aqueous phase which contained the RNA was transferred to another tube and an equal volume of chloroform added, the solution was then vortexed and centrifuged for 5 min at 10000 g. The upper phase was again removed and transferred to a fresh eppendorf and RNA precipitated by addition of 3 M sodium acetate pH 5.5 (1/10 volume) and 100% ethanol (2.5X volume). The solution was incubated at -80°C for > 30 minutes, and then centrifugation for 20 min at 10000 g. The RNA pellet was washed in 90% ethanol, lyophilized and resuspended in 45 μl of sterile distilled water.
2.15.8 Alkaline Hydrolysis

If the probe was to be used for in situ hybridization it was hydrolysed into smaller fragments (200 bp or less). The precipitated RNA probe was hydrolysed by resuspension in 5 µl of 0.4 M NaHCO₃, 0.6 M Na₂CO₃ (pH 10.2) and incubating for 30 min at 50°C. The reaction was neutralised by adding 3 M sodium acetate (pH 4.6). The RNA was ethanol precipitated again as above, washed, dried and resuspended in the required volume.

Determination of the optimum time for hydrolysis was as follows:-

\[
t = \frac{L_0 - L_f}{K \times L_0 \times L_f}
\]

\[t = \text{time in minutes}\]
\[L_0 = \text{initial fragment length in kb}\]
\[L_f = \text{final fragment length in kb}\]
\[K = 0.11 \text{ kb/min}\]

³⁵S riboprobes were counted in a scintillation counter in order to determine the effectiveness of labelling.

2.15.9 In situ Hybridization

The in situ hybridization method used has been described previously (Fazakerley et al., 1991).

Pretreatment of slides

All glassware was thoroughly washed with sterile distilled DEPC treated water and then autoclaved prior to use, and all solutions used in this technique were made under RNase free conditions. Sections which were 7 microns thick and embedded in parafin were dewaxed in xylene (15 min/RT) and hydrated through graded alcohols (1 min in each of 100%, 95%, 70%, 50%,
and 30%) containing 0.33 M ammonium acetate. The sections were then sequentially treated twice with sterile \( \text{H}_2\text{O} \) (1 min/RT), 0.2 M \( \text{HCl} \) (10 min/RT), twice with sterile \( \text{H}_2\text{O} \) (1 min/RT), 1% Triton-X100 (1.5 min/RT), twice with sterile \( \text{H}_2\text{O} \) (1 min/RT), PBS (5 min/RT), 10 \( \mu \text{g/ml} \) proteinase K in 20 mM Tris, 2 mM \( \text{CaCl}_2 \), pH 7.0 (20 min/37°C). This reaction was stopped by treating the slides with PBS, 2g/L glycine and EDTA (5 min/RT) slides were washed in PBS (5 min/RT) and sterile \( \text{H}_2\text{O} \) (3 min/RT). These tissue treatments were to optimise permeabilisation of tissues. After the permeabilisation treatments sections were treated with 0.1 M triethanolamine-\( \text{HCl} \) pH 8.0 (10 min/RT) and rinsed in PBS. Finally tissues were dehydrated through graded alcohols as before all containing 0.33 M ammonium acetate except for the 100% ethanol. The sections were then left to air dry (1 hr/RT).

Prehybridization and Hybridization

After the above tissue treatments the tissues were prehybridized (see appendix 1) for >1 hr at 65°C. Labelled probes were then added to hybridization buffer (see appendix 1) boiled for 2 min and subsequently quenched on ice before being added to the sections, hybridization occurred for 12-16 hr at 55°C.

Prior to hybridization the \(^{35}\text{S} \) labelled probe was diluted to give a concentration of \( 2 \times 10^5 \) cpm/ml whereas, biotinylated and digoxigenin labelled riboprobes were diluted to 1/100. DTT was added to radiolabelled riboprobes to give a final concentration of 10 mM, this was to protect the radiolabelled sulphur groups. To prevent dehydration tissue sections were covered with Nescofilm (Bando Chemical Industries, Japan) and incubated in a humidity chamber.

Washing of sections

After hybridization the Nescofilm was removed and sections placed in 4X SSC (with 50 \( \mu \text{M} \) DTT included for the radiolabelled riboprobes). The sections
were washed once in 0.5 M NaCl/TE. Then any unhybridized riboprobe was digested with 20 μg/ml RNase A in 0.5 M NaCl/TE (30 min/37°C). Sections were then washed four times in 0.5 M NaCl/TE (15 min/37°C), twice in 2X SSC (15 min/37°C) and twice in 0.2X SSC (15 min/55°C). After the stringency washes the final stages of detection differed depending on whether the riboprobe was labelled with ³⁵S, biotin or digoxygenin.

2.15.9.1 Detection of ³⁵S Riboprobes
After the last stringency wash at 55°C the sections were dehydrated through graded alcohols with 0.33 M ammonium acetate and left to air dry, 1 hr/RT. Once dry the sections were then exposed to β-max film (Amersham, UK) which gave autoradiographic images of areas which contain viral RNA sequences.

Viral RNA at the cellular level was detected by dipping the slides five times in succession in a 50% solution of LM1 photographic emulsion (Amersham, UK) in 0.66 M ammonium acetate at 42°C, the slides were then allowed to dry and were stored at 4°C in the dark, until development.

At appropriate times post-dipping slides were developed in a 1/5 dilution of Phenisol developer 2min/RT (Ilford, UK) and the reaction was stopped in a 1/200 dilution of glacial acetic acid (1 min/RT). The sections were fixed in 4% sodium thiosulphate in distilled water (10 min/RT) and subsequently rinsed prior to haemotoxylin and eosin counterstaining (RT), and mounting in DPX.

2.15.9.2 Biotinylated Probe Detection
Following the last stringency wash of the in situ hybridization, slides were washed three times in PBS (5 min/RT) and then incubated with avidin conjugated to FITC. Avidin has a high specificity for biotin. This was incubated for 1 hr at RT, and then washed three times in PBS, sections were mounted or secondary immunostaining was carried out after this.
2.15.9.3 *Digoxigenin* Probe Detection

After the stringency washes of the *in situ* hybridization, slides were dehydrated through the graded alcohols (1 min in 30%, 50%, 75%, 90%, 100%). The sections were then washed in 0.3% H₂O₂ in 100% methanol for 30 min at RT, then hydrated through the graded alcohols. Sections were then blocked with 2% rabbit serum in PBS for 30 min at RT, primary antibody, sheep anti-digoxigenin was incubated on each section for > 1 hr, sections were reblocked and secondary antibody (rabbit anti-sheep, biotinylated) in 2% blocking serum in PBS was incubated for 1 hr at RT. After each step sections were washed three times with PBS. ABC reagent (Vector Laboratories, UK) was used according to the manufacturers instructions. ABC reagent binds to bound biotinylated antibody, this was incubated on sections for 30 min/RT, signal was visualised by using DAB substrate (Sigma, UK) and sections were counterstained with haemotoxylin, or sections went on to be secondary immunostained.

2.16 Statistical Methods Used

Student’s t-test was employed to calculate the significance between different experimental groups when comparing levels of virus titre, cytokine transcripts and antibody isotypes.
Table 2.3 Antibodies used for staining mouse tissues (method 1).

<table>
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<tr>
<th>Clone or code</th>
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<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Primary</td>
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<tr>
<td>A53</td>
<td>BeAn viral proteins</td>
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<tr>
<td>A47</td>
<td>GDVII viral proteins</td>
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<td>Secondary</td>
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Table 2.4 Antibodies used for staining mouse tissues (method 2).

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<th>Specificity</th>
<th>Dilution</th>
<th>Source</th>
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Table 2.3 Antibodies used for staining mouse tissues (method 1).

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Table 2.4 Antibodies used for staining mouse tissues (method 2).

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Figure 2.1 Bradford protein assay to determine the concentration of purified virus to be used in a TMEV-specific ELISA. The absorbance of standard proteins was measured at 595 nm as described in the Materials and methods. The y-axis depicts the optimum density and the x-axis shows the dilution of Bradford proteins. The concentration of virus preparation was found by measuring its absorbance at 595 nm and then determining its concentration from the curve derived from the absorbances of the standards.
Chapter 3

Cytokine and antibody responses during the acute phase of Theiler's murine encephalomyelitis virus infection.
Chapter 3- Cytokine and antibody responses during the acute phase of Theiler’s murine encephalomyelitis virus infection

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<td>Introduction</td>
<td>97</td>
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<td>3.2</td>
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<td>3.2.2</td>
<td>Development of TMEV specific ELISA</td>
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<td>Results</td>
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3.1 Introduction

Over the last few years with development in RNA technology, profiles of cytokine transcripts in the CNS during experimental viral infections have been elucidated for Sindbis, LCMV and HIV viruses (Wesseligh & Griffin, 1994; Watkins et al., 1995). The ideal immune response towards a virus would be one which clears the infection with minimal tissue damage. It is particularly important that viruses are cleared from the CNS with minimal neuronal damage and dysfunction, since these cells cannot be replaced. Generally, it is thought that a cytokine response which skews towards the Th2 array of cytokines (which is associated with humoral immunity and decreased cell-mediated immunity) is the best scenario for neuronal survival, if it is effective in clearing the invading virus. The Th1 spectrum of cytokines is generally associated with increased cell-mediated immunity and antibody of the isotype IgG2a. This inflammatory response can be very damaging, therefore it is often considered that a predominantly Th1 cytokine response in the CNS would be negative and destructive (Mossman et al., 1986; Abehsiraamar et al., 1992; Heinzel et al., 1995; Romagni, 1996; Sedlik, 1996; Constant & Bottomley et al., 1997; Umetsu & DeKruyff, 1997).

Cytokine mRNA’s elevated during the acute phase of Sindbis virus infection include IL-1β, TNFα, IL-4, IL-6 and IL-10. These are exclusively produced by cells of the CNS as opposed to infiltrating leukocytes (Wesseligh & Griffin, 1994). mRNA levels for IFNγ and IL-2 were also elevated in the CNS during Sindbis virus infection and these were associated with infiltrating mononuclear cells. Levels of IL-4 and IL-10 were at higher levels than IL-2 and IFNγ, suggesting that a Th2 cytokine profile dominates in the CNS of mice infected with Sindbis virus (Wesseligh & Griffin, 1994). Cytokine profiles in the brains of patients with AIDS dementia were examined and it was found that levels of TNFα were elevated (Wesseligh et al., 1993). There was no increase in IL-4, IL-6 or IL-10, and no distinctive Th1 or Th2 profile could be determined (Tyor et al., 1990, 1992, 1993). Intracerebral infection of
adult mice with LCMV usually results in death. This is associated with an elevation in mRNA levels for TNFα, IL-1α, IL-1β, IL-6 and IFNγ (Campbell et al., 1994).

The levels and type of antibody generated during a CNS viral infection may be important in either prevention or pathology. Antibodies of the IgG1 subclass are associated with a Th2 response which is documented to be anti-inflammatory, in contrast to this antibodies of the IgG2a subclass are linked to cell mediated immunity and in some cases tissue damage (Peterson et al., 1992; Karpus et al., 1995). In the case of Theiler's virus there is data indicating that antibodies generated in resistant C57Bl/6 mice are predominantly of the IgG1 subclass, whereas, IgG2a antibodies are dominant in susceptible SJL/J mice (Peterson et al., 1992). This would suggest a Th2 and a Th1 bias in these mouse strains, respectively.

The objective of this chapter was to determine the CNS cytokine and serum antibody profiles during the acute phase of Theiler's virus infection, in Balb/c, CBA and SJL/J mice. The objective of Chapter 4 was to investigate the same profiles during the chronic, persistent phase of Theiler's virus infection.

3.2 Experimental design

To examine CNS cytokine profiles and serum antibody levels in mice infected with Theiler's virus four different strains of mice Balb/c, CBA, SJL/J and SCID (severe combined immunodeficient) mice were inoculated ic with 10⁴ PFU of TMEV (BeAn). Different mouse strains have differences in their susceptibilities (see Section 1.10) (Lipton and Dal Canto, 1979, Nicholson et al., 1995). Balb/c mice are resistant to chronic disease whereas, CBA mice are intermediate in susceptibility to chronic disease (Lipton and Dal Canto., 1979, Simas & Fazakerley, 1996) and SJL/J mice are susceptible (Lipton and Dal Canto., 1979). Three to four mice were sampled on days 3, 7, 10, 14, 21 and 35 post infection. At each time point half a brain and half a spinal cord were
snap frozen and RNA was prepared from these tissues. The other halves were frozen in isopentane, and sections were cut on a cryostat. Sections were then immunostained for cellular infiltrates, adhesion molecules and cytokines. Sera were also taken from the animals and the specific anti-viral isotypes generated were determined by ELISA (see materials and methods).

3.2.1 Techniques for which total RNA was purified

RNA prepared from brains and spinal cords was used in a dot blot assay to determine levels of viral RNA, and to investigate levels of cytokine transcripts in an RNase protection assay (RPA). RPA is a novel and sensitive method for detection and quantitation of specific RNA species. The basis of the RPA is solution hybridisation of a radio-labelled, anti-sense RNA probe to its target in a complex mixture of total RNA (extracted from the tissues/samples in question). Samples then undergo digestion to degrade unhybridised probe, and are then run on a polyacrylamide gel. The gel is subsequently exposed to film (Figure 3.1) which is then scanned for accurate quantitation of the bands of interest. RPA has several advantages over other techniques which include, 1) it is highly specific and very sensitive, 2) it is 100+ fold more sensitive than Northern blotting, due to the solution based hybridisation as opposed to a filter based hybridisation, 3) it is more tolerant of partially degraded RNA, 4) multiple probes can be used in a single assay, therefore multiple species of RNA can be detected simultaneously, 5) many samples can be investigated in a single assay, 6) relative quantitation is possible because equimolar amounts of probes are used in the same reaction at the same time.

3.2.2 Development of a TMEV specific isotyping ELISA

TMEV (BeAn) was purified by caesium chloride banding following several rigorous purification steps as described in the material and methods. The optimum concentration of virus used in the ELISA was obtained by serially diluting the antigen preparation and coating it onto ELISA plates. A dilution of 1:10 of the antigen preparation gave a high absorbance on a linear section
of the dilution curve (Figure 3.9) and was therefore chosen as the dilution of antigen to be used. The actual concentration of antigen used was 5 μg per well as determined by a Bradford’s protein assay, as detailed in the materials and methods, (Figure 2.1).

Four different manufacturers ELISA plates were tested, however, only one, immulon 4 gave a good positive, reproducible result with a low background. This type of plate was used in all assays.

Further ELISAs were undertaken to determine the optimal working concentration of isotype specific secondary antibodies and the optimal concentration of test serum to be used in the assays. Pooled sera from CBA mice at 28 days post infection, was serial diluted to obtain the appropriate dilution to be used in the assay, 1:100 was the dilution chosen (Figure 3.10). The optimal concentrations of secondary antibodies were previously determined in the lab and were as follows anti-IgM 1:500, anti-IgG1 1:500, anti-IgG2a 1:1000, anti-IgG2b 1:500. As TMEV was grown on BHK-21 cells, BHK-21 cell lysates were used as a control antigen to determine the background absorbance. Sera from animals inoculated with SFV were used as a negative controls.

The ELISA experiments were not carried out as accurately as they might have been therefore the value of these experiments is questionable. In these ELISA experiments for convenience a standard 1/100 dilution of serum was used for all samples. However, if ELISA experiments were to be carried out more thorough, the total immunoglobulin level in individual samples should be determined by a separate ELISA. Therefore when reading the ELISA data this point should be remembered.
3.3 Results

A large amount of data is presented in this chapter. In an attempt to clarify this, the major findings are restated at the end of each section and are marked * and printed in italics.

3.3.1 Levels of viral RNA in brains and spinal cords

Previous studies in a number of mouse strains have indicated that peak levels of viral RNA are generally present in the brain at 7 days post infection with no significant differences between resistant and susceptible strains (Simas and Fazakerley, 1996). Fig 3.2 shows the levels of viral RNA in the brains of Balb/c, CBA, SJL/J and SCID mice. Virus was detectable in all strains at the earliest sampling point day 7 or day 10. No or minimal virus was detectable after this time in 9/9 brains from Balb/c mice sampled at days 14, 21 and 35. Similarly, only 1/8 SJL/J mice had detectable brain virus after day 10. In contrast, individual CBA mice were highly variable at days 10, 14 and 21 although all 3 animals sampled at day 35 had no detectable virus. In SCID mice brain virus titres increased from day 7 to 21. At this time point SCID mice started to appear moribund and die. Lack of an effective B or T cell response in SCID mice therefore results in unrestricted viral replication and death.

Previous studies have indicated that virus persists in the spinal cords of SJL/J mice and not in brains. This was confirmed by N-PCR. 6 out of 6 SJL/J mice had detectable levels of persisting virus in their spinal cords on days 21 and 35 post infection.

- *Balb/c and SJL/J mice clear viral RNA from brain by day 14.*
- *Individual CBA mice vary in their ability to clear brain virus.*
- *Virus persists in the spinal cords of SJL/J mice.*
3.3.2 Cytokine Levels in the brains of Balb/c mice

In the brains of Balb/c mice between days 3-10 mRNAs were detected for a number of cytokines including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p35, IL-12p40, IL-13, IFNγ, TNFα, TNFβ and TGFβ (Figure 3.3 A-O). There were clear differences in the levels and the time points at which peak expression of different cytokines occurred. Levels of the inducible IL-2 and IL-12p40 were high by the earliest sampling time point, day 3 post infection. These were the only cytokines that were almost maximal as early as day 3 (Figure 3.3 C, J). Other cytokines which were elevated as early as day 3 included IL-1α, IL-1β, IL-6, IL-13, IFNγ, TNFα, and TNFβ (Figure A, B, F, K-N). Cytokines which peaked by day 7 post infection included IL-6, IL-10, IL-12p40, IL-13 and IFNγ. After this time these cytokines decreased and returned to basal levels by day 14 (Figure 3.3 F, H, K, L). At day 10 post infection levels of IL-1α, IL-1β, IL-4, IL-5, TNFα, TNFβ and TGFβ were maximal (Figure 3.3 A, B, D, E, M-O). The largest amounts of mRNA were for the pro-inflammatory cytokines, including IL-1α, IL-1β, TNFα, and TNFβ (Figure 3.3 A, B, M, N, Table3.1) and the lowest levels of transcripts were for the Th2 cytokines IL-4, IL-5 and IL-10 (Table3.1). TNFα levels returned to basal by day 21 post infection, however TNFβ, IL-1α and IL-1β did not return to basal levels until day 35 or thereafter (Figure 3.3 A, B, M, N). IL-10 peaked at day 7, and IL-4 and IL-5 at day 10, however they all returned to basal levels by day 14 (Figure 3.3 D, E, H). There was no or relatively little consistent change from the controls in the levels of message for IL-7 although levels of this were clearly maximal at days 7 and 10 (Figure 3.3 G). Although elevated from day 3 the levels of IL-12p35 were highly variable. The differences in cytokine profiles and their levels in different strains are discussed in Section 3.3.6, and the differences between individual mice are discussed in Section 3.3.11.
3.3.3 Cytokine levels in the brains of CBA mice
Like Balb/c mice IL-2 and IL-12p40 were the first cytokines to peak being maximal by 3 days post infection (Figure 3.3 C, J). Other cytokines clearly elevated at day 3 included IL-1α, IL-1β, IL-6, IL-13, TNFα and TNFβ. At day 7 IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IFNγ and TNFα all peaked (Figure 3.3 B, D-F, H, K-M). Thereafter levels of cytokines varied between individual mice. In some animals levels declined whereas in others high levels were present on days 14 and 21. However, all 3 mice sampled on day 35 had basal levels of most transcripts. TNFβ peaked at day 10. As in the Balb/c mice the highest transcript levels were observed for the pro-inflammatory cytokines TNFα, IL-1α and IL-1β. Levels of IL-12p35 showed little or no consistent change throughout the course of infection (Figure 3.3 I). For the constitutively expressed transcripts there were increased levels of IL-7 and TGFβ, however these varied little above basal. The differences and comparisons in cytokine profiles between different strains are discussed in Section 3.3.6. Differences between individual CBA mice are discussed in Section 3.3.11.

3.3.4 Cytokine levels in the brains of SJL/J mice
As with Balb/c and CBA mice, IL-12p40 levels were maximal by 3 days post infection (Figure 3.3 J). As with Balb/c and CBA mice although IL-2 levels were maximal by day 3 these levels were dramatically reduced and were only a little above background (Figure 3.3 C). The only other transcripts clearly elevated at day 3 in SJL/J mice were IL-6, IL-7, IL-13 and IFNγ (Figure 3.3 F, G, K, L). Levels of mRNA for IL-10 peaked at day 7 and returned to basal levels by day 14 (Figure 3.3 H). IL-1α, IL-4, IL-5, IL-6, IL-7, IFNγ, TNFα, and TNFβ were all maximal at day 10 post infection and returned to basal levels by day 21 post infection (Figure A, D-G L-N). An early day 7 peak in TGFβ was observed which was not present in CBA and Balb/c mice. As with IL-2 a major difference was observed in the levels of IL-1β, which were markedly reduced in this strain compared to Balb/c and CBA (Figure 3.3 B). Levels of
IL-12p35 were maximal at day 14, and showed much less variability than levels in Balb/c and CBA mice. The differences and comparisons in mRNA expression for the cytokines between different mouse strains are discussed in Section 3.3.6., and the differences and similarities between individual SJL/J mice are discussed in Section 3.3.11.

3.3.5 Consistencies between strains

- Levels of IL-12p40 and IL-2 were maximal or near maximal by day 3.
- High levels of TGFβ were constitutively present, as were lower levels of IL-7 and IL-12p35.
- The highest levels of inducible transcripts were for IL-1α, TNFα, TNFβ.
- Maximum levels of transcripts were generally present at or between days 7 and 10.

3.3.5.1 In brains of Balb/c mice.

- The first cytokines detectable (at day 3) were IL-1α, IL-1β, IL-2, IL-6, IL-12p40, IL-13 and IFNγ.
- Maximum levels of Th1 (IFNγ) transcripts were more than 2 fold higher than maximal levels of Th2 (IL-4, IL-10) transcripts.

3.3.5.2 In brains of CBA mice

- The first transcripts detectable (at day 3) were IL-1α, IL-1β, IL-2, IL-6, IL-12p40, IL-13, TNFα and TNFβ.
- Between days 10 and 35, levels of cytokines showed large variations between individual mice.
- Maximal levels of Th1 (IFNγ, IL-12p40) and Th2 (IL-4 and IL-10) transcripts were equivalent.

3.3.5.3 In the brains of SJL/J mice

- The first transcripts detectable (at day 3) were IL-2, IL-6, IL-7, IL-12p40, IL-13, IFNγ and TGFβ.
- Transcripts for IL-2 and IL-1β were not present.
Maximal levels of IFNγ (Th1) were less than IL-4 (Th2).

3.3.6 Comparison of levels of viral and cytokine RNA in the brains of individual Balb/c, CBA and SJL/J mice

It is important to emphasise that individual animals are represented by the same symbol at each time point i.e. the red circle on day 10 represents the same animal in all the Figures for both virus and cytokines (Figures 3.2, 3.3, 3.4). Comparisons in the level of virus in the brain (Figure 3.2) with the levels of proinflammatory cytokines (Figure 3.3) indicates a positive correlation. For example:-

1) The green and blue CBA mice at day 10 (Figure 3.2B) both have high and very low levels of virus. Relative to the blue mouse the green mouse (high virus) had higher levels of IL-1α, IL-1β, IL-4, IL-5, IL-6, IFNγ, TNFα, TNFβ, and lower levels of IL-7, IL-10, IL-12p40 and IL-13.

2) The green and blue CBA mice sampled at day 14 have moderate and very low levels of virus, respectively. Relative to the blue mouse the green mouse (high virus) had higher levels of IL-1α, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p35, TNFα, TNFβ and lower levels of IL-12p40 and IL-13.

3) The green and red CBA mice sampled on day 21 have respectively very high and low levels of virus. Relative to the red mouse the green mouse (high virus) had higher levels of IL-1α, IL-4, TNFα, TNFβ and lower levels of IFNγ and IL-13.

4) In SJL/J mice on day 14 (Figure 3.2C) the blue mouse had detectable levels of virus whereas the green and red mice had none. Relative to the green and red mice the blue mouse (with virus) had higher levels of IL-1α, IL-4, IL-5, IL-6, TNFα, TNFβ and lower levels of IL-13.

Generally in all of these cases and consistently in at least 3/4 cases there is a positive correlation between presence of virus and high levels of IL-1α, IL-4, IL-5, IL-6, TNFα and TNFβ and a negative correlation with IL-13.
To investigate this further levels of IL-1α, IL-6 and TNFα were compared with the levels of virus between days 10 to 21 post infection (Figure 3.7). Clearly, the amount of these cytokines is proportional to the amount of virus.

- For all 3 mouse strains at early time points, and in CBA mice with persistence there was a clear positive correlation between the level of brain virus and the levels of transcripts for IL-1α, IL-4, IL-5, IL-6, TNFα and TNFβ, and a negative correlation between virus and levels of transcripts for IL-13.
- In general in Balb/c and SJL/J mice levels of cytokines (particularly IL-1α, IL-4, IL-5, IL-7, IL-10, and TNFα) rapidly declined following clearance of brain virus.

3.3.7 Comparisons of cytokine levels in the brains of Balb/c, CBA and SJL/J mice.

Figure 3.5 provides a comparison of the average level of individual cytokine transcripts during the acute phase of disease in the different mouse strains. There were both similarities and differences between strains. Statistical analysis was performed on the individual samples using the Student t-test, with a p value of 0.05 being taken as significant. Baseline levels were taken into consideration when statistical analysis was carried out.

3.3.7.1 Pro-inflammatory cytokines

**IL-1α.** Levels of IL-1α were consistently higher in CBA than SJL/J mice at days 3, 7 and 10 post infection (p=0.001, p=0.02, p=0.02, respectively) (Figure 3.3 A), and levels in Balb/c mice were higher than SJL/J mice at day 3 (p=0.03).

**IL-1β.** One of the most dramatic differences between mouse strains was in the levels of IL-1β. No transcript expression above background for this cytokine was detected in SJL/J mice, consequently levels of this message were significantly lower in SJL/J mice than Balb/c and CBA mice at days 3, 7, 10,
CHAPTER 3  CYTOKINES, ANTIBODIES AND THE ACUTE PHASE

14 and 21 post infection (see Figure 3.3 B). ( SJL/J:Balb/c p=0.01, p=0.002, p=0.007, p=0.01, p= 0.005 and SJL/J:CBA mice p=0.03, p=0.005, p=0.03, p=0.002, p=0.005). Maximum levels were higher (60, 40) and earlier (d7, d10) in CBA than in Balb/c mice.

IL-6. Levels of IL-6 followed a similar pattern of expression in all strains, however there were subtle differences, with SJL/J mice having increased levels at post infection day 10 compared to Balb/c mice (p=0.02), and CBA mice having increased levels on day 21 compared to both Balb/c (p=0.001) and SJL/J (p=0.02) mice. The high levels of IL-6 in 2/3 CBA mice at day 21 is consistent with the continued presence of virus in the brains of these mice at this time.

IL-12p40. There were early consistencies between the Balb/c (resistant), CBA (intermediate) and SJL/J (susceptible) mice. Levels of IL-12p40 were the first to peak in the brains of all strains of mice (Figure 3.3 J). This was apparent by day 3 post infection, the earliest sampling point. At this time levels were high or maximal in all three strains. The maximum levels of mRNA expression for this cytokine were similar in all three strains. In Balb/c and SJL/J mice levels returned to basal by day 21. In contrast, levels remained elevated at day 21 in CBA mice all of which continued to have virus detectable in their brains (Figure 3.2B).

TNFα. Levels of TNFα followed similar kinetics in all 3 mouse strains. However, SJL/J mice had significantly decreased levels on day 3 compared to Balb/c (p=0.04) and CBA (p=0.06) mice.

TNFβ. Levels of TNFβ were similar in all strains until day 35 post infection at which time Balb/c mice had elevated levels compared to CBA (p=0.03) and SJL/J (p=0.004) mice. This is interesting because in Balb/c mice (the resistant strain) at day 35 virus is undetectable. Continuing inflammation in Balb/c
mice with no detectable viral RNA has been observed (M. Brahic personal communication), this may explain the high levels of TNFβ.

3.3.7.2 Th1 cytokines (IFNγ, IL-2 and IL-12p40)

Levels of the classically defining Th1 cytokines IFNγ and IL-2 showed clear differences between strains.

IL-2. There were dramatic differences in the levels of IL-2. SJL/J mice made few transcripts for this cytokine, consequently, CBA and Balb/c mice had significantly higher levels of IL-2 than SJL/J mice at all time points. This is a similar pattern to that observed in the SJL/J mice for IL-1β.

IFNγ. There were differences in the levels of IFNγ. Maximum levels of IFNγ were present at day 7 in the Balb/c and CBA mice and declined rapidly whereas levels were maximal at days 10 and 14 in SJL/J mice and did not return to basal by day 35. CBA mice had significantly elevated levels on day 7 compared to SJL/J mice (p=0.01). However the situation was reversed by day 35 by which time SJL/J mice had the highest levels of this cytokine.

3.3.7.3 Th2 cytokines (IL-4, IL-5, IL-10)

There were differences in levels of Th2 cytokines (IL-4, IL-5 and IL-10) in the brains of the different mouse strains studied.

IL-4. Relative to both CBA and SJL/J mice, Balb/c mice had lower levels of IL-4 transcripts (Balb/c:CBA day 7 p=0.05; Balb/c: SJL/J day 10 p=0.004).

IL-10. This phenomenon was also observed for levels of IL-10, with Balb/c mice having significantly lower levels of IL-10 on day 7 when compared to CBA and SJL/J mice (p=0.03, p=0.002, respectively).
3.3.7.4 Anti-inflammatory cytokines

Although there were high constitutive levels of TGFβ in all three mouse strains, these were elevated in most mice during infection. SJL/J mice showed an early peak at day 7, when titres were lower or not elevated above background in Balb/c and CBA mice.

- In addition to the absence of IL-1β and IL-2, relative to Balb/c and CBA mice, SJL/J mice made a slow TNFα and IL-1α response, and had lower titres of IL-1α.
- SJL/J mice had a delayed but prolonged IFNγ response.
- Balb/c mice had a low Th2 (IL-4 and IL-10) response relative to CBA and SJL/J mice, whereas, Balb/c mice had higher levels of IFNγ than IL-4, this was reversed in SJL/J mice.
- SJL/J mice had an earlier peak of TGFβ expression (at day 7) than Balb/c and CBA mice.

3.3.8 Cytokine levels in the spinal cords of Balb/c mice.

As with the brains, mRNA’s for several cytokines were detected in the spinal cords of animals infected with TMEV. In Balb/c mice on day 3 levels of IL-2, IFNγ, TNFα and TNFβ were above basal levels. In the spinal cords of Balb/c mice the majority of cytokines detected peaked on day 7 post infection, these included IL-1α, IL-4, IL-13, IFNγ, TNFα and TNFβ (Figure 3.4 A, D, F, J- L). Levels of IL-6 peaked on day 10 post infection (Figure 3.4 E). The majority of cytokines returned to basal levels between days 14-21 post infection. Interestingly, in this mouse strain IL-10 remained at control levels throughout the course of infection (Figure 3.4 F). Unlike brain, no transcripts for IL-5 and IL-7 were detected. Some animals had high and even maximal levels of IL-1β, IL-2 and IFNγ at late time points (Figure 3.4 B, C, J).

During the first two weeks of infection relative to brains generally, with the exception of IL-4 levels of transcripts for all the cytokines were lower in the spinal cord than in the brain. This was pronounced (2 fold difference) for IL-
1α, IL-10 and IL-13, and was particularly striking for IL-1β, where average maximum levels in the brain at day 10 were 40 and in the spinal cords were 5 (Figures 3.3 B, 3.4 B); and for IL-6 where average maximum levels in the brains at day 7 were 34 and in the spinal cords 9 (Figures 3.3 F, 3.4 E). Constitutively levels of IL-12p35 were lower in the spinal cords (18) than in the brains (75) (Figures 3.3 I, 3.4 G).

3.3.9 Cytokine levels in the spinal cords of CBA mice.

Most cytokines did not peak until day 14 post infection, these included IL-1α, IL-1β, IL-4, IL-10, IL-12p40, TNFα, and TNFβ (Figure 3.4 A, B, D, F, H, K). At day 7 post infection IFNγ peaked (Figure 3.4 J). Levels of TGFβ were elevated from day 7 (Figure 3.4 M). Unlike brain, no transcripts for IL-5 and IL-7 were detected. At day 35 post infection levels of TNFβ, TNFα, IL-2, IL-1α IL-1β and IL-6 were high and in many of these maximal. These cytokines are pro-inflammatory cytokines and it is likely virus is persisting in the spinal cords and continues to drive the inflammatory response. Interestingly, no virus was detected in the brains of the 3 CBA mice sampled at day 35 and the levels of these cytokines were not above background in the brains at this time.

The first cytokines elevated (day 3) were IL-1α, IL-2, IL-1β, IL-12p35, IL-12p40, IL-13, IFNγ, TNFα and TNFβ. The expression of various cytokines differed between brains and spinal cords. In brains the induction of most cytokines was more rapid peaking at around day 7, this contrasted with spinal cords where peak expression of most cytokines was around day 14. In general, maximum levels were also slightly lower in the spinal cords and there was also more variability in the data. As with Balb/c mice levels of spinal cord transcripts were generally lower again with levels of IL-1β and IL-6 being the most significant. The basal levels of IL-12p35 were lower in the spinal cords than the brain and there were consistent increases in the spinal cord but not the brain following infection (Figures 3.3 I, 3.4 G). Levels of IL-1β, IL-1α and IL-6 did not show early peaks as in the brains but showed a
slow steady increase (Figure 3.3 A, B, E). This would be consistent with the later arrival of virus in this location (ic inoculation) and a slow build up of viral titres.

3.3.10 Cytokine levels in the spinal cords of SJL/J mice.
Like Balb/c mice most cytokines peaked on day 7 in the spinal cords of SJL/J mice, these included IL-6, IL-10, IL-12p40, IL-13, IFNγ, TNFα and TGFβ (Figure 3.4 A, E, F, H-K, M). IL-4 peaked at day 10. At day 3 transcripts were detected for several cytokines including IL-1α, IL-4, IL-6, IFNγ, TNFα, TNFβ and TGFβ. There was minimal or no production of transcripts for IL-1β, IL-2, IL-5, IL-7 and IL-12p35 (Figure 3.4 B, C, G). In the case of the latter there was no detectable constitutive level of this transcript. This was different to the brains of the SJL/J mice which had high constitutive levels of IL-12p35, which were as high as those in the brains of Balb/c and CBA mice. Balb/c and CBA mice had lower spinal cord levels of IL-12p35. Several cytokines remained elevated in SJL/J mice at day 35 post infection these included IL-1α, IL-6, IL-13, IFNγ, TNFα and TNFβ (Figure 3.4 A, E, I-L). Elevation of these cytokines was consistent with viral persistence (Section 3.3.1).

There were numerous similarities in cytokine expression between brains and spinal cords in SJL/J mice, however, there were also differences. As in brains levels of IL-2 and IL-1β were basal. There was little constitutive or inducible expression of IL-12p35 in the spinal cords, whereas levels were high in the brains of SJL/J mice. Levels of IL-10 were greater in spinal cords compared to brain. Maximum levels of IL-1α, IL-4, IL-6, IL-12p40, IFNγ, TNFα and TNFβ were approximately equivalent in brains and spinal cords. Interestingly, the time course of the response for most cytokines in the spinal cords of SJL/J mice is biphasic, with a reduction in titres at day 10.

3.3.11 Spinal cord: consistencies between strains
- *As in the brain high levels of TGFβ were constitutively expressed.*
As in the brain the highest levels of transcripts were for TNFα, TNFβ and IL-1α.

As in the brain maximum levels of transcripts were generally found between days 7 and 14.

In contrast to the brain no transcripts were detectable for IL-5 and IL-7.

### 3.3.11.1 Balb/c spinal cords
- The first cytokines detectable at day 3 were IL-2, IFNγ, TNFα and TNFβ.
- Most cytokine transcripts peaked early at day 7.
- Induced maximum levels for IL-1β, IL-6 and possibly IL-1α, IL-10 and IL-13 were lower in the spinal cord than in the brain.
- Constitutive levels of IL-12p35 were lower in the spinal cords than the brains.

### 3.3.11.2 CBA spinal cords
- The first cytokines detectable at day 3 were IL-1α, IL-1β, IL-2, IL-12p40, IL-13, IFNγ, TNFα and TNFβ.
- Most cytokines peaked late at day 14.
- Generally, transcription was lower in the spinal cords than in brains.
- At day 35 post infection levels of IL-1α, IL-1β, IL-2, IL-6, TNFα and TNFβ were high and in some cases maximal. This contrasted with the transcript levels in the brains for these cytokines which were all basal at this time point.

### 3.3.11.3 SJL/J spinal cords
- The first cytokines detectable at day 3 were IL-1α, IL-4, IL-6, IFNγ, TNFα TNFβ and TGFβ.
- Most cytokines peaked at day 7.
- IL-1α, IL-6, IL-13, IFNγ, TNFα and TNFβ remained elevated in SJL/J spinal cords on day 35.
- There was minimal or no transcription levels for IL-1β, IL-2 and IL-12p35.
- Early levels of IL-10 were greatly increased in the spinal cords compared to the brains.
3.3.12 Comparison of cytokine levels in the spinal cords of different mouse strains

There was a difference in the kinetics of cytokines expressed between the different mouse strains. In Balb/c and SJL/J mice most cytokines peaked early (day 7) this contrasted with CBA mice, where most cytokines did not peak until day 14. Whereas, comparison of spinal cord virus clearance in Balb/c and CBA mice correlates with the more rapid cytokine response in the former this does not seem to be case and effect since in SJL/J mice an equally rapid cytokine response does not result in viral clearance. Figure 3.6 shows the averages of the cytokine profiles during the acute phase of disease in the spinal cords of the different mouse strains. There are various differences between the cytokine levels in the spinal cords of SJL/J, CBA and Balb/c mice throughout the acute phase of Theiler’s virus infection. Generally the levels of the pro-inflammatory cytokines TNFβ, IL-1β and IL-12p40 were all much higher in the spinal cords of CBA mice than in SJL/J and Balb/c mice. These same CBA mice had viral persistence in the brain and previous studies from this laboratory (Simas and Fazakerley, 1996) have shown that many CBA mice also have viral persistence in the spinal cords at these time points. It is likely therefore that as in the brains these high levels of proinflammatory cytokines reflect viral load.

3.3.12.1 Pro-inflammatory cytokines

The kinetics of IL-1α, IL-6, TNFα and TNFβ is similar during the first 10 days post infection. On days 7 and 10 titres rise in all 3 mouse strains, after this time titres are slightly above basal in Balb/c mice, whereas they remain elevated in CBA and SJL/J mice. For example on day 10 levels of TNFα are significantly increased in CBA mice compared to Balb/c mice on days 14, 21 and 35 (p=0.009, p=0.01, p=0.08, respectively); and on day 21 and 35 SJL/J mice have increased levels compared to Balb/c mice (p=0.01, p=0.04, respectively). This would be consistent with viral persistence in the spinal cords of CBA and SJL/J mice but not Balb/c mice, and reiterates the
correlation observed between viral load and levels of these cytokines in the brain (see Section 3.3.6).

**IL-1β.** As in the brains, the levels of IL-1β in the spinal cords of SJL/J mice were dramatically lower than in the other strains at all time points post infection (Figure 3.4 B).

3.3.12.2 Anti-inflammatory cytokine

**TGFβ.** As in the brain, transcripts for TGFβ were constitutively high in all 3 strains. In all 3 strains at day 7 post infection there was an increase in expression of TGFβ transcripts. Thereafter, there was a large variation in levels of transcripts in Balb/c and SJL/J mice whereas all CBA mice maintained an elevated level (Figure 3.4 M).

3.3.12.3 Th1 cytokines (IFNγ, IL-2 and IL-12p40)

**IL-2.** The pattern of expression of IL-2 in the spinal cords was similar to that seen in the brains. Balb/c and CBA mice had comparable levels at all time points however as in the brains the levels of IL-2 were markedly decreased in the spinal cords of SJL/J mice at all time points compared to Balb/c and CBA mice (Figure 3.4 C).

**IL-12p40.** IL-12p40 levels were generally similar in all mouse strains at all time points (however, levels were elevated on days 10 and 14 in CBA mice compared to Balb/c and SJL/J mice).

**IL-12p35.** As with levels of pro-inflammatory cytokines there was an increase of IL-12p40 in all stains but this returned to basal levels in Balb/c mice on day 14 but remained elevated in CBA and SJL/J mice after this time point. Transcripts for this cytokine were detected in control spinal cords of Balb/c, and CBA mice but not in the spinal cord of SJL/J mice, and were not inducible after infection (Figure 3.3 I, 3.4 G). This may be relevant to the well
documented differential clearance of virus from the brains but not the spinal cords in this mouse strain.

**IFNγ.** Levels of IFNγ were similar in the spinal cords of all strains at all time points (Figure 3.4 I).

3.3.12.4 Th2 cytokines

**IL-4.** As in the brains there appears to be a stronger IL-4 response in CBA and SJL/J mice than in Balb/c mice. For example SJL/J mice had increased levels on day 10 compared to Balb/c mice (p=0.01), and CBA mice have elevated levels of IL-4 when compared to Balb/c mice on day 14 post infection (p=0.05).

**IL-10.** IL-10 remained at basal levels in Balb/c mice. Levels of IL-10 in the spinal cord were elevated in SJL/J mice on day 7 compared to Balb/c (p=0.03) and CBA (p=0.004) mice.

- There was a difference between mouse strains in the kinetics of the cytokine response in the spinal cord. Balb/c and SJL/J mice made a more rapid response than CBA mice.
- After day 10 levels of pro-inflammatory cytokines IL-1α, IL-6, TNFα and TNFβ were low in Balb/c mice but remained high in CBA and SJL/J mice. This reflects the persistence of virus consistently restated in the literature in CBA and SJL/J mice.
- As in the brain, SJL/J mice made a more pronounced IL-4 and IL-10 response in the spinal cord.
- As in the brain, levels of IL-2 and IL-1β were basal in SJL/J mice spinal cords.
- In the spinal cords of SJL/J mice but not Balb/c and CBA mice levels of IL-12p35 were basal.
3.3.13 Comparison of different cytokine levels in the same individual
The power of the RPA is in its ability to generate quantitative and qualitative measures of a large number of different cytokine transcripts in a single sample. This contrasts with RT-PCR which is difficult to quantitate and measures only one transcript with each assay. In general, the majority of individual mice expressed both pro-inflammatory and anti-inflammatory cytokines. Similar patterns in individual mice were observed in brains and spinal cords with the majority of individuals from all strains producing mainly pro-inflammatory cytokines early on during infection, however differences between individuals of the same strain existed on and after day 7.

Analysis of individual CBA mice suggests that in some CBA mice there is a clear polarisation of the CNS immune response towards either Th1 or Th2 cytokines. For example, on day 7 post infection in the brain of the CBA mouse depicted by the blue square there are high levels of IFNγ but low levels of IL-4. In contrast the mouse depicted by the green square at this time point shows low levels of IFNγ and higher levels of IL-4. A similar phenomenon is seen on day 10 post infection in the brains of the CBA mice. The CBA mouse represented by the green square has high levels of IFNγ (which correlates with high levels of virus see Figure 3.2), and low levels of IL-10 and IL-13 (Figure 3.3 H, K, L) whereas, the mouse represented by the red square has high levels of IL-4, IL-5 and IL-10 but low levels of IFNγ and IL-12p35 (Figure 3.3 D, E, H, I, L). In the spinal cords of CBA mice on day 10, the mouse depicted by the blue square had high levels of IL-4, IL-10 and IL-13 and low levels of IFNγ (Figure 3.4 D, F, H, I, J). However, in the mouse represented by the green square there were high levels of IFNγ but low levels of IL-4, IL-10 and IL-13 (Figure 3.4 D, I, J).

Clearly individual CBA mice appear to polarise their type 1 or type 2 response as early as day 7 to 10 post infection, in both brains and spinal cords.
• CBA mice polarise their type 1 or type 2 responses as early as days 7 to 10 post infection.

3.3.14 Cytokine Levels in SCID mice
SCID mice have a dysfunctional recombinase enzyme and consequently no specific B or T cell immune responses. In SCID mice cytokine levels in the brains and spinal cords were very low from day 3-14 post infection (fig 3.8). This indicates that the higher cytokine levels observed in immunocompetent mice between days 3-14 requires activation of specific immune responses. However, moribund animals had high levels of TNFα, IL-1α, IL-6, IL-2 and IL-12p40 indicating that these cytokines can be produced by CNS cells in the absence of specific immune responses. Although transcript levels were higher in Balb/c, CBA and SJL/J mice than in SCID mice, it is not clear whether infiltrating cells produce these pro-inflammatory cytokines themselves or cause intrinsic CNS cells to increase production, or whether there is a combination of both.

• TNFα, IL-1α, IL-6, IL-2 and IL-12p40 are produced in the infected CNS in the absence of specific immune responses indicating production by resident CNS cells

3.3.15 Antibody isotypes during the acute phase of TMEV.
In addition to cytokine profiles, antibody profiles were determined by ELISA (as described in the materials and methods) during the acute phase of the infection, in the different strains. A standard 1:100 dilution of sample sera was used in ELISA experiments. Therefore these data are not as accurate as they could be (as discussed previously Section 3.2.2). Therefore caution must be noted when reading the results for the ELISA’s

Levels of antibodies have been shown to be important during picornaviral infections, and this is the case for TMEV. In particular, it has been shown that neutralising antibody is important in controlling virus spread in the CNS.
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(Borrow et al., 1993). It has been suggested that antibody isotype correlates to the type of CD4+ T helper cell response (Peterson et al., 1991, Pope et al., 1996). Therefore, it was of interest to determine whether there was a difference in the isotype of the serum antibody response in the different strains of mice in this study.

Antibody isotypes in the serum of Balb/c, CBA, SJL/J and SCID mice were determined by ELISA. Statistical analysis using Students t-test was performed on the data.

IgM, IgG1, IgG2a and IgG2b were all generated in mice inoculated with Theiler’s virus, however, there were differences in the kinetics of response between different strains and in the levels of different subclasses produced between the strains (Figure 3.11).

There were no consistent differences in the levels of IgM antibody synthesised by SJL/J, Balb/c and CBA mice up to and including day 35 post infection (Figure 3.11). All strains of mice produced IgM antibody with the same kinetics. IgM was first detected at day 7 and levels increased to day 35, at which time this particular experiment stopped. (Data showing IgM levels at late time points post infection during the chronic phase of infection are presented in Section 4.3.3). IgM production is relatively slow compared to other viruses. For example, mice infected with alphaviruses produce IgM from day 4 to day 21 with levels peaking around day 7 (Fazakerley et al., 1993).

Balb/c mice produced significantly higher levels of IgG1, between days 14-35, compared to SJL/J (p=0.00003) and CBA (p=0.0009) mice (Figure 3.11). IgG1 is associated with the Th2 subset of CD4+ T cells. However, Balb/c mice also had increased levels of IgG2a compared to susceptible SJL/J (p=0.008) and CBA (p=0.07) mice (Figure 3.11). IgG2a is associated with the Th1 subset of
CD4+ T cells. It is interesting to note that on day 10 post infection Balb/c mice make a strong IgG1 but weak IgG2a and IgG2b response. This is consistent with an early Th2 response as previously described in resistant C57Bl/6 mice (Peterson et al., 1992).

Levels of IgG2b remained similar at all time points during the acute phase for SJL/J and CBA mice, however, there was a statistically significant increase in the levels of IgG2b produced in Balb/c compared to CBA mice between days 14-35 (p=0.01). As expected, at no time post infection could IgG1, IgG2a or IgG2b be detected in SCID mice.

In summary, Balb/c mice make a higher titre antibody response that CBA and SJL/J mice. Although not a highly detailed study, with the exception of the day 10 time point in Balb/c mice there is little indication of a polarisation of the IgG responses in the different mouse strains as found in a previous study (Peterson et al, 1992). In addition there were no differences between strains in the levels of IgM as was documented previously (Pena Rossi et al., 1991).

- Serum IgM responses were similar and slow in all 3 mouse strains.
- Balb/c mice make a higher titre anti-TMEV-IgG (IgG1 and IgG2a) response than CBA and SJL/J mice.
- Balb/c mice make a stronger IgG1 than IgG2a response at early time points.
- Virus is cleared between days 10 and 14 in Balb/c mice it is likely that a larger antibody response is partially responsible.

3.3.16 Immunostaining in CBA mice.
A study to investigate the expression of cytokine proteins was undertaken. The main objective was to compare and contrast protein expression with the amount of message generated (data generated by RPA). However, this proved to be more difficult than originally thought. This was because only a small area of a small number of samples could be examined by
immunostaining. Cytokines have very short half-lives therefore the amount of active cytokine in the CNS may be small, and difficult to detect.

3.3.16.1 Cytokines in the CNS
As a positive control, tissues from mice with chronic relapsing and remitting EAE (experimental autoimmune encephalitis) were stained. These tissues were found to be consistently positive for all the cytokines looked at (Table 2.4 and 3.2). IL-2 immunostaining was detected in the brains of mice on post infection day 3 but levels were not different from those seen in the negative control inoculated ic with PBS. TGFβ was expressed in all brains but levels were no different from the negative controls. TNFα was produced in brains between days 3-10 and was present in spinal cords as late as day 21. Brain and spinal cord levels were consistently higher than in the control animals. Figure 3.13 demonstrates a TNFα positive mononuclear cell infiltrating the CNS from a blood vessel on post infection day 7. In conclusion, the amount of staining achieved for cytokines was limited (see Table 3.2). From the low staining achieved for TNFα, which had the highest levels of message, the immunostaining technique can be judged to be far less sensitive than the RPA analysis and it is therefore not surprising that expression of other cytokines with far lower message levels were not detected.

3.3.16.2 Cellular infiltrates in the CNS
Histological examination of normal CBA mice showed no evidence of mononuclear cell infiltration or paucity of myelin indicative of demyelination. In contrast to this TMEV infected CBA mice showed a mononuclear cell inflammation in the brains and spinal cords. Mononuclear infiltrates increased most between days 7-14. This correlated with the time that virus was cleared from the CNS in some mice. Numbers of mononuclear cells had decreased by day 35 post infection by which time virus had usually been cleared. Characterisation of the infiltrating cells demonstrated that T cells infiltrated the CNS as early as day 3 post infection. Both CD4 + and CD8 +
cells were present with CD8+ cells often more numerous than CD4+ cells (see Table 3.3) (Figure 3.12). Expression of F4/80 a marker for macrophages and microglia was also detected at 3 days post infection, levels in the brain peaked at day 14 (Figure 3.12).

- CD3+, CD4+, CD8+ and F4/80+ cells all infiltrated the CNS during the acute phase of Theiler’s virus infection.

3.3.16.3 Adhesion molecules in CNS of CBA mice
CNS tissues were stained for the presence of adhesion molecules including, 1) LFA-1 (leucocyte function associated antigen) is ubiquitously expressed on all leukocytes and is involved in their translocation through capillary walls and in antigen presentation, 2) VLA-4 (very late activation antigen) this is found on activated T cells and 3) V-CAM-1 (vascular cellular adhesion molecule) an adhesion molecule found on endothelial cells involved in the recognition of leucocytes and their transfer into the CNS. Expression of all 3 of these adhesion molecules was observed in both brains and spinal cords of infected mice but not in control animals (see Table 3.4). In the brain, LFA-1 was detected on day 3 post infection and reached peak levels on days 10 and 14 and was absent by day 35. Patterns in the spinal cord were virtually identical although low expression remained at day 35. (Figure 3.12). V-CAM-1 was also first detected on day 3 post infection and was high on day 10 in the brain and days 10, 14 and 21 in the spinal cord (Figure 3.12). VLA-4 was expressed in a similar pattern to LFA-1 in the brains, however levels were lower in the spinal cords. Levels of LFA-1 and V-CAM-1 correlated with numbers of CD3+ and CD8+ cells in the CNS.

- There was up regulation of adhesion molecules VLA-4, V-CAM-1 and LFA-1 in the CNS during the acute phase of Theiler’s virus disease.
3.3.17 Clear and consistent observations

3.3.17.1 Brain and Spinal cord vRNA levels

- Balb/c and SJL/J mice clear viral RNA from brain by day 14.
- Individual CBA mice vary in their ability to clear brain virus.
- Virus persists in the spinal cords of SJL/J mice on days 21 and 35.

3.3.17.2 Common to Balb/c (3.3.17.2.1), CBA (3.3.17.2.2), and SJL/J (3.3.17.2.3) mice.

- In the brain, levels of IL-12p40 and IL-2 were maximal or near maximal by day 3.
- In the brain, high levels of TGFβ were constitutively present, as were lower levels of IL-7 and IL-12p35.
- In the brain, the highest levels of inducible transcripts were for IL-1α, TNFα, TNFβ.
- In the brain, maximum levels of transcripts were generally present at or between days 7 and 10.
- As in the brain, high levels of TGFβ were constitutively expressed, in the spinal cords.
- As in the brain, the highest levels of transcripts were for TNFα, TNFβ and IL-1α, in the spinal cords.
- As in the brain, maximum levels of transcripts were generally found between days 7 and 14, in the spinal cords.
- In contrast to the brain, no transcripts were detectable for IL-5 and IL-7, in the spinal cords.

3.3.17.2.1 Balb/c mice.

- In the brain, the first cytokines detectable (at day 3) were IL-1α, IL-1β, IL-2, IL-6, IL-12p40, IL-13 and IFNγ.
- In the brain, maximum levels of Th1 (IFNγ) transcripts were more than 2 fold higher than maximal levels of Th2 (IL-4, IL-10) transcripts.
• In the spinal cord, the first cytokines detectable at day 3 were IL-2, IFNy, TNFα and TNFβ.
• In the spinal cord, most cytokine transcripts peaked early at day 7.
• In the spinal cord, induced maximum levels for IL-1β, IL-6 and possibly IL-1α, IL-10 and IL-13 were lower in the spinal cord than in the brain.
• In the spinal cord, constitutive levels of IL-12p35 were lower in the spinal cords than the brains.

3.3.17.2.2 CBA mice
• In the brain, the first transcripts detectable (at day 3) were IL-1α, IL-1β, IL-2, IL-6, IL-12p40, IL-13, TNFα and TNFβ.
• In the brain, between days 10 and 35 levels of cytokines showed large variations between individual mice.
• In the brain, maximal levels of Th1 (IFNy, IL-12p40) and Th2 (IL-4 and IL-10) transcripts were equivalent.
• In the spinal cord, the first cytokines detectable at day 3 were IL-1α, IL-1β, IL-2, IL-12p40, IL-13, IFNy, TNFα and TNFβ.
• In the spinal cord, most cytokines peaked late at day 14.
• In the spinal cord, generally, transcription was lower in spinal cords than brains.
• In the spinal cord, at day 35 post infection levels of IL-1α, IL-1β, IL-2, IL-6, TNFα and TNFβ were high and in some cases maximal. This contrasted with the transcript levels in the brains for these cytokines which were all basal at this time point.

3.3.17.2.3 SJL/J mice
• In the brain, the first transcripts detectable (at day 3) were IL-2, IL-6, IL-7, IL-12p40, IL-13, IFNy and TGFβ.
• In the brain, transcripts for IL-2 and IL-1β were not present.
• In the brain, maximal levels of IFNy (Th1) were less than IL-4 (Th2).
• In the spinal cord, the first cytokines detectable at day 3 were IL-1α, IL-4, IL-6, IFNy, TNFα, TNFβ and TGFβ.
• In the spinal cord, most cytokines peaked at day 7.
• In the spinal cord, IL-1α, IL-6, IL-13, IFNγ, TNFα and TNFβ remained elevated in S/JL/J spinal cords on day 35.
• In the spinal cord, there was minimal or no transcription of IL-1β, IL-2 and IL-12p35.
• In the spinal cord, early levels of IL-10 were greatly increased in the spinal cords compared to the brains.

3.3.17.4 Comparisons.
• In the brain, in addition to the absence of IL-1β and IL-2, relative to Balb/c and CBA mice, S/JL/J mice made a slow TNFα and IL-1α response, and had lower titres of IL-1α.
• In the brain, S/JL/J mice had a delayed but prolonged IFNγ response.
• In the brain, Balb/c mice had a low Th2 (IL-4 and IL-10) response relative to CBA and S/JL/J mice. Whereas, Balb/c mice had higher levels of IFNγ than IL-4, this was the reverse in S/JL/J mice.
• In the brain, S/JL/J mice had an earlier peak of TGFβ expression (at day 7) than Balb/c and CBA mice.
• In the spinal cord, there was a difference between mouse strains in the kinetics of the cytokine response in the spinal cord. Balb/c and S/JL/J mice made a more rapid response than CBA mice.
• In the spinal cord, after day 10 levels of pro-inflammatory cytokines IL-1α, IL-6, TNFα and TNFβ were low in Balb/c mice but remained high in CBA and S/JL/J mice. This reflects the persistence of virus consistently restated in the literature in CBA and S/JL/J mice.
• In the spinal cord, as in the brains, levels of IL-2 and IL-1β were basal in S/JL/J spinal cords.
• In the spinal cords of S/JL/J but not Balb/c and CBA mice levels of IL-12p35 were basal.
• In the spinal cords of Balb/c mice there are low levels of IL-4 and IL-10, but high levels of IL-2 and IFNγ.
Some CBA mice clearly polarised their type 1 or type 2 responses as early as days 7 post infection.

3.3.17.5 Cytokine levels in SCID mice
- TNFα, IL-1α, IL-6, IL-2 and IL-12p40 are produced in the infected CNS in the absence of specific immune responses indicating production by resident CNS cells.

3.3.17.6 Antibody levels during the acute phase of TMEV.
- Serum IgM responses were similar and slow in all 3 mouse strains.
- Balb/c mice make a higher titre anti-IgG (IgG1 and IgG2a) response than CBA and SJL/J mice.
- Balb/c mice make a stronger IgG1 than IgG2a response at early time points.
- Virus is cleared between days 10 and 14 in Balb/c mice it is likely that a strong antibody response is partially responsible.

3.3.17.7 Immunostaining for cellular makers and adhesion molecules
- CD3+, CD4+, CD8+ and F4/80+ cells all infiltrate the CNS during the acute phase of Theiler’s virus infection.
- There was up regulation of adhesion molecules VLA-4, V-CAM-1 and LFA-1 in the CNS during the acute phase of Theiler’s virus disease.
3.4 Discussion

Cytokines play a pivotal role in the orchestration of local immune responses by regulating and stimulating the activation and differentiation of cells (Frednl, 1992; Watkins et al., 1995; Vanmeir, 1995; Navikas & Link, 1996; Wood & Seow, 1996; Aria et al., 1997). Cytokines are also involved in the induction of synthesis of adhesion molecules and in chemotaxis. The cytokine environment within the target organ influences the local immune response, the manifestation of the disease and the outcome of viral infection (Wesseligh & Griffin, 1994; Campbell et al., 1994). The precise cytokine profile within the target tissue is extremely important when considering the pathology and clinical outcome of a viral infection. Cytokine profiles which are generally considered to be optimal in clearing an infection and preventing pathology are those which constitute a Th2 response. Th2 type cytokines are anti-inflammatory and are important in generating a good antibody response with a low cell mediated response. Contrasting this the Th1 spectrum of cytokines is associated with an inflammatory response, increased cell mediated and decreased humoral immune responses (Mossman et al., 1986; Abehsiraamar et al., 1992; Heinzl et al., 1995; Romagni, 1996; Sedlik, 1996; Constant & Bottomley et al., 1997; Umetsu & DeKruyff, 1997).

3.4.1 Source of cytokine transcripts

Production of cytokine mRNA in the CNS can be either by indigenous cells including neurons, astrocytes, oligodendrocytes and microglia (Rubio et al., 1991; Torres et al., 1992, 1995; Couraud et al., 1994; Renno et al., 1995; Tchelingerian et al., 1995; Qi & DalCanto, 1996) or by infiltrating mononuclear cells including macrophages, CD4+ and CD8+ T cells (Mossman et al., 1986; Abehsiraamar et al., 1992; Heinzl et al., 1995; Romagni, 1996; Sedlik, 1996; Constant & Bottomley et al., 1997; Umetsu & DeKruyff, 1997). Infected SCID mice indicate which cytokine transcripts were elevated in the absence of specific immune responses. SCID mice lack T and B cells. At late time points (day 21) in SCID mice there were high levels of IL-1α, IL-2, IL-6, IL-12p40 and
TNFα indicating that these transcripts can be produced by CNS cells. However, between days 3-14 post infection in SCID mice all cytokine mRNA levels were very low and contrasted dramatically to the immunocompetent mice where levels of numerous cytokines were elevated. This shows that production of high levels of cytokine transcripts requires specific immune responses. It is unclear from these studies whether the high levels of cytokines in immunocompetent mice are produced by the infiltrating mononuclear cells or as a result of mononuclear cells stimulating the intrinsic CNS cells, or even as a result of high levels of circulating cytokines, or a combination of these.

It is well documented that cytokines can be produced by CNS cells after infection with a virus; this includes production of IL-1β, IL-4, IL-6, IL-10 and TNFα by astrocytes after Sindbis virus infection (Wesselingh & Griffin, 1994), and production of IL-1β, IL-6 and TNFα by cultured microglia after infection with Visna lentivirus in vitro (Ebrahimi, personal communication). In vitro experiments with microglia and astrocytes isolated from the CNS and infected with Theiler’s virus have shown production of IL-1β, IL-6 and TNFα (Rubio et al., 1991; Couraud et al., 1994; Renno et al., 1995; Qi & DalCanto, 1996).

The pattern of Theiler’s virus induced pro-inflammatory cytokine mRNA expression was similar to that previously seen in the other two acute CNS virus infections which have been studied in detail, Sindbis virus and LCMV infections (Wesselingh & Griffin, 1994; Campbell et al., 1994). In all immunocompetent mouse strains during the acute phase of infection with Theiler’s virus, IL-1α, TNFα and TNFβ were the cytokines which were most significantly elevated. These can be produced by a variety of cells of CNS and immune origin.
3.4.2.1 Course of events: Initial Events

During the acute phase of Theiler's virus disease, following i.e. inoculation of BeAn virus, virus titres rise in the brains from 3-7 days. Between days 7-10, levels of genomic RNA are generally maximal in the CNS (Simas & Fazakerley, 1996). From many previous studies mononuclear cell infiltration into the CNS is first apparent at about 4 days. As early as day 3 post infection several pro-inflammatory cytokine transcripts were elevated in both brains and spinal cords in all 3 mouse strains. These included IL-1α, IL-2, IL-6, IL-12p40, IFNγ, TNFα and TNFβ. These cytokines were elevated before the influx of inflammatory cells and are likely therefore to represent the response of CNS cells to infection. In support of this most of these early transcripts, IL-1α, IL-2, IL-6, IL-12p40 and TNFα were the same transcripts observed in SCID mice. The actions of the above cytokines vary widely from activation and stimulation of specific immune response to up regulation of chemokines and adhesion molecules involved in trafficking of activated lymphocytes.

The original stimulus for the rapid induction of these early pro-inflammatory cytokines is unknown, but in the first instance it is presumably signals generated either by damage (ic inoc.), or by the presence of virus. As in other tissues it is likely that IFNα/β is upregulated in the CNS during infection. IFNα/β is important in up regulation of MHC class I on the surface of many cells and is generally seen as one of the first lines of defence. It has been suggested that induction of CNS cytokines can be triggered by neurones secreting an active neuropeptide (Cocchiara et al., 1997). *In vivo* evidence has shown that numerous neuropeptides can induce the expression of cytokines. During the acute phase of Theiler's virus infection neurones are the primary cell type infected (Aubert & Brahic, 1995; Simas and Fazakerley, 1996). Infected or damaged neurones could therefore release a neuropeptide which in turn could stimulate other CNS cells to produce cytokines. Alternatively, IL-12 may be the stimulus for the increase in production of other pro-inflammatory cytokines. IL-12 is the first cytokine upregulated after many
viral infections (Germann and Rude, 1995). Again, IL-12 could be activated by damage, viral products, viral replication or factors such as interferons. IL-12 displays a multitude of biological effects with its central role being the regulation of innate and specific immune responses. This is exemplified by its ability to stimulate NK cells, Th1 cells and CTLs.

Active IL-12 is comprised of two subunits, p35 and p40. Transcripts for p35 were constitutively present in brains of all 3 mouse strains and at lower levels in the spinal cords of Balb/c and CBA mice but were not detected in the spinal cords of SJL/J mice. Transcripts for the p40 subunit were not constitutive but were rapidly induced following infection. In most animals in all strains and in both brains and spinal cords, maximal or near maximal titres of IL-12p40 transcripts occurred early at day 3. High levels of IL-12p40 were observed before high levels of IL-2 and IFNγ consistent with a role for IL-12 in the generation of Th1 cell responses.

In many viral infections the first mononuclear cells to be recruited are natural killer (NK) cells. This is also true for CNS infections as is described for LCMV (Christian et al., 1996; Zajac et al., 1996). As mentioned above IL-12 is important in the activation of NK cells which in turn produce IFNγ (Orange & Biron, 1996), which has a plethora of immunological effects and is required for Theiler's virus clearance (Chapter 5, Fiette et al., 1995). IL-12 expression is also known to induce TNFα, which also been shown to have a direct anti-viral role. High levels of IL-12 also has an affect on inhibiting the development and stimulation of CD8+ T cells (Orange et al., 1995). Its expression during Thieler's virus infection may be important in influencing CTL responses, which have been shown to be important in clearance of this virus (Larsson-Sciard et al., 1997).

Early induction of TNFα has been suggested to contribute a direct role to the control of virus infections (Paya et al., 1990). TNFα can up regulate adhesion
molecule expression and stimulate differentiation of T cells and macrophages, resulting in an increase in infiltrating mononuclear cells. It is well documented that TNFα has many effects including cytotoxicity towards neurones and oligodendrocytes, an ability to stimulate the production of neurotoxic products which include nitric oxide, platelet activating factor, prostaglandins and other metabolites of arachadonic acid, and induction of Th1 and inflammatory DTH responses (Beulter, 1993). The cells which produce TNFα are predominantly macrophages, T cells and astrocytes.

It is well documented that IL-1α and IL-1β are produced by macrophages and astrocytes. IL-1α and IL-1β are biologically more or less equivalent pleiotropic factors that act locally as well as systemically. IL-1α and β are involved in the up regulation of other cytokines including TNFα and IFNγ, and are mediators of B and T cell responses. IL-1α transcripts were consistently upregulated to high levels in both brain and spinal cords of all 3 mouse strains and transcript levels correlated with virus titres. Furthermore, IL-1α transcripts were produced by SCID mice. In contrast, IL-1β transcripts were not produced in SCID mice and although induced in the brains and spinal cords of Balb/c and CBA mice there was little or no up regulation in the brains or spinal cords of SJL/J mice.

3.4.2.2 Course of events: Early events.
Balb/c mice clear Theiler's virus infection within two weeks. This is due to a combination of factors including humoral and cell mediated immune responses. Initially, intrinsic cells of the CNS produce IL-1α, IL-2, IL-6, IL-12 and TNFα, these cytokines have wide ranging affects. TNFα and IL-6 are known to have direct anti-viral roles. However, their role in Theiler's virus infection in the CNS remains unclear. TNFα and IL-6 and are also involved in the up regulation of V-CAM-1. This facilitates infiltration of activated lymphocytes into the CNS in response to chemokine gradients (Asensio & Campbell, 1997). From 7-35 days Balb/c mice make a strong antibody
response of all isotypes and a good type 1 cytokine response (IL-2 and IFNγ). As discussed previously, IL-12 is important in the activation of NK cells which produce IFNγ. IL-12 is also important in driving Th0 cells along the Th1 pathway. The high levels of IFNγ transcripts observed in Balb/c mice could reflect higher NK cell activity but since they occur at day 7 they are more likely to represent strong Th1 T cell responses and or production of CTLs. In many viral infection high or maximum levels of CTLs are present by day 7 post infection (Butz and Bevan, 1998), and CTL activity has been demonstrated to be high in the C57Bl/6 mouse strain which is resistant to TMEV persistence (Dethlefs et al., 1997; Larsson-Sciard et al., 1997).

The series of events is clearly different in SJL/J mice. SJL/J mice had a poor humoral response in comparison to Balb/c mice and had little or no expression of IL-1β, IL-2 and IL-12p35. These above factors may explain why virus is not cleared from the CNS of SJL/J mice. IL-12 has an important role in stimulating NK cells as described previously, therefore a reduction in NK cell activity may result in a decrease in IFNγ and in turn a decrease in type 1 and CTL responses, and an up regulation in Th2 type responses as seen in this mouse strain. Lack of IL-2 has clear effects on clonal expansion of CTLs which has been described previously (Larsson-Sciard et al., 1997). Interestingly, there is an early peak of TGFβ in SJL/J mice on day 7. TGFβ is an inhibitory cytokine that decreases activity of macrophages, NK, T and B cells. This may contribute towards the inefficient immune response in SJL/J mice.

CBA mice are intermediately susceptible to viral persistence, resulting in viral clearance in some animals and not in others. The present study shows that individual CBA mice display clear type 1 or type 2 cytokine profiles. It can be speculated that animals which display a good type 1 response (like Balb/c mice), make a good CTL response resulting in elimination of virus, and conversely animals which have a good type 2 response (like SJL/J mice)
make a poor CTL response resulting in viral persistence. It would be of interest to determine whether the CBA mice which had a good type 1 response also had a good CTL response and vice-versa. In the brains of CBA mice, variability in the levels of Th1 and Th2 cytokines in individual animals was observed. Several animals which had high Th2 cytokines had low Th1 cytokines and vice versa, therefore the balance of Th1/Th2 cytokines in the CNS in individual mice may account for the difference as to why some mice clear the virus whereas others develop viral persistence (this is illustrated in Section 3.3.13). CBA mice which had high levels of pro-inflammatory cytokines at day 21 post infection also had high levels of virus indicating that the virus is still driving the immune response (see Section 3.3.6).

In CBA mice there was an increase in cellular infiltrates including macrophages, CD4+ and CD8+ T cells during the acute phase of Theiler's virus infection, with infiltrates having decreased by day 35 post-infection. There was also an increase in the expression of the adhesion molecules VLA-4, I-CAM 1 and LFA-1. Interestingly, VLA-4 is induced on activated T cells by the cytokines IL-1 and TNFα. Between days 3-14 when this adhesion molecule was detected high levels of IL-1α and TNFα were present.

3.4.2.3 Course of events: Late events
In the brains of Balb/c mice there is a rapid fall in most cytokines after viral clearance. However, certain cytokines including TNFα and IFNγ, remained elevated in the spinal cords of some CBA mice. Similarly, in the brains of SJL/J mice although most cytokines returned to basal levels, several including, IL-1α, IL-6, IL-13, IFNγ, TNFα and TNFβ remained elevated in the spinal cords. The most likely explanation for this is that virus is cleared from the brains of SJL/J mice and like Balb/c mice, cytokine levels return to basal, whereas virus persists in the spinal cords of SJL/J and some CBA mice and continues to drive cytokine responses.
3.4.3 Predisposition to persistence

In all 3 mouse strains, transcripts associated with specific immune responses Th1 (IL-2, IFNγ) and Th2 (IL-4, IL-5, IL-10) were expressed but were low in comparison to levels of pro-inflammatory transcripts produced by CNS and probably also infiltrating cells (IL-1α, IL-1β, IL-6, TNFα and TNFβ). Levels of IL-2 were lower in the brains and spinal cords of SJL/J mice during the acute phase of infection compared to CBA and Balb/c mice. Levels of IFNγ were also initially low in SJL/J mice, however they eventually reached similar levels as to that which were observed in CBA and Balb/c mice. This correlates with RT-PCR data from Monteyne et al., (personal communication). This suggests that the SJL/J mice have an inefficient Th1 response relative to Balb/c mice. Other studies have demonstrated that SJL/J mice have low levels of TMEV-specific CTL precursors. This could be either cause or effect of the low IL-2 and IFNγ transcripts seen early in these mice. Levels of IL-4 and IL-10 transcripts were higher in SJL/J than in Balb/c mice during the acute phase of disease. Again this data is consistent with RT-PCR studies by Monteyne et al. (personal communication). Therefore, although SJL/J mice are documented to produce a Th1 response at late time points post infection, it appears that they generate a strong Th2 response during the acute phase. This data puts a different slant on the Th1/Th2 hypothesis in TMEV infection and suggests that Th1 cells although causing damage during the chronic persistent phase may be essential during the acute phase for viral clearance.

In addition to low early or undetectable levels of IFNγ and IL-2 SJL/J mice were deficient in levels of transcripts for several cytokines including IL-1β and IL-12p35. The absence of IL-12p35 transcripts in the SJL/J spinal cord is striking and may be one reason why virus persists in the spinal cords but not in the brains of these mice. It could be speculated that absence of IL-12p35 and therefore active IL-12 results in a poor NK cell response, in combination with lack of production of IFNγ which in turn is important for good Th1 and CTL responses, known to be vital for viral clearance. On the other hand poor
IL-12p35 may mean that there is deficient macrophage/microglia activity also known to be important in antigen presentation and viral clearance.

IL-1β is also not expressed in the brains or spinal cords of SJL/J mice. IL-1β is predominantly produced by mononuclear cells. From the lack of IL-1β it could be speculated that SJL/J mice have a lack of macrophage activity, which accounts for the inability of these mice to clear the virus. This hypothesis would be further supported by the fact that SJL/J mice also have lower levels of TNFα, a cytokine produced partly by macrophages. However, the histopathology in SJL/J mice is characterised by a florid macrophage/microglia response. Although there are large numbers of macrophages/microglia in SJL/J mice it is possible that they are not activated and this has lead to increased recruitment due to a feedback mechanism.

Deficiencies in levels of transcripts for brain and spinal cord IL-2 also occur in SJL/J mice. SJL/J mice are deficient in levels of brain and spinal cord IL-2. This cytokine is important in clonal expansion of T cells, and a deficiency in this cytokine would result in a reduction of CTLs, which have been shown to be an important prerequisite for viral clearance. Therefore, in combination with low levels of IL-1β, IL-2 and IL-12p35, SJL/J mice may have low levels of activated microglia and CTL activity.

The major differences in antibodies between mouse strains during the acute phase of Theiler’s virus infection were in the levels of IgG1 and IgG2a. Levels of IgG1 were dramatically increased in resistant Balb/c mice compared to intermediately susceptible CBA and susceptible SJL/J mice. The levels of IgG2a were not as high as the levels of IgG1 and the differences between strains were not as statistically significant. A previous study by Peterson et al (1992) demonstrated that resistant C57Bl/6 mice produced predominantly antibodies of the IgG1 subclass, and susceptible SJL/J and intermediately susceptible C3H HeY mice produced antibodies of the IgG2a subclass, during
the acute phase of Theiler's virus infection. Th1 cells are associated with a DTH and promote IgG2a synthesis with the inhibition of other IgG subclasses. In contrast to this cells of the Th2 subset promote production of IgG1 and IgE synthesis and suppress IgG2a synthesis (Peterson et al., 1992). The results of Peterson's study support a Th1/Th2 dichotomy, with susceptibility being associated with Th1 cells and resistance being associated with Th2 cells. Susceptibility to demyelination induced by Theiler's virus is generally associated with an increased DTH caused by CD4 + T cells and infiltrating macrophages (Clatch et al., 1986, 1987, 1988; Peterson et al., 1992; Gerety et al., 1994; Karpus et al., 1995; Pope et al., 1996). Further support for the association between Th1 responses and disease is provided by an adoptive transfer study. An anti-TMEV CD4+ Th1 (IL-2, IFNγ producing) cell-line when transferred into susceptible SJL/J mice which had previously been inoculated with Theiler's virus resulted in acceleration of onset of demyelination and increased areas of demyelination (Karpus et al., 1995).

The antibody profiles described in this chapter provide little support for the Th1/Th2 hypothesis with regards to Theiler's virus infection. Resistant Balb/c mice did produce more IgG1 than susceptible SJL/J mice and intermediately susceptible CBA mice. This is consistent with the hypothesis, implicating Th2 cells in clearance, however the cytokine profiles in Balb/c mice contradicts this suggest that there is more of a type 1 response in Balb/c mice during the acute phase of the disease. Also both resistant and susceptible strains produced IgG2a (which according to the hypothesis is associated with Th1 cells and demyelination). It is clear there is not a polarised IgG isotype response during the acute phase of Theiler's virus infection, but all isotypes are upregulated particularly in Balb/c mice. It is likely that the CD4+ Th1 and Th2 cells are both present and disease or resistance to disease is not mutually exclusive to one particular subset of helper cells. However, recent evidence has elucidated that animals which are MHC class II deficient (on a resistant H2-b background) which therefore lack
CHAPTER 3 CYTOKINES, ANTIBODIES AND THE ACUTE PHASE

any functional CD4+ T cells, produce lesions of demyelination (Njenga et al., 1996). This data suggests that demyelination cannot simply be explained by a Th1 versus Th2 hypothesis, and that other factors are also involved.

A previous study (Pena Rossi et al., 1991) shows that levels of IgM are higher in the spleens of C57Bl/6 mice than that of susceptible SJL/J mice on post infection day 7. However, the data presented in this chapter suggests that there is no difference in the levels of IgM in the serum between the mouse strains.

3.4.4 Concluding remarks.
Clearly there are differences between mouse strains during the acute phase of Theiler’s infection (Figure 3.14). In both resistant Balb/c and SJL/J mice neurones and microglia are the primary cell types infected. This causes an up-regulation in pro-inflammatory cytokines, interestingly SJL/J mice little or no expression of IL-1β and IL-12p35 compared to Balb/c mice, this may be indicative of a dysfunctional macrophage/microglial response (Figure 3.14). Between days 7 to 10 there is an increased in trafficking of primed CD4, CD8 and F/80 positive cells as well as an up regulation of adhesion molecules in the CNS. In summary in Balb/c there is a good anti-viral IgG response with an up-regulation of IgG1, IgG2a, IgG2b this is coupled with an increase in IL-2 and IFNγ as well as an increase in several pro-inflammatory cytokines (Figure 3.14). It was not verified as to which cell types produced individual type 1 cytokines however it could be suggested that they were produced by either CD4+ helper cells, CD8+ CTLs or a combination of both. In contrast to this the inflammatory response in SJL/J mice is not as strong, this is coupled with a poor IgG1 and IgG2a antibody response. However in SJL/J mice there is an up regulation of type 2 cytokines, IL-4 and IL-10 and an early peak in TGFβ (Figure 3.14). The results from both strains suggest that a good antibody response is important in controlling the spread of virus whereas a good perforin mediated CTL response is required for complete clearance. (see
Chapter 6, Larsson Sciard et al., 1997), however the precise role of type 1 and type 2 responses is still a matter of debate.

In conclusion, there are multiple cytokines expressed in the brains and spinal cords of mice and there are differences in antibody isotypes generated in the serum of infected mice during the acute phase of Theiler's virus infection. However, the nature of the cytokine and antibody response is a elaborate and diverse network. With the expression of different cytokines occurring at different levels and at different time points post-infection. It is clear that the cytokine response during Theiler's virus infection is multifaceted with the expression of many cytokines influencing, co-ordinating and controlling each others expression. The intricate and complex nature of the cytokine response during Theiler's virus infection adds further caution to the safety and efficacy of using a single cytokine therapy when considering treatment for a CNS inflammatory disease, such as M.S.
Figure 3.1 An RNase Protection Assay
Image A) 14 day exposure and image B) 24 hour exposure using the ML-11 and ML-26 template probe sets for various subsets.
A

Proben

344  TNFβ ➔
325  TNFα ➔
   IL-4 ➔
   IL-5 ➔
251  IL-1α ➔
228  IFNγ ➔
178  IL-2 ➔
163  IL-6 ➔
151  IL-1β ➔
100  L32 ➔

IL-10 ➔
GM-CSF ➔
TGFβ ➔
IL-13 ➔
IL-12p40 ➔
IL-12p35 ➔
IL-7 ➔
100
Figure 3.3 Levels of mRNA transcripts in the brains of Balb/c, CBA and SJL/J mice infected with Theiler's virus during the acute phase of the disease. Brain RNA samples were isolated from days 3 to 35 post infection, from mice inoculated i.e. with $10^4$ PFU of TMEV (BeAn). mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. Levels of various transcripts were determined in the brains of Balb/c (○) CBA (□) and SJL/J (▲) mice. Each individual mouse is colour coded and is represented by the same colour at the same time point in each graph. The solid pink line represents the average (n=4) level of transcript determined in uninfected control mice. The SD error bars were not included for control mice as there was minimal variation between control animals. Graph A) IL-1α, B) IL-1β, C) IL-2, D) IL-4, E) IL-5, F) IL-6, G) IL-7, H) IL-10, I) IL-12p35, J) IL-12p40, K) IL-13, L) IFNγ, M) TNFα, N) TNFβ, O) TGFβ. The x-axis represents the day post infection and the y-axis represents the intensity of the band on the gel, which is directly proportional to amount of transcript.
E) mIL-5 Brain

F) mIL-6 Brain

Day post infection

Intensity
G) mIL-7 Brain

H) mIL-10 Brain
I) mIL-12p35 Brain

J) mIL-12p40 Brain

Graphs showing intensity over day post infection for different mouse strains: Balb/c, CBA, SJL/J.
K) mIL-13 Brain

L) mIFNγ Brain

Graphs showing the intensity levels over time for Balb/c, CBA, and SJL/J strains after infection.
M) mTNFα Brain

N) mTNFβ Brain

Day post infection

Intensity

Balb/c

CBA

SJL/J
O) mTGF\(\beta\) Brain

![Graphs showing intensity over day post-infection for Balb/c, CBA, and SJL/J strains.](image)

- **Balb/c**: Intensity peaks around 15 days post-infection, then decreases.
- **CBA**: Intensity remains relatively stable with a slight increase around 15 days post-infection.
- **SJL/J**: Intensity shows a peak around 10 days post-infection, followed by a decline.
**Figure 3.4** Levels of mRNA transcripts in the spinal cords of Balb/c, CBA and SJL/J mice infected with Theiler's virus during the acute phase of the disease. Spinal cord RNA samples were isolated from days 3 to 35 post infection, from mice inoculated *i.e.* with $10^4$ PFU of TMEV (BeAn). mRNA transcripts were detected by RNase Protection Assay as described in the *materials and methods*. Transcripts were visualised by autoradiography and quantitated by densitometry. Levels of various transcripts were determined in the brains of Balb/c (○) CBA (□) and SJL/J (△) mice. Each individual mouse is colour coded and is represented by the same colour at the same time point in each graph. The solid pink line represents the average (n=4) level of transcript determined in uninfected control mice. The SD error bars were not included for control mice as there was minimal variation between control animals. Graph A) IL-1α, B) IL-1β, C) IL-2, D) IL-4, E) IL-6, F) IL-10, G) IL-12p35, H) IL-12p40, I) IL-13, J) IFNγ, K) TNFα, L) TNFβ, M) TGFβ. The x-axis represents the day post infection and the y-axis represents the intensity of the band on the gel, which is directly proportional to amount of transcript.
A) mIL-1α Spinal cord

B) mIL-1β Spinal cord
C) mIL-2 Spinal cord

D) mIL-4 Spinal cord
E) mIL-6 Spinal cord

F) mIL-10 Spinal cord

Graphs showing changes in intensity over days post infection for different mouse strains (Balb/c, CBA, SJL/J) for mIL-6 and mIL-10 in the spinal cord.
G) mIL-12p35 Spinal cord

H) mIL-12p40 Spinal cord
I) mIL-13 Spinal cord  

J) mIFNγ Spinal cord
K) mTNFα Spinal cord

L) mTNFβ Spinal cord

Intensity vs. Day post infection for Balb/c, CBA, and SJL/J.
M) mTGFβ Spinal cord

Day post infection

Intensity

Intestine

Day post infection

Intensity

Intestine

Day post infection

Intensity

Intestine
Figure 3.5  The averages of the expression of selected cytokines in the brains of Balb/c, CBA and SJL/J mice during the acute phase of infection of Theiler's virus infection. Brain RNA samples were isolated from days 3 to 35 post infection, from mice inoculated i.c. with $10^4$ PFU of TMEV (BeAn). mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. The x-axis represents the day post infection and the y-axis represents intensity. Different coloured lines represent different cytokines.
Balb/c proinflammatory cytokines in brains

CBA proinflammatory cytokines in brains

SJL/J proinflammatory cytokines in brains

Day post infection
Figure 3.6 The averages of the expression of selected cytokines in the spinal cords of Balb/c, CBA and SJL/J mice during the acute phase of infection of Theiler's virus infection. Spinal cord RNA samples were isolated from days 3 to 35 post infection, from mice inoculated i.c. with $10^4$ PFU of TMEV (BeAn). mRNA transcripts were detected by RNase Protection Assay as described in the *materials and methods*. Transcripts were visualised by autoradiography and quantitated by densitometry. The x-axis represents the day post infection and the y-axis represents intensity. Different coloured lines represent different cytokines.
Proinflammatory cytokines in spinal cord

Balb/c Proinflammatory cytokines in spinal cord

Day post infection

CBA Proinflammatory cytokines in spinal cord

Day post infection

SJL/J Proinflammatory cytokines in spinal cord

Day post infection

Intensity

0 3 7 10 14 21 35

0 20 40 60 80 100 120

0 20 40 60 80 100 120

0 20 40 60 80 100 120
Figure 3.7  Comparison of levels of vRNA with the levels of transcripts for pro inflammatory cytokines. CBA mice were inoculated i.c. with $10^4$ PFU of TMEV (BeAn) and sampled on various days post infection, during the acute phase of disease. Each symbol represents an individual animals sampled between days 10 and 21 post infection. The x-axis represents the amount of total brain RNA required to get a positive signal by a dot blot assay. The y-axis is a measure of the quantity of cytokine message. The animals with the highest cytokine levels require only a small amount of total brain RNA to detect virus, indicating that there is more vRNA in the brains of these mice. On the other hand, animals with the least amount of cytokine require greater amounts of total RNA to get a positive signal.
Relationship between levels of cytokine mRNA and viral RNA.

TNFα

IL-1α

IL-6
Figure 3.8 Levels of mRNA transcripts in the brains of SCID mice infected with Theiler's virus during the acute phase of the disease. Brain RNA samples were isolated from days 3 to 21 post infection, from mice inoculated i.c. with $10^4$ PFU of TMEV (BeAn). mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. The x-axis represents the day post infection and the y-axis is a measure of the quantity of the transcript. Each circle represents an individual mouse.
### Brains

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<th>Day max. level occurred</th>
<th>CBA max. level</th>
<th>Day max. level occurred</th>
<th>SJL/J max. level</th>
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### Spinal cords

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<th>CBA max. level</th>
<th>Day max. level occurred</th>
<th>SJL/J max. level</th>
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Table 3.1 Comparison in levels of different cytokines in the brains and spinal cords of Balb/c, CBA and SJL/J mice during the acute phase of Theiler's virus infection. The table indicates the different cytokines mRNAs which were detected during the acute phase of disease and shows the differences in their levels of expression. The figures represent the densitometry reading of the particular band of interest after normalisation.
Figure 3.9 Titration of purified viral antigen for use in TMEV specific ELISA. Immulon 4 plates were coated with purified BeAn antigen (■) and different concentrations of 10% BSA (▲). Sera were pooled from CBA mice 28 days after inoculation with 10^4 PFU of TMEV (BeAn) i.c. Anti-TMEV antibody was detected with anti-mouse total immunoglobulin conjugated to HRP as described in the Materials and methods. A dilution of 1/10 was elected to be used in the ELISA, as it gave the highest absorbance, with the lowest background.
**Figure 3.10** Dilution curve of anti-TMEV. Sera were pooled from CBA mice 28 days after *i.c.* infection with $10^4$ PFU of TMEV BeAn. The anti-TMEV IgM (▲) and total anti-TMEV immunoglobulins (■) were measured by TMEV specific ELISA as described in the *materials and methods.*
Figure 3.11 Anti-TMEV antibody isotypes in the serum of Balb/c, CBA and SJL/J mice during the acute phase of Theiler’s virus disease. Serum were collected from mice inoculated i.c. with $10^4$ PFU of TMEV (BeAn), from days 3 to 35 post infection. Antibody isotypes were determined for Balb/c ( ), CBA( ) and SJL/J ( ) by TMEV specific ELISA as described in the materials and methods. The solid line represents the background absorbance which is the mean plus 2 standard deviations of the absorbance given by a 1/100 dilution of serum from a group of mice inoculated with the non-cross reacting Semiliki Forest virus and the dotted line represents the average absorbance throughout the course of infection for individual strains.
Figure 3.12  This figure illustrates the expression of different cellular infiltrates in the brains and spinal cords during the acute phase of Theiler’s virus infection in CBA mice. Immunostaining occurred by method 1 as described in the materials and methods. The information regarding the antibodies used can also be obtained in table 2.4. Immunostaining was visualised using DAB. The brains were cut sagitally and the spinal cords were cut longitudinally, all tissues were counterstained with haematoxylin. A) CD3+ cells in the spinal cord on post infection day 7 B) CD3+ cells in the spinal cord post infection day 10, C to E expression of CD4+ in the brains on post-infection day 14. F and G, CD8+ expression in the spinal cords on post infection days 14, H to J, CD8 + staining in the brains on post infection day 14. K to M, F4/80+ cells (a macrophage marker) in the brains on post infection day 7, and O and P, F4/80+ staining in the spinal cords on post infection day 10. Magnification x 25 (A, K); x 100 (D, F, H, J, M, O); x 200 (B, C, E, G, I, L, N),
Figure 3.13 This figure illustrates the expression of different adhesion molecules and cytokines in the brains and spinal cords during the acute phase of Theiler's virus infection in CBA mice. Immunostaining was by method 1 as described in the materials and methods. The information regarding the antibodies used can also be obtained in table 2.4. Immunostaining was visualised using DAB. The brains were cut sagitally and the spinal cords were cut longitudinally. All tissues were counterstained with hematoxylin. A) LFA-1 in the brains on post infection day 10. B) V-CAM-1 staining in the brains on post infection day 10. C) mononuclear cells around a blood vessel expressing TNFα in the brain on post infection day 7 and D) expression of VLA-4 in the brains of CBA mice on post infection day 14. Magnification all x 100.
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Table 3.2 Expression of cytokines in the CNS of CBA mice during the acute phase of Theiler’s virus disease. CBA mice were inoculated with $10^4$ PFU of TMEV (BeAn) i.c., and CNS tissues were frozen in isopentane and sagittal and longitudinal cryostat sections were cut for brains and spinal cords, respectively. Immunostaining was carried out as described in the Materials and methods. Detection was visualized by DAB. Tissues were counter stained with haemotoxylin. After the day post infection + depicts mice which were inoculated with $10^4$ PFU of BeAn, and - represents animals which were mock infected with PBS. A minimum of 3 mice were stained for immunological markers at each time point studied. The various levels for the various markers was recorded as - no expression, +/- 1-2 positive cells at high power fields (x 400), + 10 positive cells at high magnification (x 400), ++ 10-30 positive cells at lower power fields (x 200), and +++ 30< positive cells at observed in lower power fields of magnification (x 100).
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**Table 3.3** Expression of cellular markers in the CNS of CBA mice during the acute phase of Theiler's virus disease. CBA mice were inoculated with $10^4$ PFU of TMEV (BeAn) i.c. CNS tissues were frozen in isopentane and sagital and longitudinal cryostat sections were cut for brains and spinal cords, respectively. Immunostaining was as described in the Materials and methods. Detection was visualised by DAB. Tissues were counter stained with haematoxylin. A minimum of 3 mice were stained for immunological markers at each time point studied. After the day post infection + represents animals inoculated with virus and - represents mock infected animals inoculated with PBS. The various levels for the various markers were recorded as - no expression, +/- 1-2 positive cells at high power fields (x 400), + < 10 positive cells at high magnification (x 400), ++ 10-30 positive cells at lower power fields (x 200), and +++ 30< positive cells at observed in lower power fields of magnification (x 100).
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Table 3.4 Expression of adhesion molecules in the CNS of CBA mice during the acute phase of Theiler's virus disease. CBA mice were inoculated with 10^4 PFU of TMEV (BeAn) i.e. CNS tissues were frozen in isopentane and sagital and longitudinal cryostat sections were cut for brains and spinal cords, respectively. Immunostaining was as described in the Materials and methods. Detection was visualised by DAB. Tissues were counter stained with haemotoxylin. A minimum of 3 mice were stained for immunological markers at each time point studied. After the day post infection + represents animals infected with virus and - represents PBS mock infected controls. The various levels for the various markers was recorded as - no expression, +/- 1-2 positive cells at high power fields (x 400), + < 10 positive cells at high magnification (x 400), ++ 10-30 positive cells at lower power fields (x 200), and +++ 30< positive cells at observed in lower power fields of magnification (x 100).
Figure 3.14  Summary of the immune response occurring in Balb/c (A) and SJL/J (B) mice during the acute phase of Theiler’s virus infection. (A & B, 1)

Neurones, macrophages and astrocytes are infected early, resulting in an increase in expression of IL-12, interferon, MHC class I and II, (A & B, 2) contributing to an increase in expression of pro-inflammatory cytokines (A & B, 3) and adhesion molecules. (A, B 4 & 5) Viral antigen is presented to primed infiltrating CD4+ and CD8+ T cells with generation of anti-viral antibodies. (A, 6 & 7) In resistant Balb/c mice there is a predominantly type 1 cytokine response and an increase in virus specific anti-viral antibodies of all IgG subclasses, resulting in clearance of virus. (B, 6 & 7) In susceptible SJL/J mice there is a predominantly type 2 cytokine response and lower antibody levels of all IgG subclasses compared to Balb/c mice, resulting in persistence of virus and demyelination (see Chapter 4). * Levels of antibody were detected in the serum as opposed to CSF.
High precursor frequency of CTL's
Virus

Low precursor frequency of CTL's
Chapter 4

Cytokine and antibody profiles during the chronic phase of Theiler’s murine encephalomyelitis virus
Chapter 4  Cytokine and antibody levels during the chronic phase of
infection with Theiler's murine encephalomyelitis virus

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4.1 Introduction

Theiler's virus disease is generally divided into two phases, the acute phase which occurs during the first three weeks post infection and the chronic phase after this time (Daniels et al., 1952; Lui et al., 1967; Lipton, 1975; Dal Canto & Lipton, 1975; Lipton et al., 1980; Tsunoda et al., 1996). The chronic phase is associated with white matter disease and demyelination. The cytokine response during the acute phase of Theiler's virus infection was discussed in Chapter 3. Theiler's virus persists in the CNS of susceptible mice and leads to demyelination (Lipton et al., 1995; Simas & Fazakerley 1996; Brahic et al., 1996; Rodriguez et al., 1996). The exact mechanism(s) of persistence and demyelination remain a matter of debate and were discussed in Section 1.11.

The immune response is known to be important in clearing and controlling Theiler's virus infection (Oleszak et al., 1995; Monteyne et al., 1997). However, the immune response has also been shown to have pathogenic actions involved in the demyelinating process (Clatch et al., 1985, 1986, 1987; Peterson et al., 1991, 1992; Gerety et al., 1994; Karpus et al., 1995; Pope et al., 1996). Investigators have suggested that CD4+ T cells of the Th1 subset are involved in demyelination and there is much evidence to substantiate this. Including 1) the inoculation of Th1 cells into susceptible SJL/J mice after infection results in an early onset of disease with an increase in extent of demyelinating lesions; 2) production of IgG1 which is associated with Th2 cells is increased in resistant mice and, IgG2a which is associated with Th1 cells is elevated in susceptible mouse strains, and therefore disease pathology (Peterson et al., 1991; Gerety et al., 1994).

CBA (H2-k) mice are intermediate in susceptibility to developing chronic demyelinating disease after intracerebral inoculation with Theiler's virus. In an earlier study from this laboratory the proportion of CBA mice which had levels of persisting virus in their CNS, between days 50 and 268 post infection,
as determined by RT-PCR-SB was 53% (16/30). The majority of animals which had detectable levels of persisting virus also displayed clinical signs of chronic disease, however 16% (5/30) of persistently infected animals displayed no clinical signs (Simas and Fazakerley, 1996).

The objective of this chapter was to investigate the cytokine and antibody profiles during the chronic phase of Theiler's virus disease; in a number of intermediately susceptible CBA mice and determine whether there is a difference in cytokine and/or antibody responses between mice which clear the infection and those in which virus persists.

4.2 Experimental design

Sera and RNA samples from a previous study performed in this laboratory were analysed. In this study, a large group of CBA mice had been inoculated intracerebrally with $10^4$ PFU of TMEV (BeAn) and animals were sampled at various time points post infection from 50-268 days post infection. At each sampling point RNA was isolated from brain and spinal cord and levels of persisting virus determined by RT-PCR and Southern blotting (see Section 2.13). The animal sampling, RNA extraction, RT-PCR and Southern blotting for CBA mice was undertaken by Dr JP Simas. These same samples were used in this study to investigate levels of cytokine transcripts by RPA (see Section 2.11). Sera were collected to determine the levels of anti-viral neutralising antibodies and levels of the different isotypes of antibodies. Sera were also taken at late time points post infection from SJL/J and C57Bl/6 mice, which had been inoculated i.c. with $10^4$ PFU of TMEV (BeAn). This was also to determine the levels of antibody isotypes by ELISA. The ELISA experiments were not carried out as accurately as they might have been therefore the value of these experiments is questionable. In these ELISA experiments for convenience a standard 1/100 dilution of serum was used for all samples. However, if ELISA experiments were to be carried out more
thorough, the total immunoglobulin level in individual samples should be determined by a separate ELISA. Therefore when reading the ELISA data this point should be remembered.

4.3 Results

4.3.1 Cytokine mRNA transcript levels during the chronic phase of Theiler's virus infection

The RPA analysis was carried out using aliquots from the same RNA samples used to determine viral persistence. Levels of mRNA transcripts for several cytokines were determined by RPA, to see whether there was a difference in profiles or distinctive Th1 or Th2 profiles in mice with viral persistence compared to those which had cleared the virus. Brain RNA samples from 6 animals with viral persistence and 6 without were assayed for the presence of mRNA for TNFβ, TNFα, IL-4, IL-5, IL-1α, IL-2, IFNγ, IL-6, IL-1β, IL-3, IL-10, TGFβ, IL-13, IL-12p40, IL-12p35 and IL-7. There were no differences in the levels of mRNAs for TNFβ, IL-4, IL-5, IL-1α, IL-2, IFNγ, IL-6, IL-1β, IL-3, IL-10, TGFβ and IL-13 between the two groups as determined by Students t-test. However, there were significantly increased levels of mRNA for TNFα (p = 0.05), IL-12p35 (p = 0.01) and IL-12p40 (p = 0.02) in mice which had persisting virus (Figure 4.1). Levels of IL-2, IL-1β and TGFβ appear to be different between the two groups when looking at Figure 4.1 however, this was not statistical significant.

4.3.2 Antibody isotypes in the serum of CBA mice with and without viral persistence.
Antibody isotype levels were obtained for CBA mice which had viral RNA persisting in their CNS, and for CBA mice which had cleared the virus (between days 50 to 268 post infection). Anti-viral IgM, IgG1, IgG2a and IgG2b were detectable in the sera of mice with viral persistence and of mice which had cleared the virus. There were no differences in the levels of IgM, between animals which had viral persistence and those which had cleared the virus (Figure 4.2A). However, there were several statistical differences between the two groups of animals, as determined by students t-test for anti-viral IgG1, IgG2a and IgG2b (Figure 4.2 B, C, D). There were significantly elevated levels of anti-viral IgG1 (p=0.07), IgG2a (p=0.049) and IgG2b (p=0.0085) in mice which had viral persistence compared to animals which had cleared the virus. Animals were all monitored for the development of clinical signs. Several animals had virus detectable in their CNS but displayed no visible clinical signs. There were no differences in the levels of antibodies in animals which had viral persistence and displayed clinical signs and those which had viral persistence but did not display clinical signs.

4.3.3 TMEV specific antibody isotypes in SJL/J, C57Bl/6 and CBA mice during the chronic phase of disease.

Antibodies to TMEV of the IgM, IgG1, IgG2a and IgG2b isotypes were also measured in susceptible SJL/J and resistant C57Bl/6 mice during the chronic phase (>50 days post infection). There were several statistically significant differences between the strains, as determined by Students t-test. Levels of IgM were significantly elevated in CBA mice during the chronic phase of Theiler’s virus infection compared to susceptible SJL/J mice (p=0.0001) and resistant C57Bl/6 mice (p=0.0001) (Figure 4.3 A). There were differences in the classically defining Th1 and Th2 associated isotypes which are IgG2a and IgG1, respectively. Both resistant and susceptible strains of mice generated equivalent levels of IgG2a antibodies, however, there were significantly higher levels of IgG1 in susceptible SJL/J, and intermediately susceptible CBA
mice compared to resistant C57Bl/6 mice (p=0.01, p=0.02, respectively) (Figure 4.3 B, C). There were no differences in the levels of IgG2b in the strains studied (see Figure 4.3D). Antibody levels in different mouse strains during the acute phase of Theiler’s virus infection are discussed in Chapter 3.

4.3.4 Comparison of the levels of cytokines with antibody isotypes in CBA mice with and without persistent infection

CBA mice with viral persistence had statistically elevated levels of the pro-inflammatory cytokines TNFα, IL-12p35 and IL12p40, and also had significantly increased anti-viral antibodies of the IgG1, IgG2a and IgG2b isotypes. The most likely explanation for these findings is the continual stimulation of the immune response by virus.

4.4 Discussion

During the acute phase of Theiler’s virus infection numerous cytokines are expressed in the brains, these range from pro to anti-inflammatory, see Chapter 3. In contrast, during the chronic phase fewer cytokines were expressed and there was a difference between animals which had viral persistence and those which had cleared the virus. CBA mice which had virus persisting in their CNS had increased levels of TNFα, IL-12p35 and IL-12p40. These cytokines are associated with Th1 responses and support the hypothesis that Th1 cells cause the demyelination. Evidence supporting Th1 cells as the effector cells in demyelination includes, 1) the acceleration of disease onset in infected recipients of a TMEV specific Th1 cell line (Gerety et al, 1994); 2) correlation of disease susceptibility with development of chronic
high levels of MHC class II-restricted virus specific DTH (Clatch et al 1985, 1986, 1987); 3) demonstration of activated Theiler’s virus specific CD4+ T cells in the CNS of infected mice (Pope et al., 1996); 4) a dramatically reduced disease incidence in mice tolerised with intact Theiler’s virus virions results in anergy of Th1 cells causing a reduction in demyelination and clinical signs (Karpus et al., 1995); 5) an increased IgG2a:IgG1 ratio in the serum of resistant mice compared to susceptible mice (Peterson et al., 1991).

Although it is probable that TNFα and IL-12p40 are produced by cells of the Th1 lineage (Germann & Rude, 1995; Romagnani et al., 1996; Sedlik, 1996) it is also possible that they are produced by intrinsic cells of the CNS. It has been shown that astrocytes produce TNFα in vitro (Rubio et al., 1991,1993). It is also possible that these two cytokines are produced by infiltrating macrophages or other as yet uncharacterised cell types (Germann & Rude, 1995, Beutler, 1992). The presence of these pro-inflammatory cytokines in the CNS during persistent infection suggests that the constant immune stimulus provided by the virus results in their upregulation, however the precise cell type which produces them is not known. It has been well documented that cytokines play an important destructive role during a persistent viral infection and upregulation of several pro-inflammatory cytokines may cause direct pathology. For example high levels of TNFα can be destructive to oligodendrocytes (Dofferhoff et al., 1991; Raine, 1994).

TNFα is also upregulated in other latent and persistent viral infections. Infection of the trigeminal ganglion with HSV 1 results in expression of TNFα, IFNγ and IL-10 two months post infection (Halfod et al., 1996). Cytomegalovirus (CMV) infection results in increased levels of TNFα and IL-2 at late time points post infection (Fietze et al., 1994; Geist et al., 1994). Studies by Shearer et al, (1994) have implicated the importance of the cytokine response during infection with HIV. There is a switch from Th1 type cytokines (IFNγ and IL-2) during HIV infection to Th2 (IL-4, IL-10) with the
onset of AIDS dementia, however in the later stages of dementia there is a decrease in IL-4 and an increase in TNFα which is though to be one of the major mediators of the wasting syndrome associated with the later stages of AIDS dementia. Shearer hypothesised that Th1 cells are associated with increased levels of cell mediated immunity, and CTLs which are protective, however when the switch is made from Th1 to Th2, humoral immunity takes over from cell mediated immunity and is thought to be less protective and the final stages of dementia set in. This hypothesis was supported by two lines of evidence which include 1) many HIV exposed individuals from a high risk group generated HIV specific CTLs and remained seronegative despite several exposures (Shearer, manuscript in preparation) and also 2) preimmunisation of macaques with SIV results in a strong cell mediated response with no antibody production. After tolerisation these animals were resistant to infection, even when inoculated with high doses (Tyor et al., 1992). This illustrates the importance of the protective cell mediated immunity to HIV. In vitro studies of a vascular endothelial cell line infected with Cocksackie B virus results in the establishment of a persistent infection with the subsequent production of TNFα. This may contribute to the direct pathology caused by this virus in cardiac muscle and CNS tissue in vivo (Conaldi et al., 1997). The above examples illustrate TNFα’s important functional role in the persistent phase of many viral infections. The role of this cytokine is further discussed with regards to the acute phase of Theiler’s virus infection in Chapter 3.

In the present study, relative to mice which had cleared the infection there was clearly an increase in levels of anti-viral IgG1, IgG2a and IgG2b in CBA mice which had a persistent infection. This is not consistent with the Th1 hypothesis for demyelination. A likely explanation for this is those animals which had a persistent infection had a continuous stimulus to their immune system inducing higher levels of antibody of all isotypes during the chronic phase of Theiler’s virus induced disease. In a study of CBA mice these same
sera showed an increase in neutralising antibody titre in those animals with a persistent infection as determined by *in situ* hybridisation between days 50-268 (Simas & Fazakerley, 1996).

There was no single isotype which was particularly upregulated during the chronic phase of in CBA mice. This was not as expected because previous investigations have reported an upregulation in IgG1 levels in resistant C57Bl/6 mice and an upregulation of IgG2a in susceptible SJL/J mice (Peterson et al., 1992). These reports have suggested a Th1 versus Th2 dichotomy with IgG1 associated with Th2 cells, viral clearance and resistance, and IgG2a associated with Th1 cells, viral persistence and susceptibility. The antibody isotypes generated in CBA mice with and without viral persistence did not correlate with the Th1/Th2 hypothesis, because IgG1, IgG2a and IgG2b antibody isotype levels are all up regulated, in animals with a persistent infection.

In this study when looking at differences between strains of mice, there was only an increase in anti-viral IgG1 isotype in SJL/J and CBA mice compared to C57Bl/6 mice at late time points post-infection. However, the implications and relevance of having an increase in IgG1 is associated with Th2 cells, and it had been suggested by Miller's current model for demyelination that Th1 cells are partly responsible for the demyelination, and therefore from this isotype evidence it can be suggested that Th2 cells are also present during the demyelinating phase of the disease.

It is thought that the cytokine response during the acute phase of Theiler's virus infection plays an important role in determining viral persistence, and CBA mice are more variable in their cytokine responses between days 10-35 in brains and spinal cords compared to the other mouse strains (see *Chapter 3*). Therefore the cytokine array during the acute phase may play a important role in determining whether virus persists or not and leads to demyelination.
and disease. This could be examined by trying to manipulate the cytokine responses.

CBA mice which are intermediate in susceptibility to viral persistence and therefore demyelinating disease leave an intriguing question to be answered. Why do mice of an inbred mouse strain with exactly the same genetics lead to differences in susceptibility to developing demyelinating disease? The role of the immune system in individual CBA mice has been discussed above. However, there are other reasons rather than the immune system which may explain the differences in susceptibility in CBA mice. The sex of the animal may influence the outcome of viral infection. This was illustrated by a study by Kappel et al., (1991) who demonstrated that males were more likely to develop demyelinating disease as opposed to females. It has been suggested that females have a different hormonal milieu when compared to males, the ability of a virus to persist may be linked to hormonal status of the infected host i.e. depending of what time of the estrous cycle the female is at upon inoculation, or depending on whether the female is pregnant when inoculated may have an important influence. Oestrogen, a steroid hormone produced predominantly by females, can have effects on humoral and cell mediated immunity. I.e. inoculation of female mice with LCMV results in a fatal leptomenigitis mediated by CD4+ T cells. Males are less susceptible however males become susceptible if treated with oestrogen (Muller, 1995).

Psychological status of the host may be an important factor. Stress may have an effect on the outcome of viral infection. Animal heirarchies exists in groups of animals, the position of an individual within this hierarchy and the associated stress may affect ability to clear virus. Both these suggestions are speculative and there is little evidence to support them. However, it is known that when an animal is stressed, steroid hormones are released which can regulate the immune response (Hermann 1994, 1995). Animals which experience restraint stress are differentially affected with regards to
pathogenesis during experimental influenza virus infection. Virally infected animals which are subjected to physical restraint stress were observed to recover from influenza infection. This was documented to be due to the increased levels of circulating glucocorticoids when compared to lower levels of glucocorticoids in the unrestrained control group. Glucocorticoids have a role in modulating the immune response and restricting inflammation, therefore they increase the animals chances of survival (Hermann, 1993). Glucocorticoids also play an important role in EAE infection. EAE infected rats which underwent physical restraint stress had significantly suppressed clinical signs and a quicker recovery, compared to the unrestrained control group (Kuroda, et al., 1994). In a separate study glucocorticoids were also directly implicated in the spontaneous recovery of rats from EAE (Mac Phee, 1989). Adrenalectomy of these rats results in fatality but if adrenalectomized animals are treated with steroids they develop the same disease course as the control group. These above studies highlight the importance the physiological endocrine balance may have in affecting the clearance of Theiler’s virus. Also when an animal is stressed increased levels of TNFα are produced by the HPA. This also may have important implications in Theiler’s virus pathology, as discussed in Chapter 8. Variability in the immune response may also arise in CBA mice due to a number of factors. It is accepted that i.c. inoculation results in the introduction of part of the inoculum into the blood stream. It has now been shown that Theiler’s virus priming of the immune system occurs via the spleen and this favours a Th2 immune response, which is associated with IL-4, IL-5, IL-6 and IL-10 and a good IgG1 response (Peterson et al., 1992). Therefore the variabilities in viral persistence may be due to the variability of individual CBA mice in splenic priming of their immune response.

In conclusion, there is a increase in levels of IgM and IgG1 when comparing strains at late time points post infection. SJL/J (susceptible) and CBA (intermediately susceptible) have increases in levels of IgG1 when compared
to C57Bl/6’s (resistant). Levels of IgM are elevated in CBA mice when compared to SJL/J and C57Bl/6 mice. Evidence from the cytokine profiles and ELISAs imply that CBA mice with viral persistence have significantly elevated levels of TNFα, IL-12p35, IL-12p40, IgG1, IgG2a and IgG2b when compared to animals which have cleared the virus, therefore implicating that there are both Th1 and Th2 responses occurring during the demyelinating process.
Figure 4.1 Levels of cytokine mRNA transcripts in the CNS of CBA mice with and without viral persistence at late time points post infection. CBA mice were inoculated with $10^4$ PFU of TMEV (BeAn) i.e., and sampled between days 50 and 268 post-infection. Viral persistence was determined by RT-PCR and Southern blotting. This technique has a sensitivity such that 33 molecules of vRNA could be detected. Cytokine mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. Total RNA was isolated from the brains of CBA mice which had cleared the virus (○), those which had viral persistence (●) and from uninfected control mice (▪). Graph A, represents the ML-11 probe set and graph B, represents the ML-26 probe set.
Figure 4.2 Levels of specific anti-viral antibodies in the sera of CBA mice with and without viral persistence at late time points post infection. CBA mice were inoculated with $10^4$ PFU of TMEV (BeAn) i.c., and sampled between days 50 and 268 post-infection. Viral persistence was determined by RT-PCR and Southern blotting. The anti-TMEV antibody isotypes were determined by ELISA at a 1/100 dilution as described in the materials and methods. Levels of various isotypes were isolated from the brains of CBA mice with persistence (■) and without persistence (□). The solid line represents the background absorbance which is the mean plus 2 standard deviations of the absorbance given by a 1/100 dilution of serum from a group of mice inoculated with the non-cross-reacting Semliki Forest virus. The antiviral IgM OD reading for mice with viral persistence and for mice which had cleared the virus was 0.158 and 0.145, respectively (p=0.4, by Students t-test). The antiviral IgG1 OD reading for mice with viral persistence and for mice which had cleared the virus was 0.187 and 0.158, respectively (p=0.07, by Students t-test). The antiviral IgG2a OD reading for mice with viral persistence and for mice which had cleared the virus was 0.215 and 0.127, respectively (p=0.049, by Students t-test). The antiviral IgG2b OD reading for mice with viral persistence and for mice which had cleared the virus was 0.225 and 0.143, respectively (p=0.009, by Students t-test).
Anti-viral IgG1

Anti-viral IgG2a

Anti-viral IgG2b
Figure 4.3 Anti-TMEV antibody isotypes in the serum of Balb/c, CBA, C57Bl/6 and SJL/J mice during the acute and chronic phases of Theiler's virus disease. Serum were collected from mice inoculated i.e. with \(10^4\) PFU of TMEV (BeAn), from days 3 to 268 post infection. Antibody isotypes were determined for Balb/c (●), CBA(●), C57Bl/6 (●) and SJL/J (●) by TMEV specific ELISA as described in the materials and methods. The solid line represents the background absorbance which is the mean 2 standard deviations of the absorbance given by a 1/100 dilution of serum from a group of mice inoculated with the non-cross reacting Semiliki Forest virus.
IgG2a

IgG2b
Chapter 5

The roles of IFN$\alpha/\beta$ and IFN$\gamma$ in mice infected with the BeAn strain of Theiler’s murine encephalomyelitis virus.
Chapter 5 - The roles of IFNα/β and IFNγ in mice infected with the BeAn strain of Theiler's murine encephalomyelitis virus

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5.4 Discussion 159
5.1 Introduction

The interferon family of cytokines consists of a series of evolutionarily conserved molecules, which were discovered on the basis of their anti-viral activity (Isaacs and Lindenman, 1957). The interferon family can be subdivided into two classes. The two classes are distinct from each other and are simply called type I interferons and type II interferons (Weissmann and Weber, 1986).

Type I interferons (IFN) which include IFNα, IFNβ and IFNω are encoded by a cluster of over 20 genes (Weissmann and Weber, 1986). In contrast, type II IFN (IFNy) is structurally unrelated to type I IFNs and is encoded by a single gene ifng (Shepard, 1981). The two classes of IFN bind to separate receptors. Class I IFNs bind to a multi-subunit receptor with one receptor subunit which is fundamental for ligand binding (Uze et al., 1990; Muller et al., 1992; Novick et al., 1994). The type II IFN receptor is composed of two subunits one which is again essential for ligand binding (Aguet et al., 1988). The other is a species specific accessory subunit which is essential for function (Hemmi et al., 1994; Soh et al., 1994).

IFN molecules mediate their effects upon binding to their receptors via the JAK/STAT signalling transduction pathway (Darnell et al., 1994; Schindler and Darnell 1995). The JAK/STAT pathway transcriptionally regulates a variety of genes which are essential for initiating the effects of IFNs. As well as their anti-viral effects IFNs also have a wide array of other functional effects which include an involvement in cell growth, haematopoiesis, anti-tumour activities and wide effects on humoral and cellular immune responses (DeMaeyer and DeMaeyer-Guignard 1988; Sen and Lengyel, 1992; Farrar and Schreuber, 1993).
Genetic manipulation has made it possible to construct mice which have no functional type I or type II IFN receptors. This was achieved by homologous recombination. There are two separate strains of mice, one which lacks a functional receptor for IFNα/β (Muller et al., 1994) and the other which lacks a functional receptor for IFNγ (Huang et al., 1993), designated by IFNαβR°/° and IFNγR°/°, respectively.

The generation of these mice has enabled researchers to ascertain the roles of the different classes of IFNs with regards to viral, bacterial and parasitic infections (Huang et al., 1993; Kamijo et al., 1993). The importance of both classes has been investigated in viral infections including LCMV, VSV, VV, SFV and TMEV (Fiette et al., 1995, Van den Broek et al., 1995). IFNαβ has been shown to play an important role in the early stages of all the above viral infections, and is important in preventing viral spread. On the other hand IFNγ seems to be of little importance until there is the generation of a specific immune response. Once the specific immune response is generated IFNγ plays an essential role in clearing virus from the host in both TMEV and VV infections.

A gene near ifng locus has been associated with Theiler’s virus persistence in the SJL mouse strain, and IFNγ is considered to be a prime candidate in determining viral persistence due to its immunoregulatory properties (Bureau et al., 1992). The objective of this chapter was to assess the roles of IFNαβ and IFNγ following infection with the BeAn strain of TMEV.

5.2 Experimental design

Experiment 1
Following i.c. inoculation with 10⁴ PFU of TMEV (BeAn), three IFNαβR°/° and three WT 129 mice were sampled at each of days 3, 5 and 7 post-infection. At each time point brains, small intestine, heart and muscle were fixed in 10%
formal saline for histological purposes. These tissues were cut and used for
ISH and immunocytochemistry to determine the areas of viral spread, tropism and to examine cell death. Sera were also taken to determine levels of antibodies.

Experiment 2
Ten IFNγR<sup>0/0</sup> and ten WT129 mice were inoculated <i>i.c.</i> with 10<sup>4</sup> PFU of TMEV (BeAn). All animals were sampled at day 60 post infection. At this time point half of the brain and spinal cord was snap frozen in liquid nitrogen and RNA was subsequently extracted. This brain and spinal cord RNA was used in an RNase protection assay to determine levels of different cytokine mRNA transcripts (as described in Section 2.11), and in a nested PCR to determine whether virus persisted in these tissues (as described in Section 2.13). The other halves of the brains and spinal cords were frozen in isopentane for cryostat histology. Tissues were then cut and subsequently used for immunostaining to determine the cellular tropism of any persisting CNS virus. Serum was also taken to assess levels of neutralising antibody and the isotypes of virus specific antibodies. These were determined by neutralisation assay (Section 2.8) and a series of ELISAs (Section 2.14), respectively. The ELISA experiments were not carried out as accurately as they might have been therefore the value of these experiments is questionable. In these ELISA experiments for convenience a standard 1/100 dilution of serum was used for all samples. However, if ELISA experiments were to be carried out more thorough, the total immunoglobulin level in individual samples should be determined by a separate ELISA. Therefore when reading the ELISA data this point should be remembered.
5.3 Results

5.3.1 Clinical signs in IFNαβR\(^{0/0}\) mice inoculated with TMEV (BeAn).

Animals were assessed daily to see if there was any development of clinical disease. IFNαβR\(^{0/0}\) animals showed signs of acute TMEV disease as early as day 3 post-inoculation with all animals showing signs of encephalitis including photophobia. By day five post inoculation all IFNαβR\(^{0/0}\) mice appeared extremely unwell, they were hunched and grouped together with their fur looking stark, by day 7 post infection the IFNαβR\(^{0/0}\) mice had started to die and the surviving animals were all moribund. By day 8 post inoculation all animals had died. In contrast, at all time points studied the infected WT 129 mice remained well.

5.3.2 Tropism of TMEV in IFNα/βR\(^{0/0}\) mice.

The tropism of the virus varied greatly between the IFNαβR\(^{0/0}\) and wild type animals. As early as day 3 post infection IFNαβR\(^{0/0}\) animals had virus in the gray and white matter tracts of the CNS, and by day 5 the virus had spread throughout the CNS. This was different from the wild type mice which only developed focal infection of the gray matter. Therefore this suggests that IFNα/β is essential to prevent virus from entering cells of the white matter during the acute phase of infection Figure 5.1.

5.3.3 Clinical signs of IFNγR\(^{0/0}\) mice inoculated with TMEV (BeAn)

The clinical signs seen in IFNγR\(^{0/0}\) mice inoculated with TMEV contrasted dramatically to that seen in the IFNαβR\(^{0/0}\). Of the 10 IFNγR\(^{0/0}\) animals inoculated only 3 developed clinical signs. Animals started to develop clinical signs at 2 weeks post inoculation. Animals which developed clinical signs during the acute phase continued to show clinical signs during the chronic
phase of disease, and had hind limb paralysis until they were sampled at day 60 post-infection. The control group of infected WT129 mice remained well at all time points post infection.

5.3.4 Cytokine profiles in IFNγR°/° mice inoculated with TMEV (BeAn)

RNA was made from CNS tissues and examined for mRNA transcripts for various cytokines including IFNγ, TNFβ, TNFα, IL-4, IL-5, IL1α, IL-2, IL-6, IL-1β, IL-3, GM-CSF, IL-10, TGFβ, IL-13, IL-12p40, IL-12p35 and IL-7. At day 60 post infection, there was no statistical difference in the levels of message for any of the cytokines between the IFNγR°/° mice and the control WT129 mice, as determined by Student’s t-test (Figure 5.2 and Table 5.1).

5.3.5 Neutralising antibody levels and specific antibody isotypes generated during the chronic phase of TMEV infection in IFNγR°/° mice.

As detailed in the Introduction, neutralising antibody has been shown to be important in controlling the spread of TMEV infection, therefore it was of interest to discover if there was any difference in levels of neutralising antibodies and specific antibody isotypes between the IFNγR°/° mice and the control WT129 mice. By neutralisation assay IFNγR°/° mice had a significantly (Student’s t-test) increased amount of neutralising antibody compared to the control WT129 mice (P=0.0001) (Figure 5.3). The different anti-viral isotypes of antibodies generated during infection with TMEV were determined at day 60 by ELISA. IgM, IgG1, IgG2a, and IgG2b antibodies specific for TMEV were significantly elevated in the IFNγR°/° mice when compared to control WT129 mice as determined by Student’s t-test (IgG1 p=0.0001, IgG2a p=0.049, IgG2b p=0.006 and IgM p=0.001) (Figure 5.4 and Table 5.2). These data indicate that IFNγR°/° mice have elevated levels of all antibody isotypes during the chronic phase of disease (>60 days post-infection). This can probably be attributed to
CHAPTER 5  TMEV INFECTION AND THE ROLES OF IFN'S

continual stimulus generated by persisting virus within the CNS (see Section 5.3.6).

5.3.6 Persistence of virus in the CNS of IFNγR0/0 mice

It has been suggested that susceptibility to TMEV induced demyelination is associated with a genetic loci near the ifng gene therefore it was of interest to determine whether Theiler’s virus persisted in the CNS of inoculated IFNγR0/0 mice or whether virus was cleared independently of IFNγ. RNA was made from CNS tissues and examined for viral sequences by nested PCR.

The technique of nested PCR was developed for the detection of TMEV genome. This assay was developed as it offers a high degree of sensitivity for viral genome, and it also offers confirmation of the identity of the first round RT-PCR amplification product. Initially, first round RT-PCR was undertaken using primers 2A and 2B, see Section 2.13. Subsequently second round nested PCR was undertaken using primers N1 and N2. Primers were designed by using the published sequence data (Pevear et al., 1987). Figure 5.5 shows the RT-PCR products of the external (408 bp) and the internal (182 bp) nested primer sets analysed by an ethidium bromide stained agarose gel (lanes, 6, 8, 10, 12, 13, 14, 15). The specificity of the RT-PCR and nested PCR products were confirmed by Southern blot analysis. The sensitivity of this assay was calculated to be such that 3 molecules of viral RNA could be detected per brain (lane 15). This method is of great significance for the future study of TMEV pathogenesis and persistence as it offers an assay with an increased sensitivity as opposed to first round RT-PCR with subsequent Southern blotting, and therefore produces a more accurate readout of whether virus is persisting in the CNS.

TMEV sequences were detected in all ten of the brains and all ten of the spinal cords examined from IFNγR0/0 mice at day 60. In contrast, sequences were
only found in three of the WT129 animals brains and spinal cords sampled at this time (Figure 5.6). As a point of interest it was noted that the IFNγR0/0 animals which had visible clinical signs had the greatest amounts of virus, since this could be detected after first round RT-PCR.

5.4 Discussion

5.4.1 The role of IFNα/β during Theiler’s virus infection

IFNα/β is known to be important during the acute phase of a virus infection (Nemes et al., 1969; Hilleman, 1970; Sen and Ransohoff, 1993; Van den Broek et al., 1995). Mice which lack the IFNα/β receptor are extremely susceptible to a number of viral infections including VSV, SFV, VV and LCMV, even at very low inoculation doses (Van den Broek et al., 1995). There are several examples of IFN-induced proteins with anti-viral properties these include, Mx proteins which inhibit for example influenza virus (Samuel, 1991), p68 protein kinase which is important in a wide range of RNA and DNA viruses (Pain, 1986; Galabru and Hovanessian, 1987; Samuel, 1988, 1991), but perhaps the most relevant is the 2′5′-oligoadenylate synthase system which inhibits the action of picornaviruses (Chebath et al., 1987; Rysiecki et al., 1989; Coccia et al., 1990). Picornaviruses such as TMEV induce the 2′5′-oligoadenylate synthase system. 2′5′-oligoadenylate synthase is activated by IFNα/β and causes the degradation of viral and cellular RNAs by cleaving ssRNAs on the 3′ side of the UpXp sequence (Samuel et al., 1991). It could be that in the absence of this pathway picornaviral replication continues unchecked.

One of the most striking observations in IFNα/βR0/0 mice is that TMEV produces widespread infection of cells in the white matter tracts during the acute phase of infection. A previous study indicated that grey matter areas were exclusively infected during the acute phase of infection of IFNα/β R0/0 mice (Fiette et al., 1995). The present data suggests that IFNα/β has an
important role in preventing infection of oligodendrocytes in the white matter, and that lack of a functional IFNα/β system results in susceptibility of oligodendrocytes to TMEV. This has important implications, because of the important role oligodendrocytes play in myelin production and maintenance. Viral infection of oligodendrocytes may lead to oligodendrocyte and myelin destruction. These data suggest that the IFNα/β system may play an important role in preventing demyelination, by limiting viral spread in oligodendrocytes.

IFNs α, β, and γ all promote NK cells to become cytolytically activated and proliferate. NK cells are large granular cytolytic lymphocytes which represent a large proportion of the infiltrating lymphocytes generally during a viral infection. A peak in NK cell activity and proliferation occurs in parallel with the IFNα/β response during the acute phase of a viral infection. IFNγ promotes NK cell activity once the specific immune response is activated. The increase in viral dissemination in the IFNα/βR0/0 mice during TMEV infection could be accounted for due to the lack of NK cell activity. It has been shown that NK cell activity is important early in Theiler’s virus infection (Paya et al, 1989).

5.4.2 The role of IFNγ during Theiler’s virus infection

Several genes have been linked to susceptibility to persistent Theiler’s virus infection. One of the most interesting genes is Ifg (Bureau et al., 1993). Ifg is the gene which encodes IFNγ. This gene and its product is of great interest primarily because of its known immunomodulatory and anti-viral roles. IFNγ’s functions are numerous (Farrar and Schriber, 1993), and include the activation of macrophage, involvement in T cell proliferation (Gajewski et al., 1989), upregulation of MHC molecules expression on APC’s and other, upregulation of adhesion and costimulatory molecules involved in T cell
activation and generation of antibodies of the IgG2a subclass (Snapper and Paul, 1987).

Pullen et al (1994) reported that a neutralising monoclonal antibody directed against IFNγ resulted in exacerbation of TMEV induced disease in a susceptible mouse strain. Just after this thesis was started Fiette and colleagues (1995) demonstrated that TMEV infection of IFNγR0/0 mice on a resistant H2b genetic background resulted in viral persistence and chronic white matter disease. The present data confirms these results.

Virus was able to persist in IFNγR0/0 (H-2b) mice. Complete viral clearance occurred in the control wildtype animals. It has been well documented that TMEV clearance is MHC class I restricted and is associated with the action of CD8+ T cells (Pena Rossi et al., 1991; Rodriguez et al., 1993; Pullen et al., 1993; Fiette et al., 1993; Azoulay et al., 1994). Lack of a functional IFNγ system may prevent the generation of significant numbers of CTLs or may cause a decrease in the expression of MHC class I molecules, either or both of these could explain why the virus is not cleared. IFNγ plays a direct role in antigen presentation by activation of antigen processing via the proteosome (Driscoll et al., 1993; Gaczynska et al., 1993; Boes et al., 1994). Lack of IFNγ could therefore prevent or impair antigen presentation to CD8+ T cells. In this regard infection of IFNγR0/0 mice with LCMV results in an impairment in CTL responses (Van den Broek et al., 1995). It would be of interest to further characterise the importance of CTL responses in IFNγR0/0 mice and discover whether there was any impairment of the response generated towards TMEV.

Virus persists and demyelination occurs in the IFNγR0/0 mice indicating that IFNγ is not required for demyelination. Studies in IFNγR0/0 mice infected with LCMV and VV have shown a specific antibody profile with increase in levels of IgG1 (Van den Broek et al., 1995). It is known that IFNγ down regulates IgG1 (Snapper and Paul, 1987; Finkelman et al., 1990). However,
TMEV infected IFNγR^0/0 mice had significantly increased levels of antibodies of all subclasses and had significantly increased levels of neutralising antibody in their serum compared with the control group. Increased antibody levels in the IFNγR^0/0 mice is likely to be due to the amount of antigen in the CNS, IFNγR^0/0 mice all had persisting virus, which presumably constantly stimulated the immune response.

In conclusion IFNs play a non-redundant role in TMEV infection. IFNα/β plays an important role in acute infection. Contrasting to this IFNγ’s effects are not noticeable until the immune response is activated. IFNγ is essential for clearance of virus from the CNS, however, the exact role(s) IFNγ plays in TMEV clearance are not known. That it affects antigen presentation and CTL generation seems most likely.
Figure 5.1 Represents images illustrating the distribution of BeAn virus in the brains of IFNα/βR0/0 mice. 3-4 week old mice were inoculated with 10⁴ PFU of TMEV BeAn i.c and sampled early after infection. Viral positivity was determined by ISH using a digoxigenin labelled riboprobe which has been described previously in the materials and materials, tissues were counterstained with haemotoxilyin. A) Low magnification showing viral positivity. B to F) Individual stained virus positive cells (brown). G) Negative control. Magnification x 10 (A), x 100 (B-G).
Figure 5.2 mRNA cytokine profiles in the brains of IFNγR<sup>0/0</sup> and control WT129 mice infection with TMEV (BeAn), at day 60 post infection. All mice were inoculated <i>i.c.</i> with 10⁴ PFU of TMEV (BeAn). mRNA cytokine levels were determined by RNase Protection Assay as described see materials and methods, and measured by densotometry. Graph A represents ML-26 which is a template probe set for different cytokines including IL-10, GM-CSF, TGF-β, IL-13, IL-12p40, IL-12p35, IL-7 and L32. Graph B represents the ML-11 template set which includes probes for TNFβ, TNFα, IL-4, IL-5, IL-1α, IL-2, IFNγ, IL-1β, IL-6, IL-3. Both ML-11 and ML-26 probe sets had a probe for L32 which is a ubiquitously expressed ribosomal protein. The L32 band was used as an internal control and all samples were standardised against the density of their L32 band. INFγR<sup>0/0</sup> were represented by (●) and control WT 129 mice were all represented (○).
Intensity

TNFβ, TNFa, IL-4, IL-5, IL-1a, INFg, IL-6, IL-1b, IL-3

IL-10, GM-CSF, TGFβ, IL-13, IL12p40, IL-12p35, IL-17

Intensity

TNFβ, TNFa, IL-4, IL-5, IL-1a, INFg, IL-6, IL-1b, IL-3

IL-10, GM-CSF, TGFβ, IL-13, IL12p40, IL-12p35, IL-17
Figure 5.3 TMEV-specific neutralisation antibody titres present in the serum of IFNγR0/0 and WT129 mice at 60 days post-infection. All mice were inoculated i.c. with 10^4 PFU of TMEV (BeAn). The x-axis represents time and the y-axis represents the 50% neutralisation titre. The neutralisation titres were calculated as described in the materials and methods. The graph shows the reciprocal of the neutralisation titre (e.g. if 1/100 dilution of serum gave a 50% in reduction of plaque formation it is shown as 100 on the y-axis)
Figure 5.4 Anti-TMEV antibody titres of BeAn-infected IFNγR0/0 and WT 129 mice at 60 days post-infection. Sera were collected at day 60 post-infection from IFNγR0/0 (●) and WT 129 mice (○) inoculated i.c. with 10^4 PFU TMEV (BeAn). The anti-TMEV antibody was isotypes by TMEV ELISA as described in the materials and methods. Each symbol represents the serum from an individual mouse. The solid line represents the background absorbance, which is the mean plus 2 standard deviations of the background absorbance given by a 1/100 dilution of serum from a group of mice inoculated with the non-cross-reacting Semiliki Forest virus.
Figure 5.5 Products of first round RT-PCR and second round nested PCR of BeAn viral RNA concentrations. Lane 1 and 19 φ174 DNA size markers, lanes 7, 9 and 11 show 2 pg, 200 fg and 20 fg respectively, of amplified BeAn viral RNA after first round RT-PCR. Lanes 6, 8, 10, 12, 13, 14, 15, 16 and 17 indicate 200 pg, 2 pg, 200 fg, 20 fg, 2 fg, 0.2 fg, 0.02 fg, 0.002 fg and 0.0002 fg respectively of amplified BeAn sequences after second round nested PCR. The PCR conditions and primer sequences have been described previously in the materials and methods. After second round nested PCR a product of 182 bp is visualised when 0.02 fg of vRNA is amplified indicating the sensitivity of the assay is such that 3 molecules of virus can be detected. 408 bp and 182 bp are the lengths of the products obtained with the primers from first round RT-PCR and second round N-PCR respectively. The products were visualised by ethidium bromide staining of the agarose gel.

Figure 5.6 N-PCR amplified BeAn sequences from the brains of Sv 129 and IFNγR<sup>−/−</sup> mice, at 60 days post infection. 10 fold serial dilutions of purified vRNA from 2 fg to 0.02 fg was assayed as a positive control to determine the sensitivity of the assay. The sensitivity of the assay was such that 3 molecules of viral RNA could be detected by N-PCR. From the figure it can be seen that all (n=10) of the IFNγR<sup>−/−</sup> mice had detectable levels of persisting virus in their CNS compared to only 3 out of 10 of the wild type animals examined. The products were visualised by ethidium bromide staining of the agarose gel.
Table 5.1 Statistical Analysis comparing cytokine levels in IFNγR0/0 and WT 129 mice. Statistical analysis was carried out on the standardised densitometry readings from mRNA for ML-11 and ML-26 template sets between the IFNγR0/0 mice (n=5) and WT129 (n=4) mice at day 60 post infection. The analysis indicates that there were no differences at the p<0.05 level or even at the p<0.1 level. The figures represent the densitometry reading of the particular band of interest after normalisation.

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<th>WT 129 Average</th>
<th>WT 129 SD</th>
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Table 5.2. The statistical analysis carried out on IFNγR°/° and WT129 mice, for TMEV specific neutralisation antibody and TMEV specific isotypes to determine differences between the two groups. The figure represent the OD’s at 570 nm.
Chapter 6

The role of perforin during the acute and chronic phases of Theiler’s murine encephalomyelitis virus infection
Chapter 6 - The role of perforin in the acute and chronic phases of Theiler's murine encephalomyelitis virus infection

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6.1 Introduction

CD8+ T cells and natural killer (NK) cells play important roles in clearance and protection from Theiler's virus and the demyelination induced by Theiler's virus as discussed in the Introduction (see Section 1.14.4). CD8+ T cells function by recognising target cells presenting processed antigen in their MHC class I molecules, whereas NK cells lyse a variety of target cells without the classical restriction by MHC molecules. There are 4 independent pathways which account for CD8+ T cell effects following the recognition of a virus infected cell, these include 1) degranulation leading to the release of perforin and granzymes, 2) engagement of Fas receptor on target cells with Fas ligand on CTLs, 3) surface bound TNFα and 4) the release of soluble bioactive molecules such as the cytokine IFNγ (Lui et al., 1987; Kagi et al., 1994b; 1995). The first three mechanisms generally lead to death of susceptible cells. In the case of granzymes, fas and TNFα this is by induction of programmed cell death. In the case of perforin it is by cell lysis following compromisation of the plasma membrane by the perforin pore complex. Although CTLs may employ any of the above effector mechanisms, NK cells exclusively lyse target cells via the perforin-dependant pathway (Young et al., 1986b).

The perforin system of cytotoxicity was established by isolating and cloning the perforin molecule from cytolytic lymphocytes (Podack et al., 1985; Young et al., 1986a, 1986b, 1986c). Perforin is a glycoprotein of 534 amino acids in length and has sequence homology with C9 a component of the membrane attack complex of the complement system (Shinkai et al., 1988). Following interaction with Ca^{2+} perforin has the ability to integrate into target cell membranes where the perforin molecules polymerise with each other and form aggregates which produce pores of 10-20 nm in diameter in the target cell membranes (Litchenhels et al., 1988; Podack et al., 1988). Permeablisation
of the target cell membrane results in death of the target cell and the effector cell (CTL or NK cell) moves on to continue lysing other infected target cells.

The perforin-dependant pathway of cytotoxicity has been shown to be vital in clearing LCMV from infected hosts and limiting pathology (Kagi et al., 1994). On the other hand, the ability to clear other viral infections by a non-perforin dependant pathway has been documented with vaccinia virus (Kagi et al., 1995), vesicular stomatitis virus (Kagi et al., 1995), mouse hepatitis virus (Lin et al., 1997), mouse herpes virus -68 (Usherwood et al., 1997) and murine rotavirus (Franco et al., 1997). The aim of this chapter was to determine the role of the perforin dependant pathway of cytotoxicity in Theiler's virus infection. This was undertaken by studying Theiler's virus infection in perforin deficient mice and comparing the course of infection with the infection in resistant C57Bl/6 mice.

6.2 Experimental design

Three to four-week old, female and male C57Bl/6 and genetically engineered mice deficient in perforin on a C57Bl/6 resistant (H-2b) background were inoculated intracerebrally with 10^4 PFU of TMEV (BeAn). 4-6 mice were sampled on days 3, 5, 7, 9, 11 and 60 post-infection, brains, spinal cords and sera were taken from individual animals at each time point (Section 2.5). Half of the brain and spinal cords were immediately placed into 10% formal saline and processed for histology, cut tissues were subsequently used for ISH and immunostaining to determine the areas of virus spread, the cell types infected and the mechanism by which infected cells died. From the other halves of the brains and spinal cords RNA or tissue culture homogenates were made. RNA was made to determine whether or not virus persisted at late time points post-infection, using nested PCR as described in the materials and methods. Homogenates were used in plaque assays to determine the virus load. Sera were taken to determine and compare the levels of neutralising antibodies.
and the isotypes of the antibodies (there are problems with the ELISA data see Section 3.2.2).

6.3 Results

6.3.1 Acute phase clinical signs
Animals were regularly assessed at the various time points to monitor for any development of clinical signs (as detailed in materials and methods). As early as days 3 and 5 post-inoculation there were visible differences between the groups. Mice which lacked the perforin molecule appeared moribund, hunched and their fur was ruffled. This group of mice contrasted dramatically to the C57Bl/6 animals, throughout the course of infection these animals remained well and alert at all time-points. At day 7 mice which lacked the perforin molecule showed the typical clinical signs of mice susceptible to acute Theiler’s virus infection, which included poliomyelitis and hind limb paralysis. These signs remained in all the animals which survived until the animals were sacrificed at day 60 post-inoculation.

6.3.2 Acute phase plaque assay and virus spread
The course of virus infection during the acute phase of TMEV infection was typical. A productive round of viral replication peaked at day 5 in both groups and gradually decreased from day 7 to low levels at day 11 post-infection. This general pattern of infection was seen in both groups, however there were differences between the perforin deficient and wild type mice (Figure 6.1). There were statistically significant differences as early as day three between the two groups (p=0.002), as determined by Student’s t-test. These statistical differences remained at days nine and eleven post inoculation with p values of 0.006 and 0.008 respectively. This suggests that virus replication occurred unchecked in the animals which lacked perforin when compared to the control group.
6.3.3 CNS virus spread and distribution.
The neuroanatomical distribution of Theiler’s virus sequences in perforin knock out and C57Bl/6 mice was determined by in situ hybridisation (Section 2.15.9). *ISH* uses a radiolabelled riboprobe complementary to viral genomic RNA, followed by autoradiographic and microscopic analysis. The specificity of the riboprobe was tested on aged matched non-infected murine CNS tissues and at no point was there ever positive signals detected. The virus spread in brains of infected animals was consistent with the infectious virus assays (Section 6.3.2.). It can clearly be seen from Figure 6.2 that there was more virus present at all time points in the knockout mice compared to the control group. This is consistent with the infectious virus assay (Figure 6.1.). Areas of positivity were the same in both groups however there were fewer virus positive cells observed in the control group. Positive cells were identified in the cortex, hippocampus, thalamus, hypothalamus particularly in the mammillary nucleus, substantia nigra and the brain stem. There was little or no positivity found in the cerebellum, olfactory bulb, dentate gyrus, striatum, inferior or superior colliculi, pons or white matter tracts in either the perforin knock-out mice or the control C57Bl/6 mice. Together with the virus titre data this clearly demonstrates increased levels of infectious virus in the perforin knock out mice at early time-points post-infection, when compared to the control group.

6.3.4 Chronic phase clinical signs and levels of viral persistence
As mentioned above all mice which lacked perforin displayed clinical signs at 60 days post inoculation, however, none of the wild type C57Bl/6 mice showed any signs of disease. This left the imposing question, did the perforin knock out mice which displayed clinical signs have persisting virus in their CNS? This question was addressed by analysing spinal cord RNA by nested PCR as described in Section 2.13. At day 60 post-infection 7/7 perforin knock out mice had Theiler’s virus sequences persisting in their CNS. This compared dramatically to the control C57Bl/6 mice where 0/7 animals had detectable
levels of Theiler's virus RNA. The limit of sensitivity of this assay was 0.002 fg of viral RNA, which is equivalent to 3 molecules of virus, see Figure 6.3. These results demonstrate the importance of the perforin molecule and cytolytic lymphocytes, CD8+ T cells or NK cells in the elimination of TMEV from the CNS.

6.3.5 Levels of antibodies and levels of neutralisation antibody

Neutralising antibody has been shown to be important in TMEV infection, and the subclasses of antibody produced in response to TMEV infection has been shown in some studies to be of significance, with IgG1 being associated with resistance and IgG2a being associated with susceptibility to demyelinating disease. The levels of neutralising antibody and of the different subclasses of antibodies produced during the acute and chronic phases were determined in the two groups of mice by neutralisation assay and ELISA as described in Sections 2.8 and 2.14. Figures 6.4 and 6.5 demonstrate that there were no differences in the levels of neutralising antibody and no differences in antibody subclasses between the perforin knockout mice and the control C57Bl/6 mice at any time point. These data show that lack of perforin has no effect on the level or class of neutralising antibodies produced during Theiler's virus infection. From the ELISA data obtained from mice with a persistent Theiler's virus infection it was expected that the perforin deficient mice would have elevated antibody levels due to persisting virus. However in the perforin deficient animals, antibody levels remained the same as the wild type animals. This may be explained by the fact that the amount of virus persisting in the perforin deficient animals was not sufficient enough to drive a good immunoglobulin response.

6.3.6 TUNEL evidence for apoptosis?

There is evidence to suggest that different strains of Theiler's virus have differing abilities to induce apoptosis in different cell types in vitro. GDVII has a 50 fold greater capacity to induce apoptosis than the avirulent BeAn
strain of the virus in BSC-1 and BHK-12 cell lines (Lipton et al., 1997). The ability of BeAn to induce apoptosis in perforin knock out and control C57Bl/6 mice was determined by using the TUNEL technique as described in Section 2.15.4. There were differences between the perforin knock-out mice and the wild type C57Bl/6 mice. The mice which lacked perforin had an increase in levels of apoptosis, with cell death peaking at around day 7 post inoculation. This coincided with the high levels of infectious virus in the CNS. After day 7 the levels of cell death diminished to background levels. The area in which cell death was most obvious was the hippocampus, particularly the virally infected hippocampal neurones (see Figure 6.6). These data are the first to show that an avirulent strain of Theiler’s virus induces programmed cell death in vivo.

6.4 Discussion
This study was undertaken to identify the contribution of perforin-dependent cellular cytolysis in the pathogenesis of TMEV infection. A perforin-dependant pathway has been shown to be essential for the clearance of the non-cytopathic LCMV whereas non perforin-dependant pathways are essential in clearing cytopathic viral infections such as VSV, VV and SFV (Kagi et al., 1995). These cytopathic viruses are cleared either via one of the other pathways used by CTL’s, or by the generation of specific neutralising antibodies. It was of interest to discover what role perforin played in TMEV infection, primarily because TMEV is cytopathic during the acute phase of the disease but establishes a non-cytopathic phase during chronic infection.

There was no difference in the brain regions infected during the acute phase of the disease between the perforin deficient and control mice. Virus remained exclusively in grey matter areas. However, the levels of virus were greater at all times post-infection in the perforin deficient mice. This may be accounted for by the fact that at early time points post-infection when virus levels were at their highest in both the perforin deficient and C57Bl/6 mice,
NK cells are likely to be particularly important. Unlike CTLs which lyse infected cells by a variety of mechanisms, NK cells exclusively lyse cells via a perforin-dependent pathway. The early increased levels of virus in the perforin-deficient mice compared to the C57Bl/6 mice is likely to be attributable to the lack of NK cell activity. This is supported by the fact that it has previously been demonstrated that NK cells play an important protective role during the acute phase of infection with TMEV (Paya et al., 1989). The precise role of NK cells in the demyelinating process remains as yet unknown, therefore it would be interesting to determine whether perforin deficient mice had an increased or reduced degree of demyelination during infection with Theiler’s virus infection.

It could be argued that early virus load is an important factor in determining the clinical disease phenotype. At early time points post infection (days 3 and 5) perforin deficient mice had an increase in virus load in their CNS which could have resulted in an increase in the levels of cell lysis or programmed cell death leading to clinically apparent disease. No clinical signs were seen in the C57Bl/6 mice with lower CNS virus titres.

From day 7 and there after levels of infectious virus decreased in both the perforin-deficient and C57Bl/6 mice. This may be attributable to the generation of either an antibody, a specific non-perforin dependant cellular immune response to TMEV, or both. Although perforin deficient mice have CTLs which cannot lyse cells via the perforin pathway they still have normal levels of Fas dependant cytotoxicity and produce cytokines (Lowin et al., 1994; Walsh et al 1994a, 1994b). Various cytokines are known to modulate the activities of CTLs in other viral models. It has been demonstrated that TNFα released by CD8+ T cells can inhibit gene expression of hepatitis B virus (Guidotti et al., 1995) and has also been shown to act against cytomegalovirus (Pavic et al., 1993). Other cytokines such as MIP-1α and MIP-1β have been shown in vitro to be produced by CD8+ T cells and have a suppressive affect
on HIV infection (Cocchi et al., 1995). Perhaps the best recognised cytokine involved in anti-viral activity produced by CD8+ T cells is IFNγ (Kundig et al., 1996; Ruby et al., 1997). It has been shown that CD8+ T cell mediated clearance of murine rotavirus does not involve perforin, fas or IFNγ, therefore clearly there are other anti-viral mechanisms present (Lin et al., 1997). During CNS infection with Theiler’s virus it has been noted that macrophages, CD4+ T cells, and neutralising antibody as well as CTL’s infiltrate the CNS (Oleszak et al., 1995).

Theiler’s virus infection of perforin deficient mice resulted in persistence of virus during the chronic phase of the disease. This compared dramatically to the C57Bl/6 mice where virus was cleared by approximately 11 days post-infection. Although virus levels decreased to very low levels in the perforin deficient animals they were never eliminated, indicating that perforin dependant cytotoxicity is essential for the clearance of Theiler’s virus from the CNS. Lack of perforin leads to low levels of persisting virus which in turn may lead to demyelinating disease.

In summary, these data suggest that functional perforin is a prerequisite for Theiler’s virus clearance from the CNS. Lytic NK cells (which are perforin dependant for function) most likely contribute to disease prevention in the early phases of the acute diseases by decreasing viral load. CTL’s which function via perforin had little or no direct role in primary demyelination associated with TMEV infection of this mouse strain, except as a mediator of virus elimination. In these studies perforin has been shown to play an essential role in the acute phase of the disease by facilitating a reduction in viral load, and in the chronic phase of disease by its requirement to eliminate virus from the CNS.
Figure 6.1 Brain virus titres in Perforin deficient and C57Bl/6 mice during the acute phase of infection with TMEV (BeAn). Perforin deficient mice (●) and C57Bl/6 mice (○) were inoculated i.c. with $10^4$ PFU of TMEV (BeAn). Each symbol represents the brain virus titre of an individual mouse. The dotted line represents the limit of detection of the plaque assay. The red and green dashes represent the average of the brain virus titres for each group.
Figure 6.2  Neuroanatomical distribution of BeAn RNA sequences in the CNS of perforin deficient and C57Bl/6 mice during the acute phase of infection. Autoradiographic images demonstrate the spread of BeAn viral RNA in sagital sections of brains from perforin deficient and wild type C57Bl/6 mice inoculated with $10^4$ PFU of TMEV (BeAn) at 3-4 weeks old. ISH was the technique used to detect viral sequences using a $^{35}$S-labelled riboprobe complementary to virus RNA sequences. Areas of positivity appear black in colour. Numbers on the x-axis represents the days post infection, and the y-axis represents the group of animals inoculated.
Figure 6.3  N-PCR amplified BeAn sequences from the spinal cords of C57Bl/6 and perforin deficient mice, at 60 days post infection. Purified vRNA of 2 fg and 0.02 fg was assayed as a positive control to determine the sensitivity of the assay. The sensitivity of the assay was such that 3 molecules of viral RNA could be detected by N-PCR. From the figure it can be seen that all (n=7) of the perforin deficient mice had detectable persisting virus in their CNS. None (n=7) of the wild type animals examined had detectable viral RNA. The N-PCR products were visualised by ethidium bromide staining of the agarose gel.
Figure 6.4  TMEV-specific neutralisation antibody titres in the sera of perforin deficient and C57Bl/6 mice during the acute phase of TMEV infection. Perforin deficient (•) and C57Bl/6 (○) were inoculated i.c. with $10^4$ PFU of TMEV BeAn. The x-axis represents time and the y-axis represents 50% of the neutralisation titres. The neutralisation titres were calculated as described in the materials and methods. The graph shows the reciprocal of the neutralisation titre (e.g. if 1/100 dilution of serum gave a 50% in reduction of plaque formation it is shown as 100 on the y-axis).
Figure 6.5  Anti-TMEV antibody titres of perforin deficient and C57Bl/6 mice during the acute and chronic phases of disease. Sera were collected at day 3 to day 35 post-infection from perforin deficient (●) and C57Bl/6 mice (■) inoculated i.c. with $10^4$ PFU TMEV (BeAn). The anti-TMEV antibody was isotyped by ELISA as described in the materials and methods. Each symbol represents the serum from an individual mouse. The solid line represents the background absorbance, which is the mean plus 2 standard deviations of the absorbance given by a 1/100 dilution of serum from a group of mice inoculated with the non-cross-reacting Semiliki Forest virus.
Figure 6.6  Immunostaining for viral proteins and TUNEL staining as a marker for apoptosis after infection of perforin deficient mice which were inoculated with the BeAn strain of Theiler's virus. Perforin deficient mice were inoculated with $10^4$ PFU of BeAn, i.e., and sampled on days 3, 5, 7, 9 and 11 post infection. CNS tissues were snap frozen in isopentane and cyrostat sections were subsequently cut. Immunostaining and the TUNEL assay were performed on a number of tissues as described in the materials and methods. Viral staining is visualised by DAB (brown) and TUNEL staining is visualised by BCIP/NBT (blue/black), tissues were counterstained with methyl green as described in the materials and methods. A-B) Double stained hippocampal neurones stained brown for virus and blue black for TUNEL positivity (x25). C-E) Double stained hippocampal neurones stained brown for virus and blue black for TUNEL positivity (x100).
Chapter 7

GDVII Infection
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7.1 Introduction

Strains of Theiler's virus are generally divided into two distinct subgroups, the avirulent subgroup and the neurovirulent subgroup. These were discussed in the Introduction. The avirulent subgroup consists of the BeAn, DA, Yale, and WW strains (Daniels et al., 1952; Lipton et al., 1975; Dal Canto & Lipton, 1975; Lehrich et al., 1976), whereas the neurovirulent subgroup contains the GDVII and FA strains (Theiler and Gard, 1940b; Liu et al., 1967; Lipton, 1980). Over the past two decades most research has concentrated on characterising the genetics, immunology and pathology of the avirulent subgroup, because it is the strains in this subgroup which induce demyelination, and infection of laboratory mice with these strains is used as an animals model for MS. Less work has been undertaken on the characterisation of the neurovirulent strains.

Following intracerebral inoculation of all strains of neonatal and adult mice the neurovirulent GDVII and FA strains cause severe encephalitis (Lui et al, 1967), resulting in fatality in all mice several days post inoculation. Clinical signs associated with GDVII infection include circling behaviour, breathing difficulties, phototaxis, ruffled fur and flaccid paralysis (Theiler and Gard, 1940b; Olistky, 1945). An in depth study discovered that dose and route of inoculation were important factors in affecting clinical outcome after GDVII infection (Lui et al., 1967). Intracerebral inoculation was found to be the most lethal route of inoculation with an LD_{50} of 0.1 PFU. Intranasal, intraperitoneal and oral routes of inoculation were less efficient, resulting in a lower incidence of CNS disease, but these still demonstrated the neurovirulent properties of the GDVII strain.

After i.c. infection GDVII virus is detected in several areas of the brain and spinal cord these include, the cerebral cortex, in particular the deep layers (IV, V, VI), basal ganglia, thalamus, hypothalamus, predominantly in the
mamillary nucleus, substantia nigra, pons, and also in the anterior thalamic nuclei, and pyramidal layers of the hippocampus and the grey matter of the spinal cord in both the dorsal and ventral areas and particularly in the ventral motor neurones (Stroop et al., 1981; Simas et al., 1995). Neurones are the predominant cell type infected by GDVII, but astrocytes and oligodendroctes can also be infected (Simas et al., 1995). Infection of neurones and astrocytes was lytic, but infection of oligodendroctes was a rare event and appeared to be restricted.

No work has been done to characterise the CNS cytokine response during GDVII infection and this is the main objective of this chapter. The levels of programmed cell death (apoptosis) occurring in the CNS of mice after infection with GDVII were also measured.

7.2 Experimental Design

Three experiments were carried out. Experiment A was to determine the average time of death of animal after GDVII infection. Experiment B was carried out to examine cytokine transcript levels in the CNS and antibody levels in the serum of mice inoculated with GDVII. Experiment C to determine the level of infectious virus in the CNS of mice after infection with GDVII. Experiment A identified post infection day 5 as the average time of death after GDVII infection. Therefore to investigate the cytokine profiles and levels of apoptosis in the CNS after GDVII infection 20 mice were inoculated with $10^4$ PFU of TMEV (GDVII) i.c. Three to five mice were sampled on days 1, 2, 3, and 4 post-infection. At each sampling point brains were divided sagitally with one half being used for RNA purification and subsequently used in an RPA, to determine the cytokine profiles (see materials and methods). The other half of the brain was fixed in 4% phosphate buffered formal saline, and cut for immunostaining to determine viral spread and the ability of virus to induce apoptosis in the cell(s) it infected. The same was done for the two
separate halves of the spinal cords. Sera were also collected to determine if there was any generation of antibodies by ELISA as previously described in the materials and methods. In experiment C, 20 mice were inoculated with $10^4$ PFU of TMEV (GDVII) i.c., animals were sampled on days 1-5 and whole brains and spinal cords were snap frozen and used to determine infectious virus titres by plaque assay.

### 7.3 Results

#### 7.3.1 Clinical signs and virus titres in the CNS of mice following GDVII infection (experiments A and C)

Animals were observed daily for clinical signs associated with CNS disease. These were first apparent at 2 days post infection. All animals showed characteristic signs of acute encephalitis which included phototaxia, circling behaviour with occasional fitting. The fur of most animals appeared ruffled, and some mice displayed breathing difficulties and flaccid paralysis of the limbs. Clinical signs worsened until the animals died. Animals began to die at 3 days post infection and all were dead by 5 (Figure 7.1). The levels of infectious virus in the CNS were determined by plaque assay. Brain virus titres increased rapidly and peaked at 3 days post infection. Levels of infectious virus remained consistently high at days 4 and 5 post-infection (Figure 7.2A). Titres were more variable in the spinal cords as compared to the brain but titres increased with time and reached similar levels to that observed in the brains (Figure 7.2B).

#### 7.3.2 Cytokine and antibody levels (experiment B)

The levels of mRNA for different cytokine transcripts in the brains and spinal cords at different time points post infection were determined by RNase protection assay (see materials and methods). Several cytokines were probed for including TNFβ, TNFα, IL-4, IL-5, IL-1α, IL-6, IFNγ, IL-1β, IL-10, TGF-β,
IL-13, IL-12p40, IL-12p35 and IL-7. There were few clear differences observed during the time course of GDVII infection (Figure 7.3). Changes in the levels of pro-inflammatory cytokines in the brains of infected animals were the most noticeable. Levels of TNFα were low at days 1 and 2 post infection but were elevated dramatically at days 3 and 4 post infection (Figure 7.3 A). This pattern was also observed for IL-1α and IL-12p40 transcripts, but lower levels were observed for these cytokines (Figure 7.3 B, E). Increased levels of IFNγ and IL-2 were also observed on days 3 and 4 post infection (Figure 7.3 C, D). There was no consistent difference in the levels of any of the other cytokine transcripts probed for. The kinetics of the upregulated cytokines, correlated with the virus titres.

Expression of cytokine transcripts in the spinal cords was limited compared to the brains. TNFα, IL-12p40 and IL-2 were elevated in the spinal cords on post-infection days 3 and 4 (Figure 7.4 A, B, C), however, there was no differences in any of the other transcripts at any of the time points. Perhaps an explanation for this is that the virus is directly inoculated into the brain therefore the cytokine response here starts immediately, whereas virus does not reach high levels in the spinal cords until day 4 post-infection. This may account for the response in the spinal cord being slower. Sera from animals were examined for levels of antibody by ELISA. No virus specific antibody was detected. This was as expected because levels of specific neutralising antibody are normally not detected before day 7 post infection (Lipton and Gonzalez-Scarano, 1978).

7.3.4 Viral tropism and levels of apoptosis

The CNS histopathology induced by GDVII was studied by a combination of immunocytochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). The objectives were to determine the levels of programmed cell death (apoptosis) after GDVII infection, the areas
affected and the cell type undergoing cell death, and whether any cells undergoing cell death were infected with virus. The areas of double stained positivity for virus and TUNEL were variable and included cerebral cortex, in particular the deep layers (IV, V, VI), basal ganglia, thalamus, hypothalamus, predominantly the mammillary nucleus, substantia nigra, pons, and also the anterior thalamic nuclei, and the pyramidal layers of the hippocampus. Virus staining was exclusively in the cytoplasm, and TUNEL positive cells had the characteristic appearance of cells undergoing apoptosis with condensation and marginalisation of chromatin in the nuclei and the occasional appearance of small satellite apoptotic bodies (Figure 7.5).

Viral antigen positive cells were widely distributed and appeared to be predominantly neurones. Classification of infected cell type was undertaken by morphological analysis. Areas of TUNEL positivity corresponded with areas of viral positivity, and many cells were double-labelled for viral antigen and TUNEL (Figure 7.5), however some cells were negative for TUNEL and positive for virus and vice versa (Figure 7.5), some infiltrating monocytes also stained positive for TUNEL (Figure 7.5). Throughout the course of GDVII infection as the levels of infectious virus increased and the virus spread throughout the CNS, levels of apoptosis increased. These were maximal at day 4 post infection. It was at this time point that messages for TNFα, IL1α and IL-2 were also maximal.

7.4 Discussion

Fatal encephalitis is the clinical outcome following intracerebral inoculation of the GDVII strain of Theiler’s virus regardless of the genetic background of the mouse. This contrasts with the avirulent TO strains (Theiler and Gard, 1940b; Lui et al., 1967; Lipton, 1980). GDVII has a LD50 of 0.1 PFU after intracerebral inoculation. Animals start to die around day 3 post infection (Lui et al., 1967; Simas et al., 1995). The GDVII strain of Theiler’s virus is more
neurovirulent than any of the TO strains, infecting approximately ten times more cells than the avirulent subgroup (Aubert and Brahic, 1995). GDVII has the ability to spread rapidly through the grey matter layers of the brains of infected mice, virus spreads from cell body to cell body and along neuronal pathways (Stroop et al., 1981; Simas et al., 1995). It was originally thought that neurones exclusively were infected with GDVII (Lui et al., 1967; Stroop, 1981), however more detailed studies have shown that astrocytes and to a lesser extent oligodendrocytes can also be infected by GDVII (Simas et al., 1996).

Numerous cytokine messages were upregulated during GDVII infection these included those for TNFα, IL-1α, IL-12p40 and to a lesser degree IL-2 and IFNγ. However, that for TNFα was the most dramatically upregulated. High levels of TNFα have been associated with a phenomenon called TNFα shock (Trgovcich et al., 1996). This has been observed in neonatal mice inoculated with another neurotropic virus, Sindbis virus (Trgovcich et al., 1996). Therefore, it is possible that animals inoculated with the GDVII strain of Theiler’s virus undergo TNFα shock. It was also noted that TNFα was dramatically elevated in neonatal mice inoculated with Theiler’s virus see Chapter 8.

Other pro-inflammatory cytokines were also upregulated including IL-1α and IL-12p40, both of these cytokines are produced by macrophages and can upregulate the production of TNFα or be themselves upregulated by TNFα. These cytokines may contribute to the disease pathology either directly or indirectly.

Apoptosis has been observed in many persistent viral infections in vivo, including HIV encephalitis, HTLV-1 associated myelopathy, and its animal model HAM rat disease and recently apoptosis has also been observed in oligodendrocytes in MS patients and in animals with experimental allergic encephalitis (Petito et al., 1994; Umehara et al., 1994; Seto et al., 1995; Tomaru
et al., 1995; Yoshiki et al., 1995; Gray, 1996; Masliah et al., 1996; Shearer et al., 1996). It has also been well documented that infection of cultured cells with a wide variety of viruses including herpesviruses, parvoviruses, retroviruses, paramyxovirus, myxoviruses, alphaviruses and picornaviruses result in activation of a programmed cell death pathway (Johnson et al., 1992; Ishii et al., 1993; Kawanishi et al., 1993; Koga et al., 1994; Morey et al., 1993; Laurent-Crawford et al., 1991; Meynard et al., 1992; Ohno et al., 1994; Rey-Cuille, 1994; Esolen et al., 1995; Takizawa et al., 1993, Fesq et al., 1994; Hinshaw et al., 1994; Levine et al., 1993; Ubol et al., 1994; Tolskaya et al., 1995; Jeurissen et al., 1992; Auwaerter et al., 1995., Allsopp et al., 1998). Various in vitro studies have shown that inoculation with BeAn and GDVII causes apoptosis in a variety of cell lines (Jelachich et al., 1995; Jelachich et al., 1996; Obuchi et al., 1997; Tsunoda et al., 1997), however this has not been characterised in vivo. To determine whether programmed cell death is an important phenomenon in vivo during picornavirus infection, levels of apoptosis were determined during GDVII infection of mice. Apoptosis was observed in the CNS of mice inoculated with GDVII when compared to controls, and the levels of apoptosis increased with the time post-infection. Therefore it could be stated that GDVII infection and its ability to induce apoptosis is directly related to mortality levels in adult mice. It may be that this cell suicide program is an important host defence mechanism in eliminating a virally infected cell, however apoptosis of irreplaceable cell types such as neurones, which are non-mitotic, may have detrimental effects. However, there are important genes which are anti-apoptotic, p35 and iap in baculovirus (Clem et al., 1997; Vucic et al., 1997), and Elb in adenovirus (Han et al., 1996; Querido et al., 1997). These anti-apoptotic genes may allow virus to replicate and, or persist. It may be that these vital cellular anti-apoptotic genes are switched off or over-ridden during GDVII virus infection of neurones resulting in an increase in apoptosis and therefore in host mortality. The ability of the neurovirulent GDVII strain of Theiler’s virus to induce cell death may be a direct result of their ability to overcome a protective host factor and therefore induce
apoptosis. GDVII virus induced apoptosis in vivo could be partially responsible for increased mortality.

Poliovirus, a representative of the picornaviridae encodes products which prevent apoptosis (Leonmonzon & Dalakas, 1995; Raikhlin & Agol, 1995) therefore BeAn infection may encode a genetic component that prevents apoptosis. This could explain why very little cell death is seen in BeAn infection in comparison to the levels seen in the infection with GDVII.

Many viruses have the ability to induce apoptosis in vitro and in vivo as discussed above. The mechanism by which apoptosis is induced is variable depending on the type of infecting virus. It has been well documented that TNFα has a plethora of effects, but perhaps one of its most interesting and relevant is its ability to induce apoptosis. (Beutler, 1993; Tartaglia et al., 1993; Alderson et al., 1994; Takahashi et al., 1994; Smith et al., 1994; Daniels & Krammer, 1994; Wiley et al., 1995). TNFα functions through two receptors TNFR60 (p55-60) and TNFR80 (p70-80), these receptors are expressed on a variety of tissues including haematopoetic cell types, although membrane expression is independently regulated and may differ considerably depending on cell type (Lazdins et al., 1997). Once the receptor is activated it has the potential to induce apoptosis. Recently, the ability of TNFα to induce apoptosis has been documented during HIV infection. TNFα has been shown to induce apoptosis of CD4+ T lymphocytes from HIV infected individuals (Clerici et al., 1997). The ability of TNFα to induce apoptosis of virally infected cells may be more general. It can be hypothesised, given the correlation between TNFα and apoptosis, that during GDVII infection TNFα induces apoptosis.

Virus induced apoptosis has been shown to be an important factor in other viral diseases such as that produced by Chicken anemia virus where infection results in complete destruction of the thymic cortex in hatchlings (Noteborn &
Koch, 1994, 1995), also measles virus induces apoptosis in thymocytes (Esolen et al., 1995; Fugier-Vivier et al., 1997; McQuaid et al., 1997), also several retroviruses are capable of inducing apoptosis in culture (Laurent-Crawford et al., 1991; Ohno et al., 1994; Rey-Cuille, 1994), however, apoptosis of CD4+ T cells and neurones in AIDS pathogenesis appears to be indirect as these cells are largely uninfected (Estus et al., 1994; Ameisen et al., 1995).

In summary, this chapter illustrates the ability of GDVII to induce apoptosis in neurones in vivo. Levels of TNFα are dramatically increased with time post infection and this correlates with mortality levels. Whether there is a relationship between levels of TNFα and amount of apoptosis is as yet still to be further characterised.
Figure 7.1 Mortality profiles in Sv 129 mice infected with the neurovirulent GDVII strain of Theiler's virus. Animals were inoculated with $10^4$ PFU of TMEV (GDVII) i.c., and monitored daily. The x-axis represents day post infection and the y-axis represents the number of living animals.
Figure 7.2  Brain and spinal cord infectious virus titres of Sv 129 mice after GDVII infection. Sv 129 mice were inoculated with $10^4$ PFU of TMEV (GDVII) i.c, and levels of infectious virus was determined for A) brains and B) spinal cords by plaque assay, which has been described in the materials and methods. The limits of detection of this assay were $10^{1.7}$ and $10^2$ PFU/g for brains and spinal cords, respectively.
Figure 7.3 Levels of mRNA transcripts detected from the brains of Sv 129 mice infected with the GDVII strain of Theiler's virus. Brain RNA samples were isolated from days 1 to 4 post infection, from mice which had been inoculated i.c. with 10⁴ PFU of TMEV (GDVII). mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. Levels of various transcripts were isolated from the brains of Sv 129 mice. The x and y axis represents day post infection and intensity, respectively. The curves represents the average level of transcript, and the horizontal solid line indicates the control level of that particular cytokine.
Figure 7.4 Levels of mRNA transcripts detected from the spinal cords of Sv 129 mice infected with the GDVII strain of Theiler's virus. Spinal cord RNA samples were isolated from days 1 to 4 post infection, from mice which had been inoculated i.c. with $10^4$ PFU of TMEV (GDVII). mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. Levels of various transcripts were isolated from the spinal of Sv 129 mice. The curve represents the average level of transcript, and the solid horizontal line indicates the control level of cytokine message.
Figure 7.5 Immunostaining for viral proteins and TUNEL staining as a marker for apoptosis after infection with the GDVII strain of Theiler’s virus. Animals were inoculated with $10^4$ PFU of GDVII, i.e., and sampled on days 1, 2, 3 and post infection. CNS tissues were snap frozen in isopentane and cryostat sections were subsequently cut. Immunostaining and the TUNEL assay were performed on a number of tissues as described in the materials and methods. Viral staining is visualised by DAB (brown) and TUNEL staining is visualised by BCIP/NBT (blue/black), tissues were counterstained with methyl green as described in the materials and methods. A) Wider spread staining for virus in the brain (x4). B) Red arrow points to a double stained neurone double labelled for virus (brown) and TUNEL (blue/black) staining (x25). C) Red arrow points to a double stained neurone labelled for virus and TUNEL staining (x100). D) Individually stained TUNEL positive cell (blue/black) and individually stained cell labelled for virus (brown) (x100). E) Blue arrow points to cells undergoing apoptosis with condensed nucleus and apoptotic bodies (x100). F) Blue arrow points to single TUNEL positive cell, brown are points to single stained virus infected cell.
Chapter 8

Cytokine levels in neonates after infection with
Theiler’s murine encephalomyelitis virus
## Chapter 8 - Cytokine levels in neonates after infection with Theiler’s murine encephalomyelitis virus

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8.1 Introduction

It is well documented that there is a noticeable change in the susceptibility of rodents to neurotropic virus infections during the first few weeks post birth (Sigel, 1952; Johnson et al., 1971; Fleming, 1977; Ogata et al., 1991). There are numerous neurotropic viruses where susceptibility to clinical signs or disease is age related, examples include Sindbis, Japanese encephalitis, St. Louis encephalitis, Herpes simplex, Yellow fever, Measles, Eastern equine encephalitis, Bwamba fever, West Nile, Semiliki forest and Theiler’s murine encephomyelitis viruses (Theiler, 1940; O’Leary et al., 1942; Lennette & Koprowski, 1944; Melnick et al., 1951; Sigel, 1952; Johnson, 1964; Flemming, 1977; Penney & Wolinsky, 1976; Rodriguez et al., 1983b; Steiner et al., 1984; Swoveland & Johnson, 1989; Ogata et al., 1991). In all of these viral infections, infection of neonates or suckling animals results in a fatal encephalitis, however, in contrast to this infection of weaned animals results in a non-lethal infection. Why are levels of susceptibility linked to age? Many arguments have been considered, however it seems likely that developmental maturity of the CNS plays an important role (Pathak et al., 1976; Pathak & Webb, 1978; Fazakerley et al., 1993; Oliver et al., 1996, 1998; Oliver & Fazakerley, 1997) whereas the maturity of the specific immune response is documented to have no effect (Johnson et al., 1972; Griffin et al., 1976; Flemming, 1977; Schneider-Schaulies et al., 1989; Swoveland & Johnson 1989; Sharpe et al., 1989; Fazakerley et al., 1993). However, the contribution of these two factors may be different in different viral infections.

Infection of neonates with Theiler’s virus results in fatal encephalitis which is associated with high CNS virus titres and an extensive mononuclear cell inflammation (Wroblewska et al., 1977; Penney & Wolinsky 1979; Rodriguez et al., 1983) similar to that seen after infection with the neurovirulent GDVII strain of Theiler’s virus in adults (Lui et al., 1967; Stroop et al., 1981; Simas thesis, 1994). The studies in this chapter investigated the expression of
various cytokine transcripts in the CNS to determine if there were any differences between Theiler’s virus infected and mock infected neonatal animals.

### 8.2 Experimental design

Cytokine profiles in neonatal mice were determined for animals at different time-points post-infection by RPA (as previously described see material and methods). Seven day old (P7) mice were inoculated with $10^4$ PFU of TMEV (BeAn) intracerebrally. From previous pilot experiments it was known that suckling animals inoculated with TMEV die around day seven post-infection, therefore animals were sacrificed on days 2, 4 and 7 post infection. When animals were sacrificed whole brain was taken and snap frozen. RNA was later purified from this and used in an RPA. Sera were also collected to determine antibody profiles by ELISA (as previously described see materials and methods).

### 8.3 Results

#### 8.3.1 Clinical signs in neonatal mice (P7)

Infected animals showed signs of encephalitis around day 4 post infection, and were moribund by day 7, by day 8 post infection all animals had died (Figure 8.1). Virus was distributed extensively throughout the brains of the infected mice in both grey matter and white matter tracts (Figure 8.2). There were also major histopathological changes observed with areas of extensive necrosis and a massive cellular infiltrate (Figure 8.3), as early as day 2 post infection.
8.3.2 Cytokine expression

RPA allows the detection of various cytokines including TNFβ, TNFα, IL-4, IL-5, IL-6, IL-1α, IL-1β, IFNγ, IL-2, IL-10, TGFβ, IL-13, IL-12p40, IL-12p35 and IL-7. During infection of neonates the expression of different cytokines varied at different time points and between individual mice. Proinflammatory TNFα was expressed in the largest amounts and its expression was varied in different mice at different time points. There was very little difference between the levels of TNFα at day 2 between the virus infected and mock infected mice, however at day 4 and 7 post infection there was a massive increase in levels of mRNA for TNFα in the animals infected with Theiler’s virus compared to controls (Figure 8.4 B). It is at these time points that levels of virus are at their highest. By this time virus had spread throughout the CNS. This was accompanied by infiltrates of mononuclear cells, and after these time points animals start to die.

Levels of TNFβ, IL-4, IL-6 and IL-1α followed similar kinetics to that seen with TNFα, with very little difference in expression between infected and mock infected animals at day 2 post infection. However, by day 4 and 7 levels of TNFβ, IL-4, IL-6 and IL-1α were increased compared to the controls (Figure 8.2 A, C, D, E). The expression of these cytokines on days 4 and 7 were not as high as that seen for TNFα. By days 4 and 7 post infection levels of all 5 of these cytokines had increased and were higher than controls, (apart from IL-6 which had returned to the same levels as that seen in the control animals) (see Figure 8.2 A, C, D, E). There was no detectable difference in the expression of mRNA for any of the other cytokines transcripts probed for. At days 4 and 7 post infection there was no detectable anti- TMEV IgM, IgG1, IgG2a or IgG2b in the serum of these mice.
8.4 Discussion

Neonatal animals infected with Theiler's virus usually die around seven days post infection, at this time a massive mononuclear cell infiltrate is observed with widespread viral distribution in the CNS (Penney & Wolinsky 1979; Rodriguez et al 1983b). In contrast to this in adult mice (3-4 weeks old), CNS infection is usually focal (Simas and Fazakerley, 1996). In most mouse strains these foci of infection are cleared by an activated immune response 2-3 weeks post infection (Oleszak et al., 1995; Montenye et al., 1997). There has not yet been any clear cut explanation as to why this is the case for Theiler's virus although various hypotheses have been suggested, which include 1) how developed the CNS is, at the time of infection, 2) the ability of the neonatal animal to constitute a fully functional immune response and 3) the TNFα shock hypothesis as has been described for Sindbis virus (Trgovcich et al., 1997).

During the first two post natal weeks, there are major changes in CNS cells. During this time period many physiological developments occur which include synaptogenesis, axonogenesis and myelination (Larramendi, 1969; Altman, 1971, 1972b, Rees et al., 1976; Landis, 1983). It has been suggested that viral age-related neurovirulence may be due to the differential ability of viruses to interact with cells of the CNS in the developing and mature mouse. These changes could affect viruses at any one or more of a variety of levels including viral entry, replication, maturity and release.

The widespread virus distribution in the developing brain could be due to a differential affinity of the virus receptor, or a change in the expression of the virus receptor between neonatal and adult animal, which has been suggested for Semiliki Forest virus (Altman, 1971, 1972b; Pathak et al., 1976; Rees et al., 1976; Helenius et al., 1980; Marsh & Helenius, 1989; Kielian, 1995). In the developing brain endocytosis occurs at post synaptic membranes (Altman, 1971, 1972b; Rees et al., 1976; Landis, 1983), this could result in non-specific...
uptake of virus, and in older animals the virus may rely on receptor mediated endocytosis to enter the target cell. This may explain why several viruses including SFV can be taken up by the immature neurones and not by adult neurones.

After entering the cell the process of virus replication has to be supported by the host cell, it is well documented for a number of viruses that replication relies on the ability of the infected cell to produce smooth membranes (Dales & Siminovitch, 1961; Amako & Dales, 1967; Caliguri & Tamm, 1970; Schlesiner & Malfer, 1982; Katch et al., 1986; Kuge et al., 1986; Guinea & Carrasco, 1990). In the immature CNS there is an abundance of smooth membrane production due to the amount of axonogenesis and synaptogenesis occurring during the first two weeks postnatal (Altman, 1971, 1972b; Rees et al., 1976; Landis, 1983). Theiler's virus may use this smooth membrane production for viral replication. As the CNS matures the production of smooth membranes decreases, and this is one possible mechanism which could result in restriction of viral replication.

It is unknown which developmental changes within the CNS and at what level they occur whether it be entry, replication or release actually affect the spread of Theiler's virus infection. A more neuroanatomical study with this virus must be undertaken as has been done for other virus systems (Oliver & Fazakerley, 1997).

Whether or not neuronal maturation has an effect on BeAn infection, it has no effect on infection with the more neurovirulent GDVII strain of TMEV. This causes widespread neuronal destruction irrespective of age of animal (Stroop et al., 1981; Simas Thesis, 1994, Aubert et al., 1995). This is due to differences in viral sequence/genome between the two strains. Differences in receptor binding site between the two strains have already been documented on the viral capsid, this may explain why different strains of the same virus have
differing abilities to spread in different mouse strains (Kilpatrick et al., 1991; Lou et al., 1996; Seto et al., 1996).

100% mortality has been described before in neonates, infected with low levels of Sindbis, Semiliki Forest and West Nile viruses, whether inoculated intracerebrally, intranasally or orally. This was also true for Theiler’s virus. Death in neonatal mice was attributable to virus induced encephalitis because virus replicates to high titres in the brains of the infected animals, this is due to the permissive of murine CNS tissues for Theiler’s virus growth (Penney & Wolinsky, 1979; Rodriguez et al., 1983). However, other immunological, metabolic, endocrine and physiological parameters are also likely to be involved.

The role of LPS (bacterial endotoxin) in the pathogenesis of septic shock has been studied extensively (Dofferhoff et al., 1991; Vanderpomp et al., 1995). Endotoxic shock does not seem to exert its effects on the host directly but rather elicits the production of host factors, including cytokines IL-1α, IL-6 and TNFα which may inturn lead to shock and death of the host (Chirigos & Desimone; Glauser, 1996). The cytokines produced by LPS are all proinflammatory and all play important roles in shock, however, TNFα is the most important mediator of shock. The cytokines TNFα, IL1α and IL-6 which are expressed in response to stress activate the Hypothalamus-Pitutary-Adrenal (HPA) axis which in turn causes a cascade of cytokine and hormonal upregulation which results in severe metabolic and haemodynamic dysfunction (Tracey et al., 1989; Jaattela, 1991). It is possible that a virus could, like an endotoxin, mediate shock. It has been reported that infection of neonatal mice with the TRSB strain of Sindbis virus results in death, the animals which died had an increase in levels of TNFα and the steroid hormones adrenocorticotropic hormone and corticosterone (Trgovcich et al., 1997). The production of TNFα, adrenocorticotropic hormone and
corticosterone are therefore associated with the phenomenon of “shock”, and may be causing the death of the animal.

Although hormone levels after Theiler’s virus infection were not measured, levels of mRNA for TNFα in the CNS were, and these were dramatically elevated. It can be hypothesised that TNFα elicits a shock phenomenon in young mice after Theiler’s virus infection through stimulation of the HPA axis (Sapolsky & Meaney, 1986, Trgovcich et al., 1997). Levels of TNFα were also measured in adult mice which is discussed in Chapter 3. Interestingly, although levels of TNFα are high during the acute phase of infection in adult SJL/J, Balb/c and CBA mice, they are not as high as those seen in neonatal animals. It can be stated that levels of TNFα are highest in the brains of the CBA mice on post infection day 7 when compared to the SJL/J and Balb/c mice, and it is in adult CBA mice that mortality levels are highest during the acute phase of infection, compared to any other strain (15%). This suggests that although only a small percentage of adult CBA mice die during the acute phase of infection when compared to the neonatal mice, these adult mice may also be dying due to TNFα related shock.

Previous studies with the endotoxin LPS have shown that C57Bl/6 (H2-b) mice which are normally resistant to TMEV persistence and chronic disease become increasingly susceptible treated with LPS (Pullen et al., 1995). LPS stimulates the HPA to produce TNFα, IL-1α and steroids, which results in an increase in susceptibility and demyelination. It could be speculated that increased levels of TNFα, IL-1α, IL-6 and steroid hormones in (resistant) C57Bl/6 mice caused by LPS, results in an increase in susceptibility due to induced shock or perhaps increased levels of apoptosis of infected cells due to higher levels of TNFα. Levels of apoptosis in neonatal mice were not looked at, however as levels of apoptosis correlated with levels of TNFα in GDVII infection, it is possible that neonatal mice die of overwhelming neuronal distraction due to increased levels of TNFα causing widespread apoptosis.
The consequences of proinflammatory cytokine induction and the TNFα shock response may constitute a previously unrecognised aspect of Theiler’s virus induced disease in neonatal mice which could lead to the mortality observed in this infection.

In summary, neonatal mice die after BeAn infection, with widespread distribution of virus throughout the CNS. This spread of infection may be attributable to CNS cell maturation. However, it is also noted that levels of TNFα are exceptionally high therefore animals may be dying as a result of septic shock. This TNFα production is a previously unrecognised aspect of Theiler’s virus pathology.
Chapter 9

General Discussion
9.1 Cytokine and antibody profiles during the acute phase of Theiler’s virus infection 197
9.2 Cytokine and antibody profiles during the chronic phase of Theiler’s virus infection 199
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9.6 Cytokines expressed in neonates 202
The presented data has been discussed in detail at the end of each chapter therefore the aim of this chapter is to summarise the basic findings and to suggest areas of future research. The immunology of the avirulent BeAn and neurovirulent GDVII strain of Theiler’s virus, has been investigated in detail in different mouse strains. Chapter 3 investigated the cytokine and antibody profiles in different mouse strains inoculated with BeAn i.c. during the acute phase of infection. Chapter 4 examined the cytokine and immunoglobulin profiles in CBA mice which had viral persistence and compared the levels to those of animals which had cleared the virus during the chronic phase of disease. Chapter 5 and 6 characterised the roles of type I and type II interferons and perforin during Theiler’s virus infection. Chapter 7 investigated the cytokine profiles in the brains of adult mice inoculated with the GDVII strain of virus and examined the levels of ongoing cell death (apoptosis) in the tissues of the CNS. Cytokine profiles in the CNS and mortality levels of Theiler’s virus infected neonatal mice were examined in Chapter 8.

The pathogenesis induced by Theiler’s virus is complex. Initially virus predominantly infects neurons, this mediates an acute encephalitis in all strains infected. Encephalitis is followed by a rapid cell mediated response with a slower humoral response. In resistant strains virus is cleared, however in susceptible strains which have an inadequate immune response virus persists at low levels in oligodendrocytes, in white matter tracts of the CNS. It is during infection of oligodendrocytes that chronic demyelinating disease occurs. Over the past decade much evidence has accumulated to suggest that CD4+ T helper cells and the cytokines produced by them are the key effector during the demyelination process (Miller et al., 1987; Gerety et al., 1994; Karpus et al., 1995). It is thought that in persistently infected cells antigen is presented in the CNS which is recognised by circulating CD4+ Th1 cells, these cells then produce cytokine which mediate the recruitment of macrophages/microglia. These large phagocytic cells elicit more proinflammatory cytokines and free radicals which further contribute to myelin damage. Initially the autoimmune component of Theiler’s virus induced demyelination was not recognised however recently new
evidence has emerged which suggests that there is both B and T cell epitopes to CNS myelin at late time points during Theiler’s virus disease (Miller et al., 1997). This thesis is very pertinent to current research because it investigated cytokine profiles during the chronic demyelinating disease, but more importantly it determined cytokine profiles during the acute phase of Theiler’s virus disease in different mouse strains, which may explain why virus persists and therefore causes demyelination, in susceptible mice. The cytokine information elucidated during the acute phase of disease extended on the information which was previously known and suggested reasons as to why virus may be persisting and causing demyelinating disease.

9.1 Cytokine and antibody profiles during the acute phase of Theiler’s virus infection.

Cytokine and antibody profiles were determined in several different mouse strains during the acute phase of Theiler’s virus infection including Balb/c (resistant), CBA (intermediately susceptible), SJL/J (susceptible) and SCID mice. There were several similarities in different mouse strains in the levels of pro-inflammatory cytokines expressed during acute disease. During the first two weeks post infection IL-1α, TNFα, TNFβ, IL-12p40’s expression was up regulated in all immuocompetant strains. This correlated with the infiltration of mononuclear cells into the CNS. Production of cytokines in the CNS was not associated with indigenous CNS cells, as determined by examining cytokine levels in SCID mice inoculated with Theiler’s virus. Despite there being similarities in the proinflammatory cytokines during the acute phase of disease there were also differences between the strains. Interestingly, levels of IL1β were undetectable in the brains and spinal cords of SJL/J mice. SJL/J mice also had decreased levels of IL-2 when compared to CBA and Balb/c mice, however levels of IL-10 and IL-4 were increased in SJL/J mice when compared to Balb/c mice. Antibody levels were also different between the strains. All strains produced similar levels of IgM, however Balb/c mice produce high levels of IgG1 and IgG2a when compared to CBA and SJL/J mice. The above data suggests that Th1 cells may be important in clearing
the virus in the resistant Balb/c strain which had higher levels of IL-2, IFNγ and IgG2a when compared to the susceptible SJL/J strain. Conversely the SJL/J strain produces a predominantly Th2 response during the acute phase of disease with low levels of IgG2a and higher levels of IL-4 and IL-10 when compared to Balb/c mice. Therefore, indicating that although Th1 cells have been implicated in the chronic demyelinating phase of Theiler’s virus they appear to play a protective role in Balb/c mice during the acute phase of disease.

All the above data was determined using CNS tissues, however no data was obtained about cytokine profiles in peripheral immunological tissues such as the peripheral lymph nodes (more specifically the cervical lymph nodes which drain antigen from the brain) and in particular the spleen. All these tissues are important sites for T and B cell priming. It is unlikely that T or B cell priming occurs in the CNS, as no evidence for this phenomenon exists. Therefore although the cytokine profiles were determined in the brains and spinal cords it is unlikely that the cytokines generated in the CNS are going to effect T helper of CTL generation in the periphery. Therefore the cytokines detected in the CNS which are produced by infiltrating cells are unlikely to have an effector T or B cell functions but are rather more likely to act locally as chemoattracts or local effector molecules. Therefore, in light of this it would be of interest to determine the cytokine profiles of the spleen and the regional lymph nodes in the different mouse strains to elucidate what is occurring in the periphery. Isolation of particular T cell subsets from the blood of different mouse strains and characterisation of their cytokine profiles would elucidate a more specific set of results and may determine the likelihood of particular subsets involved in disease pathogenesis or viral clearance.
9.2 Cytokine and antibody profiles during the chronic phase of disease

Levels of TNFα, IL-12p35 and IL-12p40 were upregulated in CBA mice persistently infected with Theiler’s virus when compared to CBA mice which had cleared the virus, therefore suggesting that Th1 cells are active during the chronic phase of Theiler’s virus disease. On the other hand anti-viral isotypes IgG1, IgG2a and IgG2a were all up-regulated in animals with a persistent infection therefore indicating that the anti-viral antibody profile does not agree with the Th1/Th2 hypothesis. Therefore it is likely that both Th1 and Th2 cells are present during the chronic phase of disease and whether one subset is exclusively associated with the demyelinating process still has to be characterised.

A way of determining whether a particular T helper subset is involved in the demyelinating process during chronic infection with Theiler’s virus would be to manipulate the T helper cell response. It has been well documented that the use of drugs (pentoxifylline), parasites (Schistosome eggs) and monoclonal antibodies (B7-1) affect the T helper subsets. The maturation of CD4+ T precursor helper cells is complex and they mature along two separate pathways. Both Th1 and Th2 cells produce different cytokine profiles with IFNγ being produced predominantly by Th1 cells and IL-4, IL-6 and IL-10 are predominantly produced by Th2 cells. The type of T helper cell response being elicited can have important implications in pathology. Upregulation and expansion of T cells of a particular subset is dependant on certain signals. The first signal is the binding of the TcR with the MHC associated antigen, the second signal is provided by costimulatory molecules on the antigen presenting cells. Emerging is the importance of a family of costimulatory molecules called the B7 family. B7-1 (CD80) is exclusively on APCs and is involved in the interaction between the CD28 or CTLA-4 molecules on Th1 cells. B7-2 is predicted to have a similar role as B7-1 except for Th2 cells. Experiments in animals inoculated with EAE and a monoclonal
to B7-1 causes a decrease in Th1 cells and an increase in Th2 cells which results in the prevention of onset of clinical signs. On the other hand, treatment with a monoclonal to B7-2 causes the reverse and results in increased disease severity. Therefore implicating Th1 cells in the pathology of the disease. It would be of great interest to use these monoclonals in Theiler’s virus research to determine the exact role of the Th1 and Th2 cells.

Interestingly, TNFα and IL-12 are up regulated in mice with a chronic persistent infection and therefore during demyelination. TNFα has been implicated in causing demyelination in vitro however IL-12’s direct role in demyelination has not been investigated. Therefore it would be of fascination to further investigate the direct role of these specific cytokines during the demyelinating process. This is now possible through the use of genetically engineered mice which are deficient in the receptors for TNFα and also for IL-12. Nitric oxide has also been implicated in demyelination therefore its role in Thieler’s virus induced demyelination could also be determined through the use of iNOS deficient mice.

9.3 The roles of interferon

Interferons are very important in most if not all viral infections due to their anti-viral properties. The role of IFNα/β was discovered to be in protecting oligodendrocytes (the myelin maintaining and producing cell) from infection, as lack of functional IFNα/β results in extensive viral dissemination through out white matter areas in the brains, and eventually death of the infected animal. IFNγ’s role, although not vital, appears to be essential if virus is to be cleared from the CNS of infected animals. This could be due to IFNγ important functions in innate and specific immunity with its involvement in NK, CD4+ Th1 cell and CD8+ T cell activities. However, the precise role of IFNγ is yet to be discovered. Further characterisation of the immunology of the IFNγ R°/° mice would be instrumental in determining the precise roles of
IFNγ in Theiler’s virus infection. This could include an in depth time course study exploring the expression of Theiler’s virus specific CTLs, T helper cells and antibodies.

9.4 The roles of perforin

The presence of perforin during Theiler’s virus infection is also essential to prevent wide spread dissemination of virus throughout the CNS during the acute phase of disease, probably due to its important role in NK cell activity. The rapid dissemination during the acute phase of Thieler’s virus disease posed an interesting question and that is, what is the role of NK cells during Theiler’s virus infection? The role of NK cells in Theiler’s virus could be examined by infecting NK cell deficient mice and following the course of infection to see whether virus infection was cleared controlled or increased. Perforin mediating killing is only one type of CTL killing. There are several other mechanisms CTLs use to mediate their killing such as Fas and TNFα. The roles of Fas and TNFα could now be examined due to the generation of knock out mice. If this data was obtained then it would be possible to ascertain the exact roles that different types of CTL killing fulfil during Theiler’s virus infection. Perforin is equally important during the chronic phase of disease in eliminating the virus. Virus persists in perforin deficient mice whereas it is cleared in infected wild type mice, illustrating the importance of perforin for viral clearance. Although virus persists in perforin deficient mice levels of demyelination were not looked at, therefore it would be of interest to determine how extensive the demyelination was in perforin deficient animals, and to establish whether perforin was ultimately required in the demyelination process.
9.5 GDVII infection

GDVII causes a fulminant encephalitis in mice of all genetic backgrounds. The GDVII strain of virus disseminates quickly throughout the CNS infecting predominantly neurons of the grey matter. Levels of infectious virus increased in parallel with levels of TNFα, TNFβ, IL-2, IL-12p40 and IL-6 in the brains of animals infected with GDVII. Interesting cells undergoing programmed cell death correlated with the increase in levels of infectious virus and pro-inflammatory cytokines.

9.6 Theiler’s virus infection in neonates

Infection of neonatal mice with Theiler’s virus resulted in widespread dissemination throughout the CNS. Virus levels correlated with levels of TNFα, IL-1α, IL-6, TNFβ. Increases in the levels of TNFα were the most different from the PBS inoculated control group. It has previously been suggested that high levels of TNFα are associated with a shock phenomenon which results in death of animals. Therefore hypothetically mortality in neonates may be due to the fact that they have very high levels of TNFα and are therefore are dying from TNFα shock.

TNFα is massively up regulated in neonatal mice infected with BeAn and adults infected with GDVII. This massive up regulation in TNFα correlates with massive cell death which eventually leads to death of the animal. The implication of TNFα in septic shock have been described previously. However its implications in Theiler’s virus infection has not been noted. It would be of interest to determine the levels of steroid in the serum of neonatal animals infected with BeAn and adults infected with GDVII because these are also indicators of septic shock and would further substantiate the evidence that these animals are dying form septic shock. The importance of TNFα in inducing cell death in neonatal animals infected with BeAn and in adults
infected with GDVII could be further investigated by inoculating TNFα receptor deficient mice. If the neonatal mice recovered for BeAn infection and adults recovered from GDVII infection that would then decipher whether TNFα was the essential component for triggering cell death which gradually leads to death of the animal.
Figure 8.1  Mortality levels in neonatal mice infected with TMEV (BeAn). Neonatal mice (P7) were inoculated with $10^4$ PFU of TMEV (BeAn) i.c., and observed daily. The x-axis represents day post infection and the y-axis represents the number of living animals.
Figure 8.2  Autoradiographic images of BeAn sequences in a neonatal mouse brain on post infection day 7. Neonatal mice brains which had been inoculated with Theiler’s virus were snap frozen on post infection day 7, and sagital sections were subsequently cut and ISH performed using a $^{35}$S labelled riboprobe, which has been described previously in the materials and methods.

Figure 8.3  Represents photomicrographs illustrating the histopathology of BeAn infection in an neonatal mouse brain. Neonatal mice were inoculated at 7 days of age, and animals were sampled and at 7 days post infection. Viral positivity was determined by ISH using a $^{35}$S labelled riboprobe which has been described previously in the materials and methods, tissues were counterstained with hemotoxylin and eosin. The photomicrograph shows the widespread infection with perivascular and parenchymal mononuclear cell infiltration.
Figure 8.4 Levels of mRNA transcripts detected from the brains of neonatal mice infected with the BeAn strain of Theiler's virus. Brain RNA samples were isolated from days 2, 4 and 7 post infection, from neonatal mice which had been inoculated i.c. with $10^4$ PFU of TMEV (BeAn), when they were seven days old. mRNA transcripts were detected by RNase Protection Assay as described in the materials and methods. Transcripts were visualised by autoradiography and quantitated by densitometry. Levels of various transcripts were isolated from the brains of neonatal (●) and uninfected control neonatal (○) mice. The x and y axis represent day post infection and intensity, respectively. The dotted line represents the average level of transcript, and the solid line indicates the control level of that particular cytokine. A) TNFβ B) TNFα C) IL-4 D) IL-6 and E) IL-1α.
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Appendix 1
(reagents listed in alphabetical order)

**Denhardt’s Solution (50X)**

Ficoll 5g
Polyvinylpyridoline 5g
BSA 5g

Dissolve the above in distilled water and make up to 500 ml.

**ELISA Coating Buffer**

\[
\begin{align*}
\text{Na}_2\text{CO}_3 & \quad 21.1 \text{ g/L pH 9.6} \\
\text{NaHCO}_3 & \quad 16.8 \text{ g/L}
\end{align*}
\]

To prepare 20 ml of carbonated buffer mix 1.6 ml Na\_2CO\_3 with 3.4 ml NaHCO\_3 and make up to 20 ml with distilled water.

**Growth Medium (BHK cells)**

Tryptose phosphate broth 50 ml (10%)
New born Calf serum 50 ml (10%)
Add above to Glasgow's modified Eagles medium (GMEM) and mix thoroughly. Store at 4 ̊C.

**Hybridization Buffer**

The following is prepared per 25 slides (allowing 25 ul/slide).

\[
\begin{align*}
\text{Deonised Formamide} & \quad 400 \mu\text{l} \quad (50\%) \\
\text{ISH Salts (20x)} & \quad 200 \mu\text{l} \quad (50\%) \\
\text{Denhardt’s (50%)} & \quad 80 \mu\text{l} \quad (5\%) \\
\text{Dextran sulphate (50%)} & \quad 80 \mu\text{l} \quad (5\%) \\
\text{ssDNA (10.5mg/ml)} & \quad 20\mu\text{l} \quad (263 \mu\text{g/ml}) \\
\text{tRNA (10 mg/ml)} & \quad 20\mu\text{l} \quad (250 \mu\text{g/ml}) \\
\text{Heparin (2000 U/ml)} & \quad 8 \mu\text{l} \quad (20 \text{ U/ml}) \\
10\% \text{ SDS} & \quad 8 \mu\text{l} \quad (0.1\%) \\
\end{align*}
\]

Prepare on use of day.

**Phosphate buffered formal saline (4%)**

\[
\begin{align*}
\text{NaCl} & \quad 8.75\text{g} \\
\text{Na}_2\text{HPO}_4\cdot\text{H}_2\text{O} & \quad 4\text{g} \\
\text{NaH}_2\text{PO}_4 \text{(anhyd)} & \quad 6.5\text{g}
\end{align*}
\]
Make up to 1 L with distilled water, pH to pH 6.7 with HCl and autoclave. Usually diluted 1/10 prior use.

Prehybridization Buffer

<table>
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<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
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<tr>
<td>Deonised Formamide</td>
<td>400 µl</td>
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<tr>
<td>ISH Salts (20x)</td>
<td>200 µl</td>
<td>(50%)</td>
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<tr>
<td>Denhardt’s (50%)</td>
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<td>(5X)</td>
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<td>ssDNA (10.5mg/ml)</td>
<td>20 µl</td>
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<tr>
<td>tRNA (10 mg/ml)</td>
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<tr>
<td>Heparin (2000 U/ml)</td>
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<tr>
<td>10% SDS</td>
<td>8 µl</td>
<td>(0.1%)</td>
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<tr>
<td>Sterile distilled water</td>
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