Role of Brain Microglial Cells in Neuropathogenesis of Maedi-Visna Virus Infection

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Declaration

I hereby declare that the dissertation entitled "Role of Brain Microglial Cells in Neuropathogenesis of Maedi-Visna Virus infection" 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.

Bahram Ebrahimi

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Abbreviations

AIDS	acquired immunodeficiency syndrome
ALSV	avian leukosis and sarcomaviruses
AP	alkaline phosphotase
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
β-NacGal	beta-N-acetyl-D-galactose
CA	capsid
CNS	central nervous system
CAEV	caprine arthritis-encephalitis virus
CD	cluster of differentiation
CMI	cell-mediated immunity
CPE	cytopathic effect
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocytes
DAB	3,3'-diaminobenzidine tetrahydrochloride
DIG	digoxingenin
DIV	days in vitro
DLS	dimer linkage site
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EAE	experimental allergic encephalitis
EBV	Epstein-Barr virus
ECM	extracellular matrix
E. Coli	Escherichia coli
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
EIAV	equine infectious anaemia virus
Env	envelop
EtBr	ethidium bromide
FCS	foetal calf serum
FGF	fibroblast growth factor
FIV	feline immunodeficiency virus
FITC	fluorescein isothiocyanate
Gag	group-associated antigens
GalC	galactocererobroside
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte macrophage-colony stimulating factor
gp	glycoprotein
HBBS	Hank's buffered salt solution
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HTLV	human T-cell lymphotrophic virus
ic	intracerebral

ICA	intracytoplasmic type A
ICAM	intercellular adhesion molecule
ICE	interleukin converting enzyme
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IN	integrase
LCA	leukocyte common antigen
Kb	kilobase
LCMV	lymphocytic choriomeningitis virus
LFA	late function antigen
LME	L-methyl ester
LN	lymph node
LTR	long terminal repeats
MAb	monoclonal antibody
μg	microgram
μl	microlitre
M	molar
mM	millimolar
μM	micromolar
ml	millilitre
MA	matrix
MBP	myelin basic protein
MGC	multinucleated giant cells
mRNA	messenger RNA
MHC	major histocompatibility complex
MIP	monocyte inflammatory protein
MMTV	mouse mammary tumour virus
MS	multiple sclerosis
M-tropic	macrophage tropic
MVV	maedi-visna virus
MW	molecular weight
MuLV	murine leukaemia viruses
N/A	not applicable
NBT	nitroblue tetrazolium
NC	nucleoprotein complex
NES	nuclear export signal
NGF	nerve growth factor
NLS	nuclear localisation signal
NRE	negative response element
NSI	non-syncytium inducing
ORF	open reading frame
Ova	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PB	primer binding
PBL	peripheral blood lymphocytes
PDGF	platelet-derived growth factor
pH	potential hydrogen
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PCD	programmed cell death
PCR	polymerase chain reaction
PDL	poly-D-lysine
PDO	poly-D-ornithine
PFA	para-formaldehyde
pi	post-infection
PKC	protein kinase C
PLP	proteolipid protein
PNK	polynucleotide kinase
PNS	peripheral nervous system
POL	polymersae
PR	protease
pr	precursor
PWM	pokeweed mitogen
RANTES	regulated upon activation normal T cell expressed and secreted
REV	regulation of expression of viral proteins
RNA	ribonucleic acid
RNase	ribonuclease
RPM	revolutions per minute
RRE	rev response element
RT	reverse transcriptase
SCP	sheep choroid plexus
SDW	sterile distilled water
SFV	semliki forest virus
SI	syncytium inducing
SIV	simian immunodeficiency virus
SPA	sheep pulmonary adenomatosis
SRV	simian retrovirus
SSC	standard saline citrate
SU	surface
Ta	annealing temperature
Tat	transactivator of transcription
TAR	transactivation response element
TCA	tri-carboxylic acid
TF	transcription factor
TGF	transforming growth factor
Th	T lymphocyte helper
TM	transmembrane
Tm	melting temperature
T-tropic	T lymphocyte tropic
tRNA	transfer RNA
Tris	2-amino-2- (hydroxymethyl)-1,3-propandiol
TUNEL	terminal deoxynucleotide transferase-mediated UTP nick end labelling
U3	untranslated 3' region
U5	untranslated 5' region
VCAM	vascular cell adhesion molecule
VIF	virus infectivity factor

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ABSTRACT OF THESIS

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A prominent feature of lentivirus infections is involvement of the central nervous system (CNS).The ovine Maedi-Visna Virus (MVV) is the prototypic lentivirus, which like all other known lentiviruses, results in infection of the CNS with protracted neuropathology.The complications in deciphering the mechanisms of lentivirus-induced neuropathology arise because of discordance between extent of pathological changes and scarcity of virus antigen-positive cells in the CNS.These and other observations have lent support to the indirect bystander damage hypothesis, whereby released soluble factors of host and/or viral origin may result in extensive tissue damage.Brain macrophages/microglia are frequently viral antigen-positive in lentivirus-infected neural tissues.Primary ovine glial cultures were set up to study the cellular events that may contribute to MVV-initiated neuropathology.The ovine glial cells showed both morphological as well as phenotypic similarities to glial cells from other species. The role of microglial cells in visna neuropathology was studied further by use of highly enriched (purity > 98%) primary cultures.Ovine microglial cells showed extensive phenotypic similarities with cells of the monocyte/macrophage lineage.However, they also exhibited distinct morphological characteristics.Further, ovine microglial cultures could be productively infected with MVV *in vitro*.This was demonstrated by a combination of immunocytochemical and molecular assays.Assessment of ovine cytokine production in MVV-infected microglial cells *in vitro* demonstrated significant increases in the message RNA levels of pro-inflammatory cytokines tumour necrosis factor-α and interleukin-6.Intrathecal infection experiments carried out in immunosuppressed animals had previously demonstrated an association between early visna lesions and the virus dose. Severe combined immunodeficient (SCID) mice were used to assess whether MVV-infected microglial cells could contribute to neuropathological changes commonly associated with lentivirus infections and independent of specific cellular immune responses. Intrastriatal injection of MVV-infected ovine microglial cells into SCID mice resulted in significantly increased acute gliosis *in vivo*.The neurotoxicity of MVV-infected microglial cells in visna neuropathology was also demonstrated by *in vitro* cultures of murine neuronal cells.The supernatants from ovine microglial cells infected with MVV *in vitro* contained factors, which resulted in both morphological changes and loss of murine trigeminal cultures. Similar observations were made with a synthetic peptide homologue encoded by the MVV regulatory protein Tat.The findings in this thesis demonstrate that infection of ovine microglial cells with MVV can result in release of soluble factors, which may contribute to pathological changes associated with lentiviral infection of the CNS.

Contents

CHAPTER ONE
Introduction

1. Introduction

1.1 Historical background

In 1933, in an attempt to improve the wool industry, the government of Iceland allowed the importation of 20 Karakul sheep, a breed known for its wool quality, from Halle in Germany. After passing the quarantine checks, these imported sheep were distributed to different farms throughout Iceland. By the time the clinical symptoms of an apparently new disease were recognised, the disease had already spread to other farms in the country, culminating in the first epidemic, which occurred between 1939-52. The second epidemic occurred between 1954-65 in the western part of Iceland. The disease was eradicated by the end of 1965 because of a mass slaughter policy adopted by the government of Iceland (Pálsson, 1976).

The clinical symptoms of this new disease were first recognised by Gíslason in 1947, in adult ewes of 3 years or over, and invariably during or immediately after the lambing season. The earliest clinical sign was dyspnoea (shortness of breath or *maedi* in Icelandic). Other lung diseases, such as sheep pulmonary adenomatosis (SPA), or Jaagsiekte, and parasitic pneumonia complicated the early diagnosis of this new disease. However, unlike SPA or parasitic pneumonia, maedi-affected sheep displayed neither any noticeable nasal discharges, nor coughing (Gíslason, 1964). Affected animals succumbed to the inevitable fatal outcome of the disease, some 3-8 months after showing the first clinical symptoms. Again, this was in contrast to chronic diseases such as parasitic pneumonia (Sigurdsson *et al.*, 1952).

The rapid spread of the new disease was suspected to be due to the husbandry practices in Iceland, where large numbers of animals from different farms were housed in close proximity during winter months. This was followed by free hill grazing during summer months, where animals from different flocks were allowed to mix with each other. It was also realised that the spread occurred via nasal secretions and vertically from ewes to lambs. Retrospectively, lack of an epidemic in Germany was attributed to the different animal husbandry practised in that country (Pálsson, 1974).

After a few years, when maedi had already become prevalent on farms scattered around the country, a new clinical disease of the central nervous system (CNS) was diagnosed. This wasting disease (or *visna* in Icelandic) always occurred as a complication of maedi. Clinical visna, like maedi, was a slow disease in its onset and

progression. Both maedi- and visna-affected animals invariably followed a protracted course of slow progression to fulminant disease, resulting in inevitable death. This slowness of disease progression and rapid end-stage differed from other chronic diseases, for example paratuberculosis (Sigurdsson, 1954).

The epidemiological studies by Gíslason had already indicated that the new disease was caused by a contagious agent and that the minimum incubation time was approximately 2 to 3 years. Experimentally, maedi and visna could be transmitted to healthy animals by inoculation with infected tissues such as brain, spinal cord, lung and spleen. These and other similar observations, taken together, led to the understanding that the causative agent (s) was a virus. In 1954, Sigurdsson used the term lentivirus [from Latin *lentus* slow], to aptly describe the clinical course of maedi-visna.

Earlier work had shown that tissue culture passaged agent (s), isolated from natural cases of maedi and visna was transmissible experimentally. There was also some compelling evidence that both maedi and visna may share a common causative agent. This was based on observations that inoculations of sheep with brain homogenates from visna cases also resulted in clinical maedi and vice versa (Sigurdsson *et al.*, 1960; Sigurdadóttir and Thormar, 1964). This was further supported by the very similar cytopathic effects (CPE) of different maedi and visna isolates under tissue culture conditions (Thormar and Helgadóttir, 1965). However, it was not until 1967 (Gudnadóttier and Pálsson) that a single viral agent, which was filterable, was isolated. This newly isolated virus resulted in clinical maedi-visna when used in experimental infections of sheep. This infective viral agent was named the **M**aedi-**V**isna **V**irus (MVV).

1.2 Global distribution of MVV

Clinical symptoms similar to maedi-visna have been reported in other countries under different regional nomenclature. These include Graaf-Reinet in South Africa, Zwoegerziekte in Holland, La bouhite in France, ovine/Montana progressive pneumonia and ovine lentivirus in USA. Other pathological manifestation associated with MVV infection include non-suppurative arthritis and indurating interstitial mastitis (Cutlip *et al.*, 1985; Anderson *et al.*, 1985; van der Molen *et al.*, 1985).

The same causative agent has also been recognised in other countries worldwide. These were originally based on clinical symptoms and serological analyses, and

subsequently confirmed by sequence data (De Boer *et al.*, 1970; Cutlip and Laird, 1976; Seimen *et al.*, 1985; Sonigo *et al.*, 1985; Braun *et al.*, 1987; Watt *et al.*, 1990; Querat *et al.*, 1990; Staskus *et al.*, 1991; Sargan *et al.*, 1991 Giangaspero *et al.*, 1993).

1.3 Classification of lentiviruses

The classification of viruses used in this thesis are according to the recommendations put forward by the International Committee on Taxonomy of Viruses (ICTV) (Classification and Nomenclature of Viruses, Fifth report, 1991).

The ovine MVV is a *lentivirus* genus of Retroviridae family of viruses (Table 1.1). Retroviruses are a unique family of viruses because they contain an RNA dependent DNA polymerase (or reverse transcriptase) by which the positive single-stranded RNA genome codes for a double-stranded viral DNA (Baltimore, 1970; Temin and Mizutani, 1970). Members of the Retroviridae family of viruses share a common genome arrangement, which is the basis of their inclusion in this family: structural genes *gag* (**g**roup-**a**ssociated anti**g**ens), *pol* (**pol**ymerase), and *env* (**env**elope) are arranged in a 5′ to 3′ orientation. These structural genes are flanked by **L**ong **T**erminal **R**epeats (LTR), which contain elements for control of viral gene expression (Fig.1.1). In addition to structural genes, complex retroviruses, which include lentiviruses also have small **O**pen **R**eading **F**rames (ORF), which code for regulatory/auxiliary proteins (reviewed by Cullen, 1992).

Table 1.1 Classification of Retroviruses

Classifications are according to ICTV (1991). Abbreviations used are as follows: Human Immunodeficiency Viruses type-1/2 (HIV-1/2), Simian Immunodeficiency Virus (SIV), Caprine Arthritis-Encephalitis Virus (CAEV), Equine Infectious Anaemia Virus (EIAV), Feline Immunodeficiency Virus (FIV), and Bovine Immunodeficiency Virus (BIV).

Genus	Type species (other members)	Subgenera	Species
Mammalian type B Oncoviruses	Mouse Mammary Tumour Viruses		
MLV-related Viruses (Mammalian type C Retrovirus Group)	Murine Leukaemia Virus ,MuLV	Mammalian type C viruse	Murine sarcoma and leukaemia viruses Feline sarcoma and leukaemia viruses Gibbon ape leukaemia virus Guinea pig type C virus Porcine type C virus Woolly monkey sarcoma virus
		Reticuloendotheliosis viruses	Avian reticuloendotheliosis virus
		Reptilian type C viruses	Viper retrovirus
Type D Retrovirus Group	Mason-Pfizer Monkey Virus (Squirrel monkey retrovirus) (Langur virus, PO-1-Lu)		
Avian Type C Retrovirus Group	Avian Leukosis Virus (Avian sarcoma and leukaemia viruses,ASLV)		
Spumavirus Group (Foamy Virus Group)	Human Foamy Virus (Simian foamy virus) (Feline syncytia virus) (Bovine syncytia virus)		
HTLV-BLV Group	Human T-Cell Lymphotropic Virus Type 1;HTLV-1) (Human T-cell lymphotropic virus type 2; HTLV-2) (Simian T-cell lymphotropic virus, STLV) (Bovine leukaemia virus, BLV)		
Lentivirus Group	Human Immunodeficiency Virus	Primate immunodeficiency viruses	HIV-1/HIV-2 SIV
		Ovine/Caprine lentiviruses	MVV/CAEV
		Equine lentiviruses	EIAV
		Feline lentiviruses	FIV
		Bovine lentiviruses	BIV

1.4 Molecular biology of lentiviruses

1.4.1 Reverse transcription

The viral enzyme, reverse transcriptase (RT), is coded by the *pol* gene in retroviruses (Verma, 1977; Goff, 1990). Retroviruses use different species of cellular transfer RNA (tRNA) molecules packaged into the mature virion as primers; MVV utilises $tRNA^{Lys1,2}$ (Sonigo *et al.*, 1985; Coffin, 1990). Moreover, an essential requirement for RT-mediated reactions is the presence of divalent cations. Retroviral RTs utilise either Mg^{2+} (e.g., by MMTV, SRV, lentiviruses and spumaviruses), or Mn^{2+} (e.g., by ASLV and MuLV) (Nanduri and Modak, 1990; Tirumalai and Modak, 1990).

Retroviruses have a high mutation rate, which is partly due to lack of an editing activity by RT (Coffin, 1990; Katz and Skalka, 1990). This high error rate may have played an important part in the evolution of retroviruses. This is one of the mechanisms used by this family of viruses to evade the host immune system. It is estimated that the error rate may be as high as 1 in 1700-4000 in the case of HIV-1 (Preston *et al.*, 1988; Roberst *et al.*, 1988). Similar high mutation rates have been observed with CAEV infections *in vivo* (Turelli *et al.*, 1996). This high error rate is believed to result in generation of quasi-species of viruses. This emerging virus pool may differ greatly from the original pool of viruses at the time of infection (Payne *et al.*, 1987).

1.4.2 RNase-H ORF

The *pol* gene of retroviruses also codes for a protein with RNase-H activity. RNase-H degrades RNA in an RNA-DNA hybrid. This enzyme is involved in the removal of viral RNA during proviral strand synthesis (Moelling *et al.*, 1971; Goff, 1990)

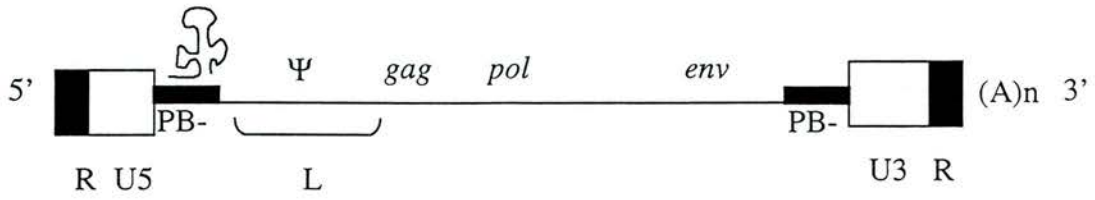
1.4.3 Proviral DNA synthesis

Reverse transcription in lentiviruses results in synthesis of a duplex viral DNA from a diploid RNA genome (Fig.1.1). Shortly after the receptor-mediated entry of virion into permissive cells, reverse transcription of viral genome takes place in the uncoated viral nucleoprotein complex (NC) within the cytoplasm. This NC is referred to as the pre-integration complex. In HIV-1, reverse transcription of viral genome can be detected within two hours of infection, and it is associated with the pre-integration complex in the cytoplasm (Farnet and Haseltine; 1991 Munis *et al.*, 1992).

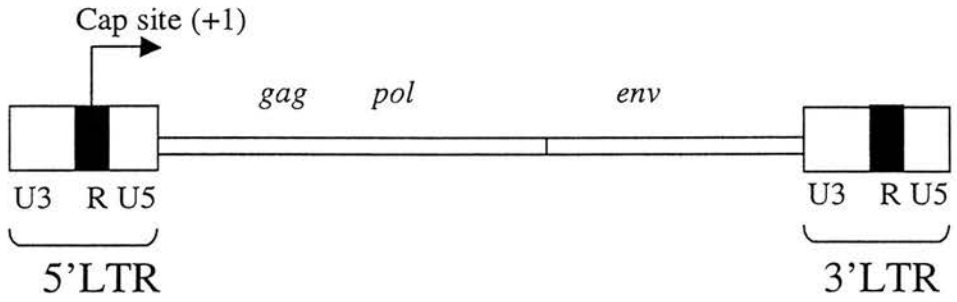
Fig.1.1 Genome organisation of Retroviruses

The genome of lentiviruses consists of two identical full-length RNA transcripts that are linked by non-covalent bonds. The LTR contains the promoter sites that drive viral transcription. The untranslated leader (L) sequence contains the dimer linkage site (DLS). The L sequence also contains the packaging sequence (Ψ). A is representative of an RNA genome, with cellular tRNA bound to the primer binding site (PB). B represents the proviral form of the genome, with cap present on all transcripts. C represents the spliced proviral genome of MVV.

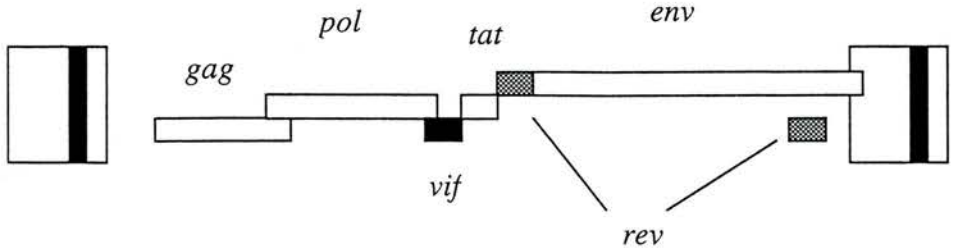
A


5'
Ψ
gag
pol
env
(A)n 3'
PB-
PB-
R U5
L
U3 R

B


Cap site (+1)
gag
pol
env
U3 R U5
U3 R U5
5'LTR
3'LTR

C


pol
tat
env
gag
vif
rev

Earlier experiments had shown that *in vitro* infection of sheep choroid plexus (SCP) cells with MVV resulted in detectable amounts of duplex proviral DNA. The newly synthesised proviral DNA may exist in different forms; i.e., both circular (open circle or type I) and linear. The newly synthesised proviral DNA (within the pre-integration complex) is transported into the nucleus, where it may be integrated into the host-cell genome. Presence of a **n**uclear **l**ocalisation **s**ignal (NLS) in the form of a nucleophilic motif on the matrix (MA) protein of HIV-1 directs the transport of pre-integration complex into the nucleus of infected cell (Harris *et al.*, 1981; Lee and Coffin, 1990).

1.4.4 Integration of proviral DNA into host-cell genome

Retroviral integration depends on the *pol* gene-derived protein, integrase (IN). This protein is released from the Gag-Pol polyprotein precursor by a proteolytic cleavage (Varmus and Brown, 1989; Brown, 1990; Goff, 1992; Whitcomb and Hughes, 1992). Biochemical analyses of sheep choroid plexus (SCP) infected *in vitro* had shown that the majority of MVV DNA existed as free and linear duplex DNA. Circular DNA constituted only 0.1-0.3 percentage of the viral DNA. Both forms were found predominantly in the nucleus of infected cells in the early phase of infection (five hours post-infection) (Harris *et al.*, 1981). There is some evidence from HIV-1 that non-integrated free DNA may be used as a template for virus gene expression, and may contribute to HIV CNS dementia (Teo *et al.*, 1997).

HIV-1 can integrate into host-cell genome. Moreover, IN mutants of both HIV-1 and CAEV are not infectious, indicating the importance of integration in life cycles of these viruses (Wiskerchen and Muesing, 1995). Similarly, MVV infection of fibroblasts *in vitro* may result in integration of virus into host-cell genome. However, the frequency of this event is very low. Recently, it has been shown that only one out of 200 infected cells was positive for integrated provirus by chromosomal *in situ* hybridisation technique (C. Woodall, per. comm.). This is in agreement with previous observations that many copies of viral DNA (100-200) per cell are found as free, non-integrated linear DNA in the nucleus of infected cells (Harris *et al.*, 1981).

1.4.5 Role of viral LTR in virus gene expression

Transcription of proviral genes in infected cells is under the control of flanking LTRs. The lentiviral 5′ LTR is involved in initiation of transcription. It contains core promoter elements CCAAT (CAT box) and TAATA (TATA box), which bind the cellular TFIID complex: a pre-requisite for initiation of basal transcription by cellular

RNA pol II. U3 also contains **n**egative **r**egulatory **e**lements (NRE), which down regulate transcription (Rosen *et al.*, 1985; Hirano and Wong, 1988; Flanagan *et al.*, 1989; 1992). Functional NRE elements have also been found in the LTR of MVV isolates (Hess *et al.*, 1985; Sargan *et al.*, 1991).

In MVV, which shows a more restricted cellular tropism compared to human and primate lentiviruses, the LTR appears less complex (Gabuzda *et al.*, 1989). The LTR of the prototypic laboratory strain of MVV, the Icelandic K1514, contains two 43-bp direct repeats with multiple non-consensus copies of the AP-1 binding site. However, consensus AP-1 (TGAGTCA) and AP-4 (GCAGCTG) binding sites are located upstream from the TATA box in K1514 LTR (Gabuzda *et al.*, 1989; Hess *et al.*, 1989, Gdovin and Clements, 1992). The proximal AP-1 site in K1514 LTR is functional. This site also plays a crucial role in mediating viral activation in macrophages (Gabuzda *et al.*, 1989).

Other, non-LTR sequences may also play a role in regulation of viral sequences. Transient transcription assays have identified a downstream region beyond the transcription start site in a North American MVV strain, OLV-CU1, required for virus-specific *trans*-activation (Campbell and Avery, 1996).

1.4.6 Temporal regulation of transcription

Infection experiments carried out *in vitro* with MVV result in a lytic cycle of approximately three days (Brahic *et al.*, 1977). The virus life cycle in fibroblastoid cells such skin fibroblasts and SCP cells is approximately 20 hours. Molecular analysis of these *in vitro* infections with MVV indicated an ordered pattern of transcription, i.e., an early phase (19-24 hours post-infection) followed by a late phase (40-72 hours post-infection). By completion of the lytic cycle, the number of MVV viral genomic RNA copies increases from 100-200 copies (24 hours post-infection), to more than 1000 copies (72 hours post-infection) (Brahic *et al.*, 1977; Haase *et al.*, 1982).

In the early phase of transcription, small, multiply spliced transcripts (1.2- and 1.6 kb or 1.4- and 1.7 kb, depending on the study) predominate in the cytoplasm with very little viral RNA detected in the nucleus. This is followed by a preponderance of unspliced genomic (9.4 kb) and singly spliced subgenomic transcripts (4.3- and 3.7 kb, and 4.9- 4.3- 4-kb, depending on study) in the late phase of transcription. In this late phase, the complete range of spliced and unspliced transcripts are found both in

Tat (formerly ORF S in MVV and *tat* III in HIV-1) is coded by a multiply spliced transcript in MVV (1.5- to 2-kb, depending on study), and predominates in the early phase of infection *in vitro*. MVV Tat is encoded from a single exon, 5′ to the *env* gene, and with a molecular weight of 10 kDa (Vigne *et al.*, 1987; Davis and Clements, 1989).

Tat influences the expression of all viral transcripts by increasing the steady-state levels of viral mRNA (Hess *et al.*, 1989). In addition to its mRNA kinetics, Tat like Rev, is also a modular protein. MVV Tat contains functional domains, with minimal similarity to HIV-1 Tat (Carroll *et al.*, 1991; Jones and Peterlin, 1994; Fig. 1.3).

In human lentiviruses (HIV-1 and HIV-2), SIV, EIAV, and BIV, Tat-directed *trans*-activation of viral genes is mediated via a **trans-activation response** (TAR) element. TAR is an RNA sequence with a stem-loop secondary structure, present in the R region of LTR (reviewed by Gardner *et al.*, 1994). Moreover, MVV genome, like CAEV and FIV, does not encode a TAR element (Carroll *et al.*, 1991; Sparger *et al.*, 1992).

There are conflicting reports with regard to the actual role of ungulate lentiviral Tat proteins in virus infection. In one study, *tat* deletion-mutants of CAEV showed greater than 1000-fold reduction in virus titres (in fibroblastoid cells), compared to the wild-type control virus. This prompted the authors to draw the conclusion that CAEV Tat was required for efficient virus replication (Saltarelli *et al.*, 1993). A second study attempted to delineate the role of CAEV Tat employing a similar genetic approach. Deletion mutants of *tat* had no effect on CAEV replication either *in vitro* (in both fibroblastoid cells and primary macrophages) or *in vivo* (Harmache *et al.*, 1995). However, a common finding in both studies was that Tat showed only a moderate *trans*-activation in CAT assays. This moderate *trans*-activation compared to the high *trans*-activation by HIV-1 Tat (>1500-fold) has been cited as the basis for suggestions that Tat is dispensable in MVV and CAEV infections (Kolson *et al.*, 1994)

Harmache and co-workers (1995) showed that partial and complete mutation of Tat ORF appeared to confer no inhibitory phenotype upon virus infection, both *in vitro* and *in vivo*. Moreover, animals infected with these constructs sero-converted, indicating that basal promoter activity was sufficient for virus replication. However, it is not clear whether *tat* mutations may affect the outcome of the clinical disease.

1.4.9 The Vif ORF

MVV *vif* (formerly ORF Q) is a homologue of HIV-1 *vif* transcript, and part of the *env* gene transcribed as a 4.8 kb message, late in transcription (Rabson *et al.*, 1985; Vigne *et al.*, 1987). MVV *vif* encodes a protein with a molecular weight of 29 kDa, which can be detected in the cytoplasm of MVV-infected cells, and was not associated with cell-free virions (Audoly *et al.*, 1992).

Deletion mutant studies on the CAEV *vif* indicate that Vif is essential for efficient virus infection *in vitro* (Harmache *et al.*, 1995). Moreover, it was shown that this defect was not at the level of reverse transcription or transcription, but rather at the late stages of virion assembly and release

Deletion mutants of CAEV *vif* were shown to result in severely impaired virus replication, critical in establishing a persistent infection and pathogenesis. The smallest mutation was near a C-terminal motif. Moreover, this motif (Ser-**Leu**-Gln-X-**Leu-Ala**) is highly conserved in all known lentiviral Vif sequences. This motif has an overall hydrophobic profile, and would be expected to interact with the cell membrane. The possible interaction of Vif through its hydrophobic moiety may play a role in virion assembly and budding (Oberste and Gonda, 1992).

There are some functional similarities between Vif proteins of HIV-1 and ungulate lentiviruses. In the absence of Vif, HIV-1 virus infectivity is greatly reduced. However, this reduction appears to be cell-type dependent (Fan and Peden, 1992; Gabuzda *et al.*, 1992; Gabuzda et al., 1994). Furthermore, Vif was shown to be required for efficient uncoating and release of viral RNA in the cytoplasm and also in complete synthesis of viral DNA (Sova and Volsky, 1993; von Schwelder *et al.*, 1993).

1.4.10 Deoxyuridine triphosphotase (dUTPase) ORF

Unlike primate and bovine lentiviruses, dUTPase-related sequences are found in EIAV, CAEV, MVV and FIV. This was based on sequence homologies with dUTPases of *E.coli* and herpesviruses (Sonigo *et al.*, 1985; Stephens *et al.*, 1986; McGeoch, 1990). Moreover, purified preparations from EIAV and FIV infected cells demonstrated dUTPase activity (Elder *et al.*, 1992).

Mutations in the dUTPse ORFs of MVV and CAEV have been reported to result in a delayed replication in caprine macrophages *in vitro* (Turelli *et al.*, 1996). However, a dUTPase mutation of MVV was shown to be dispensable for virus replication in ovine

macrophages *in vitro* and also to the outcome of the clinical disease in experimental infections *in vivo* (Petursson *et al.*, 1998).

Experimental infections of goats with CAEV with wild-type virus, deletion mutants or single-point mutants (DU-G) in the dUTPase ORF, showed no significant differences in the outcome of the clinical disease (Turelli *et al.*, 1997). However, one of THE animals infected with a dUTPase bearing a single base mutation showed a reversion (G to A) to an active dUTPase, suggesting that a functional dUTPase ORF is favoured by the virus. Using similar genetic approaches in EIAV, dUTPase function appears to be necessary for efficient virus replication in equine macrophages *in vitro* (Threadgill *et al.*, 1993). The levels of integrated viral DNA was 2- to 3-fold lower in mutant viruses than in wild-type virus. Moreover, steady-state levels of full-length viral transcripts were reduced by over 100-fold. This was found to be due to misincorporation of dUTP into the proviral genome (Steagall *et al.*, 1995).

Mutational analyses of *dUTPase* ORF indicate that lentiviruses, which encode this gene, prefer a functional protein product. However, lack of a dUTPase activity does not appear to be detrimental to infection/replication of these viruses.

1.4.11 Endonuclease ORF

By analogy with primate lentiviruses, this protease is probably involved in the cleavage of Gag-Pol precursors. Studies from HIV-1 indicate that the endonuclease is released by an autolytic cleavage from Gag-Pol dimers (Navia and McKeever, 1990).

1.5 Structural proteins of MVV

In lentiviruses, structural proteins are encoded by a set of late, singly spliced transcripts. The presence of RRE on these transcripts prevents their multiple splicing. Moreover, these mRNA species are polycistronic. Structural proteins of MVV are *gag*-coded proteins, which are involved in virion assembly and *env*-coded glycoproteins. Env glycoproteins partake in coating of mature virion, budding from the infected cell, and binding to cellular receptor for the next generation of infection (reviewed by Narayan, *et al.*, 1993) (Fig. 1.2).

1.5.1 Gag proteins

The MVV *gag*-coded proteins are translated from a single full-length genomic transcript (9.4 kb) (Vigne *et al.*, 1982, 1987). This transcript is translated into a Gag-Pol precursor protein, $Pr150^{gag\text{-}pol}$, and is post-translationally processed into virion-associated proteins. These are proteases (PR, p10); RT (p68/p61); and endonuclease

(EN, p35). A second *gag*-coded precursor, $Pr55^{gag}$ is also post-transitionally cleaved by the viral protease into protein components that form the mature viral particle. These are: major core protein or capsid (CA, p25); matrix protein (MA, p16); and nucleoprotein, (NC, p14) (Vigne *et al.*, 1982) (Fig. 1.2). Both Gag-Pol and Gag precursors are generated from the same full-length transcript, by a ribosomal (-1) frameshiting mechanism, a feature common to lentiviruses.

1.5.2 Env proteins

In MVV, *env*-coded proteins are translated from singly spliced mRNA molecules (4.3- and 3.7-kb). Unlike *gag*-coded mRNAs, which are translated on free ribosomes, translation of *env*-coded messages takes place on membrane-bound ribosomes. The product of *env*-coded transcripts is a precursor molecule, $Prgp150^{env}$ ($Pr100^{env}$). Similar to other secretory proteins, the MVV $Pr100^{env}$ is guided into the lumen of endoplasmic reticulum by presence of a signal peptide, with a nonpolar sequence, and subsequently removed by an endopeptidase in the lumen of ER. The $Prgp150^{env}$ is transported into the Golgi complex for further glycosylation/oligomerisation. By analogy to HIV-1 Env proteins, MVV $gp150^{env}$ is cleaved, by a cellular endoprotease, into the N-terminal surface (SU) glycoprotein, gp135, and the C-terminal transmembrane (TM) gp45 (Vigne *et al.*, 1982, 1987; Sonigo *et al* 1985). In MVV, the putative receptor-binding gp135 is non-covalently linked to the TM gp46, which has an anchor domain in the cytoplasmic membrane and is involved in cell fusion that follows receptor binding (Doms *et al.*, 1993). In HIV-1, extensive glycosylation (mannose, N-acetylglucosamine, galactose, fructose, and other complex carbohydrates) is important for the proper folding of gp120 and the recognition of CD4 receptor (Domes *et al.*, 1993). Moreover, proteolytic cleavage of HIV-1 $Prgp160^{env}$, the precursor equivalent of MVV gp150, is critical to virion infectivity (McCune *et al.*, 1988).

HIV-1 Pr $gp160^{env}$ assumes either a dimeric or a tetrameric quaternary structure in the lumen of ER. This multimeric structure remains as such throughout its synthesis/modification through the Golgi complex and its assembly on the virion (Weiss *et al.*, 1993). This *in situ* multimerisation of $gp160^{env}$ coincides with the ability to bind CD4 in the lumen of ER, indicating the importance of the quaternary structure in receptor binding. Moreover, disulphide bonds are also formed early in the maturation of $gp160^{env}$ (Earl *et al.*, 1991). This quaternary structure is a vital

determinant in the recognition of viral antigenic epitopes by the host immune system (Border *et al.*, 1994).

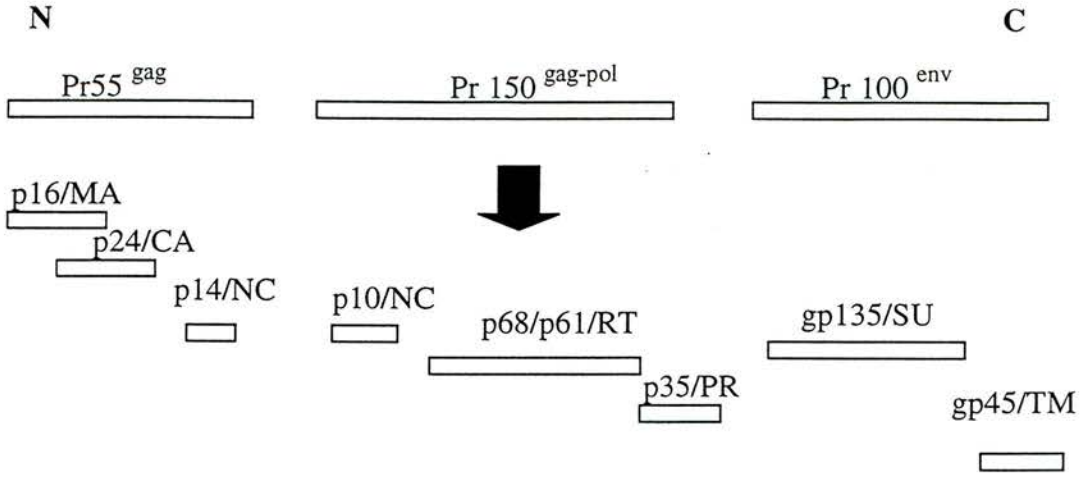
DNA sequence comparison between two closely related, MVV strains, K1514 and its antigenic variant LV1-1 demonstrated a hypervariable cluster (nucleotides 7864 through 7890), spanning 250 bp on either side of TM carboxy terminus (Braun *et al.*, 1987). These hypervariable clusters contribute to the antigenic variations (escape mutants) associated with lentiviruses (Clements *et al.*, 1981; Salinovich *et al.*, 1986). Two closely related MVV 1514 virus strains, whose sequences differed by less than one per cent, and mainly in the *env* region, differed greatly in antibody neutralisation assays (Braun *et al.*, 1987).

Extensive sequence analyses have predicated five hypervariable loops (N-terminal V1 through to C-terminal V5) within the SU subunit of HIV-1 gp120 (Myers *et al.*, 1993). The V3 loop has received the most attention, because of its importance in cell tropism of HIV-1 (Moore and Nara, 1991). Mutations in this region result in noninfectious virus (Grimalia *et al.*, 1992). The region that binds CD4 is between V4 and V5 (Myers *et al.*, 1993). Env is anchored to the cytoplasmic membrane via its non-covalent interaction with gp41, and via a hydrophobic domain in the N-terminal of gp41 (Gallaher *et al.*, 1989). Exchanges of the V3 loop convert a macrophage-tropic (M-tropic) virus (nonacidic amino acids at position 325) to a T-cell tropic virus (T-tropic) (acidic amino acids or alanine residues at position 325) (Milich *et al.*, 1993; Schulz *et al.*, 1993). Moreover, basic amino acids and hydrophobic amino acids in positions 311 and 325 of V3 confer syncyctium producing (SI) and nonsyncytium inducing (NSI) phenotypes respectively (DeJong *et al.*, 1992).

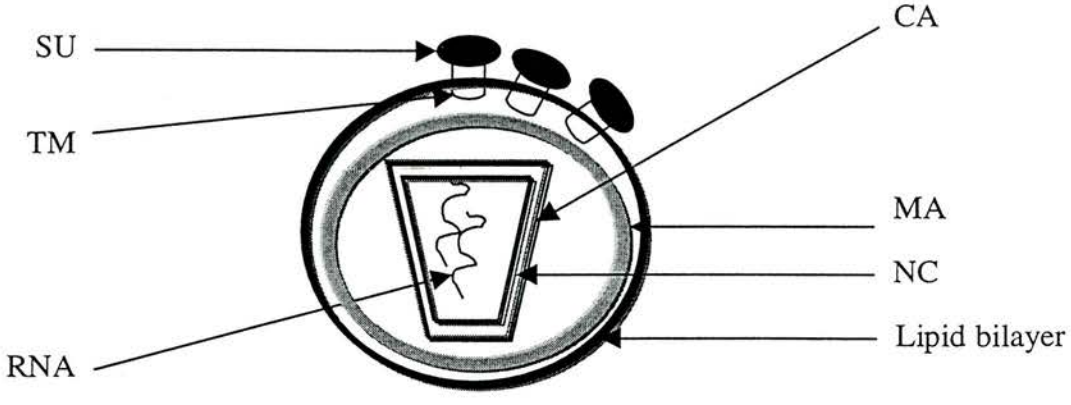
Fig. 1.2 Protein processing and virion assembly

Processing of MVV precursor proteins based on the positioning of splice donor and splice acceptor sites (A). The arrangement of a prototypic lentivirus virion is depicted in diagram B. The research on HIV-1 has shown that the gp120-derived membrane spikes are composed of oligomers rather than monomers. By analogy with HIV-1, the MVV virion core is believed to contain the diploid RNA genome, PR, RT and IN. (adapted from Narayan *et al.*, 1993).

A


N
C
Pr55 gag
Pr 150 gag-pol
Pr 100 env
p16/MA
p24/CA
p14/NC
p10/NC
p68/p61/RT
p35/PR
gp135/SU
gp45/TM

B


SU
TM
RNA
CA
MA
NC
Lipid bilayer

1.6 Cellular tropism

Cells of the monocyte/macrophage lineage are the major targets for infection and replication of lentiviruses *in vivo* (Narayan *et al.*, 1982; Hirsch *et al.*, 1991; Onuma *et al.*, 1992; Sellon *et al.*, 1992; Toyosaki *et al.*, 1993; Pederson *et al.*, 1993). The EIAV viral antigens were detected in the Kupffer cells in the liver of horses with acute EIAV infection. This was the very first report of *in vivo* infection of macrophages by a lentivirus (McGuire *et al.*, 1971).

Lentiviruses can be divided into two groups based on their cellular tropism *in vivo*. The first group includes MVV, CAEV and EIAV. These viruses appear to productively infect predominantly cells of monocyte/macrophage lineage (Narayan and Clements, 1989). The second group, which includes HIV-1, HIV-2, SIV, FIV and BIV, productively infect cells of lymphocyte lineage as well as cells of monocyte/macrophage lineage (Daniel *et al.*, 1985; Kestler *et al.*, 1990; Torten *et al.*, 1991).

Co-infection with CAEV and MVV has indicated that cells of ovine and caprine origin can sustain the productive infection of these two viruses concomitantly, both *in vitro* and *in vivo* (Jolly and Narayan, 1989). These two viruses are closely related, both antigenitically and based on their amino acid sequences. This molecular relatedness can be between 75% in the *gag-pol* genes and 80 % in the case of *env* gene (Saltarelli *et al.*, 1990). However, infection of ovine/caprine cells with these two virus groups was shown to be both group-specific and saturable (Jolly and Narayan, 1989).

MVV specific receptor (s) is yet to be fully characterised. However, one report has shown an association between the putative receptor and the **m**ajor **h**istocompatibilty **c**omplex (MHC) class II antigens (Dalziel *et al*, 1991). A second report has identified a 50-kDa protein that may either associate with and/or be part of the cellular receptor for MVV entry into the cells (Crane *et al.*, 1991).

In early asymptomatic HIV-1 infection, slow replicating non-syncytium-inducing (NSI) M-tropic HIV-1 strains predominate (Schuitemaker *et al.*, 1992). During the course of infection, there is a shift in virus strains with a preferential tropism for T-cells (Connor and Ho, 1994; Schuitemaker *et al.*, 1992). In 50% of the infected individuals, the shift to T-tropism is also concomitant with emergence of syncytium-inducing (SI) viral variants, followed by a rapid disease progression (Koot *et al.*,

1993). This preferential tropism was associated with the V3 loop of the gp120 (DeJong *et al.*, 1992). Surprisingly, the amino acid at position 325 of the gp120 which confers M-tropism (non-acidic) or T-tropism (acidic or alanine) also plays a role in whether the variants are SI or NSI (Milich *et al.*, 1993). In one study, all tissues tested for HIV-1 viral sequences were infected with predominantly M-tropic variants, regardless of tissue of origin (Donaldson *et al.*, 1994).

CD4 molecule was shown to be the major receptor for HIV-1 (Dalgleish *et al.*, 1984). However, some contradictory observations with regard to the tropism of HIV-1 for different cell types had indicated that CD4 is not the only cellular receptor mediating infection by HIV-1. For example, transient transfection of CD4 into cells of non-human origin did not render these cells susceptible to HIV. Moreover, consistent and puzzling observations were made in Africa, where groups of high-risk individuals who had been exposed to the HIV virus on numerous occasions were found seronegative. Also some groups of seropositive individuals did not progress to full-blown AIDS for a long time post-exposure and were referred to as "slow progressors" (reviewed by Weiss, 1996).

The first indications that a second receptor was involved came from observations demonstrating that soluble β-chemokines secreted by CD8+ T cells could suppress HIV infectivity (Jansson *et al.*, 1996; Cocchi *et al.*, 1996). This observation was supported by the elevated circulating chemokine levels in exposed but uninfected individuals (Paxton *et al.*, 1996). Secondly, a member of chemokine family, FUSIN (formerly LESTER) was shown to be needed for membrane fusion and entry of T cell-adapted HIV-1 strains (Feng *et al.*, 1996). This was followed by cloning and characterisation of chemokine receptors used by the M-tropic strains of HIV-1 (Deng *et al.*, 1996; Dragic *et al.*, 1996; Alkhatib *et al.*, 1996).

Chemokines are members of the cytokine superfamily. This family consists of some 50 related proteins, ranging from 68 to 120 amino acids (in the mature form). They are divided into three classes based on the cysteine amino acids arrangement within the protein (reviewed by Schall *et al.*, 1994).

1.7 Lentiviral persistence

It is not clear whether persistent infection with lentiviruses results in a latent infection or a chronic infection. According to definitions put forward in Field's Virology (1994) a transient viraemia in the acute phase of a virus infection may be followed by:

- A latent infection established when a virus persists in a non-infectious form with intermittent periods of viral reactivation and shedding. Moreover, latent viruses must cause productive infection of permissive cells, under certain conditions, i.e., they must be responsive to reactivation during the course of infection. The prototypic example of a virus causing a latent infection is the human Epstein-Barr virus (EBV), or;
- A chronic infection established when the immune system substantially reduces viral replication, but cannot clear the infection. Therefore, there is an ongoing, low level of productive infection. Hepatitis B is an example of a chronic infection.

All lentiviral infections result in a protracted period of clinical latency. This had led to the suggestions that this period of clinical latency is a reflection of viral latency. It is now accepted that lentiviral replication takes place at all stages of infection (Michael *et al.*, 1992; Bagnarelli *et al.*, 1992; Piatak *et al.*, 1993; Pantaleo *et al.*, 1993; Emberston *et al.*, 1993). This is because of availability of sensitive techniques such as polymerase chain reaction (PCR).

A consistent finding with *in vivo* infections with MVV is the low levels of cell-associated circulating virus (Pétursson *et al.*, 1976; Griffin *et al.*, 1978). This has been shown to be as low as 10^4 infected cells leaving a cannulated peripheral lymph node over a 20-day period, detected by co-cultivation assays (Bird *et al.*, 1993). Although this number could be higher with a more sensitive technique such as PCR, it is nevertheless indicative of a cell-associated and restricted infection (Brahic *et al.*, 1981 Staskus *et al.*, 1991). In acute MVV infection experiments, very few infected cells are detected in efferent lymph. This low level of infectivity appears to reflect the monocytes/macrophages and dendritic cells (DC) population (between 1 to 8%) in the lymph node (LN) (Blacklaws *et al.*, 1995). Moreover, these observations would suggest that the monocyte/macrophage and DC population in the LN is most likely to be a reservoir of infection *in vivo*.

In lentiviruses, regulatory proteins Rev and Tat determine the extent of latency in tissue culture models. A similar pattern of viral transcription was found in asymptomatic HIV-1 positive individuals (Seshama *et al.*, 1992). Activation of viral LTR via stimuli such as phorbol esters, *in vitro*, results in a transition from a latent infection to a productive infection (Folks *et al.*, 1987; Pomerantz *et al.*, 1990). However, such a clear dichotomy between latency and productive infection does not appear to exist in lentiviral infections *in vivo*.

Emberston and co-workers (1993), using *in situ* PCR combined with immunostaining techniques, detected large numbers of latently infected CD4+ lymphocytes and macrophages in the lymphoid tissues of apparently asymptomatic HIV+ individuals. However, a small population of lymphocytes (one in 100-400) was positive for both viral DNA and RNA. The infection in monocytes/macrophages was more restricted to proviral DNA. Further, Pantaleo and co-workers (1993) detected cells, which contained both viral DNA and RNA.

Therefore, a consensus has been established with regard to state of lentivirus infection *in vivo* and that is, a greater number of cells are positive for proviral DNA than previously thought. Moreover, a small proportion of these infected cells are transcriptionally active at levels similar to those found in productive infection (Brahic *et al.*, 1981; Staskus *et al.*, 1991; Emberston *et al.*, 1993; Pantaleo *et al.*, 1993). Hence, it has been suggested that a state of "cellular latency" or "restricted latency" exist *in vivo*, based on the criteria that viral regulatory transcripts (*rev/tat*) are detected with minimal productive infection.

1.7.1 Cellular and viral determinants of latency

The host range of viruses is determined by both cellular and viral factors. The restriction imposed by the cellular factors upon the infective agent may operate at different levels. This restriction could be at an early step, for example, lack of appropriate receptor (s) for virus entry. Lack of appropriate molecular machinery, to interact and upregulate viral gene expression may contribute to defective infection. Incompatible intracellular processing pathways necessary for assembly and release of infective progeny may also contribute to a defective infection/latency. Viral determinants too could play a role in latency/productive infection. These can include viral sequences outside the *env* region, for example within the LTR, or the site of integrated provirus.

An example of cellular restriction placed upon lentiviral infection is demonstrated by immature precursor cells. Precursor bone marrow cells show a reduced ability to support a productive infection, compared to cultured macrophages (Narayan *et al.*, 1982; Gendelman *et al.*, 1985,1986). This block in MVV transcription was linked to the cellular maturation of monocyte into macrophages (Brahic *et al.*, 1981; Gendelman *et al.*, 1986). Similarly, maturation of blood-derived monocytes into macrophages *in vitro* resulted in increased permissiveness to CAEV and EIAV infections (Anderson *et al.*, 1983; Maury, 1994).

The association between cellular maturation/differentiation and lentivirus life cycle is via induction of cellular transcription factors (TF), for example AP-1complex, which binds the LTR elements (Gabuzda *et al.*, 1989). Similar observations regarding the importance of LTR elements as determinants of virus infectivity have been reported for HIV-1 infection (reviewed by Ignatius Ou and Gaynor, 1993).

Not all cells of monocyte/macrophage lineage appear to respond equally to lentiviral infection. Some reports have demonstrated the inability of certain tissue macrophages to support a productive infection by MVV, for example the Kupffer cells (Gendelman *et al.*, 1984). This has led to the suggestion that perhaps only a subset of cells of the monocyte/macrophage lineage have the ability to support a productive infection by lentiviruses. There are no data at present to support this hypothesis.

The restriction imposed upon viral replication may be due to the intracellular processing of viral components. MVV and CAEV can productively infect ovine primary macrophages and goat synovial membrane epithelial cells with similar kinetics. However, CAEV failed to replicate productively in ovine SCP cells. This was found to be due an abnormal proteolytic processing of envelope glycoproteins (Cheblounе *et al.*, 1996). Such intracellular processing may have implicationsfor the outcome of clinical disease. Infection of SCP by two closely related MVV molecular clones, KV1772-kv72/67 and LV1-1KS1 resulted in similar kinetics of infection. However, LV-1-1KS1 replicated less efficiently in primary macrophages. Furthermore, intracerebral inoculation of sheep with both clones gave a completely different picture; clone KV1772-kv72/67 resulted in characteristic visna lesions in the CNS, whereas LV-1-1KS1 failed to do so (Torsteinsdóttir *et al.*, 1997).

It is known that cells of different lineages may process intracellular proteins differently. For example, intracellular processing/assembly of MVV in fibroblasts and macrophages is rather cell-type specific. MVV infection of fibroblasts results in

assembly of viral particles on the plasma membrane. However, in macrophage infections with MVV, virus assembly takes place within intracytoplasmic vacuoles (Dubois-Dalcq, *et al.*, 1979; Lee *et al.*, 1996). In HIV-1 infection of human glial cells, it has been shown that lack of a productive infection may be due to a block in the intracellular processing of the gp160 (Shahabuddin *et al.*, 1996). Similarly, infection of primary macaque macrophages with two molecular clones SIVmac219/17E (M-tropic), and SIVmac219 (infects macrophages but less efficiently) which differ only in the Env region resulted in differential processing of viral gp160 in macrophages. However, viral gp160 processing was identical for both molecular clones in lymphocytes (Stephens *et al.*, 1995).

Other example of cellular restriction placed upon virus replication is HIV-1 infection of astrocytes *in vitro*, which results in a persistent infection with preponderance of multiply spliced transcripts, mainly *nef* and *rev* (Tornatore *et al.*, 1994). Infected monocyte/macrophage cells can be activated into a productive infection after stimulation, for example by phorbol esters. This results in a productive infection by appearance of predominantly genomic and sub-genomic transcripts (Muesing *et al.*, 1985; Pomerantz *et al.*, 1990). In contrast, in an attempt to stimulate viral gene expression in infected astrocytes by cytokines, only an increase in multiply spliced transcripts was detectable. There was no (detectable) increase in the levels of genomic and sub-genomic transcripts (Tornatore *et al.*, 1994). The precise reason for this latency is not known.

Apart from the restriction imposed upon the virus by viral/cellular factors, a major component in the outcome of any viral infection is the role of host-derived immune responses toward the invading pathogen.

1.8 Immune responses to MVV

The first stage in targeted (specific) elimination of a viral pathogen, is the recognition of invading pathogen. Specific immune activation requires the presentation of antigen in the context of MHC molecules (Zinkernagel and Doherty, 1974). Specific (MHC-restricted) cellular immune responses are initiated after MVV infection *in vivo*. This is in the form of antibody production (via CD4+ T help), and $CD8^+$-mediated effector functions (Griffin *et al.*, 1978; Bird *et al.*, 1993; Lee *et al.*, 1994; Blacklaws *et al.*, 1995).

1.8.1 Humoral immune responses

Following *in vivo* infection with MVV, an initial viraemia and antigenaemia (mainly p25) is initiated. Of the 20 or more antigens recognised by immune serum, Env and Gag antigens are the most prominent (Pétursson *et al.*, 1976; Bird *et al.*, 1993).

Neutralising antibodies were among the first specific immune responses observed in MVV-infected animals, at 2-3 months post-infection. (Sigurdsson *et al.*, 1960). These antibodies remain detectable for years after seroconversion. Generation of neutralising antibodies against MVV Env glycoproteins does not result in elimination of virus, but prevents the re-infection and release of infectious progeny virions. Moreover, coincident with appearance of neutralising antibodies is a reduction in circulating virus (Pétursson *et al.*, 1976; Griffin *et al.*, 1978).

The generation of specific immune responses against MVV were best illustrated by cannulation of regional peripheral lymph nodes, where initiation of immune responses takes place. The first antibodies are generated against viral p25 (capsid or major core protein). The detection of soluble p25 immune complexes as early as 2 days may be indicative of virus replication (Blacklaws *et al.*, 1995). These early antibody responses closely resemble the humoral immunity in HIV-1 infections (Stramer *et al.*, 1989).

Kinetic studies have shown that neutralisation by MVV-specific immune serum is inefficient (Kennedy-Stoskopf and Narayan, 1986). Treatment of viral particles with neuraminidase, which removes sialic acid side chains on Env glycoproteins increased the efficiency of neutralisation *in vitro* (Huso *et al.*, 1988). This suggests that carbohydrate side-chains may obscure antigenic epitopes, making antibody binding/neutralisation less efficient.

The mode of action of MVV neutralising antibodies is dependent on the cell type used *in vitro*. In fibroblast cultures, neutralising antibodies prevent both binding of the virion to the receptor and replication of already internalised viral particles. On the other hand, these antibodies enhanced virion entry in macrophages via Fc receptors (FcR), and inhibition of virus replication was intracellular, presumably via degradation in the phagosomes (Jolly *et al.*, 1989).

The HIV-1 epitopes that are used by the immune system to generate neutralising antibodies are diverse. The major epitope used for neutralisation is near CD4 binding site on gp120 (Kang *et al.*, 1991). Moreover, HIV-1 neutralising antibodies were

reported to be, by comparison to those generated against MVV/CAEV, more efficient in preventing infection *in vitro* (Nara *et al.*, 1987). However, HIV-1 neutralisation assays were carried out on laboratory-adapted T cell lines. In light of new discoveries in differential receptor usage by T-tropic and M-tropic virus strains, use of such assays may result in artificially high values. This has been complemented by the poor neutralisation obtained with sera from infected individuals, and use of mAbs to neutralise primary HIV-1 isolates (Moore, 1995).

The poor neutralisation capacity of mAbs generated against the most antigenic domain of gp120 (V3 loop), is because the V3 loops are less immunogenic in oligomeric gp120 than monomeric gp120. Moreover, the bulk of V3 loop is cryptic in M-tropic strains of HIV-1. This is significant, since M-tropic viruses predominate in the early phases of infection (Bou-Habib *et al.*, 1994).

The observations made on the antigenicity of HIV-1 gp120 would suggest that topology of intact envelope glycoproteins has a significant bearing on the efficiency of neutralisation *in vivo*. This in turn questions the apparent potency of neutralising antibodies observed *in vitro* and in prevention of disease *in vivo*. For example, in SIVmac infection of rhesus monkeys, neutralising antibodies are detected and may provide some protective immunity (Zhang *et al.*, 1988).

In MVV, a 24 amino acid hydrophobic stretch at the amino terminus of TM domain has been identified which mediates fusion. Antisera from some infected animals with MVV and CAEV and mAbs generated against this peptide prevented fusion *in vitro* (Crane *et al.*, 1991). In HIV-1, anti-fusion antibodies may function by dissociating gp120-gp41 complex, rendering the virion inactive (Poignard *et al.*, 1996).

1.8.1.1 Infection-enhancing antibodies

Non-neutralising infection-enhancing antibodies are generated in infection with lentiviruses, which operate via FcR (Jolly *et al.*, 1989; Robinson and Michell, 1990). In HIV-1, a correlation has been suggested between the appearance of enhancing antibodies and progression to disease (Kliks *et al.*, 1993). Similarly, enhancing antibodies are detected in MVV infections (Jolley *et al.*, 1989).

1.8.1.2 Antibody-dependent complement-mediated (ADC) cytotoxicity

Complement fixing antibodies can be detected at 2-3 weeks after inoculation with MVV (de Boer, 1970). In HIV-1, the C1q-binding sites on the gp41 have been mapped (Thielens *et al.*, 1993). Moreover, structural similarities have been identified

on gp120 and C1q, and C1q can compete with gp120 for binding to gp41. This may result in release of gp120 from the virions (Stoiber *et al.*, 1994).

1.8.1.3 Antibody-dependent cell cytotoxicity (ADCC)

Cells with cytotoxic potentials may bind opsonized particles via FcRs, resulting in death of infected cells, by a mechanism referred to as antibody-dependent cell cytotoxicity (ADCC). In HIV-1, antibodies triggering ADCC are predominantly of IgG_1 isotype, and to a lesser extent of IgG_2 isotype. These neutralising antibodies are directed against the Env proteins (Ljunggren *et al.*, 1988). MVV-specific ADCC activity has not been detected in immune serum from infected animals (Rayburn, 1992).

Natural killer cells, which are part of non-specific immune responses, may interact via their surface FcγRIII with the IgG antibodies generated against the viral antigens. Cell killing via NK cells is mediated by the perforin pathway. The precise reason for lack of an ADCC killing in MVV infection is not known. However, there is a correlation with loss of ADCC activity and disease progression in HIV-1 infection. It is known that NK activity is dependent on cytokines such as INF-γ and IL-12 and that monocyte/macrophage cells are the main source of IL-12. Therefore, loss of NK/ADCC activities may be linked to the loss of monocyte/macrophage functions in progression to clinical disease.

There are no reports on NK activity in MVV infection. It may be that ADCC-fixing antibodies in MVV-infections differ from those in primate lentivirus infections. It is proposed that lack of ADCC activity in MVV-infected animals correlates with the lack of serum IgG_2 (Rayburn, Ph.D thesis, 1992). It is plausible that the NK activity is not initiated in MVV infection due to: a) lack of optimum cytokine environment; b) because of dysfunctional monocyte/macrophage cells, or; c) an inherently low NK activity in ungulates.

1.8.2 Cellular immune response in MVV infections

An increase in CD8+ lymphocyte population is observed in the lungs, CNS, and synovial fluids of MVV-infected animals (Lujan *et al.*, 1993; Harkiss *et al.*, 1991; Torsteinsdottir *et al.*, 1992,1994). However, these CD8+ T cells do not appear to be readily cytolytic as prior *in vitro* culture in the presence of IL-2 is necessary for detection of CTL-mediated lysis (Blacklaws *et al.*, 1994; Lee *et al.*, 1994).

The precise role of CD8+ lymphocytes in control of MVV infection is unclear. Antibody depletion of $CD8^+$ cells *in vivo* prior to experimental infection has been carried out. Preliminary data suggest that depletion of CD8+ cells does not result in an increase in infected cells in peripheral lymph nodes. Although depletion experiments were successful, a concomitant increase was observed in the frequency of precursor CTLs (CTLp). It is not clear what role, if any, this population may play in control of virus infection. However, depletion of CD4+ cells resulted in a decrease in virus-infected cells in efferent lymph (B. A. Blacklaws, personal communication).

Delayed type hypersensitivity (DTH) responses are CD4+ T cell driven. In certain viral infections, for example Measles virus, there is a defect in DTH response during the immunosuppressive phase of acute measles (reviewd by Griffin *et al*, 1992). Ungulate lentiviruses do not infect T lymphocytes. Cell-mediated immunity (CMI) in the form of $CD4^+$ T cell proliferation to viral antigens is continually detected after MVV infection *in vivo* (Rayburn, Ph.D. thesis, 1992; Bird *et al.*, 1993; Blacklaws *et al.*, 1995). These proliferative responses are predominantly against Gag antigens (Rayburn, Ph.D. thesis, 1992). Moreover, MVV infection does not appear to result in significant reductions in DTH responses, recall antigens or mitogens (Pyrah *et al.*, 1996; P. Bird, unpublished data).

Cytotoxic T lymphocyte (CTL) activities mediated by CD8+ T cells in HIV-1 infected individuals are readily detected, prior to appearance of neutralising antibodies, and correlate with temporary clearance of viraemia in primary phase of infection (Borrow *et al.*, 1994). These primed CTLs can be lytic at low ratios of one CTL to 1000 infected CD4+ cells (Yang *et al.*, 1997). Suppressive activities of CD8+ T cells can also be nonlytic (Walker *et al.*, 1986). The CTL responses in HIV-1 mediate the suppression of virus replication by both lytic and nonlytic mechanisms (Yang *et al.*, 1997). In $CD8^+$-mediated nonlytic pathway, release of chemokines, for example macrophage inflammatory protein-1 (MIP-1) and releases upon activation normally T cell expressed and secreted (RANTES), results in suppression of virus replication (Yang *et al.*, 1997).

1.8.3 Non-specific (innate) immune responses in MVV infections

Non-MHC-restricted innate immune responses are the first line of defence against pathogens. These are soluble factors, such as interferons, NK cells and cells of mononuclear phagocytes lineage, which include macrophages.

Macrophages are the main cell-type permissive to all lentiviruses *in vivo*. Moreover, macrophages isolated from MVV-infected animals can be targets for CTL killing (Lee *et al.*, 1994). It is therefore feasible to assume that loss of infected monocyte/macrophages because of CTL activity may compromise the innate immunity against pathogens and result in disease.

1.9 Why do lentiviruses persist?

Despite rigorous immunity mounted against lentiviruses by the host immune system, the infection persists, culminating in clinical disease and inevitable death. Viruses, during their co-evolution with their hosts, have evolved a myriad of strategies to evade the host immune system.

1.9.1 Immune determinants of viral persistence

1.9.1.1 Antigenic variants

Humoral immune responses mounted against lentiviruses do not appear to be effective in eliminating the virus (see above). The envelope glycoproteins are the most prominent targets for neutralising antibodies, since they are the most exposed viral components that take part in initial receptor-binding activities. However, the kinetics of neutralisation would suggest that these antibodies are inefficient in their mode of action. This is partly due to the extensive glycosylation of envelope glycoproteins. In small ruminant lentiviruses, treatment of viral particles with neuraminidase, which removes sialic acids, resulted in enhanced neutralisation by immune sera. Desialylation also rendered the virus more prone to protease digestion (Huso *et al.*, 1988). This observation would imply that carbohydrates on the viral surface are important in both protecting viral proteins from digestion by proteases and in protecting the virus from rapid neutralisation by antibodies

In HIV-1, heavily glycosylated gp120 results in diminished neutralisation by anti-envelope antibodies. The shedding of viral envelope proteins may also be used as a decoy to evade the immune system. Since the topology of oligomeric gp120 is a vital determinant for neutralisation, therefore, the shed gp120 may result in a deviated humoral response against the monomeric form of gp120. Moreover, the V3 loop is a dominant immunogen in generating antibodies. However, this region is also highly variable in its amino acid sequence, resulting in antigenic variants that may become escape mutants (reviewed by Poignard *et al.*, 1996).

The error prone RT of lentiviruses can also result in antigenic variants (drift) that may escape the host immune system. The best example of antigenic drift is the episodic EIAV. In EIAV infection, emerging virus variants are proceeded by quasi-species-specific neutralising antibodies. These result in diminished viraemia, before the emergence of new escape variants (Rwambo *et al.*, 1990).

MVV may employ antigenic drift as a method of escaping the host immune system. However, the importance of this strategy in persistence and pathology of MVV is unclear. In long-term experimental infections with MVV, the emergence of antigenic variants was a rare event: an estimated 16% escaped type-specific antisera (Thormar *et al.*, 1983; Lutley *et al.*, 1983). However, use of antisera may underestimate the rate of antigenic drift in these experiments. Epitope-overlapping mAbs raised against MVV gp135 and tested on strain 1514 and its antigenic mutants (LV-1 to LV-6 isolated from a single infected animal) showed the existence of antigenic variants, whereas polyclonal anti-gp135 sera failed to detect these differences (Stanley *et al.*, 1987).

Gag-derived proteins are also highly immunogenegic in MVV (Rayburn, 1992). However, unlike anti-gp135 mAbs, mAbs raised against the major core protein of MVV failed to detect antigenic variants (Stanley *et al.*, 1987).

Hypergammaglobulinaemia and increased B cell activation occur in HIV-1 infections. The carboxyl terminus of gp41 has the capacity to induce poly-clonal B-cell activation in normal B lymphocytes, causing them to differentiate into immunoglobulin-secreting plasma cells. This immunogenegic domain is part of the transmembrane region of gp41, and therefore not involved in receptor-mediated infection of permissive cells (Chirmule *et al.*, 1990).

1.9.1.2 CTL repertoire usage

Cell mediated immune responses take place in all lentiviruses, and to varying degrees. However, these cellular immune responses, like humoral immune responses are unable to clear the infection.

In HIV-1 infections, patients may be categorised into slow progressors or fast progressors, with different underlying determinants, for example second receptor usage, or the genotype of MHC alleles. In one study, the fast progressors had a vigorous and oligoclonal expansion of T cells, whereas slow progressors had a less vigorous, but broader T cell repertoire (Pantaleo *et al*, 1994). Anti-Gag CTL

responses were detected in 75% of subjects compared to 26% against Env and Nef (Bourgault *et al.*, 1991). Moreover, in a longitudinal study, Gag-specific CTLp frequencies early in infection were paralleled by increasing numbers of HIV-1 infected $CD4^+$ T cells (Klein *et al.*, 1995). However, some reports have shown lack of a consistent correlation between disease severity and CTL frequencies (O'Toole *et al.*, 1992).

Since HIV-1 has been shown to have a high turnover *in vivo*, it is also possible that the CTL repertoire generated against viral epitopes is lagging behind emergence of new viral variants. There are, however, no reported studies on the epitope usage by CTL in MVV infection.

1.9.2 Viral determinants of persistence

Viruses can evade the immune system by different mechanisms, for example, by infecting immunologically inert sites such as the nervous system. This is best exemplified by herpes simplex, which persists in the sensory ganglia.

Viral proteins are processed intracellularly and presented to $CD8^+$ T lymphocyte in the context of MHC-I. Cells bearing such complexes are recognised and may be lysed by $CD8^+$ CTL. Several viruses interfere with the intracellular pathway/expression of MHC-I and therefore evade the immune system. For example, IPC47 of herpes simplex which sits on TAP molecule in the endoplasmic reticulum and thereby interferes with MHC pathway. Other example is the EBNA1 of EBV which contains a 20 amino acid stretch of glycine/alanine, that interferes with cellular proteasome machinery (reviewed in Fields Virology, 1994).

Virus-coded proteins may also have direct immunosupressive properties. Within the v*if* ORF of MVV, a sequence was identified that showed high similarity to the immunosuppressive sequence from the retroviral transmembrane protein p15E. Synthetic peptides of this sequence inhibited lymphoproliferation stimulated by either IL-2 or T-cell antigen receptor. These peptides also inhibited the enzymatic activity of protein kinase C (PKC) (Ruegg *et al.*, 1990).

There are no indications that infection with ruminant lentiviruses results in downregulation of MHC-I. However, there is some evidence that HIV-1 can downregulate the expression of MHC-I on the surface of infected cells. Reduced expression of MHC-I may result in enhanced killing by NK cells. However, co-culture of HIV-infected cells with NK cells showed little or no killing. Since killing

by NK cells is also dependent on the presence of cytokines, it is postulated that diminished NK-mediated killing is partly due to impaired cytokine production (Zheng and Zucker-Franklin, 1992).

1.10 Pathology of MVV infection

An inevitable result of a protracted infection with all lentiviruses is organ-specific pathology. The pathology in MVV infections may manifest as interestitial pnumonia (maedi), arthritis of joints, mastitis and leukoencephalomyelitis (visna). Henceforth, the clinical disease associated with the CNS because of MVV infection will be referred to visna.

The first reports of both natural and experimental visna were by Sigurdsson and co-workers (1957, 1962). Extensive description of neuropathology was carried out by intracerebral (i.c.) infections of animals (Georgsson *et al.*, 1976).

The earliest sign of clinical visna is cachexia. This is followed by change by progressive ataxia (fore- and hind limb), and subsequently hind leg paralysis within a few months of showing emaciation. There appears to be no loss of appetite until and during the last stages of clinical disease. Sometimes the disease progresses in waves with slight intervening remission. The clinical disease may manifest as early as six months post-infection (p.i), although this may depend on breed susceptibility (Georgsson *et al.*, 1976; Watt *et al.*, 1990). The end-stage fatal disease is concomitant with fine trembling of facial muscles, blindness and seizures, all signs of extensive CNS involvement.

1.10.1 Histopathology of visna

All lentiviral infections can result in CNS pathology. However, the extent of neuropathies may differ among lentiviruses. (Sigurdsson *et al.*, 1956; 1994McIlwraith and Kitchen; 1978Bredesen *et al.*, 1988; Letvin and King, 1990; Dow *et al.*, 1990; Gonda *et al.*,1994).

The most striking feature of MVV neuropathology is the extensive lymphocytic infiltration, which is in the form of perivascular infiltrates or "cuffs", around blood vessels and in brain parenchyma. Leptoemeninges of both brain and spinal cord show inflammatory reaction. The brain lesions are asymmetric and widespread, however, certain anatomical regions show a predilection for lymphocytic infiltrates. These include the areas immediately adjacent to ventricular system in sub-ependyma. Consequently, lesions are found more frequently in the white matter than in the grey

matter. Lesions seldom present in cerebral or cerebellar cortex and if present are in the form of perivascular cuffs spreading from overlying meningitis. The choroid plexus is frequently inflamed with lymphoid follicle formation and often with germinal centres. Glial nodules are rare, and if present seem to be focal and widespread and can be randomly located. The end stage visna may result in liquifaction necrosis and loss of neuroparenchyma (reviewed by Petursson *et al.*, 1976).

Visna pathology associated with the peripheral nervous system (PNS) appears to be a rare event, and may arise as a result of inflammatory infiltrates from the adjacent meninges. However, Sigurdsson and co-workers (1962) reported isolated inflammatory foci in peripheral nerves at some distance from the nerve roots, with isolated cases of degenerative myelinated tracks.

Like MVV, HIV-1 infection may also result in neuropathology. The first isolations of HIV-1 from the brains of AIDS patients were reported by Shaw and co-workers (1985) and Ho and co-workers (1985). HIV encephalopathies include HIV-encephalitis (presence of HIV virus in brain parenchyma), and AIDS-dementia complex or HIV-associated dementia (both refer to clinical syndrome). A hallmark of the CNS disease is the presence of multi-nucleated giant cells (MGC). MGC are formed by the fusion (syncytium) of infected macrophage-type cells in the brain parenchyma (reviewed by Glass and Johnson, 1996).

Intravenous infection of rhesus macaques with SIV also results in CNS lesions similar to visna. The CNS infection with SIV is an early event, as virus can be detected within seven days post-infection. At the earliest stage, the infection localised mainly to perivascular $CD68^+$ mononuclear cells. Moreover, early viral replication coincided with neuropathological changes, consisting of gliosis, perivacular cuffs, microglial reactivity and MHC-II up-regulation. Interestingly, early CNS changes were more widespread than infection of macaques via i.c. route. Similarly, infections of rhesus macaques with either SIVmac or SIVsm (sooty mangabey) also result in encephalopathies with MGC (Lackner *et al.*, 1991). MGC are prominent in HIV-1 and SIV infections, but rare in visna, and non-existent in FIV and CAEV (reviewed by Georgsson, 1994).

1.10.2 Demyelination

The initial lesions in all lentiviruses appear similar. These consist of meningitis, perivascular infiltration, especially of the deep white matter, and inflammation of the choroid plexus.

Myelin abnormalities are present in lentiviral infections of the CNS. In visna, myelinated nerve fibres with only minimal changes may traverse through inflammatory foci. The dissociation of myelinated nerve fibres by inflammatory exudate may cause loss of myelin intensity (white pallor). However, complete destruction of myelin is only observed in necrotic lesions. Moreover, sharply demarcated plaques of primary demyelination may also occur. The initial lymphocytic infiltrates in visna are similar to CNS lesions observed in experimental allergic encephalitis (EAE) and lymphocytic choriomeningitis virus (LCMV). In visna, demyelination appears to be secondary in appearance, i.e., damage to myelin after initial damage to the neurones or axons, although occasionally plaques of primary demyelination similar to multiple sclerosis (MS) are also noted. Primary demyelination, if present, is a late manifestation of visna infection (Georgsson *et al.*, 1994).

Unlike visna neuropathology, vacuolar myelopathy is a common finding in HIV-1 neuropathies. Involvement of white matter in HIV results in leukeoencephalopathy, and diffuse myelin pallor. Diffuse myelin pallor does not appear to be due to loss of myelin, and may correlate with dementia. Leukoencephalopathy may include myelin breakdown, macrophage infiltration and presence of MGC. Inflammatory foci in visna brains include gliosis. Similarly, both astrocytosis and microgliosis are common findings in HIV-1. B cell lymphomas are associated with neurological complications with HIV and SIV infections. There are no lymphomas in visna infection of the CNS. (reviewed by Glass and Johnson, 1996).

1.11 Lentiviral CNS tropism

In visna-infected brains, the phenotypic assignment of virus-infected cells has been based on cellular morphology. However, use of antigenic markers such as CD68 and *Ricinus cummunis* agglutinin-1 (RCA-1) for cells of monocyte/macrophage lineage, has confirmed the identity of virus-infected cells (Torsteinsdottir *et al.*, 1992; Brodie *et al.*, 1995). The highly focal and restricted visna infection in CNS complements the

restricted pattern of viral gene expression observed in other tissues, for example maedi lungs, and lymph nodes.

In contrast to a low frequency of productively infected cells, MVV viral RNA structural can be detected in a variety of cell types, including blood endothelial cells (Haase, 1985; Zink *et al.*, 1994; Brodie *et al.*, 1995). Both viral DNA and RNA were found in infected cells within the inflammatory foci. Viral antigen-positive cells constituted a smaller number of infected cells when compared to viral RNA-positive cells. Viral RNA was also detected in cells that stained positive with markers for oligodendrocytes and astrocytes (Stowring *et al.*, 1985).

Sequence analyses of HIV-1 viruses isolated from different tissues from AIDS patients show presence of virus quasi-species. However, HIV-1 isolates from brain tissues show a preponderance of M-tropic viruses. Unlike blood-derived isolates, brain-derived HIV-1 isolates are predominantly non-cytopathic inducing (NSI), grow well in macrophages, and are less susceptible to neutralising sera (Ball *et al.*, 1994; Brew *et al.*, 1996).

In both HIV and SIV infection of the CNS, viral antigen- and/or nucleic acid-positive cells were predominantly stained with markers for monocytes and macrophages. Moreover, in HIV encephalopathies, viral transcripts and antigens are also detected in a variety of cell populations in the virus-infected brains. These include astrocytes, oligodendrocytes, blood endothelial cells, and lymphocytic infiltrates (Wiley *et al.*, 1986; Li *et al.*, 1991).

1.12 Route of lentiviral entry into the CNS

The earliest lesions after MVV infection of CNS are small subependymal inflammatory foci, usually around a venule or small vein in the parenchyma. These initial "cuffs" are composed of lymphocytes (50-70%), plasma cells and monocytes/macrophages (10%), some of which are virus-positive (Haase, 1985; Torsteinsdottir *et al.*, 1992). Although the perivascular cuffs in HIV-1 neuropathies are minimal compared to visna, nevertheless, some of the mononuclear inflammatory cells in such cuffs are also positive for viral proteins and transcripts (Wiley *et al.*, 1986). Similar observations have been made with SIV infections (Li *et al.*, 1991).

These and similar observations prompted Peluso and co-workers (1985) to suggest that entry of lentiviruses into the CNS took place by infected monocyte/macrophages

(and possibly T cells with primate lentiviruses and FIV) via a "Trojan horse" mechanism.

The extravasation of cells from systemic circulation in to neuroparenchyma is a multi-step process, and requires the involvement of adhesion molecules on both the endothelium and migrating cell (reviewed by Springer, 1990). Interestingly, in EAE model of MS, induction of vascular cellular adhesion molecule-1 (VCAM-1) on endothelial cell of BBB has been shown to facilitate the infiltration of lymphocytes in to the parenchyma (reviewed by Miller and Karpus, 1994).

1.12.1 Role of Blood-brain barrier in lentiviral neuropathology

The presence of viral antigens/transcripts in endothelial cells of the blood-brain barrier (BBB) indicates that these cells may be used as a route of entry by viruses from blood into the CNS. Endothelial cells of BBB have been shown to harbour lentiviral proteins and transcripts (Wiley *et al.*, 1986; Zink *et al.*, 1994; Brodie *et al.*, 1995). Furthermore, damage to BBB integrity has been reported in HIV-1 neuropathies, which may correlate with AIDS complex dementia (reviewed by Glass and Johnson, 1996).

Damage to the BBB resulting in vascular permeability has been documented in several virus infections of the CNS, for example measles virus infection of the CNS (reviewed by Gosgriff, 1989). Similarly, damage to the BBB has been demonstrated in early lesions of MS (Kermode *et al.*, 1988). The precise mechanism of how damage is done to the enodothelial cells of the BBB in MS is not known. The damage to the BBB could be due to direct infection by a virus, by certain cytokines, or by antibodies against the endothelial cells of the BBB (reviewed by Miller and Karpus, 1994).

Lymphocytic choriomeningitis virus (LCMV) is an *Arenavirus* genus of Arenaviridae. In adult mice, infection of the CNS with LCMV results in severe pathological changes mediated by CD8+ T cells. Treatment of these mice with mAbs against CD8+ T cells protects mice against disease. It is believed that virus specific CD8+ T cells damage the infected endothelial cells of the BBB resulting in cerebral oedema (Buchmeier *et al.*, 1980).

In a murine model of MS, infection of a resistant strain (BALB/c) with an avirulent mutant of a neurotropic strain of Semliki Forest virus (SFV-A7) results in MS. Intraperitoneal infection of mice with SFV-A7 resulted in infection of vascular

endothelium and perivascular neurones in cerebellar and spinal cord white matter. Presence of viral antigens correlated with damaged BBB (Soliu-Hänninen *et al.*, 1994).

Therefore, either direct or indirect damage to BBB may contribute to neuropathology. Viral antigens and transcripts have been detected in lentiviral infections of the CNS (*ut supra*). However, the close proximity of perivascular macrophages to the basement membrane of BBB may complicate the accurate identification of infected cells because of intimate association of perivascular cells with the basal lamina of the vessels. For example, in a SIV infection of rhesus monkeys, both endothelial cells and perivascular cells appeared positive for viral proteins. However, use of double immunostaining demonstrated that the majority of infected cells also stained positive for $CD68^+$ monocyte/macrophages (Lackner *et al.*, 1991).

1.13 Independent evolution of lentiviruses in the CNS

Virus recovered from the brain of patients with HIV-induced neuropathological disease shows envelope sequences different from the virus in the blood of the same individual (Power *et al.*, 1994). In one study, none of the V3 sequences obtained from brain tissues was found among those from lung, peripheral blood mononuclear cells (PBMC), and lymph node samples (Hughes *et al.*, 1997). Moreover, comparison of cloned sequences from the brain and spleen tissues from demented and non-demented patients showed two amino acid substitutions in the V3 loop (histidine at position 305 and proline at position 329) that correlated with neurotropism and the clinical HIV dementia (Power *et al.*, 1994). Although other studies show a similar trend in the sequences of V3 with its relation to neurotropism, there appears no conserved sequence in this region that would confer universal neurotropism upon invading viruses (Hughes *et al.*, 1997). Nevertheless, these results imply an independent evolution of neuropathic strains of HIV-1 in the brain compartment. Moreover, these and similar analyses have indicated that CNS infection by HIV takes place early after infection, and may be prior to commencement of clinical AIDS.

Korber and co-workers (1994) hypothesised that the reason for the preponderance of M-tropic variants, with respect to the V3 sequences derived from nonlymphoid tissues such as brain, is because viral infection of such tissues occurs early post-infection, and at a time when the viral population is relatively homogenous in the V3 region. Moreover, as disease progresses, quasi-species from lymphoid tissues may undergo

more rapid sequence changes in regions such as V3, which is under selection pressures such as neutralising antibodies and CTL. This is apparent from the rapid changes in the population dynamics of HIV-1 in the PBMC after treatment with antiviral protease inhibitors (Ho *et al.*, 1995). However, cellular constraints, independent of immune pressures may also contribute to the selection of viral variants in non-lymphoid tissues, for example the CNS. This is best explained by the restricted infection of MVV in monocyte cells prior to maturation/differentiation into macrophages.

Infection of macaques with SIVmac239, a molecular clone of T-tropic SIVmac, results in productive infection of lymphocytes that can invade the CNS. Viral DNA was recovered from the brain tissues of these animals. However, PCR analyses of recovered *env* sequences revealed that a minimal virus replication had occurred *in vivo*, and only one animal showed a change to M-tropic virus sequences. Moreover, in spite of immunosuppression and AIDS, these animals did not show overt neurological diseases (Stephens *et al.*, 1995). Interestingly, multiple sequential intracerebral passage of SIVmac239 in the brains of rhesus macaques resulted in the selection and replication of an M-tropic neurovirulent strain, SIVmac239/17E-Br (Sharma *et al.*, 1992). This neurovirulent strain differed by only 16 amino acids from the parent strain, located in the *env* region. Similar neurotropic determinants have been located in the *env* sequences of HIV-1 isolates (Power *et al.*, 1994).

Taken together, these observations demonstrate that cells of monocyte/macrophage lineage are most consistently associated with both neurotropism and neurovirulence.

1.14 Immune status of the CNS

Historically the CNS has been regarded as an immune-privileged site. This has been because of a) low to undetectable levels of MHC molecules in the parenchyma; b) lack of a demonstrable lymphocytic drainage; and c) seclusion from the peripheral immune system by a physical barrier, i.e. the blood-brain barrier (BBB). These features may contribute to the impaired (delayed) rejection of xenografts transplanted in the CNS. However, use of more sensitive techniques, together with availability of better reagents have demonstrated that although the CNS may be a less active site in immunological terms, it is by no means an immune privileged organ. This is apparent from the trafficking T lymphocytes into the CNS, and detectable levels of MHC molecules on cells of neuroparenchyma, including endothelial cells of the BBB.

Moreover, antigens injected or grafted intrathecally (i.c) may be transported to the deep cervical lymph nodes.

In the non-inflamed ovine CNS, constitutive MHC-I expression was detected on endothelial cells in grey and white matter, choroid plexus and ependyma. Moreover, MHC-I was also present but to a lesser extent, on pericytes, meninges, and apparently pia-arachnoidal cells. On the other hand, MHC-II expression was less widespread, and mainly detected on pericytes and on microglial cells in the white matter. Some subependymal glial cells were also positive for the surface expression of MHC-II. Glial cells were found negative for surface expression of both MHC class-I and II (Georgsson *et al.*, 1997).

Therefore, the presence of MHC molecules (class-I and II) in the CNS is indicative of potential antigen presentation to both CD4+ and CD8+ T cells, and therefore potential targets for immune-mediated pathology.

1.14.1 Immune determinants of visna neuropathology

There are several observations, which imply that CNS lesions in visna are immune-mediated. This appears to be via a cell-mediated immunity (CMI), supported by the observations that virus-infected cells can support a mixed lymphocyte reaction *in vitro*. The CMI profiles of infected sheep showed a correlation with the CNS lesion formation (Griffin *et al.*, 1978, Torsteindóttir *et al.*, 1992). Moreover, pre-treatment of animals with anti-thymocyte sera plus cyclophosphamide, results in a generalised immunosuppression. Subsequent infection these animals with K1514 strain of Visna did not result in a significant CNS lesion formation, when compared with untreated controls (Nathanson *et al.*, 1976). However, this treatment could only be sustained for a short period due to inherent problems with this approach. Nevertheless, indications were that immune responses directed toward the virus did play a role in early lesion formation.

Free virus could be isolated from cerebrospinal fluid (CSF) of visna-infected animals some 30 days p.i, with a progressive decline in the isolation of virus. In a similar study, free virus could be detected from the CSF within two weeks of infection, which also declined with time. No virus could be isolated from CSF after four months p.i. This was concomitant with the maximum levels of neutralising antibodies in the CSF. The ratio of CSF/serum neutralising antibodies varied from ¼ to eight. In one case, lack of CSF neutralising antibody was correlated with severe clinical visna.

Moreover, CSF pleocytosis has been shown to correlate with lesion severity in visna (Petursson *et al.*, 1976; Griffin *et al.*, 1978; Nathanson *et al.*, 1979; Georgsson *et al.*, 1979).

1.14.1.2 Role of humoral immunity in visna neuropathology

Since demyelination is observed in Visna infections of the CNS, it was postulated that this might be due to generation of anti-myelin antibodies. No anti-myelin antibodies were detected in Visna infections (Nathanson *et al.*, 1981). Antibody-mediated pathology has been reported in CAEV infection, where high titre antibodies to Env, but not Gag antigens have been shown to correlate with the severity of arthritic joints. However, there is no reported evidence that such a mechanism may play a role in the CNS pathology (Bertoni *et al.*, 1994; Perry *et al.*, 1995).

In HIV-1 infection, a cross-reactivity has been observed between gp41 antibodies with human astrocytes and astrocytoma cell lines. Since intrathecal anti-gp41 antibodies are also present in some 50% of AIDS patients, it was therefore suggested that such antibodies may play a role in virally induced autoimmune disease. A panel of mAbs against gp41 was used in a detailed study of brain sections of apparently normal HIV- patients, and AIDS patients, with or without dementia. Cross-reactive anti-gp41 epitopes were present in all brain tissue, irrespective of presence or absence of HIV. However, there was a significant increase in the number of immunoreactive cells in HIV^+ samples (Spehar and Strand, 1994). Similar observations have been made with anti-gp120 antibodies, which cross-react with human brain proteins (Trujillo *et al.*, 1993). However, it is not clear what role, if any, these antibodies may play in the CNS pathology.

1.14.1.3 Role of CD4+ T cells in visna neuropathology

Autoimmunity may be mediated by an antibody-independent, CD4+ T cells (DTH) mechanism. For example, CD4+ T cell-mediated EAE, which results from introduction of CNS antigens (brain and spinal cord homogenates), into the periphery. EAE has been used as a murine model for the relapsing-remitting model of human MS. Generation of encephalitogenic CD4+ T cells to major myelin proteins, for example myelin basic protein (MBP) and proteolipid protein (PLP) results in EAE via release of pro-inflammatory cytokines, for example macrophage inflammatory protein 1α (MIP-1α). These factors in turn cause the chemoattraction and activation of monocyte/macrophage cells which in turn result in myelin damage via a bystander

mechanism. Adaptive transfer of MBP-reactive CD4+ T cells into naive mice elicits disease, and antibody depletion of Vβ8+ cells *in vivo* reduces the severity of disease in mice (reviewed by Miller, 1994).

Generation of naturally occurring encephalitogenic T cells may be a result of similarities between self-epitopes and non-self, viral epitopes. An amino acid similarity between the Visna polymerase and myelin prompted the suggestion that visna-induced demyelination may be due to molecular mimicry. However, a synthetic peptide encompassing the shared amino acid homology between Pol and myelin protein failed to induce a cell-mediated response (Weise and Cranegie, 1988).

1.14.1.4 Role of $CD8^+$ T cells in visna neuropathology

Although the CD4/CD8 ratio in systemic circulation does not change significantly after infection with MVV (~2), there is a decrease in CD4/CD8 ratio (~0.8 - 1) in CSF. This almost reversal of ratios was more pronounced in neuroparenchyma, where CD4/CD8 ration was in the order of 0.8 compared to approximately two in perivascular cuffs, implying a differential transport of CD8+ T cells into the parenchyma (Torsteinsdottir *et al.*, 1992). Since MVV-infected cells can be targets for CTL *in vitro*, therefore, presence of $CD8^+$ cells may contribute to neuropathology.

CD8+ T cells can directly contribute to neuropathology. Theiler's murine encephalomyelitis virus (TMEV) is an *Enterovirus* genus of Picornaviridae family and has been used a model for the chronic-progressive forms of human MS. TMEV infection results in lifelong persistence in the CNS. In susceptible SJL/J ($H\text{-}2^s$) mice, infection is characterised by CNS perivascular inflammatory infiltrates of mononuclear cells and primary demyelination (reviewed by Miller and Karpus, 1994). Immunosuppression with mAbs against CD4 or CD8 molecules decreases the number and extent of demyelination in suceptible mice (Welsh *et al.*, 1987; Rodriguez and Sriram, 1988). TMEV productively infects oligodendrocytes. Mice with $H\text{-}2^b$ haplotype are resistant to TMEV and clear the infection. Disruption of the Aβ gene of MHC-II molecules in $H\text{-}2^b$ mice [$H\text{-}2^b(Ab^o)$], which abrogates MHC-II mediated functions resulted in the persistence of virus in the CNS with demyelinating lesions, which co-localised with virus-specific CD8+ T cells These results also indicate that CD8 + T cells can function in the absence of Th cells (Njenga *et al.*, 1996).

It is therefore possible that CD8+ T cells generated as a result of Visna infection may play a role in neuropathology *in vivo*, culminating in neurological deficits. However,

there is no direct evidence to correlate the presence of CD8+ T cells with lesion formation in lentiviral-associated neuropathies. Moreover, there are limited reports of lentivirus- infected oligodendrocytes and neurones, which may act as cellular targets for CTL activity.

The discrepancy between the frequency of productively infected cells and extent and character of pathological lesions indicates that mechanisms other than the direct effect of the virus may be involved in neuropathies associated with lentiviruses, referred to as "bystander" damage. For example, in experimental infections with Visna, the number of viral antigen-positive cells was very low, even in areas showing the highest density, i.e., choroid plexus. This was estimated to be 1 in 10^3 to 1 in 10^4 cells (Hasse *et al.*, 1977; Georgsson *et al.*, 1989). The discrepancy between infected cells and pathology is most profound in autopsy of brain tissues from HIV+/AIDS patients, where sparse pathology was found in samples from patients that had profound cognitive impairment (reviewed by Budka, 1989).

1.14.1.5 Activated cells may cause bystander damage

In MVV infection of the CNS, the perivascular infiltrates are mainly of CD4+ T cell phenotype (Torsteindottir *et al.*, 1994). However, perivascular infiltrates in visna are not unique to the brain vasculature. In chronic arthritis because of MVV infection, an overall increase in all T cell subsets in synovia has been observed, which was concomitant with an increased MHC-II expression on cell layer lining the synovium (Anderson *et al.*, 1994). Moreover, in a similar study, T cells were found to have an IL-2R-negative phenotype (Wilkerson *et al.*, 1995). Similarly, a population of $CD8^+$/MHC-II^+/IL2-R^- T cell was detected in the lymph node of acutely infected animals (Blacklaws *et al.*, 1995)

In HIV-1 infection, impaired proliferative responses to recall antigens, pokeweed mitogen (PWM), anti-CD3- or anti-CD2-antibody treatments have been reported (Hofmann *et al.*, 1989).

A lack of response by lymphocytes to various stimuli has been reported in immunosuprression caused by Measles virus. Measles virus is able to block mitogen-induced proliferation of PBMC (McChesney *et al.*, 1988). The decrease in proliferation was not due to lack of T cell growth factor, IL-2, but rather a defect in the expression of IL-2Rα subunit (Bell *et al.*, 1997). Moreover, the IL-$2R^-$ phenotype of PMBC in the measles study appeared to have no adverse effect on the secretion of

cytokines, for example IL-2 and IFN-γ from T cells (Bell *et al.*, 1997). It is noteworthy that one of the major inducers of adhesion molecules on BBB is INF-γ, in particular ICAM-1. Moreover, IFN-γ is a potent inducer of both MHC-I and II molecules, and activates monocyte/macrphages, NK cells and CTL.

A nonglycosylated protein with a molecular weight of 54-64 kDa, named lentivirus-interferon (LV-IFN; also refereed to as lentiferon), has been identified from co-cultures of MVV-infected macrophages and lymphocytes, with similar biological activities to IFN-α and IFN-γ. Neither the infected macrophages nor lymphocytes alone could produce LV-IFN. Moreover, use of monoclonal antibodies to MHC-II blocked production of LV-IFN. The activated phenotype of macrophages (MHC-II+) observed in LN, maedi lungs, arthritic joints and visna might be a function of LV-IFN, because direct infection of macrophages with MVV does not result in increased expression of MHC-II. Intracerebral infusion of crude LV-IFN preparation into visna-infected brains increased the CNS lesions. Paradoxically, LV-IFN also inhibited viral transcription and differentiation of monocytes into macrophaes (Narayan *et al.*, 1985; Zink and Narayan, 1989).

Whether the cytotoxicity is mediated by LV-INF, other cytokines or the source of this cytokine (lymphocytes or infected macrophages) is not known. The complexity of biological properties associated with LV-IFN is most probably due to several factors in the supernates from the co-cultures of lymphocytes and infected macrophages.

1.15 Do lentiviruses infect astrocytes?

In visna, there is a single report of viral transcripts detected in astrocytes (Stowring *et al.*, 1985). However, this observation has not been substantiated (Georgsson *et al.*, 1989).

An early description of paediatric AIDS was demonstrated by the rare presence of viral particles in astrocytes (Epstein *et al.*, 1984). Moreover, the non-productive infection of paediatric astrocytes has been linked to the presence of the early viral transcripts *nef*. It is therefore, proposed that astrocytes may function as a reservoir of infection in HIV-1(Erfle *et al.*, 1991).

HIV-1 infection of human foetal astrocytes and astrocytoma cell lines can result in a persistent and non-productive infection. However, infection of primary cultures of human astrocytes has been negative (Sharpless *et al.*, 1992).

1.16 Do lentiviruses infect oligodendrocytes?

In visna, there is a single report of viral transcripts detected in oligodendrocytes (Stowring *et al.*, 1985). However, more recent studies have failed to show viral products in oligodendrocytes (Georgsson *et al.*, 1989; Brodie *et al.*, 1995).

In HIV-1 infections of the CNS, oligodendrocytes appear to be targets for neuropathy. Although cultured oligodendrocytes may support a productive infection *in vitro*, direct proof of infected oligodendrocytes *in vivo* remains elusive (Sharpless *et al.*, 1992; Albright *et al.*, 1996).

1.17 Do lentiviruses infect neurones?

There are no reports that lentiviruses directly infect neuronal cells. For example, intracerebral infection with a highly neurovirulent strain of visna did not result in infections of neurones (Georgsson *et al.*, 1989).

Neuronal damage is frequently observed with lentiviral neuropathies. In visna, shrinkage and sclerosis of nerve cell bodies or swelling and/or chromatolysis, sometimes accompanied by stallitosis, is occasionally noted in inflammatory foci, but may also occur independently of lesions (Gerogsson *et al.*, 1976). In HIV-1 infection of the CNS, extensive loss of neuronal complexity of up to 50% has been reported (Masliah *et al.*, 1992).

1.18 Visna-meditated CNS pathology

Lentivirus-mediated neuropathology in the absence of specific immune responses has been demonstrated by use of *in vitro* models and in transgenic mice. For example, expression of HIV-1 genome under the human neurofilament-L (NF-L) promoter in mice, resulted in expression of the transgene in neurones concomitant with neurological syndrome (Thomas *et al.*, 1994). In similar experiments, neural expression of HIV-1 *env* under the NF-L promoter in transgenic mice also resulted in abnormal dendritic swellings in neurones. Interestingly, reactive astrocytosis, a common finding in lentivirus infections of the CNS, was also observed (Berrada *et al.*, 1995). Furthermore, intra-cerebral inoculation of HIV-1 infected monocytes into SCID mice resulted in gliosis and neuronal injury (Persidsky *et al.*, 1996).

The MVV *env* gene has also been studied as a transgene in sheep (Clement *et al.*, 1994). In these experiments, transgenic sheep were constructed that expressed the *env* gene (also containing *rev*) under the control of MVV LTR. Three animals were identified that expressed the Env glycoproteins (and Rev protein). Interestingly,

monocytes from transgenics did not express the Env glycoproteins until differentiation into macrophages. These animals had shown no obvious clinical abnormalities by the time these results were published. There has been no update on the progress of this study.

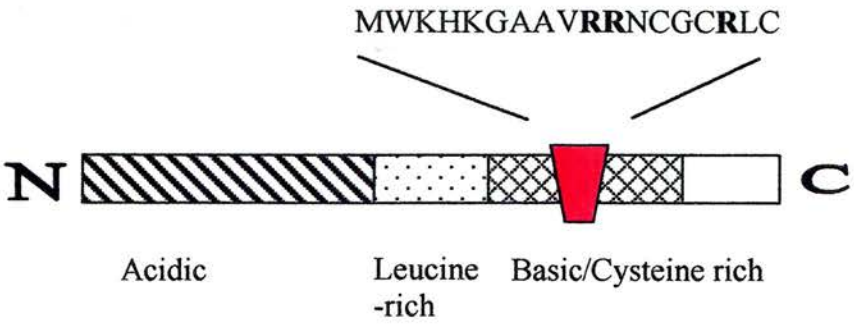
1.18.1 Neurotoxicity of Visna Tat protein

The MVV Tat protein is a 10 kDa protein, which predominates in the early phase of infection *in vitro* (see section 1.4.8). Use of synthetic peptide homologues of Visna Tat has led to the identification of an arginine-rich domain with neurotoxic properties. This neurotoxic domain resides in the N-terminus of the protein (Fig.1.3). Similarly, a neutrotoxic domain has also been identified in the HIV-1 Tat protein (Hayman *et al.*, 1993; Philippon *et al.*, 1994).

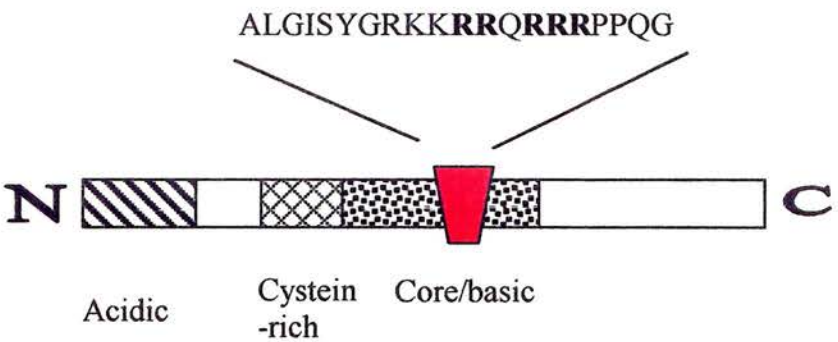
Fig.1.3 The neurotoxic domains of MVV and HIV-1 Tat proteins

Both MVV and HIV-1 Tat proteins contain neurotoxic domains, which are schematically represented in red. A common feature of neurotoxin domains is the presence of arginine residues (depicted in bold-type). The neurotoxic domain of MVV is an arginine-rich stretch of 18 amino-acid peptide. N and C correspond to amino- and carboxy-termini respectively.

Visna Tat protein



HIV Tat protein



1.19 Breed susceptibility to visna

The susceptibility to clinical visna appears to be breed-specific. In contrast to Icelandic sheep, which are highly prone to visna, European breeds very rarely develop visna. Maedi is by far the most common clinical outcome of infection with MVV in British breeds (N. Watt, pers. comm.). Intracerebral inoculation of Icelandic strain of MVV, K1514, into British breeds (Corriedale, Dorset, and Hampshire) resulted in a transient localised CNS inflammation and demyelination, followed by scar formation (astrocytosis) and recovery. Although virus could be isolated from the brains of these animals up to one year post-infection, no viral RNA could be detected (Zink *et al.*, 1987).

1.20 Summary

Host-specific lentivirus infections may result in an insidious and protracted persistent infection, with the inevitable tissue-specific pathology. This protracted course of infection has been demonstrated to be partly due to the restricted replication of viral genome in cells of monocyte/macrophage lineage.

The presence of virus-positive cells has been associated with tissue pathology. The CNS is one of the main organs that is both infected and pathologically affected in all lentiviral infections. The pathology in the CNS is directly linked to the virus infection. Although T-tropic lentiviruses may infect the CNS in primate lentiviruses and FIV, overwhelming evidence indicates that M-tropic lentiviruses are the main virus variants that infect the CNS early in the course of infection.

MVV does not infect T- or B-lymphocytes and it appears to be solely an M-tropic lentivirus. Moreover, MVV, like other lentiviruses results in CNS pathology (visna), and with similar, although not identical, brain lesions.

The pathological appearance of the CNS lesions because of visna infection (and other lentiviruses) would indicate that immune mediated responses contribute to neuropathology. This is feasible, at least for the initial stages in the course of infection. This specific immune-mediated damage in the CNS may be either direct, for example by CTL, or indirect via release of soluble factors, for example cytokines. The protracted course of infection would also suggest that these virus-specific immune-mediated responses might contribute to restricted replication of lentiviruses.

The low frequency of productively infected cells in the CNS, the presence of lesions removed from sites of inflammatory lesions and virus antigen-positive cells have lent

support to the bystander damage hypothesis. Therefore, the neuropathology because of lentivirus infections may be categorised into two interconnecting mechanisms:

1.20.1 Immune-mediated neuropathology

Specific immune-mediated responses may result in tissue damage. The presence of CD8+ T cells in visna CNS is an example. However, tissue damage by antigen-specific T cells may also result in bystander damage, via release of cytokines. This is best exemplified by murine EAE models of MS. In addition, the presence of cytokines in the CSF of AIDS patients would support this hypothesis. Damage to the cells of BBB either directly or indirectly, may have an additional role in CNS pathology. However, the presence of CNS lesions concomitant with severe immunosuppression in primate lentiviruses would suggest that mechanisms other than specific immune responses might play a role in disease progression in the CNS.

1.20.2 Virus-meditated bystander neuropathology

The pattern of CNS lesions and a direct correlation with infected cells and neuropathology prompted suggestions that virus infection *per se* may also contribute to neuropathology. This proposal has been strengthened by both *in vitro* and transgenic models, where virus-encoded gene products can contribute to neuropathology in the absence of immune effector cells.

Cells of monocyte/macrophage lineage have been shown to harbour viral antigens. Microglial cells are believed to be the CNS equivalent of tissue macrophages and therefore susceptible to lentivirus infection. The HIV-1/SIV neuropathies suggest that brain microglial cells may play a crucial role in disease pathogenesis.

The mono-tropism of MVV for cells of monocyte/macrophage lineage makes this lentivirus an ideal model to study the role of brain microglial cells in lentivirus infections of the CNS.

1.21 Aims of the project

At the start of this project, there was no published report on the role of brain microglial cells in visna neuropathogenesis/pathology. Therefore, the aims of this research project were as follows:

1. Establishment and phenotypic characterisation of primary ovine glial cultures with emphasis on microglial cells
2. Susceptibility of enriched ovine microglial cultures to visna infection *in vitro*
3. Functional alteration of microglial cells after MVV infection with emphasis on role of cytokines as mediators of visna pathogenesis/pathology
4. Neurotoxicity of microglia as a result of infection with MVV
5. Consequences of MVV-infected cells in brain parenchyma in the absence of specific immune responses

CHAPTER TWO
Materials and Methods

2 Materials and Methods

2.1 Chemicals

The chemicals and reagents used in experimental procedures were obtained from the following sources, unless stated otherwise: Sigma Chemical Company, Poole, Dorset; Gibco/BRL, Paisley, Strathclyde; Amersham International, Amersham, Buckinghamshire; Boehringer Mannheim, Lewes, East Sussex; Northumberland Biologicals Ltd., Cramlington, Northumberland; Promega Ltd., Southampton, Hampshire.

The composition of buffers and stock solutions are given in the appendix.

2.2 Molecular biology

2.2.1 Media for bacterial growth

Bacterial cultures were grown in Luria Bertani (LB) broth or on LB agar plates. The LB medium was 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.171 M NaCl at pH 7.5. For LB plates, 1.5% (w/v) agar was added to the LB medium. Cultures were made selective by the addition of appropriate antibiotics at 50 μg/ml, (stock solution at 50 mg/ml, filter-sterilised and stored at –20 °C), unless stated otherwise. Autoclaved LB/agar solution was allowed to cool down to approximately 40-50 °C, before the addition of antibiotic (s).

2.2.2 Bacterial transformation

Commercially obtained competent cells kept at -70 °C were thawed on ice. Plasmid DNA at varying concentrations (20 –200 μg, and in no more than 15 μl) were added to the cells. This was incubated on ice for 30 minutes. Cells were heat-shocked at 42 °C for one minute, and placed immediately on ice for 2 minutes. One ml of pre-warmed LB medium was added to the transformed cells and incubated at 37 °C, with shaking (200 rpm) for one hour. Alternatively, a richer medium [Bacto trypton (2%); Bacto yeast extract (0.5%); NaCl (10 mM); KCl (2.5 mM); $MgCl_2$ (10 mM); $MgSO_4$ (10 mM); and glucose (20 mM)] can be used instead of LB medium. The incubation step allows for the expression of antibiotic-resistant gene. The tube was microfuged at top speed for five seconds, and the pellet resuspended in 100 μl of LB medium, pre-warmed at 37 °C. Twenty-five and 75 μl aliquots were placed on agar plates with appropriate antibiotic (s). Plates were left right side up for 15 minutes at 37 °C, for

the medium to be adsorbed into the agar. Plates were then inverted and incubation was continued overnight.

2.2.3 White/blue colony selection

To select for recombinant colonies, 5-Bromo-4-chloro-3-indoly-β-D-galactopyranoside (X gal) was used. X-gal stock solution was dissolved in N-N´-dimethyl formamide at 40 mg/ml, and 25 µl was spread on top of each LB agar plate, prior to application of bacteria and was allowed to diffuse into the agar for one hour at room temperature. The commonly used inducer, isopropoyl-β-D-thio-galactopyranoside (IPTG) was not requird in TA cloning experiments, since the competent bacterial strain INVαF´ [endA1, recA1, hsdR17 (r-k, m+k), supE44, 1-, thi-1, gyrA, relA1, f80 lacZΔM15Δ (lacZYA-argF), deoR+, F´] (Invitrogen), does no express the *lac*I repressor.

2.2.4 Hirt DNA isolation

Unintegrated provirus was extracted from infected cells by the modified method of Hirt (1967). Infected sheep chondrocyte monolayers grown in T25 tissue culture flasks were rinsed with phosphate-buffered saline (PBS), treated with one ml of lysis buffer (100 mM Tris-HCl pH8.0, 1 M NaCl, 10 mM EDTA, 0.2% SDS) for 10 minutes at 37 °C, and then overnight at 4 °C. Cell extracts were centrifuged at 15000 x g at 4 °C. The supernatant was treated with proteinase K (100 µg) overnight at 37 °C. DNA was extracted twice with phenol/chloroform, followed by precipitation with 1/10 volume of 3-M sodium acetate (pH 5.2), and 2.5 volumes of ethanol. After centrifugation in a bench-top microfuge at 13000-rpm (12000 x g) for 20 minutes, DNA pellet was dried and resuspended in 100 µl of sterile distilled water (SDW). The concentration of DNA was determined by spectrophotometry at 260nm, and adjusted to one mg/ml.

2.2.5 Small-scale plasmid preparation ("minipreps")

Qiagen™ columns (Hybaid Ltd.) were used for isolation of plasmid DNA. The procedure is based on the alkaline lysis method of Birnboim and Doly (1979). Briefly, after harvesting overnight cultures (5 ml), the bacteria were lysed in NaOH/SDS, and in the presence of RNase A. This is based on the principle that SDS denatures the cellular proteins, while alkaline conditions cause the chromosomal and plasmid DNA to denature. The lysate was neutralised by the addition of acidic

potassium acetate. The high concentration of the salts causes denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate, while shorter plasmid DNA renatures and stays in solution. The denatured plasmid DNA was eluted with 80 µl of SDW. The minipreped plasmid DNA was analysed by restriction enzyme digests and sequencing.

2.2.6 Restriction enzyme digests

DNA at 1-5 µg was digested with appropriate restriction enzymes, and under the assay conditions recommended by the suppliers. Restriction digested DNA was checked by running an aliquot (2-5 µl) on an agarose gel.

2.2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used for qualitative/quantitative analysis of DNA (plasmid minipreps, enzyme digests, PCR bands) and RNA samples. Sigma Type I low IEE agarose was used for casting gels. Gels were cast at concentrations of 1-2% (w/v), in 1 x TAE buffer plus 0.5 µg/ml of ethidium bromide (EtBr). Samples were mixed with 0.2 volume of 5 x loading buffer [15% Ficoll 400 (w/v) plus 50 µg of Orange G (Sigma)], prior to loading in individual wells cast in the gel. The gel was run in running buffer (1 x TAE), at 80-100 volts (5 volts/cm). Electrophoresis was allowed to continue until the dye front had travelled at least 2/3 of distance along the length of the gel.

For qualitative analysis of RNA, samples were treated as outlined for DNA samples, except that RNA samples were heated to 80 °C, for 3 minutes and snap-cooled on ice for a further 3 minutes, prior to loading into gel. This treatment prevents RNA molecules assuming a secondary structure.

Nucleic acid bands were visualised by UV transillumination at 302 nm. An imager was used for obtaining permanent records.

2.2.8 Alkaline transfer of nucleic acids to hybridisation membranes

To determine the specificity of DNA bands after electrophoresis, gels were transferred to Hybond-N+™ (Amersham) or Genescreen Plus™ (NEN, Du Pont). The alkaline transfer protocol is based on the method of Southern (1975). The gel was pre-soaked in denaturing/transfer solution (0.4N NaOH), on an orbital shaker for 20 minutes. The gel was laid on a wick of 3MM Whatmann paper connecting the two reservoirs on either side of an electrophoresis tank. The gel was then overlaid with a pre-wetted membrane, two sheets of gel-sized 3MM paper. This construction was then overlaid

with a pile of paper towels. A weight (~ one Kg) was placed on top of the pile to assist the capillary motion of DNA through the gel and onto the membrane. The transfer was allowed to proceed overnight. After the transfer, the blot was rinsed with 2 x SSC and dried in a vacuum oven at 80 °C. At this stage, the blots can be kept indefinitely prior to hybridisation.

2.2.9 Southern (blot) hybridisation

Pre-hybridisation and hybridisation were carried out at 45 °C in a Hybaid™ hybridisation oven. The pre-hybridisation and hybridisation solution were identical, except that hybridisation solution also contained the radiolabelled probe. The hybridisation solution was prepared from pre-made stock solutions and used freshly. The hybridisation solution contained 1 x Denhardt's solution, 2 x SSC, 1% SDS and 100 µg of salmon sperm DNA (see appendix).

After incubation for one hour, the pre-hybridisation solution was replaced with the hybridisation solution (containing the probe), and the incubation was carried on overnight. Blots were subsequently washed with 0.5% SSC, on an orbital shaker for 15 minutes at room temperature. This was followed by a 30 minutes wash at 50 °C in a stationary water bath. The blots were dried in a vacuum oven at 80 °C for one hour. At this stage, either the DNA bands were visualised by X-ray autoradiography or image analysed using a phosphoimager.

2.2.10 Radio-isotope labelling of oligodeoxynucleotide probes

Gene-specific oligonucleotide primers were end-labelled with T4 polynucleotide kinase (PNK) (NBL) in the presence of Redivue™ ^{32}P γ ATP (Amersham, Cat. No.AA0068). The PNK assay contained one microlitre of gene-specific primer (100 pM), 2.5 µl of 10 x PNK buffer [700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, and 50 mM DDT; provided with enzyme], 19 µl of SDW, 0.5 µl (5 U) of PNK, and 2 µl (20 µCi) of radio-isotope. The mixture was incubated at 37 °C for one hour, followed by heat-inactivation at 70 °C for 5 minutes. The labelled probe was kept at -70 °C and used within two weeks of synthesis.

2.2.11 Radio-isotope incorporation

The extent of isotope incorporation was monitored by tri-carboxylic acid (TCA) precipitation. One microlitre of labelled probe was spotted onto a GF/C filter (Whatmann). This represents the total count. Another microlitre of labelled probe was diluted with one ml of 10% ice-cold TCA and left on ice for 10 minutes. Using a

Biorad vacuum manifold, the second filter was washed through a filter paper with five ml of absolute alcohol, followed by five ml of ice-cold 10% TCA. This represents the incorporated count. Both filters were dried at 65 °C for 15 minutes. Dried filters were placed in microfuge tubes, to which one ml of Hisafe™ scintillation liquid (Wallac Beta counters) was added. A Wallac™ Micro-Beta counter was used. The percentage of incorporated radioisotope ranged between 15-30%. It is recommended that at least 10^6 counts per blot be used. Routinely, five microlitres of labelled probe was used in five ml of hybridisation solution per blot.

2.2.12 X-ray autoradiography

Probed blots were autoradiographed by exposure to X-ray film (XOMAT-AR™, Kodak), either at room temperature, or at −70 °C. In cases of PCR products, this was limited to overnight exposures at room temperature. An automated processer was used to develop X-ray films.

2.2.13 DNA molecular size markers

Bacteriophage λ DNA, cut with EcoRI and HindIII, was run at the final concentration of one microgram with as molecular size marker. This commercially-obtained reagent (BRL) gives DNA fragment sizes of 21226, 5146, 4973, 4268, 3530, 2027, 1904, 1709, 1375, 947, 831, 564, and 125 base pairs.

2.2.14 Gene Cloning

Prior to cloning the gene of interest, the PCR product was run on a 2-3% low-melting temperature gel (LMG; Sigma) at 4 °C. Bands of interests were visualised under UV illumination, cut out into a microfuge tube and genecleaned.

The Geneclean II™ kit (BRL) was used for gene cleaning prior to cloning procedures. This technique is based on properties of silica matrix, which binds nucleic acids. Gene of interest, amplified by primer-specific PCR, was run on an LMG, cut out, and placed in a microfuge tube. To this, three volumes of six molar sodium iodide (NaI) were added and the mixture was vortexed to dissolve the gel fragment. Five microlitres of Glassmilk ™ per microgram of DNA was added, mixed, and kept on ice for five minutes, with occasional mixing. The tube was microfuged at full speed (13000 rpm), for 5 seconds, and supernatant removed. This centrifugation was repeated once more to remove any residual NaI solution. The pellet was washed three times with 400 µl of New Wash ™, first by vortexing, followed by microfugation at

top speed for five seconds. The final spin was repeated to remove any residual New Wash. The pellet was resuspended in 20 µl of Sigma water, kept at 50 °C for 2-3 minutes, and then spun at 30 seconds. Approximately 15 µl of the supernatant was removed carefully. This (15 µl) suspension contained the "cleaned" DNA. Cleaned DNA was checked by agarose gel electrophoresis by running an aliquot (2 µl).

2.2.15 TA cloning

The TA™ cloning kit (Invitrogen) was used for cloning of PCR fragments, after gene cleaning. The TA system takes advantage of non-template dependent activity of DNA polymerase, ***Thermus aquaticus*** (Taq pol) (GibcoBRL, Cat. No.18038-026). This enzyme is heat-stable and therefore synthesizes DNA at elevated temperature from single-stranded templates in the presence of a primer. The enzyme lacks any 3´ to 5´ proof reading activity, which results in addition of a single deoxyadenosine to the 3´-end of the DNA molecule during the PCR reaction. The TA plasmid vector contains single 3´-end deoxcythymidine overhangs, allowing for the ligation of PCR fragment into the plasmid in the presence of T4 DNA ligase.

2.2.15.1 Ligation of PCR products into TA plasmid

Cleaned PCR fragments were ligated into the TA plasmid. This was achieved by incubation of five microlitres of cleaned gene with one microlitre of vector (fragment to vector ratio of 1:1), one microlitre of ligase buffer, and one microlitre of ligase (1U/µl) in a total assay volume of 10 µl. A second ligation assay contained a PCR fragment to vector ratio of 2:1, to improve the probability of ligation. The assay mixes were incubated at 12 °C overnight. For transformation, 10 µl of ligation assay mix was added to 50 µl of INVαF´ OneShot™ bacteria (Invitrogen).

2.2.16 RNA isolation

RNeasy™ spin columns (Qiagen) were according to the manufacturer's recommendations for isolation of total RNA. Total RNA was eluted from RNeasy columns with 20 µl of Sigma water. The eluted RNA was either kept at –70 °C, or kept on ice for immediate synthesis into complementary DNA (cDNA). To check the integrity of isolated RNA, an aliquot was run on a 2% agarose gel (see section 2.2.7). The presence of two distinct bands corresponding to the 18- and 28-S ribosomal RNA was indicative of an intact preparation, otherwise the preparation was deemed degraded and therefore discarded.

The eluted material from RNeasy column also contains between 5- to 10% DNA contaminants. Most primers were designed to span introns, which results in a distinction between RNA-derived cDNA and genomic DNA. However, to prevent any false signals from genomic DNA in semi-quantitative analyses of mRNA-derived cDNA, the isolated RNA samples were pre-treated with DNase I (RNase-free; Pharmacia Biotech, cat. No. 27-0514-01), prior to reverse transcription in order to degrade any contaminating genomic DNA.

2.2.16.1 DNase I treatment of cellular RNA

To isolated cellular RNA (10 µl/~ 2 µg), the following was added: five µl of 5 x RT first strand/RT buffer [250 mM Tris-HCl (pH 8.3 at room temperature); 375 mM KCl, 15 mM $MgCl_2$; provided with the enzyme]; 1.5 µl of random hexamers (1nM); 2.5 µl of dNTPs mix (1mM); one µl of DTT (50 mM); three µl of Sigma water, and 0.5 µl (4 U) of DNase I. The assay mixture was centrifuged at top speed for five seconds and incubated at 37 °C for 15 minutes. The DNase I was heat-inactivated at 80 °C for five minutes. The reaction mix was microfuged briefly, and snap-cooled on ice for three minutes, prior to reverse transcription.

2.2.16.2 Reverse transcriptase (RT) of cellular RNA

Reverse transcription involves the synthesis of DNA from an RNA template. This reaction was performed in the presence SuperScript™ II RNase H^- Reverse Transcriptase (Life Technologies, Cat. No.18064-014). This enzyme is purified from *E. coli* containing the *pol* gene of Moloney murine leukaemia virus (M-MuLV). The enzyme has a 3´ to 5´ polymersae activity without an RNase H activity. The enzyme is genetically engineered by the introduction of a single point mutation in the RNase

H active centre. This modification prevents the degradation of RNA in the first strand cDNA synthesis, resulting in more full-length cDNA molecules and greater yields of first strand cDNA.

To the DNase I-treated RNA mixture, RNasin (1 µl, 4U) (GibcoBRL), and RT (one µl, 200U) were added. The mixture was incubated at 45 °C for one hour. The enzyme was heat-inactivated at 95 °C for five minutes. The synthesised cDNA library was kept at –20 °C for long-term storage.

2.2.17 Housekeeping gene

cDNA synthesis was checked by amplifying a housekeeping gene. This was achieved by amplification of the gene for the ubiquitous alpha sub-unit of (Na^+/K^+) ATPase pump (Woodall *et al.*, 1994). The amplification of ATPase gene was used to check for contaminating genomic DNA, integrity of RNA preparation and cDNA synthesis as well as the base line for semi-quantitation of stimulus-dependent genes, such as cytokines.

Sense primer: 5' **GCTGACTTGGTCATCTGC**
Anti-sense primer: 5' **CAGGTAGGTTTGAGGGGATAC**
Internal probe: 5' **CATCCCCTGCTGGAAGACGGAATT**
Expected PCR fragment size: 167 bp Mg Cl_2 (final concentration): 3.5 mM
Hot start: 80 °C, 5 min PCRR : 95 °C 1 min, 55 °C 1 min, and 72 °C 2 min, 35 cycles

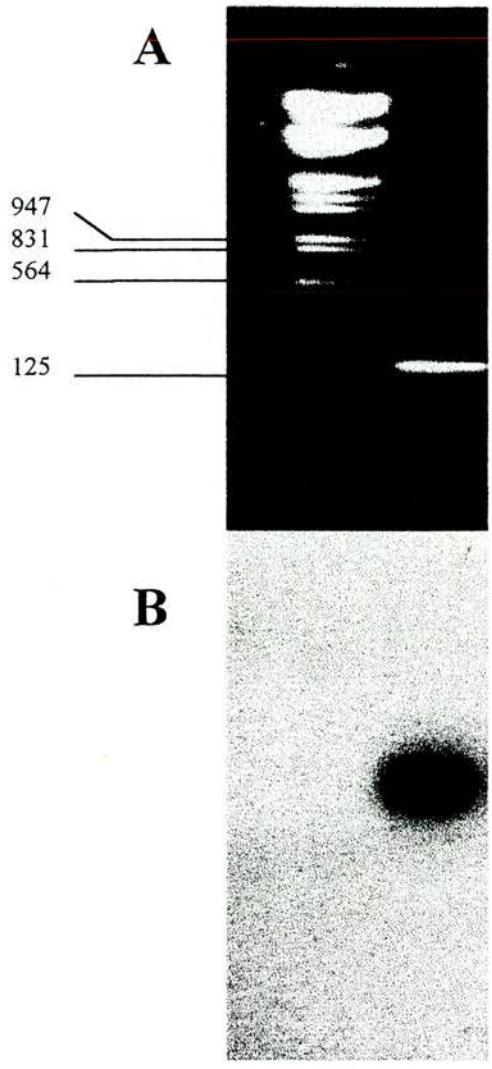


Fig. 2.1 ATPase gene amplification

The ovine housekeeping gene, ATPase, was used as the internal control. Total cellular RNA was isolated by RNeasy method. The DNAse-1-treated RNA was amplified using the standard PCR protocol. The resolution of PCR product on agarose gel resulted in a single band of 167bp (A). Subsequent Southern blot analysis using the gene-specific internal probe gave a single band (B). Numbers on the left panel refer to nucleotide base pairs of the marker DNA.

Lane M: λ cut with HindIII and EcoRI

Lane 2: Total RNA isolated from ovine CNS

2.2.18 Polymerase chain reaction (PCR)

PCR was carried out in 0.5-ml microfuge tubes, using an Omnigene™ (Hybaid) PCR machine. Each PCR reaction contained input cDNA, 100 pM of each primer (sense and anti-sense), dNTPs mix (final concentration of 100 μM), $MgCl_2$ at varying concentration (1 to 3.5 mM final concentration), and PCR buffer (see appendix). The total assay volume was 49 μl. The PCR mix was overlaid with 30 μl of mineral oil (Sigma). To prevent mispriming, the PCR mix was "hot-started" at 80 °C for five minutes, prior to addition of *Taq pol* at final concentration of one unit per reaction tube. After the addition of the enzyme, the PCR cycles were carried out at varying cycle numbers.

2.2.18.1 Primer design

Oligodeoxynucleotides were synthesised by Oswel DNA services, Department of Chemistry, King's Builidings, West Mains Road, Edinburgh. Gene-specific primer sets were designed based on the following guidelines.

1. 50-60% G+C composition.
2. Melting temperatures (Tm) of similar values, between 55-80 °C (Tm ~ 2 °C per A and T, and 4 °C for G and C).
3. No complementarity between 3 ´-ends; to prevent primer-dimers.
4. No runs of Cs or Gs at 3 ´-ends; to prevent mispriming.
5. No palindromic sequences within primers.
6. Ideal length of 15-22 bp.

2.2.18.2 Theoretical optimum annealing temperatures

The annealing temperature (Ta), is one of the important variables in a PCR reaction: too low Ta results in non-specific binding of primers and false positive results, whereas too high Ta may result in reduced/loss of product. Therefore, use an optimum Ta will result in maximum product yield. Theoretical optimum annealing temperatures (Ta^{OPT}) were calculated according to the following formulae (Rychlik *et al.*, 1990).

$$Ta^{OPT} = 0.3\ Tm^{primer} + 0.7\ Tm^{product} - 14.9 \quad \text{(i)}$$

$$Tm^{primer} = \Delta H \times 1000/\Delta S + R \times \ln(c/4) - 237.15 + 16.6 \log [K^+] \quad \text{(ii)}$$

$$Tm^{product} = 81 + 0.4\ (\%G + \%C) + 16.6 \log [K^+] - 675/l \quad \text{(iii)}$$

$$R \times \ln(c/4) = 46.7$$

Tm^{primer} is the calculated melting temperature of the product, and is based on the nearest-neighbour model of Borer and co-workers (1974), and thermodynamic values of Breslauer and co-workers (1986). ΔH and ΔS are the enthalpy and entropy for helix formation, repectively (Breslauer *et al.*, 1986) (see appendix). The total molar concentration of the annealing oligonucleotides, c, was determined empirically to be 250 pM (Rychlik *et al.*, 1990, 1991). R (1.984) is the molar gas constant and l is the length of the PCR product in bp. [K+] is the concentration of potassium ions in the PCR reaction, and is 0.05M.

Calculated Ta^{OPT} values were used as a guideline to arrive at a consensus annealing temperature (Table 2.1). This allows for use of identical conditions for most, if not all primer sets.

Primer set	Calculated Ta^{OPT} (°C)	PCR product at Ta52 °C	PCR product at Ta55 °C
Gag 1	56	+	+
Gag 2	54	+	+
Pol 1	52	+	+
Pol 2	51	+	+
Env 1	53	+	+
Env 2	52	+	+

Table 2. 1 Comparison of calculated empirical and annealing temperatures

Using the guidelines in designing of primer sets, similar, theoretical annealing temperatures (51- 56 °C) were obtained. This is exemplified by primer sets for MVV genes. PCR assays were performed for MVV genes, with Hirt DNA as input viral DNA. As table shows, use of two different annealing temperatures did not result in loss of PCR products. The standard PCR parameters were used for amplification of MVV genes.

Code	Set	Sequence	Polarity
M6109	Gag 1	TAGAGCATGGCGAAGCAAGGCT	+
M6110		TCCTGCTTGCAAATTTACAATAGG	-
M6111		AATGCCCATAGACAGTTCCCTTCTG	P
M6112	Gag 2	TTCCAGCAACTGCAAACAGT	-
M6113		TCCTTCTGATCCTACATCTC	+
M6114		AAGAATACTGAGAGGTTATGAAG	P
M6115	Pol 1	CTATGTTGTAACAGAAGCACCA	+
M6116		TGGTATTGTAAAATATGCATC	-
M6117		TATTATCCCTTCCTAATAC	P
M6118	Pol 2	ATAGTAAATGGCATCAAGATGC	+
M6119		TCCCGAATTTGTTTCTACCC	-
M6120		CATTGGCAAGTGGATT	P
M6121	Env 1	CCTCCTTATTGGAGTGCAAG	+
M6122		CCCACTGTTATATTATGTCTT	-
M6123		TATCAGTATACACCCTCATGGAATTG	P
M6124	Env 2	ATAATAGCTGCTGCAGGAG	+
M6125		ATTTIAGCCAIGAGAACCA	-
M6126		CACTATTGTGTAACCTCAA	P

Table 2.2 MVV PCR primers

The MVV-specific primer sets were designed by comparison of all published MVV sequences (Sargan *et al.*, 1991; Braun *et al.*, 1987; Querat *et al.*, 1990) and the closely related CAEV sequence (Saltarelli *et al.*, 1990) using the PILEUP programme. The British EV1 sequence was used as the consensus sequence. In cases of sequence heterogeneity, the universal base inosine (**I**) was chosen as the preferred substitution. Primer sequences are given in 5 ´ to 3 ´ orientation. Plus/ minus designation corresponds to forward and reverse primers respectively. P refers to primer sequence, used as probe for Southern blot hybridisation.

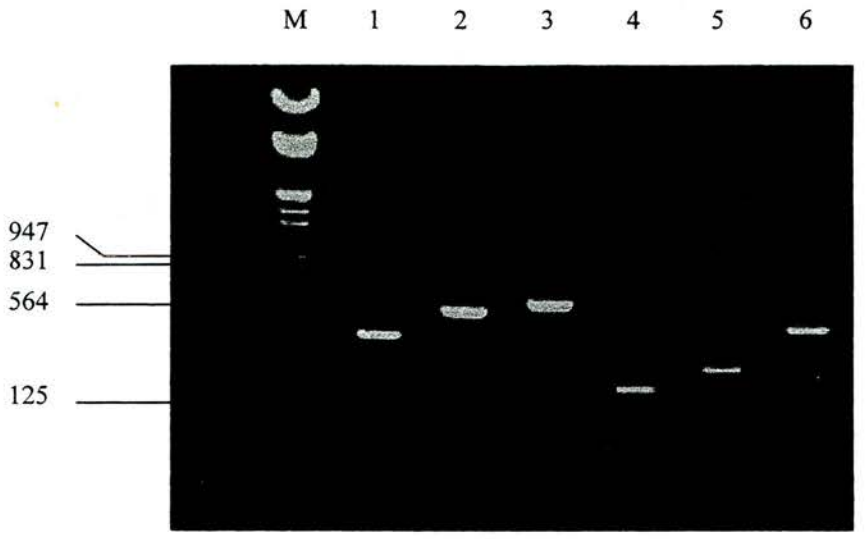


Fig. 2.2 Amplification of MVV genes

The specificity of MVV PCR primers to amplify Visna genes were tested on virus infected sheep chondrocytes. HIRT DNA isolated from EV1-infected chondrocytes was used to amplify MVV genes using the standard PCR protocol. Ten microlitre aliquots from each PCR reaction was run on an agarose gel satined with EtBr as depicted in the image.

M: λ DNA cut with HindIII and EcoRI

Lane 1: Gag-1 primer set

Lane2: Gag-2 primer set

Lane3: Pol-1 primer set

Lane 4: Pol-2 primer set

Lane 5: Env-1 primer set

Lane 6: Env-2 primer set

Table 2.3 Cytokine PCR primers

Primer for IL-1β, IFNγ, TNFα, TGFβ1, and GM-CSF were provided by C. Woodall (Caledonian University, Scotland) . Primers for IL-10 and IL-12 were provided by B. Dutia (University of Edinburgh, Scotland). These primers were used in PCR assays for ovine cytokines performed on cellular RNA isolated from ovine microglial cells

Cytokine	Forward (sense) primer	Reverse (antisense) primer	Probe
IL-1β	TACGAACATGTCTTCCG	CCAGTTAGGGTACAGGACCAG	CGATGAGCTTCTGTGTGATGCAGC
TNFα	ATGAGCACCAAAAGCATGATCC	GAAGAGCGTGGTGGCTCC	AGGAGGTGCTCTCCAACAAAGCA
IFNγ	TGAAATACACAAGCTCCTTC	TCACCTTGATGAGTTCATTGA	GCAAGACATGTTTCAGAAGTTCTTGAACGG
TGFβ1	GCCCTGGACACCAACTACTG	TCAGCTGCACTTGCAGGAG	CCTTCCGGAAGTCAATGTAGAGCTG
IL-6	ATGAACTCCCCTCTTCACAAGCG	CGGGATCCTACTTCATCCGAATAGCTCTC	AAATGACACCACCCCAAGC
GM-CSF	AGTCCTCAAGAGGATGTGGC	GCGATCTGTGAGGTAAGCTT	AACGACAGCACTGACACTGCTGCTGTG
IL-10	ATGCCACAGGCTGAGAAC	GTTCACAGAGAAGCTCAG	ACGTCCGAGCTGCCTTCGGC
1L-12	AGATGCTGGACAGTACACTC	CAATGGGCAGGCTCTCCTC	GCTCGTGGCTGACAGCAATCAGTACT

Fig. 2.3 PCR assays of ovine cytokines

Cloned genes in plasmid vectors were used as positive controls for primer sets for ovine cytokine genes. Plasmid DNA was used in each PCR reaction, using the standard protocol (see appendix). The PCR products were run on agarose gel (A). The specificity of each reaction was checked by Southern (blot) analysis (B).

M: λ DNA cut with HindIII and EcoRI

Lane 1: IL-1β

Lane 2: TNFα

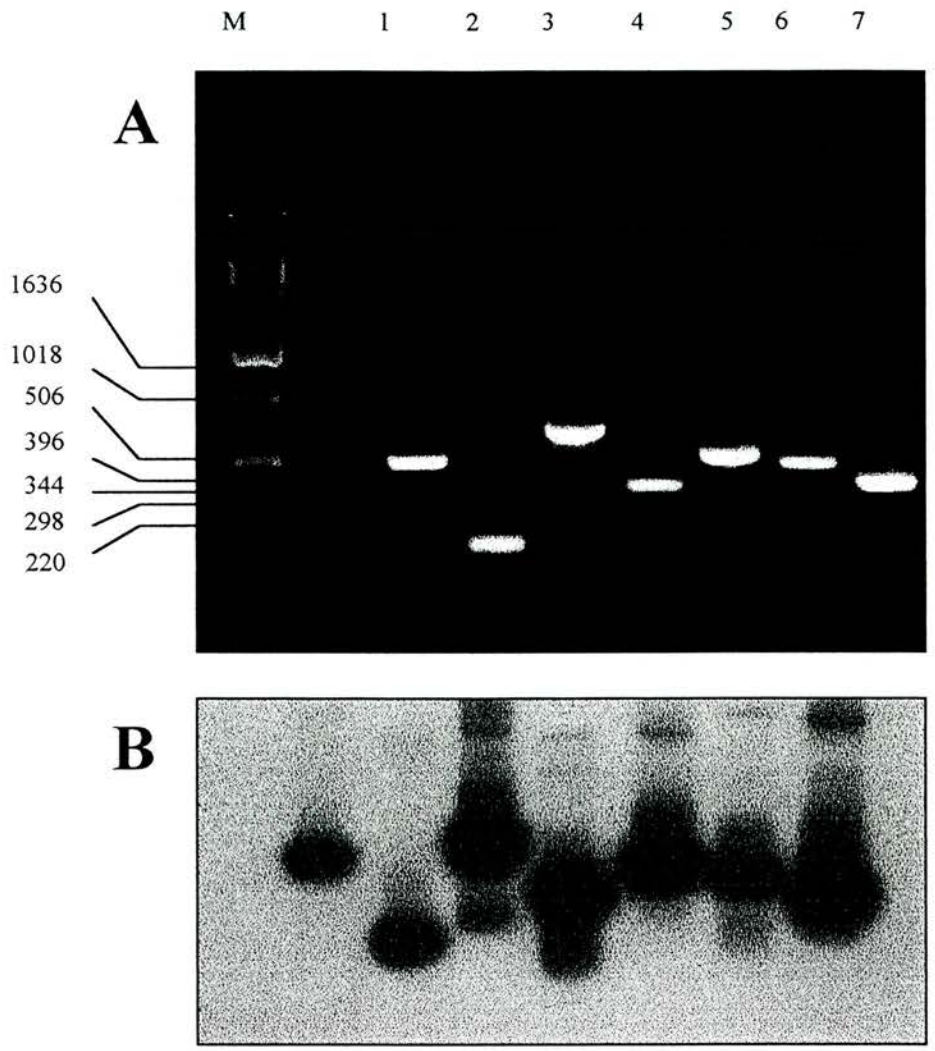
Lane 3: IL-6

Lane 4: TGFβ-1

Lane 5: GM-CSF

Lane 6: IL-10

Lane 7: IL-12


M
1
2
3
4
5
6
7
A
1636
1018
506
396
344
298
220
B

2.2.19 Cycle Sequencing

Automated sequencing was carried out by Mr. Ian. Bennet, Department of Veterinary Pathology, University of Edinburgh. Use of fluorescin-cojugated primers results in generation of labelled PCR fragments. The laser diode component of the sequencer emits radiation at 785nm, which results in electron excitation of labelled PCR fragments, in turn emitting radiation. This radiation is detected, amplified and finally digitised into a signal.

2.3 Cytospining of cells

Floating cells were collected into a 15 ml Falcon tube, centrifuged at 1200 rpm for five minutes and rinsed once with PBS. Cells were then cytospun onto Biobond™ - coated slides, using a Shandon Cytospin II, at 500 rpm for five minutes. Cytospun cells were chemically fixed with 4% PFA/PBS for 20 minutes, and rinsed and maintained in PBS. Fixed cells can be kept in PBS at 4 °C for a week.

2.4 Biobond treatment of slides

Microscopy glass slides were pre-soaked in 2% Deacon for one hour, and rinsed thoroughly in several washes of distilled water. Slides were then dried at 40 °C. Six ml of Biobond™ (British Biocell International) (2% solution in acetone for 4 minutes) was added to 300 ml of acetone, to which the slides were added. Slides were then rinsed in two washes of distilled water (five minutes each), and air-dried. Treated slides can be kept indefinitely at room temperature.

2.5 Animals

Where necessary, animal experimentation was carried out under qualified supervision and in accordance with the guidelines set out by the Home Office, UK.

2.5.1 Lambs

Lambs of mixed breeds (Texel, Black face, and Cheviot) with the average age of three days (not older than 7 days old) were obtained from the Moredun Research Institute, the Bush estate, Edinburgh, Scotland.

2.5.2 Mice

Adult female mice (between 4 to 6 weeks of age) of MF1 genotype were provided by the small animal house, department of Veterinary Pathology, University of

Edinburgh, Scotland. Adult female mice of SCID genotype were provided by the Department of Medical Microbiology, University of Edinburgh.

2.6 Tissue culture

Tissue culture procedures were carried out in category II hoods. Cells were cultured and maintained in plastic flasks and plates. Cells were maintained at 37 °C/5% CO_2 in humidified incubators. All tissue culture media were treated with benzylpenicillin (100 international U/ml), streptomycin (100 µg/ml), and Fungizone (2.5 mg/ml) unless stated otherwise. Tissue culture media were either autoclaved at 121 °C/20 minutes or sterilised through a 0.22 µ filter.

2.6.1 Sheep chondrocyte cultures

Sheep chondrocytes from synovial membrane were propagated in Dulbecco's minimum essential medium (DME), and as adherent monolayers in plastic culture flasks (Corning, UK). The medium was supplemented with 8% (v/v) of heat-inactivated foetal calf serum (FCS), penicillin/steptomycin, and 2 mM L-glutamine. Once confluency was observed, cells were removed by trypsin treatment (0.05% in versene). This constituted removal of culture medium, three rinses with versene, followed by incubation with trypsin until cells were no longer adherent. Trypsin was inactivated by addition of 5x volume of culture medium (DME/FCS). Cells were removed from the flask and collected by centrifugation, using a bench-top centrifuge (1500 rpm, 5 minutes). For re-seeding, cells were cultured at 30% confluency.

2.6.2 Virus titre determination

Sheep chondrocytes were used for virus titration. Trypsinised cells were resuspended in DME/8% FCS, and seeded into wells of a flat-bottomed 96-well plate (Nunclon, Denmark), at a density of 1 x 10^4 cells per well in 100 µl volumes. The cultured cells were incubated for a few days until a confluency of 80% was attained. Viral samples to be titrated were serially diluted in DMED/2%FCS and 50 µl were added to each well. Each dilution was tested as a triplicate. Mock-infected control was medium without the virus. Plates were cultured for two days, and further supplemented with 50 µl of DMED/2%FCS per well. The incubation was continued for another five days, or until CPE in the form of syncytia was apparent. Cells were fixed and stained with Giemsa's stain. Virus titres were calculated by using the Reed and Muench (1938) methods, which determines the 50% tissue culture infectious dose ($TCID_{50}$).

2.6.3 Giemsa staining

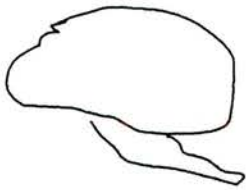
Culture medium was first removed and cells were fixed for 10 minutes with 100 µl of ice-cold acetone:methanol, at a ration of 1:1. The fixative was removed and replaced with 100 µl of 1% potassium dichromate (w/v) solution for 30 minutes. This was removed and replaced with the fixative again for 10 minutes. After the removal of the fixative, cells were air-dried for five to 10 minutes. Giemsa's stain (Gurr's improved R66, BDH, UK), freshly diluted with water, was added in 100 µl volumes to each well, and for five minutes. The stain was then removed and replaced with water for one minute, after which the water was removed and plates air-dried. The CPE was examined microscopically to determine the $TCID_{50}$.

2.6.4 Ovine glial cultures

The brain stem was the source of glial cultures. This region included diencephalone, mesencephalone, and metencephalone (excluding the cerebellum), and myelencephalone (excluding cervical portion of spinal cord caudal to obex). After lethal injection, brain stem was removed and placed in Leibovitz, medium (GibcoBRL, Cat. No. 41300-021), supplemented with benzylpenecillin (100 U/ml), strptomycin (100 µg/ml), and fungizone (2.5 mg/ml). The meninges and major blood vessels were removed. The tissue was cut into approximately 1-2 mm fragments and repeatedly triturated through a 10-ml glass pipette. The suspension was spun at 500 rpm in a bench-top centrifuge for one minute. The supernatant, which contains the glial cells was removed and kept. This process was repeated four times. The collected supernatants were pooled, and centrifuged at 1200 rpm for three minutes. Cells were suspended in DME, supplemented with N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES) (10 mM), sodium bicarbonate (NaHCO3) (10 mM), 10%FCS (heat-inactivated), and antibiotics (see above). Resuspended cells were seeded at high density. Routinely, each brain stem yielded 1-2 x 10^6 cells. Cells were cultured on poly-D-lysine treated plastic dishes (35 mm), and incubated at 37 °C/5% CO_2, and in a humidified atmosphere. Cultures are extremely vibration-sensitive at this stage, and consequently were left undisturbed for four days.

2.6.4.1 Microglial cultures

Due to the high density of cultured cells, a significant number of microglia remained as floating cells, and therefore, did not become adherent. The culture supernatant was removed and replaced with fresh medium. The removed supernatant, which is a rich



Remove brain stem and make single cell suspension

Mixed glial cultures

Re-suspend cells in 14 ml of DEM/ 10 % FCS and place in 9x 35 mm Petri dishes (1.5 ml each) Leave for 4 days

Replace medium every three days
Phenotypic staining

Mature oligodendrocytes

Re-suspend in 4 ml of DME/10% FCS in T25 flask and leave for 4 days
Remove supernate, spin and re-suspend in UltroserG
Plate on laminin-coated plates, replace medium every 2 days
Phenotypic staining

Enriched microglial cultures

Re-suspend in 4 ml of DEM/10%FCS in a T25 tissue culture flask
Leave for 4 days

Remove supernate and place in 2x 35 mm Petri dishes
Adhere by Incubating for 15 min at room temp, remove supernate and replace with fresh medium
Gradually reduce FCS to 2%

Infection experiments

Phenotypic staining

source of microglial cells, was plated on plastic tissue culture dishes (35 mm), and incubated for 15 minuets in a tissue culture incubator. This short incubation allows the adherence of microglial cells with the minimal adherence of fibroblasts and other glial cells, for example astrocytes. The medium was then removed, and adhered cells were gently rinsed once with serum-free medium and cultured with DMEM/2% FCS plus supplements (HEPES, $NaHCO_3$, penicillin, streptomycin, and fungizone). The reduced FCS proved useful in prevention of rapid growth of fibroblast-like cells.
For immunostaining procedures, microglial cells were plated at 1×10^3 -10^4 per plate. For total RNA isolation, microglial cells were seeded at 1×10^5 per plate. The medium was replaced once every 3 days.

2.6.4.2 Oligodendrocyte cultures

Ovine oligodendrocytes were cultured from mixed glial cultures. Four days after the initial seeding, the supernatant, which contained microglial and oligodendrocytes, was removed. Pooled supernatants from 4 plates were placed in a 15-ml Falcon™ tube, and using a bench-top centrifuge was spun at 1200 rpm for three minutes. The Pellet was re-suspended in one ml of Ham's F-14/ULTROSER® G medium (4% v/v) (Gibco BRL, Cat. No.091-25950H) and plated on laminin-coated, 35-mm Petri dishes. ULTROSER™ G is a serum substitute. The contaminating microglial cells were removed by L-methyl ester.

2.6.4.3 Astrocyte cultures

Ovine astrocytes remain as adherent cells after the initial seeding of mixed glial cells. Four days post-seeding, the medium was replaced with fresh DME/10% FCS plus supplements, and every three days thereafter.

2.6.5 L-methyl ester

Microglial cells can be selectively removed from glial cultures by treatment with L-methyl ester (LME). Cells were rinsed three times with serum-free DMEM, and then treated with LME (7.5 mM in serum-free DME). Treated cells were incubated between 2 hours to overnight. The medium was removed and cells were rinsed once with serum-free medium. Finally, depending on type of cells being cultured (oligodendrocytes or astrocytes), the appropriate medium was added, and incubation continued.

2.6.6 Poly-D-lysine (PDL)

To increase the adherence of mixed glial and microglial cultures, culture dishes were treated with PDL. Tissue culture grade PDL (Sigma, cat. No. P7280) was diluted in sterile distilled water (SDW) at 0.2 mg/ml, and filter-sterilised. Culture dishes were coated with PDL for 30 minutes at room temperature. PDL was then removed, and culture plates rinsed once with SDW and air-dried for two hours inside a tissue culture hood. PDL plating was carried out afresh on the day of culture. Unused PDL solution was kept at -20 °C.

2.6.7 Poly-D-ornithine (PDO)

PDO was used for coating of culture dishes prior to laminin coating. This procedure was used in culturing of ovine oligodendrocytes and murine neuronal cultures. PDO (Sigma, cat. No. P8638) was diluted in 0.15 M borate buffer (4.6g of boric acid in 500 ml of SDW, pH 8.4 with 5N NaOH) to give a final concentration of 0.5 mg/ml and sterilised through a 0.22 μm filter. Culture plates were coated with PDO overnight and at room temperature. The following day, PDO was removed, and culture dishes were rinsed once with SDW and air-dried as for PDL treatment. Unused PDO was kept under sterile conditions at 4 °C, and used within two weeks.

2.6.8 Laminin coating

Natural mouse laminin [1 mg/ml in 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, Gibco BRL, Cat. No. 23017-015] was used. Laminin was kept as 20 μl aliquots at –20 °C. For laminin coating, previously PDO-treated and dried culture dishes (35-mm) were used. A 20-μl aliquot was thawed on ice for a period of 1 hour; quick thawing of laminin may result in gelling. Thawed laminin was diluted into one ml of serum-free Hank's buffered salt solution (HBSS) without Ca^{+2} and Mg^{+2} (Sigma, cat. No. H8389), and mixed by repeated pipetting. Using a 200-μl Gilson™ pipette, 100-200 μl of diluted laminin was dispersed onto each dish (35-mm). This was achieved by creating a circle within the dish, and with a diameter of approximately 30-mm. This leaves a 3-4 mm gap to the wall of the dish. Care was taken to prevent making any contact with the wall of the dish. Laminin-coated dishes were placed in a tissue culture incubator for at least three hours. After the removal of laminin, plates were rinsed with Ham's F-14 with Saito's, and kept moist with Ham's F-14Saito' to prevent plates drying out, prior to seeding of cells.

2.6.9 Neuronal cultures

Murine trigeminal neurones were cultured from MF1 mice. Trigeminal ganglia were removed and placed into L-15 medium, supplemented with penicillin and streptomycin. After the removal of connective tissue and nerve fibres, ganglia were cut into small (1 mm) fragments. These were placed in a 35-mm petri dish with Ham's F14 medium. Collagenase (stock solution at 1.25% and stored at –20 °C, Sigma, cat. No. C3180), was diluted 1/10 in the medium to give a final concentration of 0.125% (v/v). The Petri dish was placed in a tissue culture incubator for two hours. The digested tissue was place in a 15-ml Falcon™ tissue culture tube, and spun for one minute at 1000 rpm in a bench-top centrifuge with breaks off. The supernatant was removed as much as possible, and discarded. The cell pellet was resuspended in trypsin [stock solution at 1%, kept at -70 °C and diluted 1/10 afresh in HBSS (without Ca^{2+} and Mg^{2+}) to give a final trypsin concentration of 0.1% (v/v)] (Worthington Biochemical Corporation, New Jersey, USA, code TRLVMF) and incubated in a 37 °C water bath for 20 minutes. Trypsin was neutralised with 5 ml of Saito's medium. Cells were pelleted by centrifugation as above, re-suspended in one ml of Saito's medium. Using a flamed Pasteur pipette, the pellet was triturated repeatedly to give a single cell suspension. Re-suspended cells were plated in 35-mm tissue culture Petri dishes, and incubated overnight. Routinely, neurones from four mice were cultured per single 35-mm Petri dish.

2.6.10 Saito's medium

Saito's medium was used for culturing of neuronal cells (reviewed by Bottenstein, 1984) (see Table 2.4). Stock Saito's solution was made up and kept as 11.1 ml aliquots at -30 °C. To make Saito's working strength solution, one aliquot (11.1 ml) was thawed and added to 500 ml of ready made Ham's F-14 solution, and filter-sterilised. The medium, now referred to as Saito's medium, was supplemented with five ml of L-glutamine (200 mM, GibcoBRL, Cat. No. 25030-024), with penicillin and streptomycin.

Due to the less adherent property of neurones compared to fibroblasts and Schwann cells, trigeminal neurones remain predominantly as floating cells in the supernatant or loosely adherent cells. Using a P1000 Gilson™ pipette, one ml of the supernatant was removed, with care, and placed into a 15-ml Falcon™ tube. Cells were spun at 1000 rpm for one minute. The supernatant was removed and the pellet re-suspended

in one ml of Saito's medium, supplemented with recombinant nerve growth factor (NGF) (stock solution at 10 µg/ml and kept at –70 °C, diluted in the medium to give a final concentration of 2 ng/ml) (R&D systems, Abingdon, UK, Cat. No.256-GF-100). Cells were cultured in laminin-coated, chambered 35-mm Petri dishes (Greiner Labortechnik, Germany).

2.6.11 Ham's F-14-based tissue culture media

Ham's F14 (Imperial Laboratories, UK, Cat. No. 3-791-35) is a carbonate-based medium, and was used for culturing of neuronal cells. The Ham's F-14 powder (with L-glutamine and without NaHCO3), was diluted in 500 ml of SDW, and stored in 50-ml aliquots at –30 °C. To make 1 x F-14 medium, 450 ml of SDW was added to one defrosted 50-ml (F-14) aliquot, plus one gram of NaHCO3, and pH adjusted to 6.7. Ham'sF-14/horse serum, was used in rinsing of laminin-coated Petri dishes. Heat-inactivated normal horse serum (Sigma, cat. No. H1138) was added to working strength Ham's F-14, to give a final concentration of 10% (v/v), and supplemented with antibiotics.

Supplement (stock solution)	Volume used (ml)	Diluent	Source (Cat. No.)
Path-O-Cyte 4	50	*N/A	ICN Biochemicals (81001)
Putrescine (40 mg/25 ml)	50	SDW	Sigma (P 5780)
Progestrone (1 mg/1.6 ml)	0.5	£EtOH	Sigma (P6149)
L-Thyroxine (2 mg/5 ml)	5	EtOH	Sigma (T0397)
Sodium selenite (1 mg/2.6 ml)	0.5	$PBS	Sigma (S9137)
Tri-iodo-thyronine (16.8 mg/50 ml)	5	EtOH	Sigma (T6397)

Table 2.4 Saito's solution

*: Not applicable
£: Ethanol (absolute alcohol)
$: Phosphate buffered saline

2.7 Immunohistochemistry

The following gives an account of immunohistochemical procedures used in this thesis. The antibody diluent, where applicable, was 1% (v/v) of normal goat serum (NGS) in PBS. The blocker was 3% NGS/PBS. For detection of intracellular antigens, permeabliseation of cellular cytoskeleton was carried out to allow the entry of antibody into cytosol. This was achieved by treating cells with 0.3% (v/v) Triton X-100 in PBS (TX100/PBS) for five minutes at room temperature. The fixative was 4% (w/v) para-formaldehyde in PBS (PFA/PBS). This solution was prepared freshly on the day of experiment by dissolving PFA in PBS, which was incubated at 60 °C for 45 minutes. To speed up dissolving of PFA, 10N NaOH was added drop-wise to the PFA/PBS solution. The dissolved solution was then equilibrated to 37 °C by incubation for one hour in a water bath. Where applicable, cells were fixed for 20 minutes with pre-warmed (37 °C) PFA/PBS.

All incubations were carried out in humidified chambers. In situations where light-sensitive fluorochromes were used, the incubation was carried out in humidified chambers and away from light.

2.7.1 Alkaline phosphaotase-based detection system

Alkaline phosphatase-conjugated secondary antibodies were used in detection of signals in immunostaining of infected cells and in Western blot analyses. In alkaline phosphatase (AP) detection system, the substrate (BCIP, also referred to as X-phosphate or X-phos), in the presence of nitrotetrazolium salt (NBT) is converted into an insoluble blue precipitate.

2.7.2 Immunostaining of cell cultures

Cells grown on 35-mm Petri dishes were used for immunostaining. For phenotypic characterisation of cells, immunostaining was carried out at 7-10 days post culture. All procedures were carried out at room temperature. Antibodies were either used neat, or diluted. Cells were first rinsed with pre-warmed PBS (37 °C) and with extreme care as not to dislodge any cells. For cell surface markers, live cells were first incubated with the primary antibody for one hour at room temperature, rinsed with PBS as above, and fixed with PFA/PBS. The fixative was removed and cells rinsed with PBS as above, followed by a blocking step with NGS/PBS for 30 minutes. Cells were then incubated with secondary antibody for one hour. Cells were then

rinsed with PBS as above, mounted in Vectashield™ (Vector Laboratories, Cat. No.H-1000), and viewed under microscope.

For immunostaining of intracellular antigens, cells were first fixed as above, then permeabilised with Triton-X100/PBS for five minutes, before incubation with the first antibody.

2.7.3 Terminal deoxyneucleotide transferase (TdT)-mediated UTP nick end-labelling (TUNEL)

This technique was employed to investigate the mode and extent of cellular death in MVV-infected microglial cells. The TUNEL technique was performed on cells from infected cultures at four days post-infection. Floating cells were obtained by cytospin. Fixed cells were rinsed in PBS and permeabilised with X-100/PBS for 10 minutes. Cells were then treated with DNase I buffer [30mM Tris-HCl (pH 7.2); 140 mM sodium cacodylate; 4 ml MgCl2; 0.1 mM DDT] for 20 minutes, before DNase I digest (5 μg/ml) in DNase I buffer at 37 °C for 30 minutes. Cells were washed with three washes of PBS, and equilibrated with the TUNEL buffer [30 mM Tris-HCl (pH 7.2); 140 mM sodium cacodylate; 1mM cobalt chloride] for 20 minuets. Equilibrated cells were incubated with the TdT enzyme plus labelled deoxynucleotides [TdT (20U/40 μl); Digoxigenin-11-dUTP (20 μM) in TdT buffer] at 37 °C for two hours, and in a humidified chamber. The reaction was terminated with one rinse with 2 x SSC, for five minutes at room temperature. Samples were equliberated with PBS and then incubated with blocker for 30 minutes, followed by incubation with sheep anti-digoxigenin-fluorescin isothiocyonate (DIG-FITC) at 1/20 in antibody diluent for one hour at room temperature.

For double staining of infected cells with MVV, for visna p25 major core antigen and TUNEL, cells were treated with undiluted anti-p25 mAb (VPM70), after the blocking step in TUNEL labelling. Cells where treated with biotinylated goat anti-mouse antibodies (1/100) in diluent for one hour. Cells were then rinsed three times with PBS, and incubated with rhodamine-avidin conjugate (1/100) and anti-Dig FITC (1/20) for one hour. Cells where finally rinsed in three washes of PBS and mounted in Vectasheild.

Specificity	Species	Source (cat. No.)
NF-M	Rabbit anti-rat	$
GFAP	Rabbit anti-cow	DAKO (Z0334)
A2B5	Rat anti-mouse	*BM (N1300016)
O4	Mouse monoclonal	BM (1518925)
Galc	Rabbit anti-mouse	Sigma (G9152)
CNPase	Mouse monoclonal	Sigma (5922;clone 11-5B)
F4/80	Rat anti-mouse	Serotec (MCAP497)
BS-I_4	N/A	Sigma (L2140)
Fibronectin	Rabbit anti-human	Sigma (F3648)

Table 2.5 Antibodies I

The rat monoclonal antibody, which recognises mouse NF-M was kindly provided by Prof. Brophy, Preclinical Veterinary Sciences, University of Edinburgh. The biotinylated lectin from *Bandeiraes Simplicifolia* BS-I isolectin B4 (BS-I4) recognises β-N-acetyl-D-Galactose (β-NacGal).

$This antibody was a kind gift from Prof. Brophy, department of Preclinical Veterinary Studies, University of Edinburgh.

*BM: Boehringer Mannheim

Monoclonal	Specificity
SBUT-6	CD1
VPM19	MHC-ClassI
VPM36	MHC-ClassII DR
VPM54	MHC-ClassII DQ
LFA-1	CD11a
IL-A15	CD11b
OM1	CD11c
VPM65	CD14
VPM70	P25 (MVV major core protein)
*R158 and R159	Anti-Tat

Table 2.6 Antibodies II: ovine monoclonal antibodies

Ovine monoclonal antibodies raised against ovine monocyte/macrophages were obtained from the Monoclonal Unit, Veterinary Pathology, University of Edinburgh. All monoclonal antibodies were provided as tissue culture supernatants and were used undiluted.

* Antisera raised against synthetic visna Tat peptides were kindly provided by Dr. Harkiss. The anti-Tat polyclonal antibodies were used at various dilutions (1/0-1/100) in Western blots.

2.7.4 Immunohistology of SCID brains

SCID mice were sacrificed by lethal injection of pentobarbitone (100 µl), and perfused with 10 ml of saline. Perfused brains were fixed in 50 ml of 40% para-formaldehyde/0.05% (v/v) gluteraldehyde. The entire brain was processed into 50 µm free-floating sections. The cut sections were rinsed four times in PBS/Triton X-100 (0.3%) incubated for one hour in 20% (v/v) rabbit serum. Sections were incubated overnight at 4 °C in the primary antibody. For staining with F4/80, the dilution of first antibody was at 1/1000, and for GFAP staining at1/2000. Sections were then washed twice in PBS/Triton X-100, followed by incubation for one hour with the secondary antibody. This was biotinylated rabbit anti-rat (1/50) for F4/80 and for goat anti-rabbit for GFAP (1/200). Sections were rinsed twice in PBS, and incubated for one hour with the ABC Elite™ for F4/80at a dilution of 1/50, and ABC Standard for GFAP at a dilution of 1/100. This was followed by two washes with PBS, a 10-minute incubation with the substrate, DAB. Sections were finally washed four times with PBS, placed onto gelatin-coated microscopic slides and left to air dry for one week. Dried sections were dehydrated in alcohol washes, rinsed in xylene, and permanently mounted in DPX.

2.8 Polyacrylamide gel electrophoresis (PAGE)

Proteins were analysed by SDS-PAGE. This method was used as a pre-requisite to western blot analysis (see appendix). Samples were mixed with loading buffer, boiled for 5 minutes and run on the gel. Rainbow molecular weight markers (Amersham, Cat. No. CFA 756), spanning 14.3 kDa to 200 kDa in protein weights.

2.9 Western blot analysis

Western blotting was employed to analyse proteins in culture supernatants. Using a semi-dry blotting apparatus (Biorad), proteins were transferred onto a nitrocellulose-based Hybond- C membrane (Amersham). Gels were briefly rinsed in transfer buffer [25 mM Tris (pH 9.5)/20% methanol]. Three sheets of 3MM filter paper, pre-soaked in transfer buffer, were placed on the blotter, followed by the pre-soaked hybridisation membrane. The Gel was placed on top of membrane, followed by another three pre-soaked 3MM filters. The transfer was carried out at an electrical current of 120 mA for one hour. All of the following incubations and rinses were carried out on an orbital shaker. Blots were blocked with PBS/5% Marvel (fat-free protein powder) at 4 °C, overnight. They were then treated with the first antibody, either used neat or

diluted in PBS/1% Marvel for one hour at room temperature. Blots were washed in PBS/1% Marvel (five washes over 30 minutes) followed by incubation with AP-conjugated secondary antibody as per first antibody.

Blots were then washed with PBS/1% Marvel (as above) and finally rinsed in two washes of 0.1M Tris-base (pH 9.5) over 10 minutes. Blots were developed by treatment with freshly prepared substrate mix [9 ml of Tris-HCl (pH9.5), one ml of NBT, 100 µl of BCIP, and 100 µl of 2 M $MgCl_2$]. After the appearance of protein bands (within 10 minutes), blots were rinsed with excess water and air-dried.

2.10 Inoculation of microglial cells into SCDID mice

Ovine microglial cells were inoculated into the striata of adult SCID mice. Each mouse was inoculated with 15000 ovine microglial cells in a total volume of 3 µl. The cells were injected into the right striatum of each mouse, with the left striatum serving as an internal control. All intracerebral inoculations were performed by Miss. I. Starling, Department of Preclinical Veterinary Studies, University of Edinburgh.

2.10 1 The inoculum

Microglial cells were infected five days after the initial seeding. Medium was removed and cells were rinsed with serum-free medium prior to infection with the Icelandic strain 1514K at MOI of 0.1 Control cultures were mock-infected with serum-free medium alone. Cultures were incubated overnight. Cells were rinsed three times with versene and incubated in HBSS (without Mg^{2+} and Ca^{2+}) for one hour at room temperature. Using a P1000 Gilson™ pipette, cells were dislodged from the plate by gentle and repeated pipetting of medium over the cells. Detached cells were collected into a 15-ml Falcon™ tube and centrifuged at 1200 rpm for three minutes. The pellet was re-suspended in one ml of sterile PBS. Finally, cells were counted and the density was adjusted to 5000 cells per one microlitre.

2.11. Tat and Ovalbumin (Ova) peptides

The 18 residue synthetic Tat peptide (MWKHGAAVRRNCGCRLC) and Ovalbuminpm7 peptide (ISQAVHAAHAEINEAG) were dissolved in Ham's F14 medium without serum. The dissolved peptide solution was kept at -70 °C and used at the final concentration of 27 pM.

2.12 The virus stock

The Visna virus used throughout this thesis was the Icelandic isolate, K1514. The stock virus (TCID50=$3x10^4$) was kept at -70 °C.

2.13 Transmission electron microscopy (TEM)

MVV-infected and mock-infected ovine microglial cells were harvested, washed once with PBS and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), at 4 °C for two hours. Fixed cells were then washed with cacodylate buffer and post-fixed in 1% osmium tetroxide for one hour at 4 °C. Cells were dehydrated through a gradient acetone, and embedded in Araldite. Sections (60 nm- thick) were cut and stained with uranyl acetone and lead citrate by the EM unit, Department of Preclinical Veterinary Pathology, University of Edinburgh. Sections were examined by a Philip 400 EM.

CHAPTER THREE
Phenotypic Characteristics of Mixed Ovine Glial Cultures

3.1 Introduction

A common feature of all lentiviral infections is the extensive involvement of the CNS. This is partly due to the complexity of brain as an organ, which performs complex functions. This complexity of architecture and function is a product of cellular heterogeneity and organisation.

The resident cells of the CNS parenchyma are neurones, macroglia (astrocytes and oligodendrocytes), microglial cell and blood endothelial cells. Astrocytes are estimated to make up as much as 50% of glial cell population (reviewed by Tower, 1988). In the CNS, astrocytes are divided into protoplasmic (Type 1;GFAP+, A2B5-) and fibrous types (Type 2; GFAP+, A2B5+). Protoplasmic astrocytes predominate in the grey matter and fibrous astrocytes in the white matter (reviewed by Hirano, 1985). Type 2 astrocytes (GFAP+/A2B5 +) were proposed to have been derived from oligodendrocyte- type 2 astrocytes (O-2A) progenitors, whereas the origin of type 1 (GFAP+) astrocytes is uncertain (Raff *et al.*, 1983). O-2A progenitor cells may give rise to type 2 astrocytes *in vivo* (Trotter *et al.*, 1993). However, there is experimental evidence to suggest that a distinct astroblast progenitor cell population may be the precursor to astrocytes rather than O-2A progenitors (Skoff, 1990).

In the CNS, O-2A progenitor cells proliferate in germinal zones and migrate to white matter region, where they further proliferate, differentiate, and form myelin sheaths around axons (Zerlin *et al.*, 1995). Oligodendrocyte development takes place during the late gestation and early post-natal periods, after neuronal maturation has occurred (Skoff, 1990).

Microglial cells may constitute up to 20% of glial cells in the CNS. In rodents, it has been demonstrated that microglia belong to the monocyte/macrophage lineage, seeded in the CNS early in embryonic development (Perry and Gordon, 1988).

3.2 Experimental design

The experimental approach undertaken in this chapter was aimed at establishing methods to isolate, culture and characterise primary ovine mixed glial and enriched microglial cultures. The glial cultures would then be used to determine the susceptibility of ovine microglial cells to MVV infection *in vitro*. This approach was taken to initiate tissue culture models to study neuropathogenesis of Visna virus infection.

The data presented in this chapter were repeated at least five times. Three approaches were taken in culturing of glial cells. The first approach was to stain for cell markers using mixed glial cells. Therefore, no attempt was taken to isolate any constituent cell type. The second approach was taken to enrich for microglial cultures, since these cells were intended for subsequent infection experiments and therefore intended to be highly homogenous with minimal fibroblast contamination. The third approach taken was to enrich for and drive the maturation of oligodendrocytes (see flow diagram).

3.3 Results

3.3.1 Morphology and phenotypic characteristics of mixed ovine glial culture

Mixed glial cultures were set up from the brain stem region of newly born lambs. The staining for specific cell markers were performed on five separate brain cultures. The morphology of the mixed cultures was dynamic and changed substantially with time. In the initial stages post-seeding, 0-7 days *in vitro* (DIV), most cells had a phase-bright appearance with very few cells with flat, fibroblastic morphology (Fig.3.1). At later time points post-seeding, 8-28 DIV, there was a substantial proliferation of flat, phase-dark cells that eventually formed the bed-layer. This stratification phenomenon started with a few cells sending slender processes that radiated in all directions away from the cell body. This in turn resulted in networks of cellular processes that brought cells into close physical contact with each other. This networking was also concomitant with increased proliferation (Fig.3.1).

In the early phase of mixed glial cultures (DIV 0-4), cells with astrocytic morphology were absent. However, small phase-bright cells which stained positive with A2B5 marker were detected at DIV 4-6 (Fig.3.2). The earliest presence of GFAP+ cells was at 7-9 DIV. This was concomitant with the presence of process bearing cells with astrocytic morphology (Fig. 3.2). Interestingly, the cells with astrocytic morphology also stained positive with the progenitor marker, A2B5 (Fig.3.2).

3.3.2 Oligodendrocytes in ovine glial cultures

Cells committed to oligodendrocyte lineage were detected in mixed glial cultures at early time points post-seeding (DIV 4-5). These cells were O4+/GalC-, non-adherent and constituted 10-20 cells in each 35 mm plate of glial cells (0.1-0.2%) (Fig. 3.3). In ovine mixed glial cultures, mature GalC+ oligodendrocytes were not detected. Moreover, addition of insulin at varying concentrations (1-10μg/ml) did not affect the

maturation of O4+ precursors into GalC+ oligodendrocytes (data not shown). However, non-adherent O4+ cells obtained from mixed glial cultures and plated on laminin-coated plates resulted in generation of process-bearing GalC+ oligodendrocytes (Fig.3.3).

3.3.3 Morphology and phenotype of microglial cells in mixed and enriched cultures

A cell type existed in mixed cultures that was adherent and had minimal change in appearance over time *in vitro*. Unlike flat cells, these adherent cells had a phase-bright appearance with two distinguishing features: (1) the presence of "spikes" that radiated from the cell membrane; and (2) a large, phase-dark eccentric nucleus. This cell population was later identified as microglial cells on the bases of morphology and immunostaining characteristics (Fig.3.4) (Table 3.2).

In ovine mixed glial cultures maintained in high serum (10%), clusters of proliferating $CD14^+$ cells were present atop of the bed-layer at 21-28 DIV (Fig.3 .4). At this time point, the bed layer consisted predominantly of astrocytes and fibroblasts. The clusters of phase bright cells stained positive for microglial marker, CD14 (Fig.3.5). Furthermore, on subsequent plating, these phase-bright cells gave rise to cells with microglial morphology.

Pure (> 98%) microglial cultures were set up from mixed glial cultures at four days post culture and by adherence on cultures plates. These cells had a distinct morphology (Fig.3.4). The most prominent feature of enriched microglial cultures was presence of "spikes" on cell membrane. Moreover, the morphology of these cells also changed with time. This was in the form of elongated unidirectional processes that extended up to 20 times the length of microglial cell body (Fig.3.4).

Enriched cultures of microglial cells were characterised by immunostaining with a panel of surface markers. Microglial cells stained positive with markers common to cells of monocyte/macrophage lineage rather than the phenotypic markers associated with cells derived from the neuroectoderm (Table 3.2).

3.3.4 LME is toxic to microglial cells

Microglial cells were further characterised by their susceptibility to LME treatment. In mixed glial cultures treated with LME, only microglial cells showed the toxic effects (Fig.3.6). Toxic effect of LME appeared by four hours port-treatment. This was in the form of ballooning, and was not observed in other glial cells. Moreover,

LME treatment of mixed glial cultures resulted in specific loss of microglial cells, which occurred by 24 hours post-treatment (Fig. 3.6).

Fig. 3.1 Morphogenesis of ovine glial cells in vitro

Ovine glial cells were cultured from the brain stem region of newly born lambs. Mixed glial cell suspensions were cultured in 35 mm Petri dishes. Each brain stem was cultured in nine poly-lysine-treated plates. Mixed glial cultures exhibited dynamic morphologies over time *in vitro*. The cultures constituted predominantly of phase bright cells at 0-4 DIV (A). Cellular stratification occurred at 5-7 DIV (B). Substantial stratification resulted in a bed layer of fibroblasts and astrocytes (C). Foci of phase-bright cells (microglia) were observed in confluent cultures on top of bed layer (star) at 21-28 DIV (D). White arrows indicate the presence of microglial cells in mixed cultures (magnifications at x125).

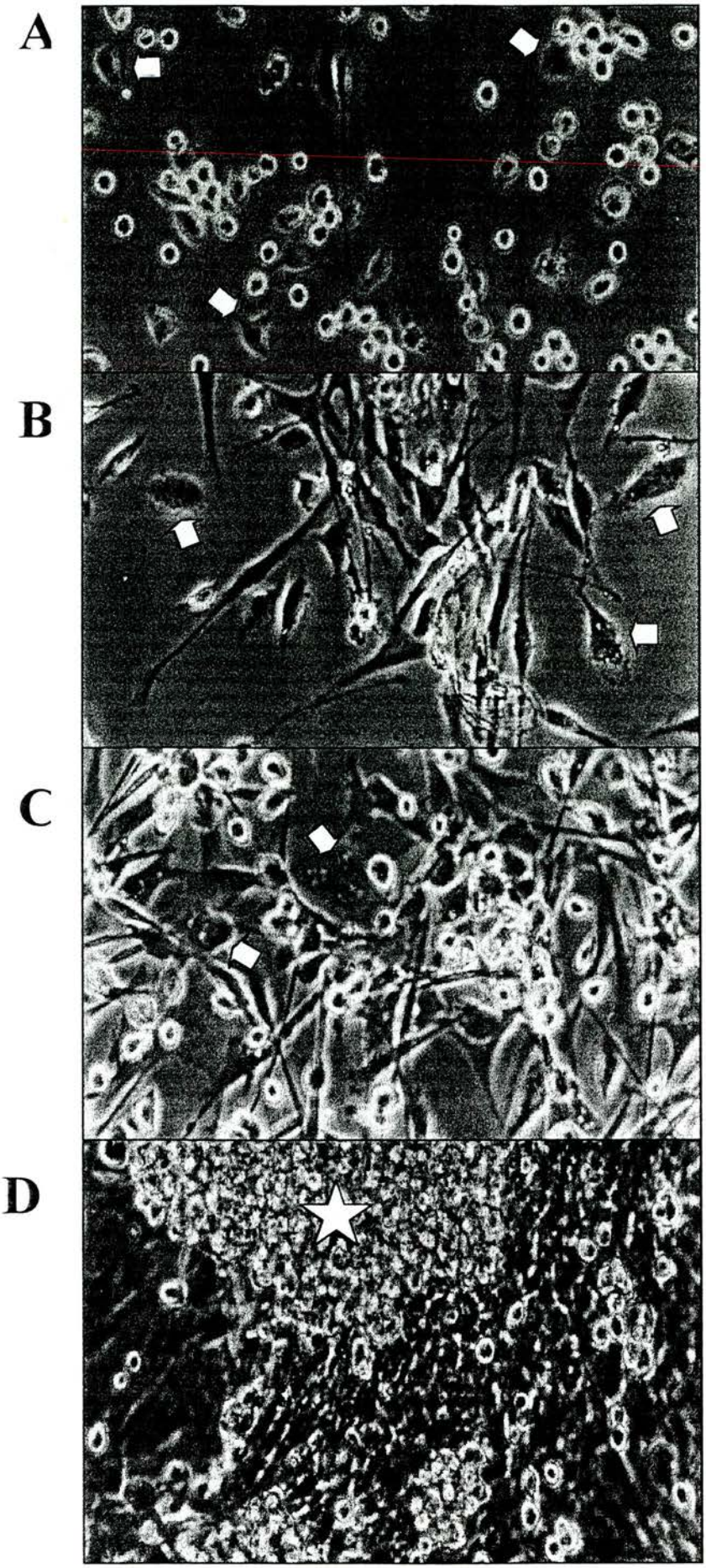

A
B
C
D

Fig. 3.2 Presence of immature A2B5 cells prior to stratification by astrocytes in mixed glial cultures

Mixed glial cells were set up as stated in the legend to Fig. 3.1. Mixed glial cultures were fixed with PFA and immunostained for different cell markers. Progenitor cells, which stained positive for A2B5, were detected in mixed glial cultures prior to GFAP staining at 4-6 DIV (A). These progenitor cells appeared to result in process bearing cells at 7-9 DIV (B). This was also concomitant with the presence of astrocytic cells, which stained positive for GFAP (C and D) (magnifications: A x 250; B and C x 200; D x 125).

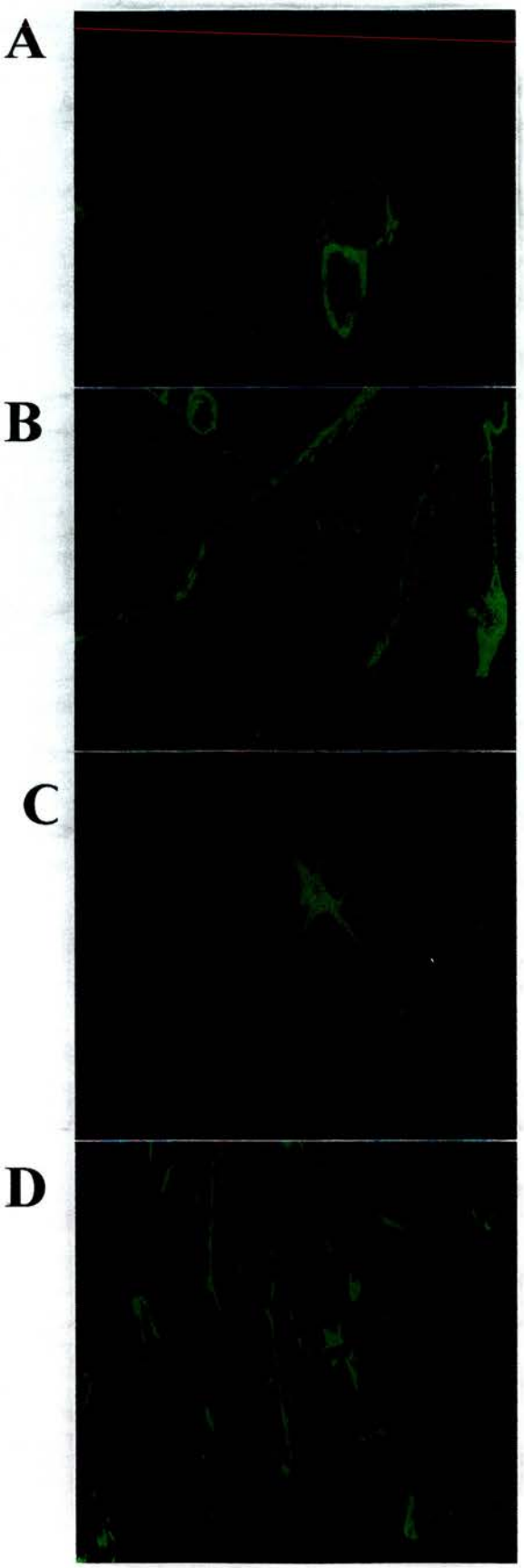

A
B
C
D

Fig. 3.3 Mature oligodendrocytes in ovine glial cultures are derived from O4+ cells

Glial cells were obtained and cultured as stated in the legend to Fig.3.1. After four days post-culture, immature oligodendrocytes (O4+) where detected as floating cells on top of bed layer in mixed glial cultures. Floating cells from mixed glial culture supernatants were collected, cytospun and stained for antibody against the O4 antigen, demonstrating lack of adherence in these cells (A). Infrequently, loosely adherent and process-bearing O4+ cells were also observed on top of bed layer in mixed glial cultures (B). Mature GalC+ oligodendrocytes with elaborate processes were observed only when O4+ floating cells were cultured on laminin-coated plates (C and D). Arrowheads point to individual cytospun cells and arrows point to elaborate cytoplasmic membranes, cultured on laminin-coated plates for two days. Magnifications: A x 250; B, C and D x 200).

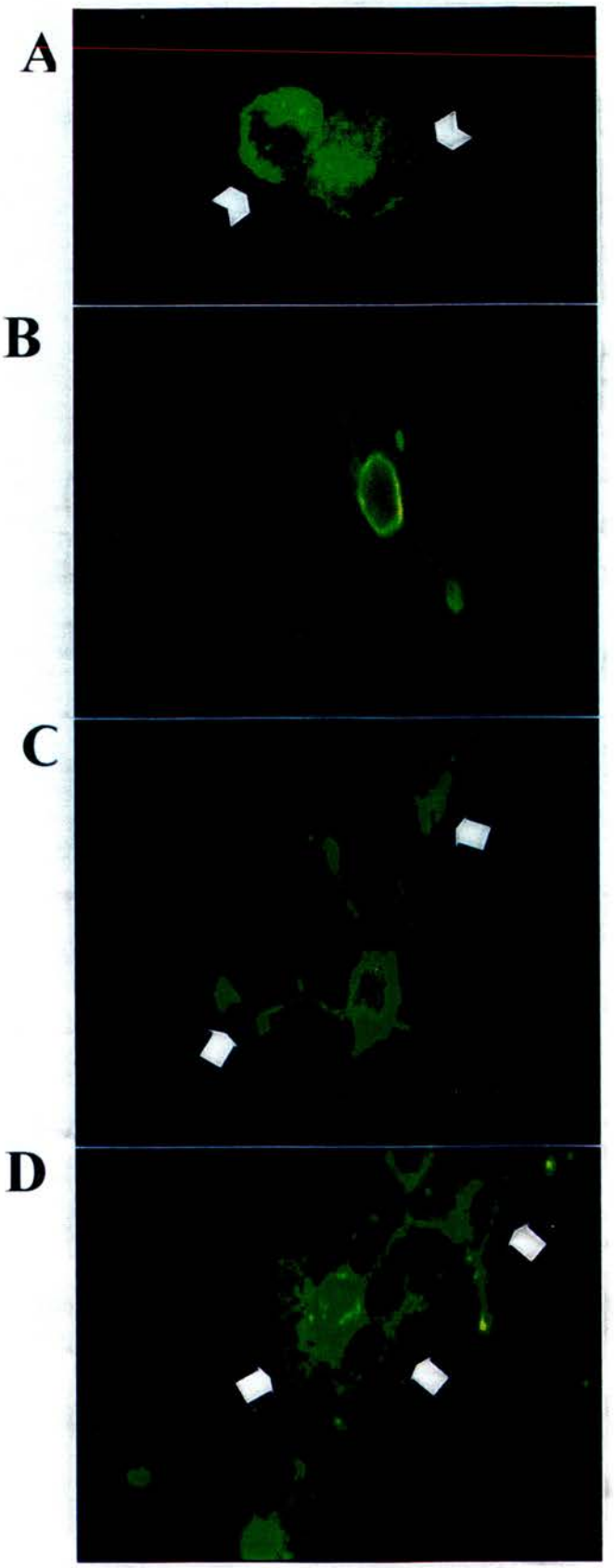

A
B
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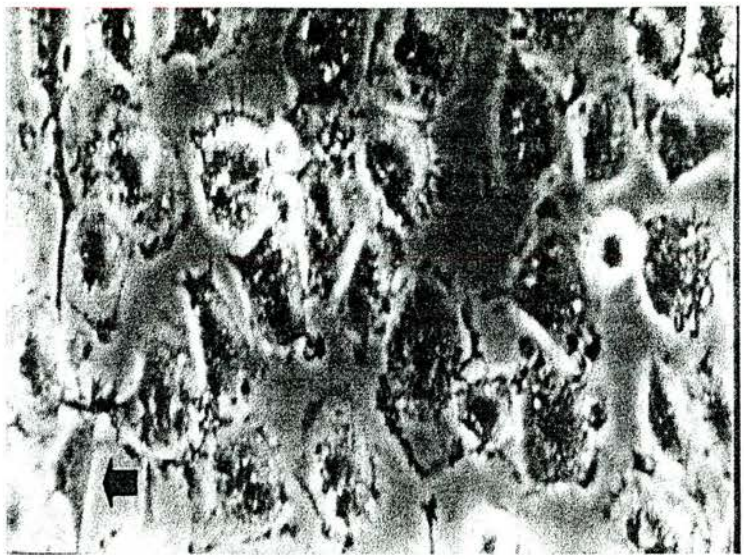
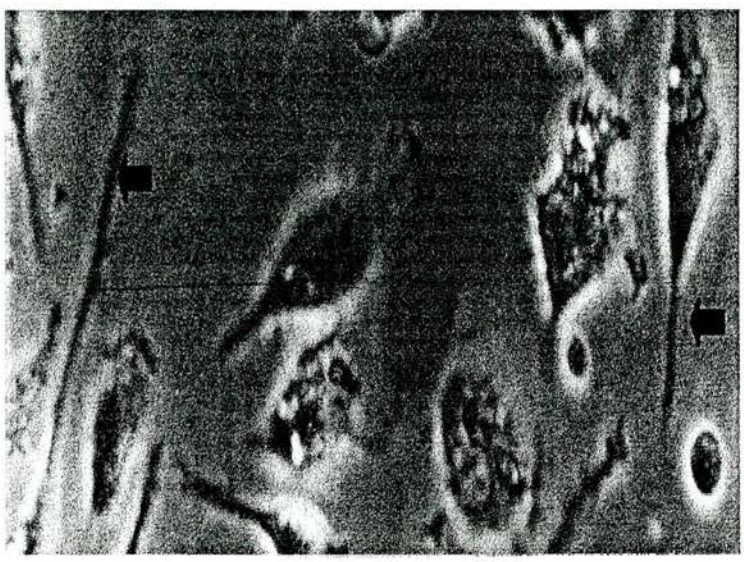
Fig. 3.4 Microglial cells possess distinct morphologies

Brain glial cells were obtained from the brain stem region. To enrich for microglial cells, mixed glial suspension from one lamb was cultured in one T25 culture flask. After four day post-culture, the supernatant was plated on 35 mm Petri dishes for 15 minutes at room temperature to allow for the adherence of microglial cells. This approach allowed for the enrichment of microglial cultures of > 98% purity, with minimal fibroblast and/or astrocyte contamination. Panel A shows live cells viewed under phase contrast microscope. The presence of fibroblast-like cells was kept to the minimum in enriched microglial cultures (red arrow). The morphology of microglial cells changed from a round appearance to cells with spinous processes (B and C). Black arrows point to the microglial spinous processes. Magnification x 200.

A



B



C

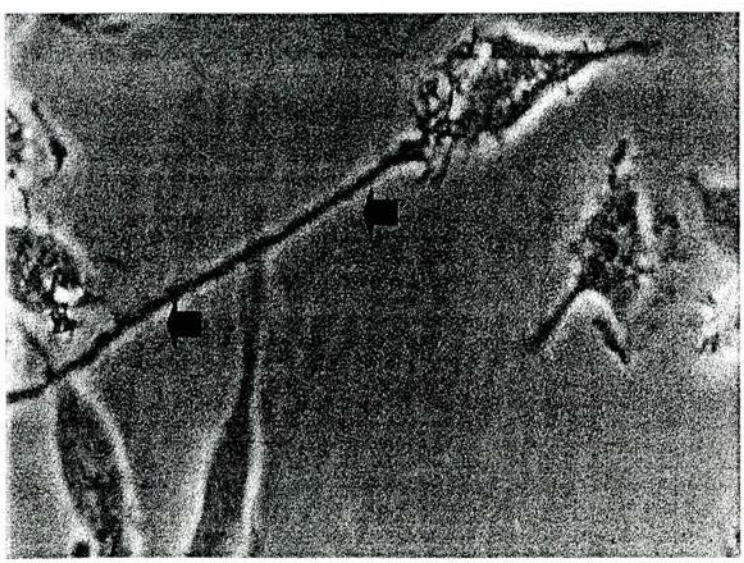


Fig. 3.5 Microglial cells are CD14 positive throughout their morphogenesis *in vitro*

Highly enriched microglial cultures were set up from brain stem region of lambs as stated in the legend to Fig. 3.4. Microglial cells were fixed and immunostained for surface expression of CD14, and detected with AP-conjugated secondary antibody. Immunostaining with CD14 localised to the membrane spikes (red arrows) and spinous processes (black arrows), the most prominent features observed on microglial cells. Panels show CD14 staining of microglial cells (A and B). Foci of microglial (immature) cells atop of bed layer also stained positive for CD14 (C). Star shows a cluster of immature microglial cells. Magnification x 200.

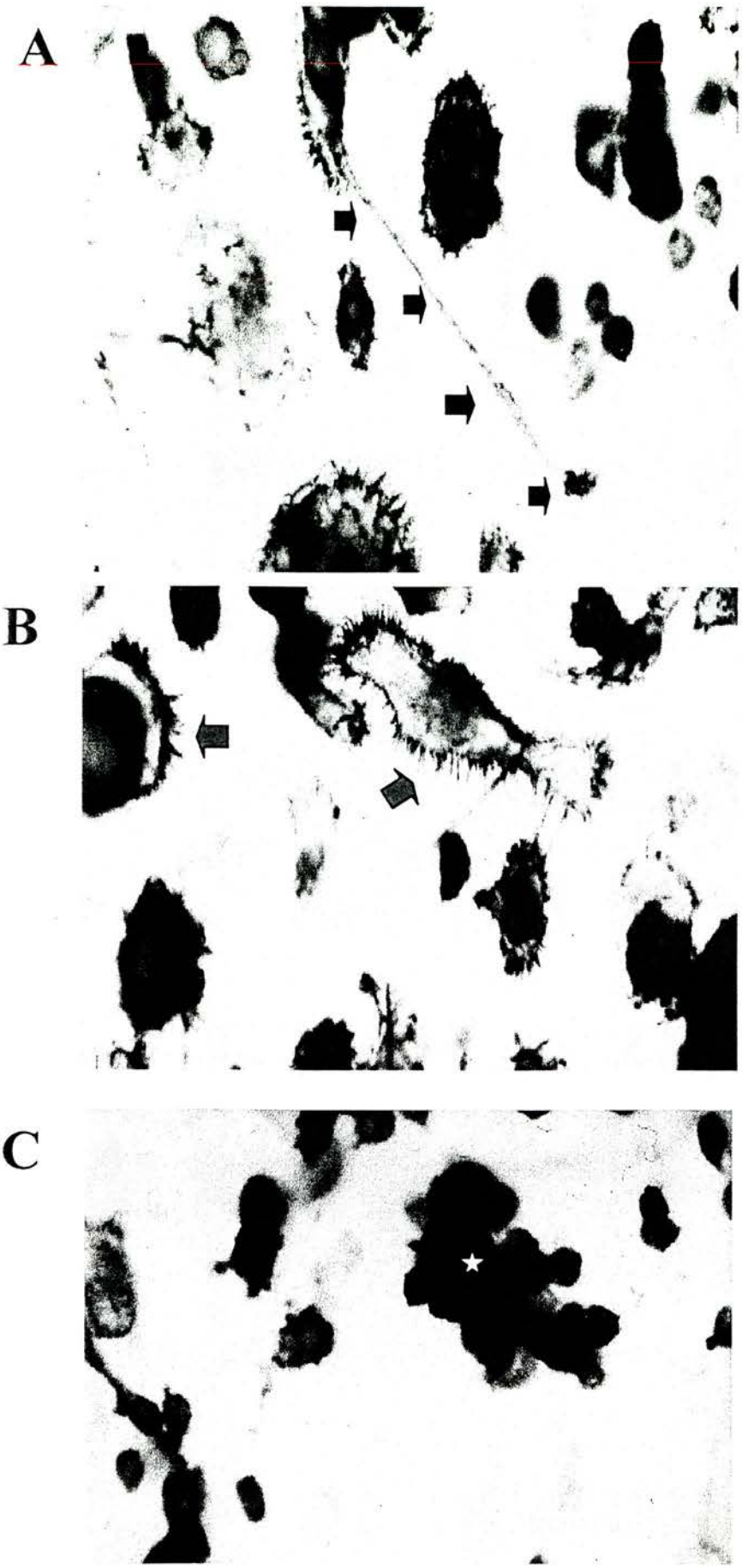
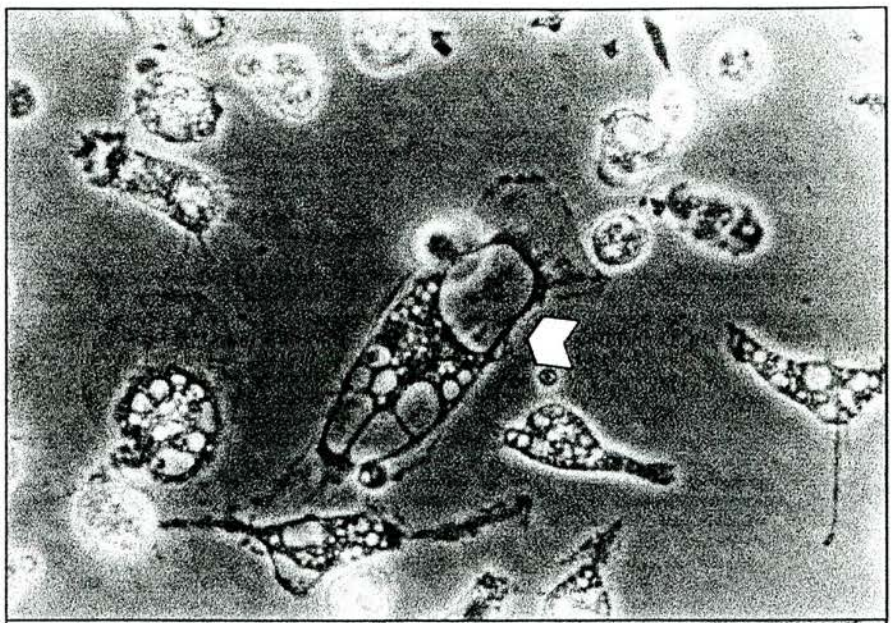

A
B
C

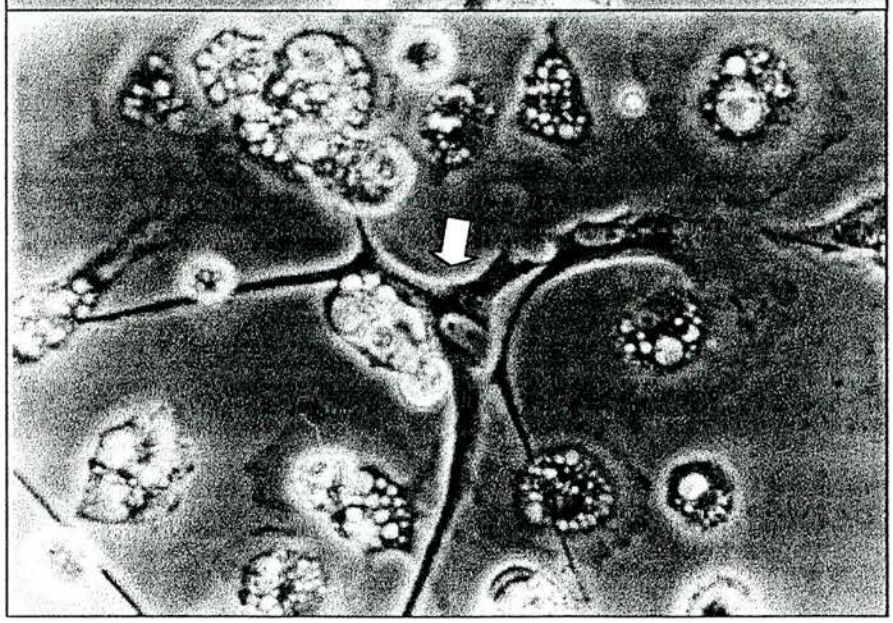
Fig. 3.6 LME treatment of mixed glial cultures

Mixed glial cultures were set up from the brain stem region as stated in the legend to Fig.3.1. Mixed glial cultures were treated with the lysosomo-tropic agent LME and morphological changes were followed by light microscopy. Only microglial cells (spike and spinous-bearing) showed the toxic effects of LME treatment. The initial sign of toxicity was intracellular ballooning (A, arrowhead). In contrast to microglial cells, cells with flat morphology did not show signs of toxicity (B, arrow). Magnification x 200

A



B



Cell Type	Cell Marker	Mixed glial cultures
Neurones	NF-M	–
Astrocytes	GAFP	+++
	A2B5	+++
Oligodendrocytes	O4	+++
	Gal C	–
	CNPase	–
Microglia	CD14	+++
	BS-I_4	+++
	F4/80	–
Fibroblasts	Fibronectin	+++
	BS-I_4	+++

Table 3.1 Phenotypic characteristics of ovine mixed glial cultures *in vitro*

Mixed glial cells were obtained and cultured as stated in the legend to Fig.3.1. Mixed glial cells were cultured in poly-lysine-treated plastic Petri dishes, fixed and immunostained for the presence of various cell-type markers at 10-14 and 21 days post seeding.

Key: -, no staining obtained; +, above background staining; +++, strong staining.

Antibody	Specificity	Microglia
SBUT-6	CD1	+++
VPM19	MHC-I	+++
VPM36	MHC-II DR	+
VPM54	NHC-II DQ	+
LFA-1	CD11a	+++
IL-A15	CD11b	_
OM1	CD11c	+++
VPM65	CD14	+++
GSI-B_4	β-NAcGal	+++
Anti-fibronectin	Fibroblasts	_
Anti-O4	Oligodendrocytes	_
Anti-Gal C	Oligodendrocytes	_
A2B5	Neuroglial progenitors	_
Anti-GFAP	Astrocytes	_

Table 3.2 Phenotypic characteristics of enriched ovine microglial cells

Enriched microglial cells were set up as stated in legend to Fig.3.4. Enriched microglial cells were fixed and immunostained with various cell markers at 7-10 days post purification *in vitro*.

Key: -, no staining obtained; +, above background staining; +++, strong staining.

3.4 Discussion

Ovine glial cultures were set up from the brain stem region of young lambs. These cultures where characterised by surface markers common to glial cells in other species.

3.4.1 Astrocytes and oligodendrocytes in ovine glial cultures are derived from precursor cells

The O-2A precursor cells (A2B5+) were detected in mixed ovine glial cultures prior to GFAP+ astrocytes and galactocerebrocide-positive (GalC+) oligodendrocytes (Fig.3.2). These progenitor cells have the capacity to become neurones, type 2 astrocytes or oligodendrocytes (Lillien *et al.*, 1988).

A2B5 is the earliest phenotypic marker, which can recognise cells of oligodendrocyte lineage. In defined medium, these progenitor cells may acquire GalC, a marker for mature oligodendrocytes. A2B5+ cells commited to the oligodendrocyte lineage develop into O4+ cells, before maturation into oligodendrocytes (reviewed by Raff *et al.*, 1989).

In ovine mixed glial cultures, presence of mature oligodendrocytes (GalC+) was not observed. This was in contrast to previously reported glial cultures obtained from caprine neural tissues (Baszler *et al.*, 1994). Use of different growth media with a combination of growth factors, such FGF, failed to drive the maturation of O4+ precursors into mature (GalC+) oligodendrocytes (data not shown).

Insulin and insulin-like growth factors (IGFI and IGFII) have been used to drive the differentiation of rodent progenitor cells into GalC+ oligodendrocytes *in vitro*. Insulin binds IGFI receptors and therefore can substitute for IGFI. Transcripts for IGFI and IGFII have been detected in rat CNS during early development. Moreover, receptors for IGF have been detected on cultured astrocytes, oligodendrocytes and O-2A progenitor cells (reviewed by Baskin *et al.*, 1988).

Based on above observations, insulin was used at varying concentrations (1-10 μg/ml) in order to derive the maturation of O4+ cells into mature oligodendrocytes. However, this approach failed to do so (data not shown).

Plating of non-adherent O4+ cells on laminin-coated plates resulted in maturation of O4+ cells into mature, GalC+ oligodendrocytes (Fig.3.3). It is known that interaction of integrins with extracellular matrix (ECM) can result in a cascade of intracellular

events that may affect cellular metabolism, proliferation and differentiation (reviewed by Damsky and Werb, 1992). Unlike β-2 integrins, β-1 integrins are mainly involved in interactions between cells and ECM. One such integrin, αvβ1, which is a receptor for laminin, is expressed by oilgodendrocyte precursors (Milner and Ffrench-Constanat, 1994). These observations may explain the maturation of ovine O4+ precursors on laminin-coated plates into GalC+ cells.

3.4.2 Ovine parenchymal microglia share phenotypic surface markers with cells of the monocyte/macrophage lineage

Enriched microglial culture of > 98 % purity were set up from the brain stem region of newly born lambs. Moreover, ovine microglial cells stained positive for markers most commonly associated with cells of monocyte/macrophage lineage (Table 3.2).

Members of β-2 integrin family have been detected on human and rodent microglial cells, both *in vitro* and *in vivo* (Akiyama and McGeer, 1990; Sedgewick *et al*, 1991; Ford *et al.*, 1995; Becher and Antel, 1996). Resting human microglial cells constitutively express CD11a (LFA-1), CD11b (Mac-1, CR3), CD11c (P150, 95, CR4), and CD18 (β-2), leukocyte common antigen (LCA, CD45), and the immunoglobulin receptor FcγRI. Moreover, the immunostaining of microglial cells for β-2 integrins has been reported to be more intense in the white matter than in the grey matter (Akiyama and McGeer, 1990).

Double immunostaining for integrins and leukocyte common antigen (LCA; CD45) has resulted in the specific identification of microglial cells within the CNS. This approach has been proposed to distinguish between perivascular macrophages ($CD45^{high}$ $CD11b/c^{+}$) and resident microglial cells ($CD45^{low}$ $CD11b/c^{+}$) (Ford *et al*, 1995).

The characteristic phenotype of *ex vivo* parenchymal microglia ($CD45^{low}$ $CD11c^{+}$) corresponded to that found *in situ* in normal, non-inflamed human brain tissues. Moreover, this phenotype differed from that of perivascular macrophages *in situ* and monocyte-derived macrophages, which were both $CD45^{high}$ $CD14^{++}$ (Becher and Antel, 1996).

The double staining approach was not employed in the phenotypic characterisation of ovine microglial cells. However, it is highly unlikely that the cultured cells were perivascular macrophages rather than microglial cells. Experiments using CD45

chimeric rats have shown that in normal, non-inflamed brain, perivascular macrophages ($CD45^{high}$ $CD11b/c^{+}$) constitute only seven percent of macrophage-type cells in the CNS (Ford *et al.*, 1996).

3.4.2.1 Presence of surface MHC molecules on cultured microglial cells

MHC molecules were detected on cultured microglial cells (Table 3.2). In normal ovine CNS, parenchymal microglial cells have been shown to express both MHC-I and II (Torsteinsdottir *et al.*, 1992; Georgsson *et al.*, 1997).

In rodent CNS, resting microglial cells constitutively express MHC-II within the white matter (Gehrmann *et al.*, 1991). Similarly, resting microglial cells in human brain tissues also express MHC-II in the white matter (Hayes *et al.*, 1988). Microglia isolated from neonatal rats, with or without IFNγ, were able to present antigen to T cells *in vitro*. Astrocytes can also be primed to present antigen by pre-treatment with IFNγ, but unlike microglial cells, they are poor antigen presenters in the absence of IFNγ (Matsumoto *et al.*, 1992). Moreover, astrocytes can be induced to express MHC-II by, for example IFNγ, however, brain microglial cells are the main parenchymal cell type that either constitutively or inducibly express MHC-II *in vivo* (Gehrmann *et al.*, 1993).

3.4.2.2 CD14 is present on ovine microglial cells in all stages of maturation *in vitro*

The cell surface marker, CD14 was detectable throughout the life span of ovine microglial cells *in vitro* (Fig. 3.5). CD14 is a receptor for the bacterial lipopolysaccharide (LPS), an endotoxin found in the outer cell wall of Gram-negative bacteria, and is expressed on monocytes and tissue macrophages (reviewed by Ziegler-Heitbrock and Ulevitch, 1993).

Human parenchymal microglia in normal CNS do not express CD14 (Ulvestad *et al.*, 1994). However, cultured adult human microglial cells have been shown to express high levels of surface CD14 (Becher and Antel, 1996). This expression of CD14 was concomitant with the morphological development of microglial cells. This is in contrast to the cultured ovine microglia, which were CD14+ regardless of their morphological development. This anomaly may be a reflection of a species difference and/or because neonatal brain was the source of ovine glial cultures rather than adult tissues.

In the inflamed CNS, there is an influx of blood monocyte-derived macrophages ($CD45^{high}/CD14^{++}$). However, the presence of CD14 on microglial cells would imply that brain parenchymal microglial cells contribute to CD14+ population in the CNS (Bercher and Antel, 1996). Interestingly, CD14+ monocytic leukeamia cells produce TNFα, whereas CD14-negative did not. This observation implies that CD14 expression may have a relevance to functional properties of microglial cells (Ford *et al.*, 1995).

3.4.2.3 Presence of CD1 on cultured ovine microglial cells

Primary cultures of ovine microglial cells stained positive for CD1 surface marker. The CD1 family comprises four (a to c) β2-microglobulin-associated glycoproteins expressed on most antigen presenting cells (reviewed by Porcelli, 1995). The presence of CD1b appears to be more widespread in ovine tissues than in human and murine tissues (S. Rhind, personal communication). CD1 immunoreactivity was also detected on apparently normal, ovine parenchymal microglial cells *in vivo* (S. Rhind, collaborative work).

3.4.3 LME toxicity: a functional assay for microglial cells

The treatment of glial cultures with the lysosomotropic agent, L-leucine methyl ester (LME) resulted in specific loss of microglial cells (Fig.3.6). The toxicity of LME is mediated by lysosomal enzymes, which convert the LME into free amino acid leucine. The build up of leucine within the organelles causes osmotic swelling and rupture of lysosomes in cells of monocyte/macrophage lineage (Thiele *et al.*, 1985).
The LME treatment of ovine microglial cultures demonstrated that these cells, in line with other tissue monocyte/macrophages, are susceptible to the toxic effects of LME. This functional assay also supports the notion that microglial cells are indeed derived from the monocyte/macrophage lineage. However, the identification of ovine microglial cells was based primarily on their phenotypic properties as determined by immunostaining for cell surface markers, and not on the LME assay. Another functional assay for cells of the monocyte/macrophage lineage would be the rosetting of red blood cells facilitated by membrane Fc receptors.

3.5 Summary

This is the first report on characterisation of primary ovine glial cultures *in vitro*. Ovine mixed glial and purified microglial cells were established from the brain stem

region of newly born lambs. The phenotypic characterisation of these cultures was carried out using a panel of antibodies. Furthermore, using LME as a functional assay demonstrated that only microglial cells were susceptible to this treatment.

Data presented in this chapter demonstrate that there is a degree of species cross-reactivity with respect to certain cell markers, for example murine O4, human fibronectin, and bovine GFAP with ovine tissues. However, F4/80 did not cross-react with ovine tissues *in vitro*, implying that the cross-reactivity is not universal.

Cells of monocyte/macrophage lineage are the main target cells by all lentiviruses both *in vivo* and *in vitro*. The morphological, functional and phenotypic characteristics displayed by cultured ovine microglial cells and microglial cells from other species imply that these cells are derived from monocyte/macrophage lineage, and therefore possible targets for infection *in vivo*.

There is a lack of direct evidence that ovine parenchymal microglial cells are targets for MVV infection. The experimental design in the next chapter was aimed at addressing the question of whether cultured ovine microglial cells can be infected with MVV.

CHAPTER FOUR
Infection of Enriched Ovine Microglial Cells with MVV

4.1 Introduction

The isolation and phenotypic characterisation of ovine microglial cells described in the previous chapter demonstrated that these cells share extensive phenotypic similarities with cells of monocyte/macrophage lineage.

The combination of phenotypic and functional similarities between brain microglial cells and other tissue macrophages, together with the tropism of all lentiviruses for cells of the monocyte/macrophage lineage had suggested that microglial cells might also be targets for lentiviruses in the nervous system (see Chapter 1). Moreover, use of brain-derived *in vitro* cultures (cellular and organotypic) has confirmed the *in vivo* observations that brain microglial cells may be targets for infection with primate lentiviruses (Takahashi *et al.*, 1996). Indeed, with dual tropic primate lentiviruses, the preponderance of M-tropic virus isolates over T-tropic viruses from infected neural tissues also supports the importance of microglia in CNS pathology.

The phenotypic assignment of MVV-infected cells *in vivo* has largely been based on morphological characteristics. For example, macrophage-like, CD68+ cells have been shown to harbour viral proteins in brains of MVV-infected animals (Torsteinsdottir *et al.*, 1992; Brodie *et al.*, 1995; Georgsson *et al.*, 1997).

There is, however, no direct evidence that MVV infects brain parenchymal microglial cells. This has been partly due lack of phenotypic markers that may distinguish between microglial cells and other monocyte-derived macrophages and lack of published protocols on isolation of parenchymal microglial cultures from ovine brains.

4.2 Experimental design

The experiments in this chapter were aimed at investigating the tropism of MVV for ovine microglial cells *in vitro*. Ovine microglial cells were cultured from the brain stem region of newly born lambs (see Chapter three). Enriched microglial cultures were infected with the Icelandic strain, K1514, at the multiplicity of infection (MOI) of 0.001. Infected microglial cultures were tested for the presence of virus by RT-PCR for the *pol* gene, by immunostaining for the viral p25 major core protein, and by electron microscopy (EM). The presence of infectious virus in supernatants of MVV-infected microglial cultures was analysed on sheep chondrocytes.

Furthermore, the possible induction of programmed cell death (apoptosis) in cultured microglial cultures as a result of MVV infection was assessed by TUNEL staining. The supernatants from both mock-infected and MVV-infected cultures were cytospun

and double stained for viral p25 and DNA fragmentation by TUNEL. The experimental infections were performed on four separate brain cultures.

4.3 Results

4.3.1 Productive infection of ovine microglial cells with MVV

MVV can infect brain microglial cells *in vitro*. Amplification of *pol* ORF from *in vitro* infected microglial cultures resulted in generation of positive RT-PCR signals (Fig.4.1). Moreover, in order to determine the type of infection, i.e., whether productive or restricted, supernatants from both infected and mock-infected cultures were tested for the presence of infectious viral particles. Supernatants from infected cultures contained infectious viral particles, the levels of which increased with time (Fig.4.2).

To characterise further the type of MVV infection of microglial cells, both infected and mock-infected cultures were immunostained with the viral major core protein, p25. Only MVV-infected cultures stained positive with p25 (Fig.4.3). Moreover, syncytia, which stained positive for p25, were also observed in infected cultures (Fig.4.3).

4.3.2 Electron microscopy of MVV-infected microglial cells

Electron microscopic (EM) studies were employed to investigate the pattern and mode of virion assembly/budding from MVV-infected microglial cells. EM studies revealed the presence of viral particles within cytoplasmic vacuoles (Fig.4.4). However, presence of budding virion from cell membrane proved to be a rare event.

4.3.3 Mode of death in MVV-infected microglial cells

Floating cells in supernatants from MVV-infected microglial cultures were analysed for viral infection (p25) and apoptosis (TUNEL). Supernatants were cytospun and used for double immunostaining for TUNEL and p25. Cytospun cells stained positive for both p25 and TUNEL (Fig.4.5). However, the presence of double positive cells was rare. Less than 4% of total dead cells stained for both p25 and TUNEL. This was in contrast to viral p25-positive cells, which constituted approximately 30% of total dead cells.

The above observations obtained from TUNEL experiments, therefore, appear to indicate that direct infection of microglial cells *in vitro* with MVV was not a pre-requisite for apoptosis.

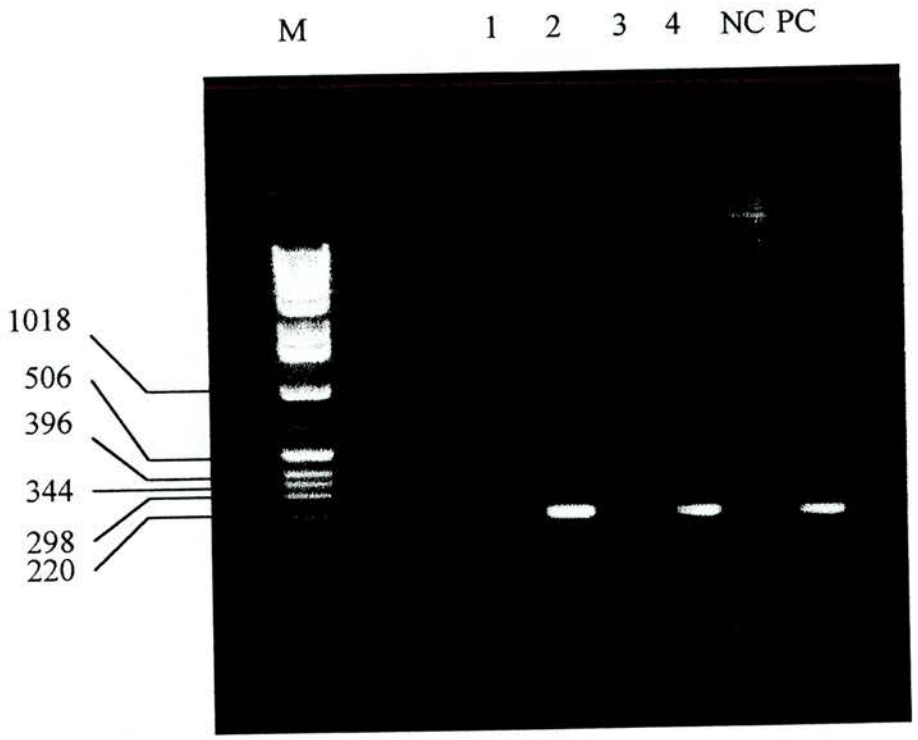


Fig. 4.1 Detection of viral *pol* gene in MVV-infected microglial cells

Enriched microglial cultures were set up as stated in the legend to Fig.3.4. Total RNA was isolated on day four post-infection, and revese transcribed. The control RNA was obtained from mock-infected microglial cultures. The MVV *pol* gene was amplified by PCR on cDNA samples. Ten microlitres from each PCR reaction was run on an agarose gel. The lanes on the gel are:

M: λ DNA marker

1: mock-infected culture (A)

2: MVV-infected culture (A)

3: mock-infected culture (B)

4: MVV-infected culture (B)

NC: negative control (Sigma water instead of input DNA)

PC: positive control (Hirt DNA obtained from MVV-infected chondrocytes)

Fig. 4.2 Kinetics of MVV infection of ovine microglial cells in vitro

Enriched microglial cultures were set up as stated in the legend to Fig.3.4. Microglial cultures (100,000 per 35 mm Petri dish) were infected with 0.001 MOI of the Icelandic strain, K1514. Supernatants form microglial cultures were harvested at different time points post-infection, and the presence of infectious virus in these supernatants was assayed on ovine chondrocytes. CPE on indicator cells included lysis and syncytium. No CPE was observed in supernatants from mock-infected control cultures.

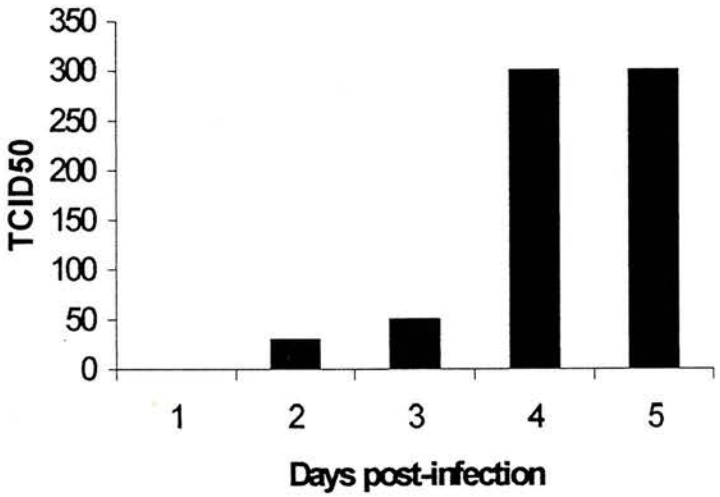

350
300
250
200
150
100
50
0
TCID50
1
2
3
4
5
Days post-infection

Fig.4.3 Immunostaining of viral p25 in MVV-infected microglial cells

Microglial cultures were infected with MVV as stated in legend to Fig.4.2. Treated cultures were immunostained on days 4-7 post-infection with the monoclonal antibody VMP70 to stain for viral p25 protein, using an alkaline phosphotase-conjugated secondary antibody. Punctate p25 immunostaining was detected in cytoplasm of infected cells (A, B, and C). Infrequently, a multinucleated giant cell (syncytium) was also detected in infected cultures (D). Mock-infected cultures did not stain positive for p25 (E). The arrows point to the nuclei of infected cells. Magnifications: A-D x 250; E x 200

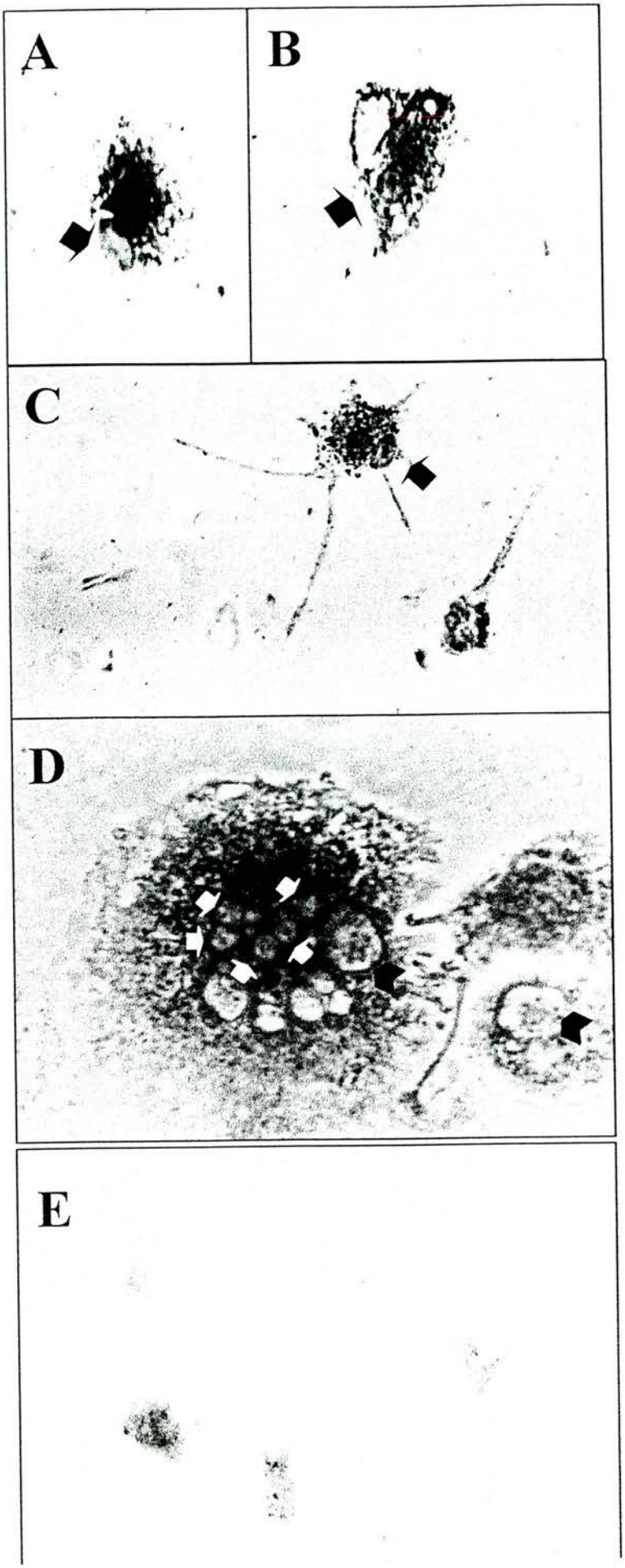
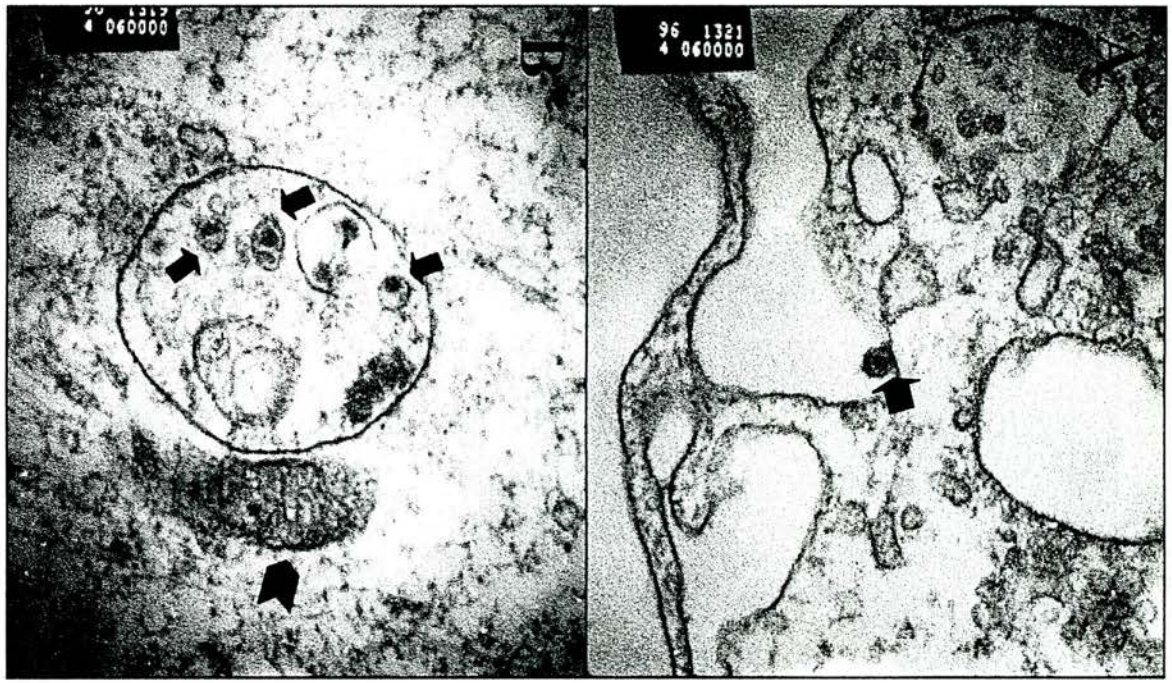

A
B
C
D
E

Fig.4. 4 Presence of virion-like structures in MVV-infected microglial cells

Microglial cultures were infected with MVV as stated in legend to Fig.4.2. MVV-infected and mock-infected microglial cells were fixed for EM studies at 5 days post-infection. Virion-like structures were observed in MVV-infected microglial cells (arrows). These structures had an approximate diameter of 100nm with an electron-dense core. These apparent MVV virions were located on cellular membrane (A) and as clusters in compartments (vacuoles) within the cytoplasm (B). The arrowhead points to a cytoplasmic structure similar to a mitochondrion. Magnification x 60,000.


4 060000
96 1321
4 060000

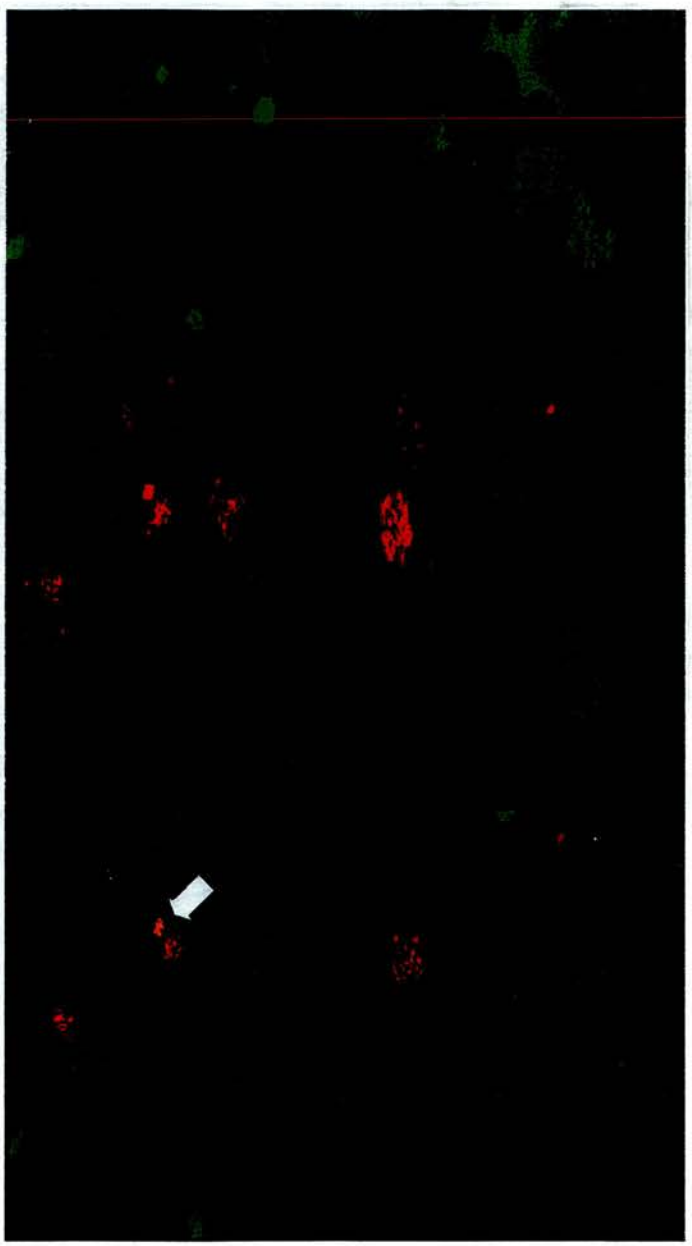
4.5 Presence of TUNEL and p25 double positive cells in MVV-infected cultures

Microglial cultures were infected with MVV as stated in legend to Fig.4.2. Floating cells from MVV-infected microglial cultures at four days post-infection were cytospun and immunostained for apoptosis by TUNEL (red) and viral p25 (green) (A and B). Double positive cells (yellow) constituted a small percentage of infected cells (C and D). Panel D represents mean +/- 1standard deviation from 10 independent microscopic fields.

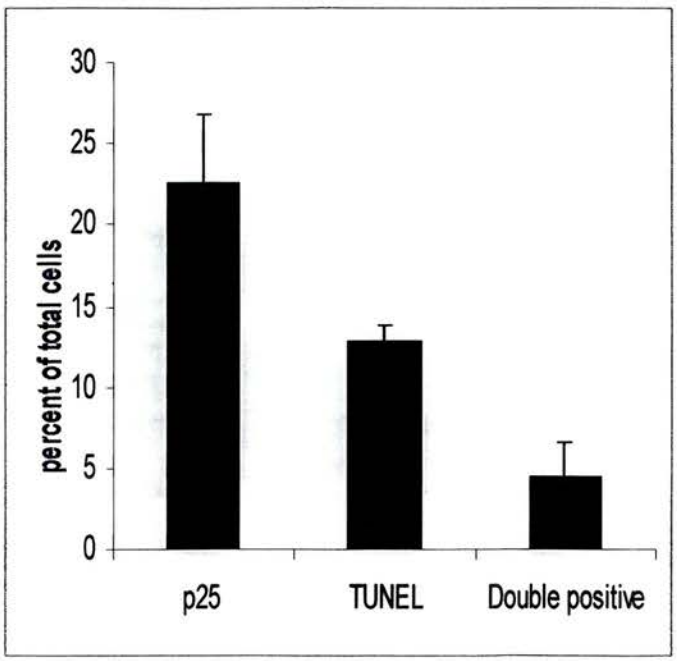
A

B

C



D


30
25
20
15
10
5
0
percent of total cells
p25
TUNEL
Double positive

4.4 Discussion

The results obtained in this chapter demonstrate that MVV could infect ovine brain microglial cells. This was based on PCR, virus titre assay, immunostaining, and EM studies.

The infection experiments in this thesis were carried out with the Icelandic strain of MVV, K1514 and at the MOI 0f 0.001. This was because the stock virus was at MOI of one. This stock virus was prepared by infection of cultured sheep skin fibroblasts, and therefore would be expected to contain fibroblast-derived cytokines and viral factors (membrane glycoproteins). Theoretically, these components (contaminants) could affect the expression of cytokines by microglial cells. The ideal infection experiment would be to use purified infectious virions with no contaminating cellular debris. However, previous attempts at purifying MVV virions by high-speed centrifugation had been unsuccessful (D.J. Roy, personnal communication). The aim of infection experiments was to analyse the potential effects of virus infection on the expression of microglial-derived cytokines. Therefore, the virus was used at 1:1000 dilution of the stock virus. This approach was expected to reduce the potential effects of cellular and viral contaminants on the expression of microglial-derived cytokines.

4.4.1 Virion assembly/budding of MVV from infected microglial cells

The morphological classification of lentiviruses is based on transmission electron microscopy applied to all retroviruses. Virion particles within cytoplasmic vacuoles were observed with MVV-infected microglial cells (Fig.4 4). This finding closely resembles the observation made with CAEV-infected caprine microglial cells *in vitro* (Baszler *et al.*, 1994).

Intracytoplasmic type A (ICA) particles, i.e., electron dense nucleocapsid without outer lipid membrane, have been reported in alveolar macrophages and monocyte-derived macrophages infected with the British maedi-visna isolate, EV-1 (Lee *et al.*, 1996). ICA particles have also been detected in EIAV, CAEV and SIV infected cells. This is in contrast to MVV-infected microglia, where no ICA particles have been observed. Similarly, no ICA particles have been reported in HIV-1 infections. Moreover, in HIV-1 infected monocytes and brain macrophages, virion assembly/budding is predominantly localised within cytoplasmic compartments, identified as Golgi elements (Ornstein *et al.*, 1988).

Viral determinants may influence the mode of virion assembly/egress in infected cells. Parallel infection of monocyte-derived macrophages with British EV-1 and

Icelandic K1514 strain resulted in different virion assembly/egress (Lee *et al.*, 1996). ICA particles were not observed in monocyte-derived macrophages cells infected with K1514. Interestingly, K1514 was also the virus isolate used in this thesis to infect ovine microglial cells *in vivo*. Therefore, it has been postulated that difference in the viral-coded sequences may influence virion production and assembly. For example, progressive deletion of HIV-1 *gag* ORF at the carboxy-terminal resulted in change of virion assembly, normally at the cell membrane, to become intracytoplasmic. The other virus-coded determinant, which may influence virion assembly, is the hypervariable *env* ORF.

Cellular determinants may also influence the mode of virion assembly/budding. Unlike monocytes, HIV-1 infection of T lymphocytes results in exclusive surface assembly of viral particles (Ornstein *et al.*, 1988). The precise reason for this anomaly is not entirely clear. However, it is known that cells of different lineage may process intracellular proteins differently. This may influence the cellular location of virion assembly/budding.

The morphological classification of lentiviruses is far from clear. SIV more closely resembles Type D than Type C retroviruses, with pronounced surface spikes, similar to Mason-Pfizer monkey virus (Munn *et al.*, 1985). However, immature HIV particles have the appearance of type C, whereas mature virion with eccentric spherical nucleoid within a conical or tubular core shell. In this context, MVV virion may share similarities with type B, C, and D during its maturation, depending on the experimental conditions employed (Lee *et al.*, 1996).

4.4.2 Mode of cellular death in MVV-infected microglia

Apoptosis, or programmed cell death (PCD), is a genetically controlled cellular event. In viral infections, PCD may limit the progeny spread. Several DNA viruses have evolved mechanisms, via expression of oncoproteins, whereby they can interfere with PCD, primarily at the p53 level (reviewed by Teodoro and Branton, 1997).

In contrast to both DNA viruses and oncogenic retroviruses, lentiviruses do not code for oncoproteins, and do not evoke cellular DNA synthesis for their progeny. Unscheduled, virus-induced DNA synthesis is one of the mechanisms that may induce PCD. However, apoptosis has been observed in HIV patients, and it has been demonstrated that T cells from such patients are sensitive to activation-induced apoptosis (Groux *et al.*, 1992). HIV-induced apoptosis bypasses the p53 pathway, and involves Fas (CD95)/FasL (CD95L) interaction. HIV/SIV Tat proteins and

HTLV Tax protein are believed to induce the expression of FasL, leading to apoptosis, via activation of interleukin converting enzyme (ICE)-like proteases. Similarly, membrane expressed HIV envelope glycoprotein has also been shown to induce PCD in uninfected CD4+ T cells (reviewed by Teodoro and Branton, 1997)

The TUNEL staining of infected cultures on days 4-5 post-infection demonstrated that only a small proportion (approximately 4%) of viral antigen positive microglial cells were also undergoing apoptosis (Fig.4.5). This observation would imply that direct infection of microglia cells is not necessarily a pre-requisite for apoptosis.

In HIV-1-infected patients, infected cells appear protected from apoptosis, whereas bystander cells show increased apoptosis in lymph nodes of infected individuals (McCloskey *et al.*, 1997). Furthermore, in myelomonocytic cells infected with HIV, the viability of cells was not affected by Fas-mAB treatment, unlike infected T cell lines (Okamoto *et al.*, 1997). These observations imply that infected myelomocytic cells are more resistant to the Fas-mediated apoptosis. Inhibition of apoptosis in HIV-1-infected cells may enhance virus production and facilitate persistent infection (Anton *et al.*, 1995). Presence of cytokines, for example TNFα may reduce the resistance of infected myelomonocytic cell to Fas-mediated apoptosis (Okamoto *et al.*, 1997).

The result obtained from infected microglial cells may imply that direct infection is not a prerequisite for cellular damage/death. HIV-1 infection of primary brain cultures has been shown to result in apoptosis of neurones and astrocytes *in vitro*. However, apoptosis was not significantly induced until one to two weeks after the time of peak virus production. This result suggested that induction of soluble factors rather than direct viral infection was responsible for apoptosis. These observations support the notion that bystander damage may take place in virus-infected tissues. Interestingly, apoptosis of neurones and astrocytes has been observed in AIDS patients with dementia at sites removed from viral positive microglial cells (Shi *et al.*, 1996).

Lentiviruses may also directly interact with apoptotic pathways. Infection of macrophages with HIV-1, *in vitro*, has been demonstrated to result in upregulation of pro-apoptotic gene, Bax, and downregulation of anti-apoptotic genes, for example Bcl-2 (Krajewski *et al.*, 1997).

4.5 Summary

Data presented in this chapter show, for the first time, that ovine microglial cells could be infected with MVV *in vitro*. Moreover, this observation is in agreement with previous reports of tropism of MVV for cells of the monocyte/macrophage lineage in other tissues, for example alveolar macrophages, and blood monocytes.

Observations made with the TUNEL method reinforce suggestions that indirect bystander cellular damage may play a role in MVV-induced neuropathology. Cellular soluble factors (cytokines) have been proposed, as part of the bystander damage model, culminating in neuropathology.

Brain macrophages/microglial cells are believed to be a major source of neurotrophic factors (neurokines) in the CNS. The presence of pathogens in the CNS, such as visna may have adverse effects on the expression of these neurokines, culminating in disease. Fibroblast growth factor-2 (FGF-2, basic FGF; bFGF) is a neurokine that is widely expressed in the CNS. This neurokine has also been implicated in vascular diseases.

There are no data available on lentiviral infections and possible changes in expression of neurotrophins in the CNS. The experimental design in chapter four was intended to clone ovine FGF-2 from brain tissues in order to generate reagents that could be used as part of a panel of assays in deciphering the visna-mediated neuropathology.

CHAPTER FIVE

Molecular Cloning of Neural Ovine Basic Fibroblast Growth Factor Gene

5.1 Introduction

There is growing evidence for the presence of growth factors, neurotrophic factors and cytokines, collectively referred to as neurokines, which maintain homeostasis in the CNS.

Glial cells (astroglia and microglia) are the major sources of brain-derived cytokines and neurokines. For example, microglial cells have been shown to be a rich source of TGF-β, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin like growth factors, and FGF-2 (Rappolee *et al.*, 1988; Shimojo *et al.*, 1991). Activated brain macrophages have also been shown to secrete nerve growth factor (NGF) (Mallat *et al.*, 1989).

Glial cells are also responsive to trophic factors. For example, microglial cells are responsive to BDNF and neurotrophin-3 (NT-3). Interestingly, the same study also showed that NT-3 induced phagocytic activity of microglial cells, indicating that these factors are closely associated with functions carried out by glial cells *in vivo*, both as autocrine and paracrine factors (Elkabes *et al.*, 1996).

FGF-2 was first described by Gospodarwicz (1974), and is a potent angiogenic agent that stimulates the proliferation of wide range of mesodermal and neuroectodermal-derived cells as well as cultured fibroblasts (reviewed by Gospodarwicz *et al.*, 1990). In addition to its effect on vascular endothelium, FGF-2 also promotes neuronal survival and increases neurite extension in foetal rat hippocampal neurones (Morrison *et al.*, 1986; Walicke, 1988).

FGF-2 immunoreactivity has been mapped in the CNS, in the neucortex and also in the pituitary (Fuxe *et al.*, 1996). Biological activities attributed to FGF-2 include differentiation, proliferation, and maintenance of cells in the CNS (Giordano *et al.*, 1992).

Although the emphasis in CNS pathology has been largely focused on "immune cytokines" such as TNFα, there is minimal data on effects such viral infection may have on brain-derived growth factors. This is particularly relevant to lentiviruses, which have the capacity to infect brain microglial cells, a cell type with magnitude of functions in the CNS. One such factor is FGF-2.

5.2 Experimental design

The aim of this chapter was to molecularly clone one such factor, FGF-2, to generate reagents in order to study the effect of MVV infection on the expression of this brain-

derived cytokine/survival factor in cultured microglial cells. Therefore, consensus PCR primers were designed from the comparison of bovine and human FGF-2 genes. Using brain tissues from an adult ewe resulted in amplification and susequent cloning of ovine FGF-2 homologue.

5. 2 Results

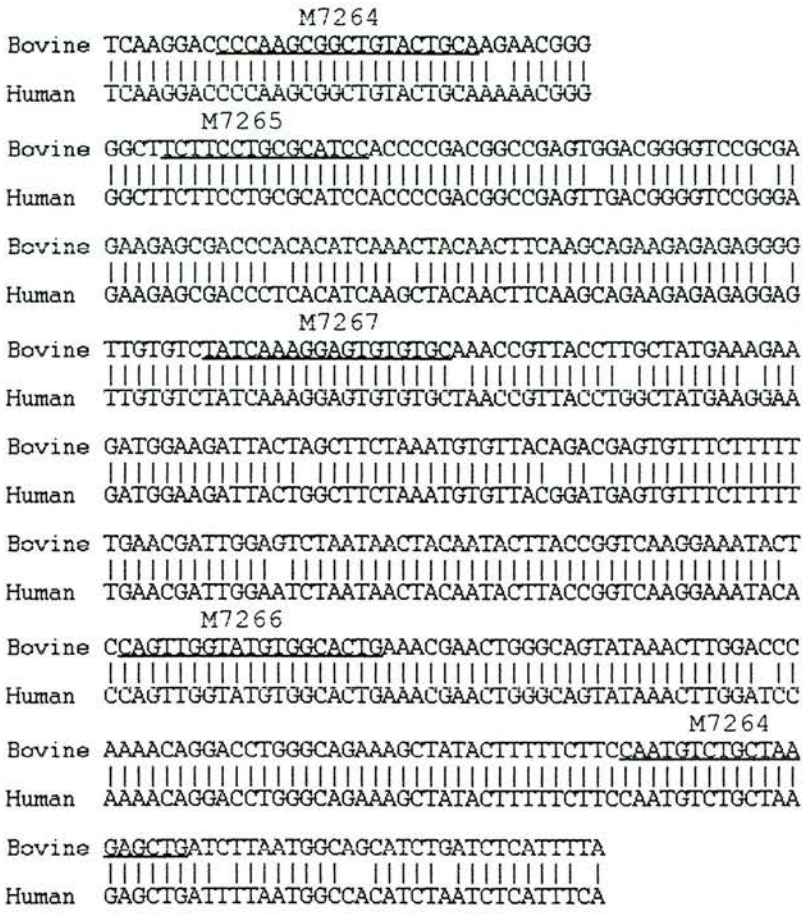


Fig. 5.1 Comparison of bovine and human FGF-2 cDNA sequences

The cDNA sequence of bovine FGF-2 (accession number m13440) was piled-up against the human FGF-2 sequence (accession number m27968), to allow for design of consensus PCR primers for amplification of ovine FGF-2 homologue. The forward and reverse PCR primers (M7263/M7264 for outer PCR and M7265/M7266 for nested (inner) PCR) were chosen from the most conserved regions and are underlined. The internal primer sequence (M7267) was used as a probe in Southern blots (see Table 5.1 for details of primers).

Primer	Sequence	Polarity
M7263	CCAAGCGGCTGTACTGCA	sense
M7264	CAGCTCTTAGCAGACATTGG	antisense
Ta^{OPT}	56 °C	
%GC	47.2	
Length of product	381 bp	
Primer	**Sequence**	**Polarity**
M7265	TCTTCCTGCGCATCCACC	sense
M7266	CAGTGCCACATACCAACTG	antisense
Ta^{OPT}	55 °C	
%GC	45.6	
Length of product	265 bp	
M7267	TATCAAAGGAGTGTGTGC	probe

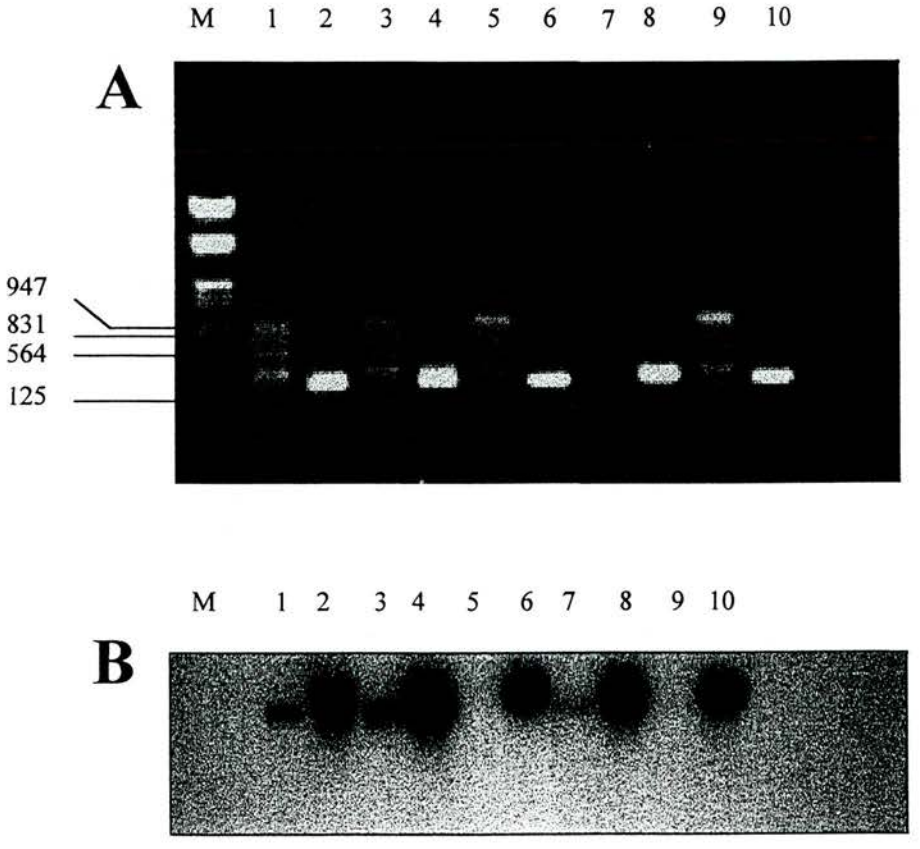
Table 5.1 Primer design and PCR parameters for cloning of ovine FGF-2 from neural tissues

The table shows the primer sequences, annealing temperatures and expected PCR products for the ovine FGF-2 homologue. The gene specific primers for ovine FGF-2 were designed from the pile-up of published bovine and human FGF-2 sequences (Abraham *et al.*, 1986a, 1986b). See Fig.5.1. for the pile-up of bovine and human FGF-2 sequences.

Fig. 5.2 Amplification of FGF-2 from ovine brain tissue

Brain tissues were obtained from different anatomical regions of an adult Cheviot ewe (aged two). Total cellular RNA was isolated using the RNeasy columns, and used for RT-PCR using FGF-2 primers designed from comparison of bovine and human sequences. One microlitre aliquots were taken from the first round of RT-PCR reactions and used in a nested-PCR with an internal primer set (see table5.1). Ten microlitre aliquots were run on an agarose gel from each PCR reaction (A). Panel B is Southern blot of the gel shown in panel A, using the sequence-specific probe M7267. The expected products generated with external primer set and the nested primer set are 381 bp and 264bp, respectively.

M: λ DNA cut with HindIII and EcoRI
Lane 1: Pituitary (external primer set)
Lane 2: Pituitary (nested PCR)
Lane 3: Olfactory bulb (external primer set)
Lane 4: Olfactory bulb (nested PCR)
Lane 5: Cerebral cortex (external primer set)
Lane 6: Cerebral cortex (nested PCR)
Lane 7: Hypothalamus region (external primer set)
Lane 8: Hypothalamus region (nested PCR)
Lane 9: Hypothalamus region (external primer set)
Lane 10: Hypothalamus region (nested PCR)


M 1 2 3 4 5 6 7 8 9 10
A
947
831
564
125
M 1 2 3 4 5 6 7 8 9 10
B

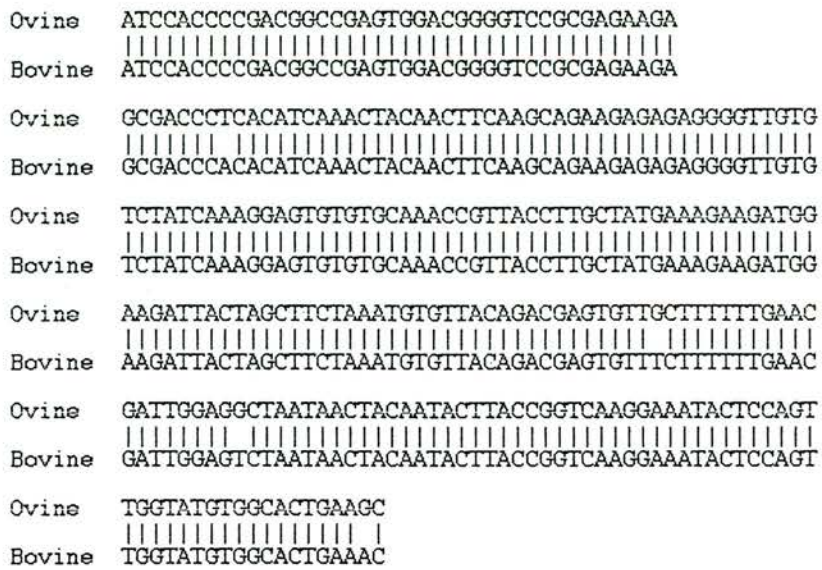


Fig. 5.3 Sequence comparison between bovine FGF-2 and the ovine FGF-2

Using consensus PCR primers derived for bovine and human FGF-2 genes, a PCR product was amplified from the brain tissue of an adult female ewe (see legends to Fig.5.1 and 5.2). The amplified PCR product was cloned into the TA cloning plasmid and sequenced. The sequence of the cloned PCR fragment was compared with the published FGF-2 sequences from different species. The pile-up is the comparison between bovine sequence (accession number m13440) and the cloned ovine gene.

	Species	% DNA similarity	Accession number
Ovine FGF-2	Bovine	98	M13440
	Human	93	M27968
	Rabbit	92	L12034
	Mouse	89	M30644
	Rat	87	X07285
	Chiken	80	M95706
	Newt	77	D89442

Table 5.2 Sequence similarities between the ovine FGF-2 homologue and FGF-2 in other species

The ovine FGF-2 homologue was cloned and sequenced from the brain tissues (see legends to Fig.5.1-3). The sequence of ovine FGF-2 gene was compared to FGF-2 DNA sequences from other species, demonstrating a high degree of evolutionary conservation in the FGF-2 gene.

5.4 Discussion

The resident brain cells are now believed to be sources for cytokines, chemokines and growth factors, which may play a role in the repair and pathology as a result of virus infection of the CNS. One such cytokine is the FGF-2.

The brain tissues obtained from different neuroanatomical sites of an adult ewe were used to clone the ovine homologue of FGF-2 gene. Using a PCR approach, an FGF-2-specific gene was amplified. The sequence analysis of the cloned PCR product demonstrated that this gene was highly homologus to the FGF-2 sequences from other species.

This is the first report of the ovine CNS-derived FGF-2 sequence analysis. Ovine FGF-2 mRNA was detected, albeit in varying amounts, in different anatomical regions of the CNS.

Most brain regions tested positive for the FGF-2 gene. However, certain regions for example the pituitary, showed an abundance of message compared to others, where nested PCR was necessary to obtain a product. This differential distribution of FGF-2 mRNA may be associated with the function and/or abundance of cells which transcribe this neurokine.

The ovine FGF-2 gene showed a high degree of sequence similarity with other published FGF-2 sequences from other species. The exceptionally highly conserved FGF-2 sequences between different species is indicative of a very strong selective pressure to maintain its biological activities. The sequenced ovine gene lacks the N-terminal putative leader peptide sequence reported in other species. This is because only the highly conserved sequences internal to the putative leader sequence was taken into consideration in designing of PCR primers. Bovine FGF-2 is a single chain, non-glycosylated protein of 16.5 kDa molecular weight (Esch *et al.*, 1985). Bovine FGF-2 is believed to be derived from a 155 amino acid protein with an amino terminal extension of nine amino acid (Met^{-9}-Ala^{-8}-Ala^{-7}-Gly^{-6}-Ser^{-5}-Ile^{-4}-Thr^{-3}-Thr^{-2}-Leu^{-1}-Pro^{+1}) not found on the mature 146 amino acid protein.

5.4.1 Relevance of neural FGF-2 to visna pathology

One of the common features of lentiviral pathology is the extensive involvement of vascular endothelium, in the form of perivascular cuffs. In the CNS, this may result in permeability of BBB and extravasation of inflammatory cells into neuroparanchyma. In AIDS, up to 30% of patients develop severe hyperplasia of

vascular endothelium and presence of spindle cells (Ensoli *et al.*, 1991). It has been shown that inflammatory cytokines (IL-1β, TNFα, and IFNγ) released from activated T cells stimulate the growth of vascular endothelia, which in turn produce chemoattractant platelet activating factor (PAF), and high levels of FGF-2 (Sciacca *et al.*, 1994; Samaniego *et al.*, 1995). Moreover, the presence of inflammatory cytokines also results in upregulation of adhesion molecules on hyperplastic endothelial cells (Kaaya *et al.*, 1996).

The inter-relationship between the FGF-2 and other cytokines/growth factors in the CNS is a complex one. Increased expression of FGF-2 in the CNS induces the expression of NGF by astrocytes, which also helps neurones to respond to NGF (Gomez-Pinilla *et al.*, 1992). Furthermore, IL-1β increases FGF-2 mRNA expression in adult rat brain and in organotypic hippocampal cultures (Rivera *et al.*, 1994).

FGF-2 in combination with PDGF has been shown to result in generation of rapidly dividing O-2A progenitor cells in adult rat optic nerve cultures. Moreover, FGF-2 treatment inhibited oligodendroglial apoptosis (Grinspan *et al.*, 1996). Further, FGF-2 immunoreactivity has been reported in microglial cells *in vivo* (Gomez-Pinilla *et al.*, 1992).

The growth promoting properties of FGF-2 may assist in remyelination in the CNS. This is significant, since damage to myelin is a common finding in lentiviral infections of the CNS. However, FGF-2 may also have adverse effects in concert with other proinflammatory cytokines in the CNS. Therefore, FGF-2 reagents described in this chapter will be used as part of a panel of cytokines to study the direct effect of MVV infection on ovine microglial cells, and its possible implication in visna neuropathology.

CHAPTER SIX
Infection of Microglia with MVV: Effect on Cytokine Gene Expression

6.1 Introduction

Data presented in chapter three demonstrated that ovine microglial cells, like microglia from other species, share extensive phenotypic markers with cells of the monocyte/macrophage lineage. Moreover, the productive infection of microglia with MVV presented in chapter four adds to previous observations with respect to the tropism of other lentiviruses for brain microglial cells.

Presence of virus-positive cells in the CNS is a prerequisite for lentiviral encephalopathies (Georgsson *et al.*, 1976; Shaw *et al.*, 1985; Shearere *et al.*, 1985; Hurtel *et al.*, 1991; Schindelmeiser and Gullotta, 1991; Dow *et al.*, 1992; Zink *et al.*, 1990; Takahashi *et al.*, 1996). However, there appears to be no correlation between lentivirus antigen-positive cells and extent of pathology in the CNS (Kure *et al.*, 1991; Petito *et al.*, 1994; Georgsson *et al.*, 1997). It is, therefore, unlikely that direct MVV infection of tissue macrophages/microglial cells (and M-tropic primate lentiviruses), is the main cause of CNS pathology. This is because of the highly restricted nature of lentiviruses *in vivo*.

A common finding in experimental infection of monocyte/macrophages with lentiviruses is that infection appears to alter, but not abrogate, the capacity of infected cells to express cellular factors and cytokines (Gruber *et al.*, 1995; Rossol *et al.*, 1992; Yoo *et al.*, 1996; Valentine *et al.*, 1992; Sasseville *et al.*, 1996; Pulliman *et al.*, 1994; Dereuddre-Bosquet *et al.*, 1997; Lechner *et al.*, 1997; Legastelois *et al.*, 1997).

Soluble factors released from activated macrophages and microglial cells, due to direct infection or via virus-coded proteins may contribute to neuropathology (Nuovo and Alfieri, 1996; Tyor *et al.*, 1992; Merrill *et al.*, 1992; Nottet *et al.*, 1996; Karsten *et al.*, 1996; Genis *et al.*, 1992; Dollard *et al.*, 1995; Tyor *et al.*, 1993; Pulliman *et al.*, 1991, Yoshika *et al.*, 1995; Nottet *et al.*, 1997). For example, proinflammatory cytokines have been detected in the CNS of patients with HIV (Nuovo and Alfieri, 1996; Burkinsky *et al.*, 1995). Interestingly, such signals co-localised with both virus-positive and virus-negative cells (Nuovo and Alfieri, 1996). Similarly, measurements of CSF from such patients have also revealed increased levels of proinflammatory cytokines and neurotoxins (Tyor *et al.*, 1992; Nottet *et al.*, 1996).

Microglial cells may constitute up to 20 % of glial cells in the CNS. Moreover, they are the main cell-type most commonly infected with primate lentiviruses *in vivo*.

6.2 Experimental design

The experiments in this chapter were aimed at asking the question of whether infection of microglial cells with MVV could result in an altered cytokine expression, which may in turn contribute to neuropathogenesis/pathology *in vivo*. Therefore, enriched cultures of ovine microglial cells were subjected to infection with MVV *in vitro*, followed by semi-quantitative RT-PCR assays for a panel of ovine cytokines.

To allow for the semi-quantitative analysis of cytokine RT-PCRs, mRNA for the housekeeping gene ATPase was used as the internal control. A series of PCR cycles were performed to obtain a calibration curve. The data presented in this chapter were obtained from four separate brain cultures.

6.3 Results

6.3.1 Analysis of gene expression in cultured microglial cells by semi-quantitative PCR

A reverse-transcriptase-PCR (RT-PCR) approach was employed to measure the levels of a panel of cytokines by cultured microglial cells. The PCR signals normalised against the housekeeping gene, ATPase. A series of PCR cycles were carried out on ATPase gene derived from microglial cells, and a cycle corresponding to the linear part of the calibration curve was chosen for subsequent PCR reactions (Fig.6.1).

RT-PCR signals were detected for TNFα, IL6 and TGFβ. However, no signals were detected for IL-1β, GM-CSF, IL-10 and IL-12, and FGF-2 in both mock-infected and MVV-infected microglial cultures (Fig.6.2). In contrast to message levels for TNFα and IL-6, which appeared to exist in minute quantities, TGFβ was expressed at relatively high quantities, exceeding the message levels for ATPase.

6.3.2 MVV-mediated alteration s in expression of cytokines by cultured microglial cells

Infection of ovine microglial cells with MVV resulted in significant up-regulation of TNFα at transcription level *in vitro* (Fig. 6.3). This direct effect of MVV on microglial cells may have implications in the neuropathology of visna. This is because TNFα has been implicated in several demyelinating diseases. Similarly, interleukin-6 mRNA was significantly elevated in MVV-infected microglial cultures (Fig.6.4).

In contrast to TNFα and IL-6, whose expression was elevated by MVV infection, the expression of message for TGFβ appeared to be reduced (Fig.6.5). Although

statistical analyses showed no significant reduction in TGFβ mRNA after infection with MVV, nevertheless, a reduced trend was observed.

Fig. 6.1 Calibration curve of RT-PCR for the housekeeping gene ATPase

A series of ATPase PCR cycles were performed on total RNA isolated from microglial cultures, in order to obtain a standard curve for the housekeeping gene. Ten microlitre aliquots from each PCR cycle was run on an agarose gel., followed by Southern blot, using an ATPase-specific probe. The blot was scanned using a phosphoimager. The graph shows the result of the Southern blot.

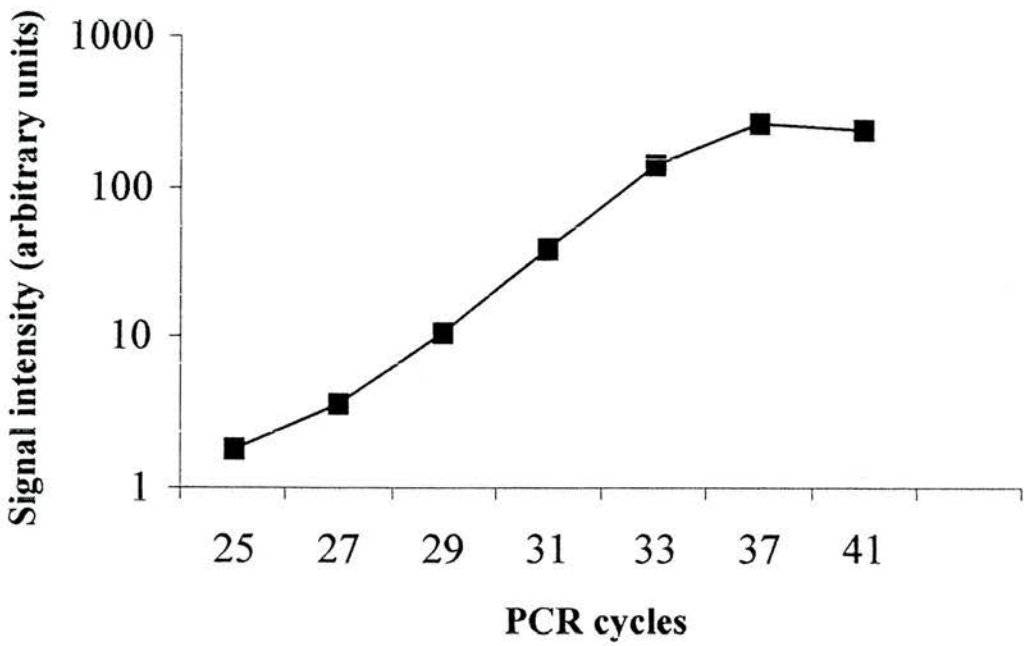

Signal intensity (arbitrary units)
1000
100
10
1
25
27
29
31
33
37
41
PCR cycles

Fig. 6.2 mRNA profiles of ovine microglial cells infected with MVV *in vitro*

Fluctuations in cytokine mRNA levels of cultured microglial cells because of MVV infection were assesed by RT-PCR. Enriched microglia (100,000 cells per culture), either mock-infected (-) or MVV-infected (+) with 0.001 MOI of K1514. Total RNA was isolated from treated cultures at four days post-infection. The identities of PCR signals were ascertained using gene-specific radioisoptope-labelled oligonucleotide primers followed by Southern blot analysis. The figure is an example of a Southern blot from one such experiment.

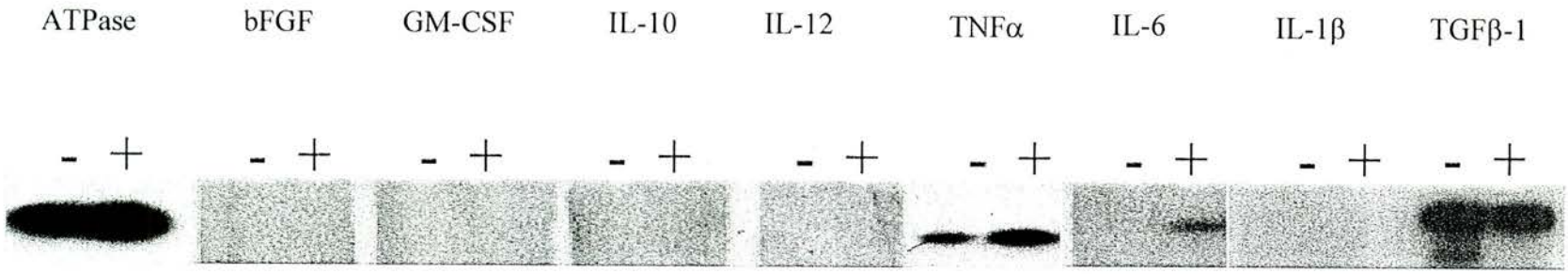

ATPase
bFGF
GM-CSF
IL-10
IL-12
TNFα
IL-6
IL-1β
TGFβ-1
- +
- +
- +
- +
- +
- +
- +
- +
- +

Fig. 6.3 Effect of MVV infection on TNFα mRNA expression by cultured microglial cells

Enriched microglial cultures were infected with K1514 at MOI of 0.001. The levels of TNFα mRNA expression in mock-infection and MVV-infected microglia (100,000 per culture) were analysed at four days post-treatment. Microglial cells were either mock-infected or MVV-infected as outlined in the legend to Fig.6.2. TNF-alpha-specific RT-PCR was performed on RNA samples from treated cultures. Ten microlitre aliquots were run on an agarose gel. The agarose gel was Southern blotted with TNF-alpha-specific probe. Probed blots were then scanned using a phosphoimager. Data were obtained from four separate brains, and presented as mean +/- 1 SD. Statistical analysis was performed by students t test.

** Denotes a significant increase in message levels ($p<0.05$) between MVV-infected and mock-infected cultures.

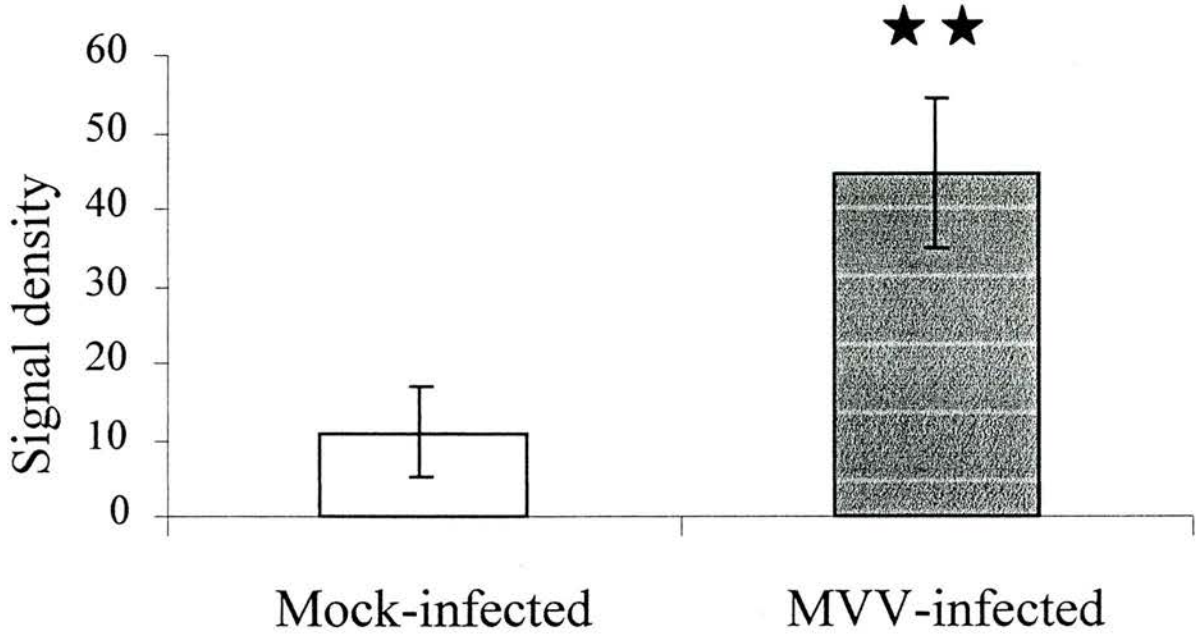

Signal density
60
50
40
30
20
10
0
★★
Mock-infected
MVV-infected

Fig. 6.4 Effect of MVV infection on IL-6 mRNA expression by cultured microglial cells

Enriched microglial cultures were infected with K1514 at MOI of 0.001. The levels of IL-6 mRNA expression in mock-infection and MVV-infected microglia (100,000 per culture) were analysed at four days post-treatment. Microglial cells were either mock-infected or MVV-infected as outlined in the legend to Fig.6.2. IL-6-specific RT-PCR was performed on RNA samples from treated cultures. Ten microlitre aliquotes were run on an agarose gel. The agarose gel was Southern blotted with IL-6-specific probe. Probed blots were then scanned using a phosphoimager. Data were obtained from four separate brains, and presented as mean +/- 1 SD. Statistical analysis was performed by students t test.

** Denotes a significant increase in message levels ($p<0.05$) between MVV-infected and mock-infected cultures.

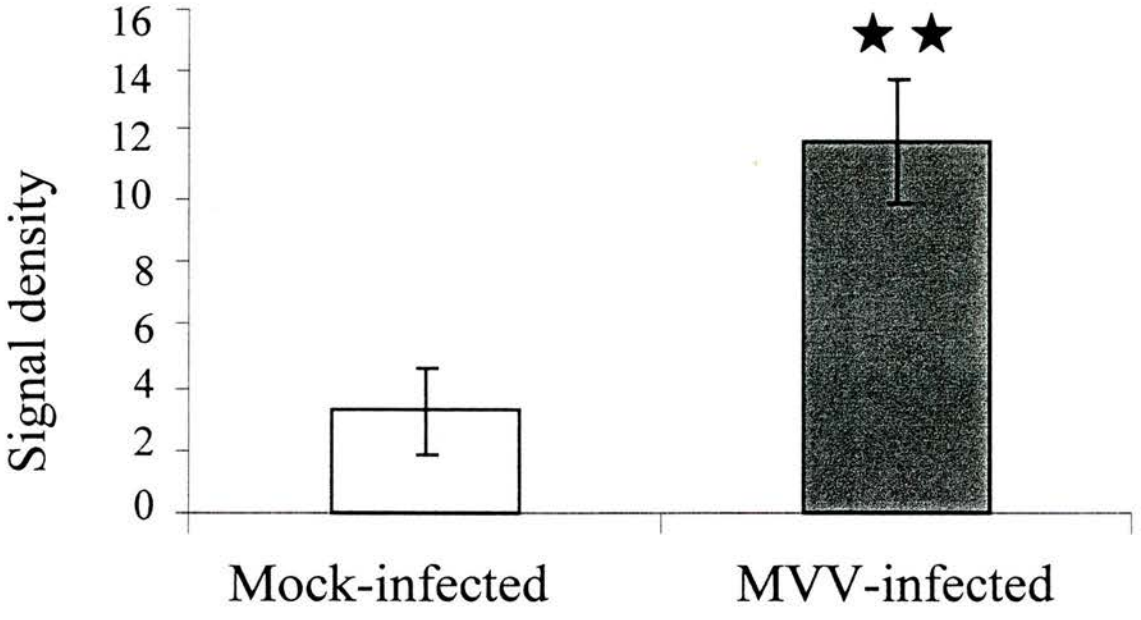
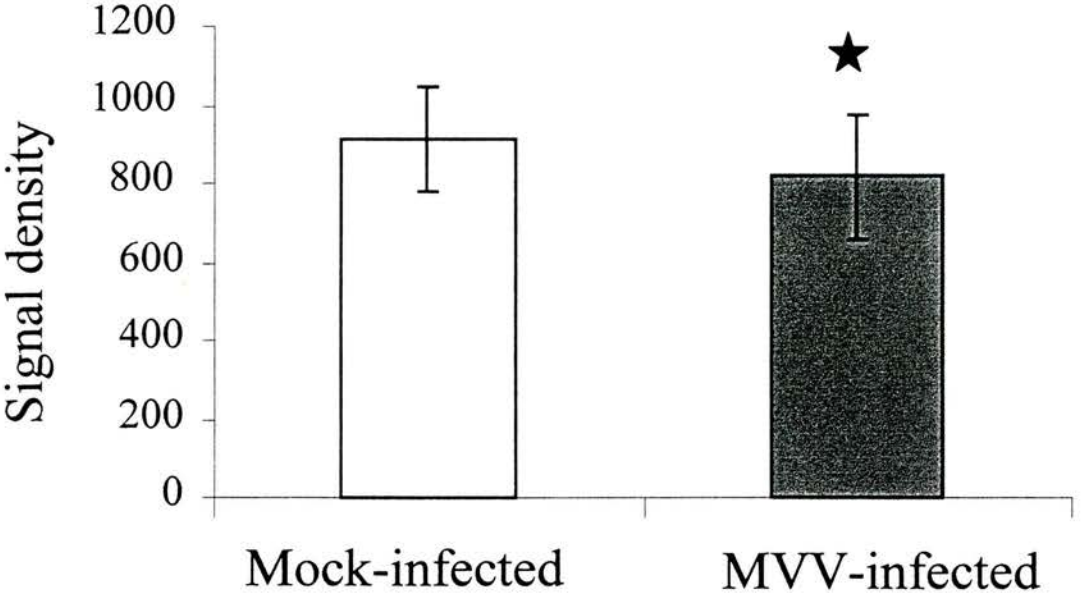

Signal density
16
14
12
10
8
6
4
2
0
Mock-infected
MVV-infected

Fig.6.5 Effect of MVV infection of TGFβ-1 mRNA expression by cultured microglial cells

Enriched microglial cultures were infected with K1514 at MOI of 0.001. The levels of TGFβ-1 mRNA expression in mock-infection and MVV-infected microglia (100,000 per culture) were analysed at four days post-treatment. Microglial cells were either mock-infected or MVV-infected as outlined in the legend to Fig.6.2. TGFβ-1-specific RT-PCR was performed on RNA samples from treated cultures. Ten microlitre aliquotes were run on an agarose gel. The agarose gel was Southern blotted with TGFβ-1-specific probe. Probed blots were then scanned using a phosphoimager. Data were obtained from four separate brains, and presented as mean +/- 1 SD. Statistical analysis was performed by students t test.

* Denotes a lack of a significant difference ($p>0.05$) between MVV-infected and mock-infected cultures.


1200
1000
800
600
400
200
0
Signal density
Mock-infected
MVV-infected

6.4 Discussion

This is the first study on direct interactions of MVV with ovine microglial cells *in vitro*. Cytokine profiling was performed on mock-infected and MVV-infected ovine microglial cells *in vitro*. Moreover, the infection of microglial cells resulted in altered cytokine phenotype. PCR signals were detected for TNFα, IL-6 and TGFβ. Conversely, under conditions used, PCR signals were not detected for bFGF (FGF-2), IL-1β, GM-CSF, IL-10 and IL-12 (Fig. 6.2). The elevated expression of TNFa and IL-6 by microglial cells and because of infection with MVV may have implications in visna pathogenesis/pathology.

TNFα is a pleiotropic cytokine that is primarily produced by activated macrophages, but also by T cells (reviewed by Tracy and Cerami, 1994). TNFα has been shown to be directly toxic to oligodendrocytes *in vitro*, which may take place in demyelinating disorders (Robbins *et al.*, 1987; Selmaj et al., 1988; Frei *et al.*, 1997). This may be of importance, because in visna neuropathology, secondary demyelination similar to MS have been observed (reviewed by Georgsson, 1994). Moreover, activation of microglial cells and astrocytes *in vitro* results in increased expression of TNFα (Lafortune et al., 199; Chao *et al.*, 1995;Lee *et al.*, 1993; Sheng *et al.*, 1995; Hetier *et al.*, 1991).

TNFα has been closely associated with HIV-induced central and peripheral neuropathies (Tan *et al.*, 1996; Neuovo *et al.*, 1996). Moreover, in murine EAE, TNFα mRNA and protein have been identified in the CNS in the acute phase of the disease (Renno *et al.*, 1995). Administration of soluble type I receptor for TNFα, as well as antibodies to TNFα and the related molecule, TNFβ, prevented the development of EAE (Ruddle *et al.*, 1990; Selmaj *et al.*, 1995).

In AIDS brains, TNFα expression has been associated with perivascular macrophages, microglia, endothelia and astrocytes (Dereuddre-Bosquet, *et al.*, 1997). Moreover, microglia isolated from SIV-infected macaques produced more TNFα when compared to uninfected controls (Sopper *et al.*, 1996). There is also some evidence that plasma levels of TNFα in HIV-infected patients correlates with disease progression (Chollet-Martin *et al.*, 1994; Than *et al*, 1997). This, however, has been disputed by others (Tan *et al.*, 1996).

Interleukin-6 (formerly B cell stimulatory factor-2, hepatocyte stimulating factor, hybridoma-plasmocytoma growth factor, and IFNβ-2), is a multifunctional

glycoprotein and a member of a large family of proteins including leukaemia inhibitory factor (LIF, formerly differentiation inhibition activator or DIA), oncostatin M (oncM), ciliary neurotrophic factor (CNTF), and IL-11 (Neddermann *et al.*, 1996). IL-6 is best characterised in the periphery as a proinflammatory cytokine, with biological functions overlapping with those of IL-1β and TNFα. IL-6 is also a potent inducer of acute phase proteins (Le *et al.*, 1989, Hirano *et al.*, 1990).

IL-6 is one of the cytokines synthesised in the CNS as part of the neurokine family of proteins. In normal CNS, IL-6 is found in ependymal cells, astrocytes, in processes adjacent or attached to the ventricles, and in cellular processes in the white matter (astrocytes and microglial cells) (Le Vine *et al.*, 1997). Cortical and hippocampal neurones also synthesise IL-6 (Ringheim *et al.*, 1995; Pousset, 1994).

Preliminary results obtained from infection of cultured caprine microglial cells with CAEV also showed an increase in IL-6 (Baszler *et al.*, 1994). However, there are conflicting reports with respect to induction of IL-6 by mononuclear cells after infection/stimulation with HIV or viral proteins (Molina *et al.*, 1990; Berman *et al.*, 1994; Navikas *et al.*, 1995, Lafreine *et al.*, 1997). Microglial cells isolated from healthy macaques produced more IL-6 than macrophages. Moreover, production of IL-6 by microglia infected with SIV increased with time, despite the fact that very few cells contained replicating virus (Sopper *et al.*, 1996).

Infection of cultured microglial cells did not appear to significantly alter the transcription of TGFβ-1 *in vitro*, although a reduced trend was apparent (Fig.6.5). Interestingly, in CAEV-infected macrophages a decrease in TGFβ-1 mRNA has been reported (Lechner *et al.*, 1997).

Cultured ovine microglial cells constitutively expressed TGFβ-1 mRNA at relatively high levels. Similarly, cells of monocyte/macrophages have been shown to constitutively express TGFβ mRNA (Biron, 1994). Constitutive expression of TGFβ-1 mRNA has also been observed in cultured astrocytes (Morganti-Kossmann *et al.*, 1992). However, human brain tissues do not appear to express TGFβ-1 protein under normal conditions (Wahl *et al.*, 1991).

The discrepancy between high levels of mRNA and translation of TGFβ-1 is because of the biology of this protein. Unlike most cytokines where induced levels of message parallels translated protein, TGFβ protein is produced as inactive precursor molecules and must be processed to become biologically active. Consequently, expression of

TGFβ can be regulated at transcriptional, translational and post-translational levels (reviewed by Biron, 1994). It is therefore difficult to draw definitive conclusions based solely on TGFβ-1 mRNA levels *in vitro*.

Immunoreactive TGFβ has been detected in the brain tissues of patients with HIV (Wahl *et al.*, 1991). TGFβ-positive cells co-localised with infected and uninfected macrophages and astrocytes in HIV-infected brains samples (Morganti-Kossmann *et al.*, 199). Both Tat and Env-derived glycoproteins of HIV have been shown to stimulate TGFβ-1 expression in glial culture. It is, therefore, postulated that release of viral components from infected cells may therefore stimulate the neighbouring glia to express TGFβ-1 (Cupp *et al.*, 1993; de Cunha *et al.*, 1995).

In maedi lungs, an increase in TGFβ-1 immunopositive cells was noted compared to control lungs. However, TGFβ-1 expression was down regulated in more advanced disease (Moreno *et al.*, 1997). This reversal of TGFβ-1 expression may parallel the degree of lymphocytic infiltration, in which case T cell-derived cytokines, for example IFNγ may play a regulatory role.

6.5 Summary

Infection of cultured microglial cells with MVV resulted in altered expression of TNFα and IL-6. These two cytokines have been implicated in the neuropathology of several viral infection of the CNS, and may also contribute to visna pathology.

The ability of MVV to induce the expression of TNFα and IL-6 may suggest that presence of virus in neuroparenchyma can contribute to pathology via bystander damage, and independent of an active immune response.

Gliosis and neuronal damage are common occurrence in lentiviral infection of the CNS. The direct effect of MVV on expression of pro-inflammatory cytokines demonstrated in this chapter, may have implications in the neuropathology of visna. For example, TNFα has been shown to result in astrogliosis and proliferation and activation of microglial cells *in vitro* (Selmaj *et al.*, 1990; Merril *et al.*, 1991).

The experimental approach in the next chapter was aimed at testing the hypothesis that whether virus-mediated responses, independent of a specific immune system can contribute to neural changes associated with visna infection of the CNS.

CHAPTER SEVEN
Infection of Microglia with MVV: Implications for Gliosis and Neurotoxicity

7.1 Introduction

In previous chapter, infection of microglial cells with MVV was shown to result in an altered cytokine profile, which may have implications in visna-mediated neuropathology.

A common histopathological feature of visna infection is astrocytosis and formation of microglial nodules, with astrocytosis being more pronounced in inflammatory foci than microglial nodules. Moreover, in visna, demyelinating lesions similar to MS have been observed (Georgsson *et al.*, 1982, 1990). In EAE models of MS, astrocytosis coincides with the onset of clinical disease and inflammation. However, astrocytosis may also persist after clinical recovery (Smith *et al.*, 1983; Aquino *et al.*, 1988).

The presence of gliosis within inflammatory foci in EAE models of MS and in visna would suggest a possible association between astrogliosis and release of proinflammatory cytokines by infiltrating lymphocytes and mononuclear cells. Moreover, cytokines (IFNγ, TNFα, IL-1α/β, IL-6, IL-7, IL-10, GM-CSF and MCP-1) have been shown to promote activation of astrocytes (Balasingam *et al.*, 1994; Giulian *et al.*, 1988; Yong *et al.*, 1991; Rostworowski *et al.*, 1997; Glabinski *et al.*, 1996; Balasingam *et al.*, 1996).

The interaction between lentiviruses and cells of the monocyte/macrophage lineage, either by direct infection and/or via release of viral and cellular factors may result in activation of these cells. For example, an activated phenotype has been associated with microglial cells in visna infected brain tissues (Georgsson *et al.*, 1997). Astrocytosis has been associated with the presence of activated macrophages/microglial cells both *in vitro* and *in vivo* (Giulian *et al.*, 1994; Balasingam *et al.*, 1996; Taupin *et al.*, 1997). Furthermore, attenuation of astrogliosis has also been correlated with reduced CD11b/CD18 (Mac-1, CR3) immunoreactivity of macrophage/microglia, suggesting a role for microglial cells in initiating astrocytosis (Balasingam and Yong, 1996).

Another feature of lentiviral pathology is neuronal damage in the absence of an apparent direct viral infection of the neurones (see Chapter 1). Infection of monocyte/macrophage cells with HIV-1 has been shown to result in release of soluble toxins when tested on brain cultures (Pulliam *et al.*, 1991; Giulian *et al.*, 1990).

Lentiviral proteins, Tat and membrane glycoproteins can also have neurotoxic properties (Gordou *et al.*, 1990; Sabatier *et al.*, 1991; Philippon *et al*, 1994; Budka *et al.*, 1989; Wu *et al.*, 1996). Interestingly, neurotoxicity is significantly enhanced in the presence of microglial cells (Lipton, 1992). Furthermore, only HIV-infected macrophages rather than HIV-infected T lymphocytes appear to produce toxins (Pulliam *et al.*, 1991). This observation suggests that cellular factors independent of viral products (but related to virus infection) might mediate neurotoxicity.

The above observations would imply that activated macrophages/microglial cells could potentially contribute to neuropathological features (gliosis and neuronal damage/loss) associated with lentiviral infections of the CNS.

7.2 Experimental design

The experiments in this chapter were aimed at deciphering the direct role of MVV-infected microglial cells in gliosis (microgliosis and astrogliosis), independent of acquired (specific) immune responses. For this purpose, mice with severe combined immunodeficient (SCID) genotype incapable of mounting specific immune responses were used. Adult SCID mice (sex and age-matched) were used. Cultured ovine microglial cells, mock-infected and MVV-infected with 0.1 MOI of the K1514 starin, were grafted into the striata of SCID mice. Brains were harvested on day three post-injection and the extent of gliosis was assessed by immunostaining for murine microglial and astrocytic cell markers using F4/80 and GFAP antibodies, respectively. The presence of microglial-derived neurotoxic factor (s) because of MVV infection *in vitro* was assessed. Primary cultures of murine trigeminal neurones were set up. Supernatants from mock-infected and MVV-infected microglial cells were harvested on day 4-5 post-infection and tested for neurotoxic factor (s) on murine trigeminal culture over a period of three days. Neurotoxicity was assessed by loss of neurones and morphological changes.

7.3 Results

7.3.1 Grafting of ovine microglial cells into SCID brains

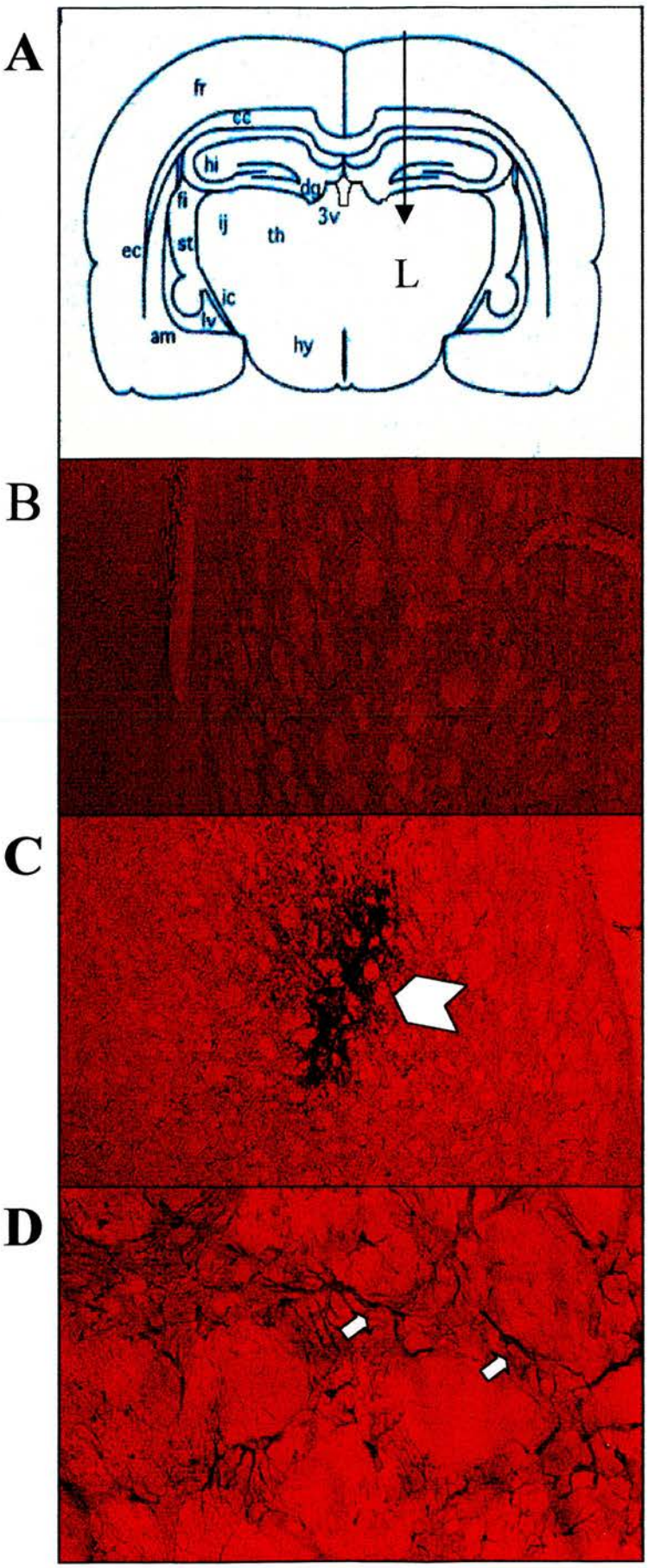
To address the possible role of MVV-infected microglial cells in activation of microglia and astrocytes *in vivo*, enriched cultures of microglial cells (>98% pure) were inoculated into the neuroparenchyma of SCID mice. Both mock-infected and MVV-infected microglial grafts resulted in increased staining of astrocytes and microglial cells in injected hemispheres at three days post-inoculation (Fig.7.1).

7.3.2 MVV-infected microglial cells initiate gliosis in SCID brains

To assess gliosis in SCID brains because of MVV infection, immunostained sections were image analysed. Significant increases ($p<0.05$) were observed in levels of F4/80 (microgliosis) and GFAP (astrogliosis) in SCID brains inoculated with MVV-infected ovine microglial cells (Figs. 7.2 and 7.3).

Fig.7.1 Intrastriatal injection of ovine microglial cells into SCID brains

Ovine microglial cells, mock-infected or MVV-infected with MOI 0.1 of K1514 and were injected into the striata of SCID mice (15000 cells per mouse in total volume of three microlitres). Brains were fixed at three days post-injection and floating sections immunostained with GFAP or F4/80. Each group consisted of three sex and age-matched adult female mice. Panel A details the site of injection into the striatum; Panels B, C and D show GFAP stained brain sections. B is the uninjected, control side, whereas C is the injected striatum. The lesion site (arrowhead) is magnified in panel D. L is the location of lesion. Abbreviations: am, amygdala; cc, corpus callosum; dg, dentate gyrus; ec, external capsule; fi, fimbria; fr, frontal cortex; hi, hippocampus; hy, hypothalamus; ic, internal capsule; ij, interventricular junction; lv, lateral ventricle; st, stria terminalis; th, thalamus; 3v, third ventricle.


A
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hy
B
C
D

Fig.7.2 Effect of MVV-infected microglial cells on murine microglia *in vivo*

Ovine microglial cells, either mock-infected or MVV-infected were injected with as stated in the legend to Fig.7.1 were injected into SCID brains (15,000 cells per brain) one day post-infection *in vitro*, and brain tissues harvested as outlined in the legend to Fig.7.1.. Sections were stained with F4/80 for murine brain microglial/macrophages. Stained sections were image analysed using the NIH IMAGE software. Each group consisted of three age- and sex-matched SCID mice.

Data presented as mean +/- 1 SD.

** Denotes a significant increase ($p<0.05$) in mice injected with MVV-infected microglial cells.

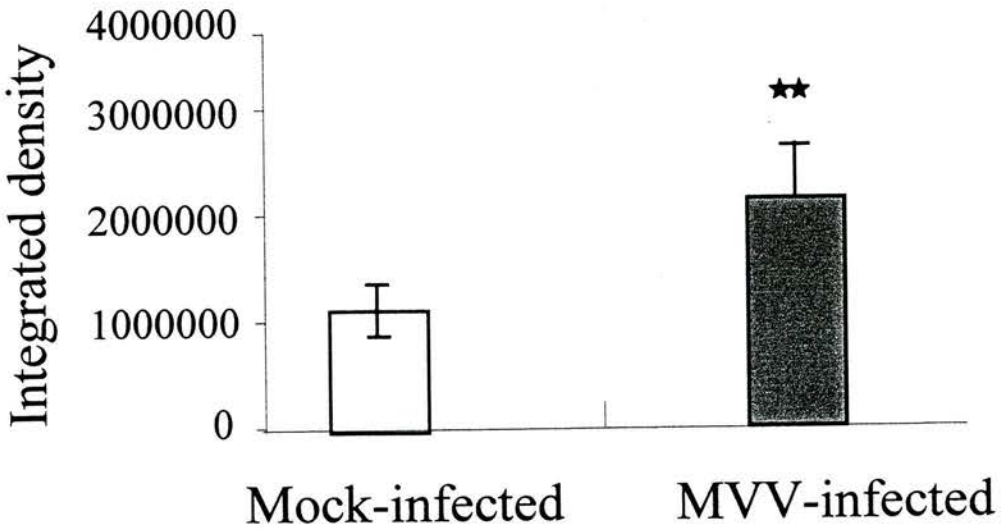
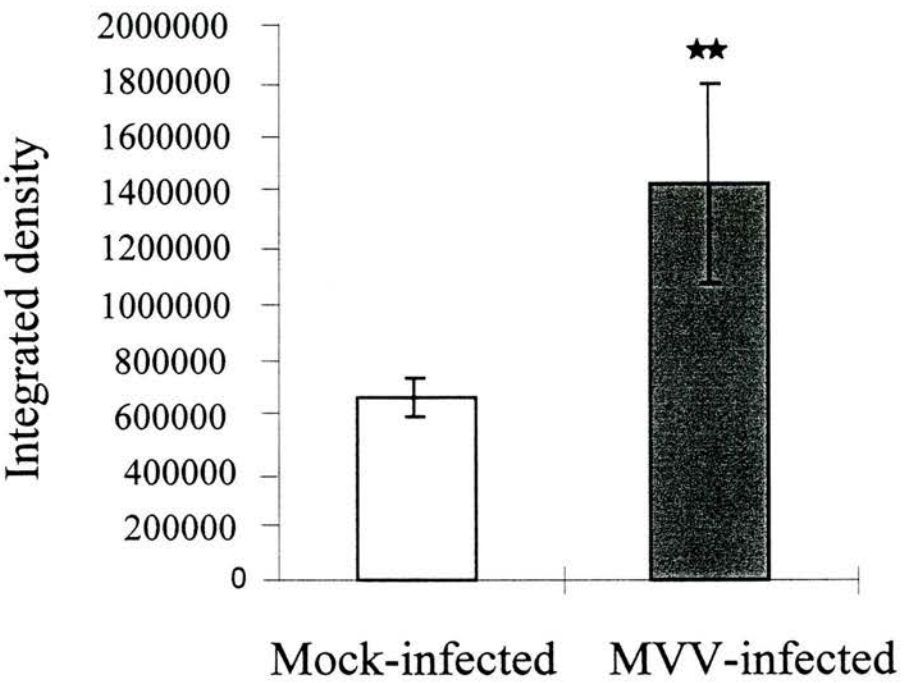

Integrated density
4000000
3000000
2000000
1000000
0
**
Mock-infected
MVV-infected

Fig.7.3 Effect of MVV-infected microglial cells on murine astrocytes *in vivo*

Ovine microglial cells, either mock-infected or MVV-infected (TCID50 = 3000) were injected into SCID brains (15,000 cells per brain) one day post-infection *in vitro*, and brain tissues harvested as outlined in the legend to Fig.7.1.. Sections were stained with GFAP for astrocytes. Stained sections were image analysed using the NIH IMAGE software. Each group consisted of three age- and sex-matched SCID mice. Data presented as mean +/- 1 SD.

** Denotes a significant increase ($p<0.05$) in mice injected with MVV-infected microglial cells.


Integrated density
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
★★
Mock-infected
MVV-infected

7.3.3 Release of neurotoxins from MVV-infected microglia

To assess the possibility that MVV infection of microglial cells may result in release of neurotoxins, supernatants from both mock-infected and MVV-infected cultures were tested on primary neuronal cultures derived from murine trigeminal ganglia. Treatment with supernatants derived from MVV-infected microglial cells resulted in loss of neurones *in vitro* (Fig.7.4). Moreover, the morphological abnormalities, for example neurite atrophy and formation of short fascicles, were apparent in cultures treated with supernatants derived from MVV-infected microglia. These microscopic abnormalities were apparent at approximately 10 hours post-treatment. In later time points, neurones treated with supernatants from MVV-infected microglial cultures also showed cytoplasmic vacuolation (Fig7.5).

7.3.4 Characterisation of MVV-mediated microglial neurotoxin (s)

The neurotoxicity experiments prompted attempts to semi-characterise the soluble neurotoxin (s) in supernatants from MVV-infected microglial cultures. To assess the possible proteinaceous nature of neurotoxin (s), supernatants from treated microglial cultures were subjected to heat treatment. However, this approach proved inconclusive in deciphering the physical nature of neurotoxin. This was probably because heat-treatment resulted in loss of nutrients and non-specific loss of neurones treated with both mock-infected and MVV-infected supernatants.

Experiments were carried out to determine the dilution necessary to sustain neurotoxicity. However, dilution of supernatants from MVV-infected microglial cultures resulted in loss of neurotoxic properties, suggestive of minute quantity of the putative neurotoxin (s) present in infected supernatants.

7.3.5 Visna Tat could replace the neurotoxicity observed with supernatants from MVV-infected microglial cultures.

To assess whether visna-derived products could also mediate neurotoxicity on trigeminal neurones, a synthetic peptide derived from the *tat* ORF of visna was used. The treatment of the arginine-rich Tat peptide and not the control peptide resulted in morphologial abnormalities similar to those observed with cultures treated with supernatants from MVV-infected microglial cells (Fig.7.6).

Western blot analyses were performed to determine the possible presence of Tat in supernatants from MVV-infected microglial cells, which may mediate neurotoxicity.

Supernatant from sheep chondrocytes infected with MVV (TCID50 = 30,000) was used as positive control for Tat. Blots were immunostained with anti-Tat peptide antibodies. However, repeated attempts failed to detect Tat-related products in supernatants from either MVV-infected sheep chondrocytes or microglial cells (Data not shown)

Fig 7.4 Neurotoxicity of MVV-infected microglial cells on trigeminal neurones

Ovine microglial cells were mock-infected and MVV-infected with MVV in vitro, as outlined in the legend to Fig.6.2. Supernatants from microglial cells were harvested at 4 days post-treatment. Primary cultures of murine trigeminal neurones were treated with neat supernatants from mock-infected (closed square) and MVV-infected (open square) ovine microglial cells. Changes in morphology and numbers of treated trigeminal neurones were observed over time under a light microscope. Data presented as mean +/- 1 SD.

** Denotes a significant ($p<0.05$) loss of neurones in cultures treated with supernatants from MVV-infected microglial cells.

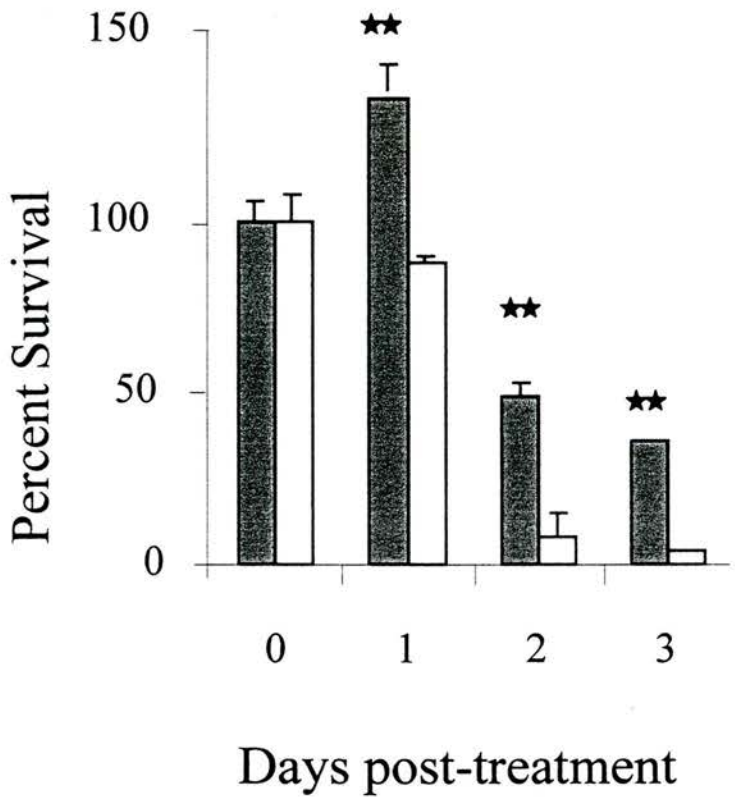

150
100
50
0
Percent Survival
★★
★★
★★
0
1
2
3
Days post-treatment

Fig. 7.5 Morphological changes in murine trigeminal neurones treated with supernatants from MVV-infected microglia

Trigeminal neurones were treated with supernatants from mock-infected and MVV-infected ovine microglia as outlined in the legend to Fig.7.4. Treated neurones were fixed on day 2 post-treatment and stained for NF-M followed by immunoflurescence microscopy, using anti-rabbit FITC-cojugate as the secondary antibody. The morphological abnormalities in cultures treated with supernatants from MVV-infected microglia included ballooning of cytoplasmic membrane (A and B; arrows), loss of arborisation (arrowheads in panel A, and C), pits in cytoplasm (arrows in D), and thickening of neurites (D). Supernatants from mock-infected microglia did not show these morphological abnormalities (E). Magnifications at x 125.

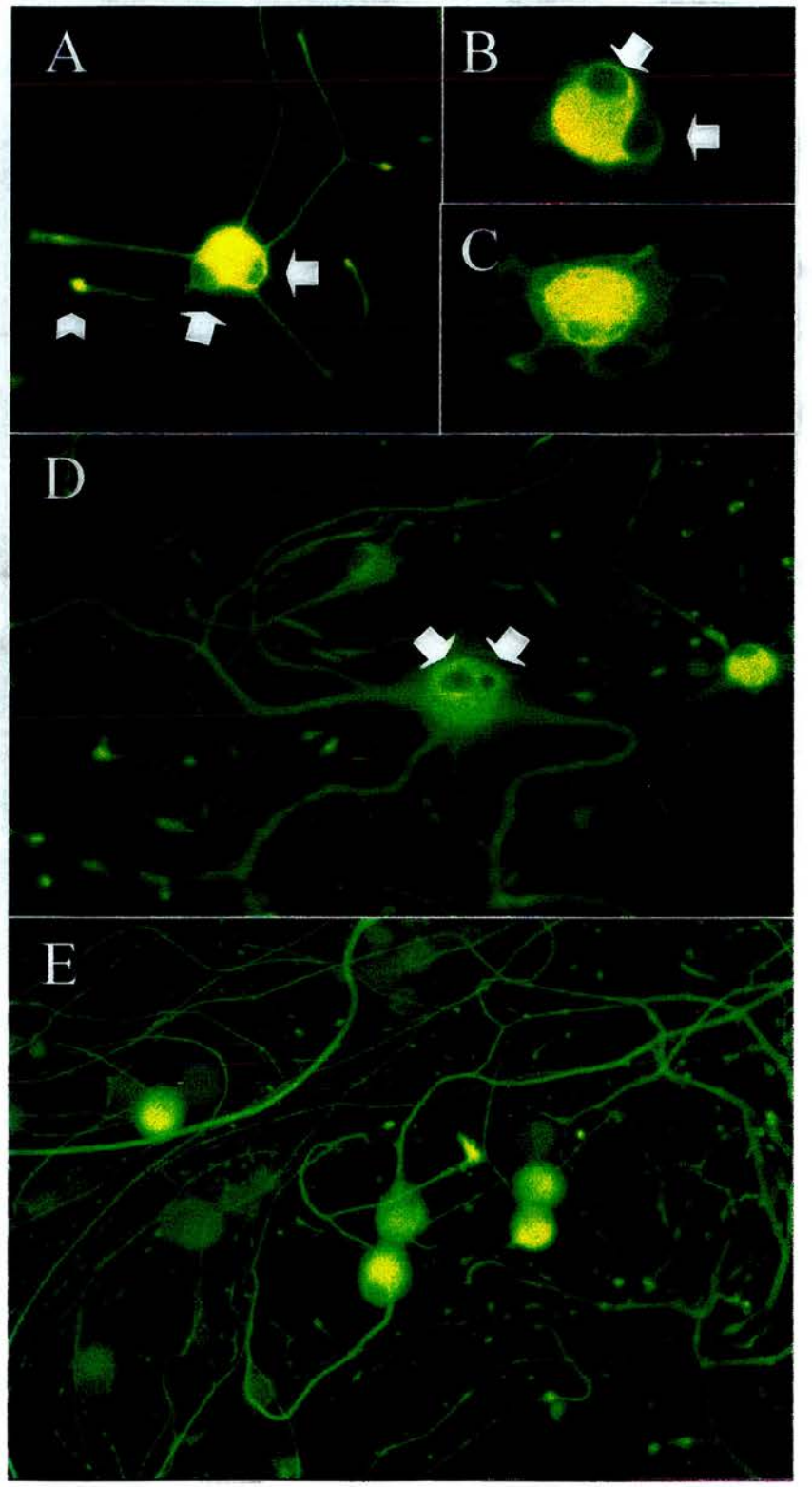
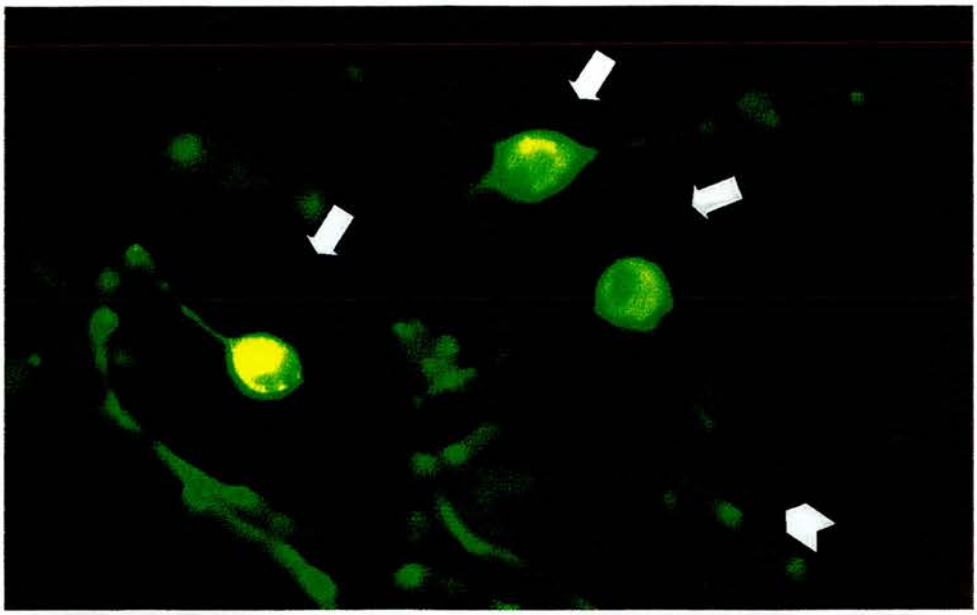

A
B
C
D
E

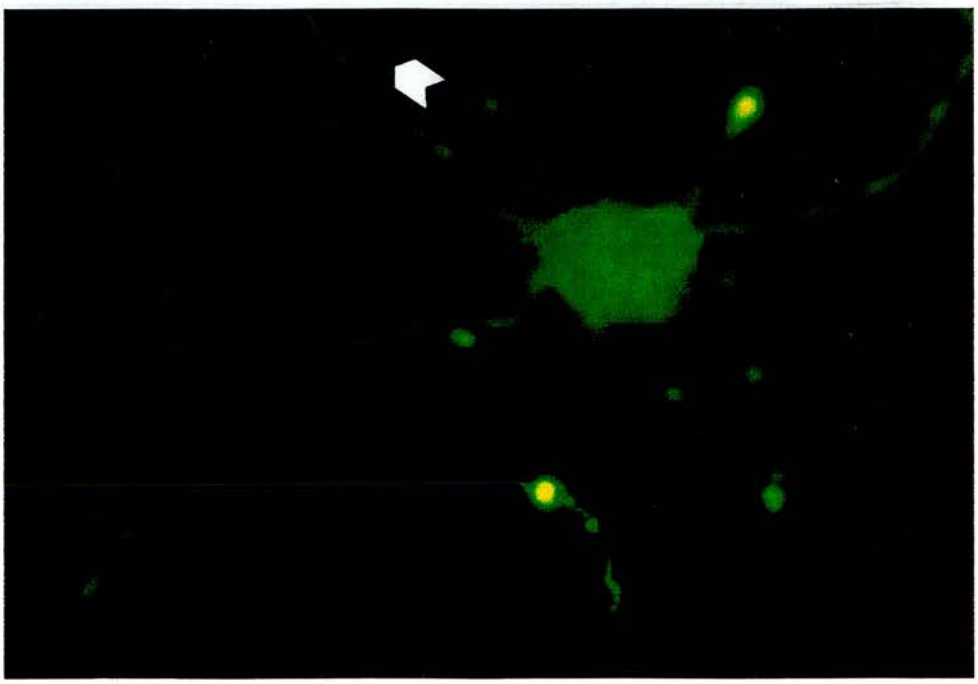
Fig. 7.6 Morphological changes associated with trigeminal neurones treated with Visna Tat peptide

Trigeminal neurones were treated with supernatants from either Tat or Ova peptides. Neurones were fixed on day 2 post-treatment and stained for NF-M, as outlined in the legend to Fig.7.5. Morphological abnormalities in cultures treated with Tat included ballooning of cytoplasmic membrane and loss of arborisation (B). Cultures treated with Ova peptide did not show these morphological abnormalities (A). Arrows indicate cell healthy looking cell bodies in Ova treated cultures. Arrowhead indicates shortening of dendrites and loss of elaborate neurite arborisation. Magnifications: A x 125; B x 200

A



B



7.4 Discussion

7.4.1 MVV-infected ovine microglial cells promote activation of microglia and astrocytes *in vivo* independent of specific immune responses

Intracerebral inoculation of SCID mice with MVV-infected ovine microglial cells resulted in apparent increased immnostaining of F4/80 (Fig.7.2) and GFAP (Fig. 7.3). The induction of gliosis in SCID mice illustrates that non-specific inflammatory responses independent of a lymphocytic infiltrate can take place in the CNS.

Similar observations have been made with HIV-1-infected monocytes inoculated intracerebrally into SCID mice (Persidsky *et al.*, 1996). Moreover, an association between gliosis and activated phenotype of resident murine microglial cells (presence of microglial nodules) was observed in the CNS of these mice (Persidsky *et al.*, 1996). This observation also indicates that a direct viral infection is not a prerequisite for activation of microglia *in vivo*.

7.4.2 Possible signals for gliosis *in vivo*

The ability of MVV to induce the expression of IL-6 *in vitro* may signal for gliosis (see Chapter 6). Over-expression of IL-6 in murine CNS has been shown to result in reactive astrocytosis and an increase in ramified (process-bearing) microglial cells (Fattori *et al.*, 1995). Moreover, in HIV/SCID experiment, activated resident murine microglia were a source of neural IL-6 (Persidsky *et al.*, 1996).

Activated astrocytes have been shown to synthesise and release the β-chemokine MCP-1 (Hayashi *et al.*, 1995). This chemokine is a potent chemoattractant for both blood-derived monocytes and microglial cells in the neuroparenchyma (Calco *et al.*, 1996; Hayashi *et al.*, 1995). Moreover, in an EAE model of MS, astrocytic expression of MCP-1 was closely associated with the clinical disease (Ransohoff *et al.*, 1993; Hulkpwer *et al.*, 1993). Other cellular sources of MCP-1 in EAE models of MS are macrophages, lymphocytes, and endothelial cells (Berman *et al.*, 1996).

TNFα has been shown to directly upregulate MCP-1 at both mRNA and protein levels (Hurwitz *et al.*, 1995). The inoculated human macrophages in SCID brains frequently stained positive for TNFα (Tyor *et al.*, 1993). Similarly, MVV-infection of ovine microglial cells elevated the expression of TNFα at the message level (see chapter 6). Moreover, intrastriatal injection of visna Tat peptide resulted in elevated levels of

endogenous proinflammatory cytokines (IL-1β, TNFα, IL-6) and iNOS (Philippon *et al.*, 1994).

Visna and HIV-1 Tat proteins have neurotoxic properties when tested *in vivo* and *in vitro* (Gordou *et al.*, 1990; Sabatier *et al.*, 1991; Philippon *et al.*, 1994; Hayman *et al.*, 1993; Nath *et al.*, 1996). These observations suggest that neurotoxicity may be mediated independent of virus infection. Use of peptide homologues have shown that Tat can be directly toxic to neurones (Strijbos *et al.*, 1995; Nath *et al.*, 1996). The neurotoxicity of lentiviral Tat has been specified to an arginine-rich motif in the basic domain. Intrastriatal injection of the arginine-rich synthetic peptide was sufficient to induce gliosis and loss of neurones *in vivo* (Hayman *et al.*, 1993; Philippon *et al.*, 1994). Moreover, Tat-mediated gliosis was significantly reduced by inhibitors of iNOS and TNFα (Philippon *et al.*, 1994; I.Starling, personal communication).

In the CNS, all three isoforms of NOS have been identified (reviewed by Yun *et al.*, 1996). Since arginine is a substrate for NOS, it has been proposed that arginine residues in lentiviral Tat may serve as substrates for NOS in generation of NO in neural tissues (Philippon *et al.*, 1994).

7.5 Summary

This is the first report to demonstrate that MVV-infected parenchymal microglial cells can promote activation of astrocytes and microglial cells in the CNS, and independent of specific immune responses. Similar results have been obtained with MVV-infected monocyte-derived macrophages injected into SCID brains (I. Starling, personal communication).

Release of neurotoxin (s) from MVV-infected ovine microglial cells mediated neurotoxicity when tested on murine trigeminal neurones *in vitro*. Interestingly, the earliest signs of neuronal damage were neurite abnormalities rather than overt loss of neurones.

CHAPTER EIGHT
General Discussion

8. 1 Introduction

The involvement of lentivirus infections of the CNS has been well documented. However, the mechanisms of neuropathology are poorly understood. Further, the underlying mechanisms may differ in infections of the CNS with host-specific lentiviruses. As outlined in the introduction to this thesis, this difference is apparent in neuropathologies observed in HIV-1 encephalitis and visna.

8.2 Consequences of MVV infection of microglial cells

Data presented in this thesis show that ovine brain microglia are closely related to cells of the monocyte/macrophage lineage and can support a productive infection with MVV *in vitro* (Chapter 3). Ovine parenchymal brain macrophages/microglia (CD68+) also stained positive for MVV antigens in experimental infections in sheep (Torstiendottir *et al*, 1992, Georgsson *et al.*, 1997).

Infection of cultured ovine microglial cells with MVV *in vitro* resulted in increased expression of TNFα and IL-6 at the transcription level (Chapter 6). Both of these cytokines (TNFα and IL-6) can potentially mediate pathological changes in the CNS (Chapter 6).

The ability of MVV to induce the expression of IL-6 *in vitro* may signal for gliosis *in vivo*, as observed in SCID experiments (Chapter 7). Over-expression of IL-6 in murine CNS has been shown to result in reactive astrocytosis and an increase in ramified (process-bearing) microglial cell (Fattori *et al.*, 1995). It is not clear whether elevated gliosis observed in SCID brains injected with MVV-infected microglia was a result of ovine microglial-derived IL-6. In HIV/SCID experiments, activated resident microglia were a source of neural IL-6 (Persidsky *et al.*, 1996).

It is also feasible that viral proteins shed by infected cells may promote gliosis. Injection of HIV-1 and visna Tat into brain parenchyma of mice resulted in inflammation and gliosis. PCR signals for proinflammatory cytokines (TNFα, IL-1α/β, IL-6), and iNOS were elevated in lesioned hemispheres, and chemical inhibition of TNFα expression resulted in a decreased lesion volume (Philippon *et al.*, 1994).

There are conflicting reports with respect to the neurobiology of TNFα (Chapter 6). Over-expression of TNFα by neurones or astrocytes in transgenic mice was sufficient to trigger chronic CNS inflammation involving a reactive glial response, infiltration

by activated T cells and macrophages, and loss of white matter and neurodegeneration (Probert *et al.*, 1997). In contrast, TNFα was found not to be directly toxic on mixed foetal glial cultures *in vitro*, but the toxicity of TNFα was found to be NO-mediated, released by astrocytes and macrophages/microglia (Chao *et al.*, 1995; Ding *et al.*, 1988). Similarly in a murine model of MS, double deficient *tnf/lta* SLJ/J (H-2^s) mice still presented with CNS lesions indicating that either TNFα and TNFβ are not essential for demyelination and/or a degree of redundancy exists in TNFα/TNFβ deficient mice (Frie *et al.*, 1997). Further, in mice infected with Theiler's virus encephalomyelitis, another model of MS, the administration of recombinant TNFα reduced the extent of demyelination without influencing the CNS virus titres (Paya *et al.*, 1990). More recently, TNFα has also been shown to have neuroprotective properties *in vivo* (Bruce *et al.*, 1996).

The anomalies observed in biological properties of TNFα are partly because of dual receptor usage by this cytokine (reviewed by Vandenabeele *et al.*, 1995). There is also lack of information with respect to possible preferential receptor usage in the CNS.

Another complicating matter is a degree of cross-species barrier that may exist between ovine and murine systems. Hence, it is also not clear if ovine cytokines can also mediate pathological changes (gliosis) in murine brains. Whether ovine TNFα and IL-6 can transduce cellular signals through receptors in murine CNS remains to be seen. Further, the receptors for ovine IL-6 and TNFα have yet to be identified. Another approach will be to inject recombinant ovine cytokines (TNFα and IL-6) into ovine or murine brains, followed by immunohistological examination of neural tissues.

8.3 The putative neurotoxin

Treatment of murine neuronal cultures with supernatants obtained from MVV-infected microglia resulted in morphological abnormalities and eventual loss of neurones (Chapter 7). Unlike HIV-1, visna is not generally regarded as a neuronal disease, based on EM studies (Georgsson et al., 1976). However, data presented in Chapter 7 warrant a closer examination of neurones in Visna infection. Perhaps the first step would be to use ovine neuronal culture instead of murine cells. Further, use of neurone specific reagents, for example antibodies against neurofilaments, and more sophisticated imaging system can help to clarify this point.

Ample evidence exists to support the neurotoxic potential of lentiviral components, for example Tat. Tat was previously shown to promote gliosis *in vivo* and neurotoxic properties both *in vivo* and *in vitro* (Gourdou *et al.*, 1990; Sabatier *et al.*, 1991; Philippon *et al.*, 1994; Nath *et al.*, 1996). Tat can be shed from infected cells (Ensoli *et al.*, 1993). Furthermore, Tat non-specifically binds cell surface at high densities (Mann *et al.*, 1991). An RGD motif in HIV-1 Tat has been proposed to meditate cellular aggregation (Kolson *et al.*, 1993). However, this motif does not exist in the sequence of K1514 strain used throughout this project. The neurotoxicity of a Tat peptide rich in arginine residues was reproduced with trigeminal neurones *in vitro* (Chapter 7). Repeated attempts with two polyclonal antibodies raised against the Tat peptide and subsequent Western blot analysis failed to demonstrate the presence of a Tat component in MVV-infected microglial supernatants.

It is not clear if Tat, possibly shed from infected microglia, contributed to neurotoxicity *in vitro*. This was because of lack of a positive control for anti-Tat antisera, for example recombinant Tat protein. If the sensitivity of Western blots was the underlying cause i.e., due to minute quantities of this protein in medium, then a more sensitive approach such as immunoprecipitation would be more appropriate.

An amine, Ntox, produced by mononuclear cells isolated from HIV-1 infected patients was neurotoxic both *in vivo* and *in vitro* (Giulian *et al.*, 1996). Interestingly, only supernatants from monocytic cell lines rather than a lymphoid cell line had neurotoxic properties *in vitro*, and was independent of viral products (Guilian et al., 1990). Other small molecules with similar characteristics, such as amino acids and their metabolites (glutamine, cysteine and QUIN) have also been shown to mediate neurotoxicity via NMDA receptor-mediated channels (reviewed by Giulian *et al*, 1990, 1996).

8.4 Microglia as antigen presenting cells: implications for visna pathogenesis

The presence of MHC surface molecules on microglial cells poses the question of antigen presentation in the CNS. This in turn has interesting possibilities, since a cellular inflammatory response is a prominent component of visna neuropathology. There are, however, conflicting reports with regard to the role of parenchymal microglial cells as APC. Parenchymal microglial cells ($CD45^{low}/CD11b/c^{+}$), isolated from adult rat brains and activated *in vivo* failed to induce proliferation of encephalitogeneic myelin basic protein-specific CD4+ T cells. This was in contrast to

perivascular macrophages (CD45high/CD11/b/c+) and thymic dendritic cells (Ford *et al.*, 1996). This observation has been contradicted by human parenchymal microglial cells isolated and characterised by a similar protocol employed by Ford and co-workers. Isolated human microglial cells supported a mixed lymphocyte reaction *in vitro* (Bercher and Antel, 1996). The notion that brain microglial cells may function as APC was reinforced by the presence of co-stimulatory molecule B7.1 (CD80) on human microglial cells (Bercher and Antel, 1996). Furthermore, microglial cells isolated from adult human brain tissues were capable of supporting a lymphoproliferative response to recall antigens (Dhib-Jalbut *et al.*, 1996). In the latter study, the isolated microglial cells were phenotyped on basis of CD45 expression. Similar observations have been made with murine resident microglial cells (Havenith *et al.*, 1998).

If brain microglia can present viral antigens similar to MMV-infected monocyte-derived macrophages, then the role of T cell immune responses in visna will become better understood.

8.5 Future work

Experiments carried out in SCID mice this thesis reinforce the usefulness of this system. Interestingly, similar observations have been made with SCID mice inoculated with MVV-infected monocyte-derived macrophages (I. Starling, personal communication).

Data obtained from SCID mice are preliminary and need to be followed up. SCID mice can be used to ask fundamental questions regarding virus-initiated mechanisms leading to pathogenesis/pathology. In addition, what are the long-term effects of injected microglial cells in SCID brains in the absence of a cellular immune response? Data presented in this thesis support the notion that virus-initiated responses, independent of acquired immune responses, may contribute to visna neuropathology.

Bibliography

Abraham, J., Whang, J., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D., and Fiddes, J. (1986a). Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *EMBO* **5,** 2523.

Abraham, J., Mergia, A., Whang, J., and al., a. (1986b). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233,** 545.

Akiyama, H., and McGeer, P. (1990). Brain microglia constitutively express beta-2 integrins. *J. Neuroimmunol.* **30,** 81.

Albright, A., Strizki, J., Harouse, J., Lavi, E., O'Connor, M., and Gonzalez-Scerano, F. (1996). HIV-1 infection of human adult oligodendrocytes. *Virology* **217,** 211.

Alkhatib, G., Combadiere, C., Border, C., Feng, Y., Kennedy, P., Murphy, P., and Berger, E. (1996). CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272,** 1955.

Anderson, L., Klevjer-Anderson, P., and Liggitt, H. (1983). Susceptibility of blood-derived monocytes and macrophages to caprine arthritis-encephalitis virus. *Infect. Immun.* **41,** 837.

Anderson, B., Bulgin, M., Adams, S., and Duelke, B. (1985). Firm udder n periparturient ewes with lymphocytic accumulations, retrovirus infection, and milk unavailable at the teat. *J. Am. Vet. Med. Assoc.* **186,** 391.

Aquino, D. A., Chiu, F. C., Brosnan, C. F., and Norton, W. T. (1988). Glial fibrillary acidic protein increases in the spinal cord of Lewis rats with acute experimental autoimmune encephalomyelitis. *J Neurochem* **51**(4), 1085-96.

Audoly, G., Sauze, N., Harkiss, G., Vitu, C., Russo, P., Querat, G., Suzan, M., and Vigne, R. (1992). Identification and subcellular localization of the Q gene product of visna virus. *Virology* **189,** 734.

Balasingam, V., Tejada-Berges, T., Wright, E., Bouckova, R., and Yong, V. (1994). Reactive astogliosis in the neonatal mouse brain and its modulation by cytokines. *J. Neurosci.* **14,** 846.

Balasingam, V., Dickson, K., Brade, A., and Young, V. (1996). Astrocyte reactivitry in neonatal mice: apparent dependence on the presence of reactive microglia/macrophages. *Glia* **18,** 11.

Balasingam, V., and Yong, V. W. (1996). Attenuation of astroglial reactivity by

interleukin-10. *J Neurosci* **16**(9), 2945-55.

Ball, J., Holmes, E., Whitewell, H., and Desselberger, U. (1994). Genomic variation of human immunodeficiency virus type 1 (HIV-1): molecular analyses of HIV-1 in seqiential blood samples and various organs obtained at autopsy. *J. Gen. Virol.* **75,** 67.

Baltimore, D. (1970). RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature* **226,** 1209.

Baskin, D., Wilcox, B., Figlewicz, D., and Dorsa, D. (1988). Insulin and insulin-like growth factors in the CNS. *Trends Neurosci.* **11,** 107.

Baszler, T., Harwood, W., Lester, K., Davis, W., and Knowles, D. (1994). Characterization of caprine microglial cells and in vitro infection with caprine arthritis-encephalitis lentivirus. *Lab. Invest.* **70,** 933.

Battula, N., and Loeb, L. (1974). The infidelity of avian myeloblastogenesis virus deoxyribonucleic acid polymerase in polynucleotide reaction. *J. Biol. Chem.* **249,** 4086.

Becher, B., and Antel, J. (1996). Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia. *Glia* **18,** 1.

Bell, A., Burns, J., and Fujinami, R. (1997). Measles virus infection of human T cells modulates cytokine generation and IL-2 receptor alpha chain expression. *Virology* **232,** 241.

Berman, M. A., Zaldivar, F., Jr., Imfeld, K. L., Kenney, J. S., and Sandborg, C. I. (1994). HIV-1 infection of macrophages promotes long-term survival and sustained release of interleukins 1 alpha and 6. *AIDS Res Hum Retroviruses* **10**(5), 529-39.

Berman, J. W., Guida, M. P., Warren, J., Amat, J., and Brosnan, C. F. (1996). Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat. *J Immunol* **156**(8), 3017-23.

Bernard, W. (1960). The detection and study of tumor viruses with the elctron micrscope. *Cancer Res.* **20,** 712.

Berrada, F., Ma, D., Michaud, J., Doucet, G., Giroux, L., and Kessous-Elbaz, A. (1995). Neuronal expression of human immunodeficiency virus type 1 env proteins in transgenic mice: distribution in the central nervous system and pathological alterations. *J. Virol.* **69,** 6770.

Bertoni, G., Zahno, M., Zanoni, R., Vogt, H., Peterhans, E., Ruff, G., Cheevers, W., Sonigo, P., and Pancino, G. (1994). Antibody reactivity to the immunodominant epitopes of the caprine arthritis-encephalitis virus gp38 transmembrane protein associates with the development of arthritis. *J. Virol.* **68,** 7139.

Bird, P., Blacklaws, B., Reyburn, H., Allen, D., Hopkins, J., Sargan, D., and McConnell, I. (1993). Early events in immune evasion by the lentivirus maedi-visna occuring within infected lymphoid tissue. *J. Virol.* **67,** 5187.

Biront, P., and Deluyker, H. (1985). *Commission of European Communities.*

Blackburn, E. (1990). Telomeres and their synthesis. *Science* **249,** 489.

Blacklaws, B., Bird, P., and McConnell, I. (1995). Early events in infection of lymphoid tissues by a lentivirus, maedi-visna. *Trends Microbiol.* **3,** 434.

Bogerd, H., Fridell, R., Madore, S., and Cullen, B. (1995). Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell* **82,** 485.

Border, S., Mitsuya, H., Yarchoan, R., and Pavlakis, G. (1990). *NIH.*

Bou-Habib, D., Roderiquez, G., Oravecz, T., Berman, P., Lusso, P., and Norcross, M. (1994). Cryptic nature of envelope V3 region epitopes protects primary monocytotropic human immunodeficiency virus type 1 from antibody neutralization. *J. Virol.* **68,** 6006.

Bourgault, I. (1991). AIDS: the difficult elaboration of a vaccine. *Rev. Infirm.* **41,** 44.

Brahic, M., Filippo, P., Vigne, R., and Haase, A. (1977). Visna virus RNA synthesis. *J. Virol.*, 74.

Brahic, M., Stowring, L., Ventura, P., and Haase, A. (1981). Gene expression in visna virus infection in sheep. *Nature* **292,** 240.

Braun, M., Clements, J., and Gonda, M. (1987). The visna virus genome: Evidence for a hypervariable site in the env gene and sequence homology among lentivirus envelope proteins. *J. Virol.* **61,** 4046.

Bredesen, D., Levy, R., and Rosenblum, M. (1988). The neurology of human immunodeficiency virus infection. *Q. J. Med.* **68,** 665.

Brew, B., Evans, L., Byrne, C., Pemberton, L., and Hurren, L. (1996). The relationship between AIDS dementia complex and the presence of macrophage tropic and non-syncytium inducing isolates of human immunodeficiency virus type 1 in the cerebrospinal fluid. *J. Neurovirol.* **2,** 152.

Brodie, S., Pearson, L., Zink, M., Bickle, H., Anderson, B., Marcom, K., and DeMartini, J. (1995). Ovine lentivirus expression and disease. Virus replication, but not entry, is restricted to macrophages of specific tissues. *Am. J. Pathol.* **146,** 250.

Brosins, J. (1991). Retroposons - Seeds of evolution. *Science* **251,** 753.

Brown, F. (1987). *Intervirology, Edmonton, Canada.*

Brown, P., Bowerman, B., Varmus, H., and Bishop, J. (1989). Retroviral integration: Structure of the initial covalent product and its precursor, and a role for IN protein. *Proc. Natl. Acad. Sci. (USA)* **86,** 2525.

Brown, P. (1990). Integration of retroviral DNA. *In* "Retroviruses-Strategies of replication" (R. Swanstrom, and P. Vogt, Eds.), pp. 19-48. Springer-Verlag, Berlin.

Bruce, A. J., Boling, W., Kindy, M. S., Peschon, J., Kraemer, P. J., Carpenter, M. K., Holtsberg, F. W., and Mattson, M. P. (1996). Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat Med* **2**(7), 788-94.

Buchmeier, M., Welsh, R., Dutko, F., and Oldstone, M. (1980). The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Adv. Immunol.* **30,** 275.

Budka, H. (1989). Human immunodeficiency virus (HIV)-induced disease of the central nervous system: pathology and implications for pathogenesis. *Acta Neuropathol* **77**(3), 225-36.

Campbell, B., and Avery, R. (1996). Sequenceanalysis and transcriptional activity of the LTR of OLV-CU1, a North American ovine lentivirus. *J. Gen. Virol.* **77,** 2999.

Capecchi, M. (1989). Altering the genome by homologous recombination. *Science* **244,** 1288.

Chao, C., Hu, S., Sheng, W., and Peterson, P. (1995). TNFalpha production by human fetal microglial cells: regulation by other cytokinse. *Dev. Neurosci.* **17,** 97.

Chebloune, Y., Sheffer, D., Karr, B., Stephens, E., and Narayan, O. (1996). Restrictive type of replication of ovine/caprine lentiviruses in ovine fibroblast cell cultures. *Virology* **222,** 21.

Chimule, N., Kalyanaraman, V., Saxinger, C., Wong-Staal, F., Ghrayeb, J., and Pahwa, S. (1990). Localization of B-cell srimulatory activity of HIV-1 to the carboxyl terminus of gp41. *AIDS Res. Hum. Retroviruses* **6,** 299.

Chollet-Martin, S., Simon, F., Matheron, S., Joseph, C. A., Elbim, C., and Gougerot-Pocidalo, M. A. (1994). Comparison of plasma cytokine levels in African patients with HIV-1 and HIV-2 infection. *Aids* **8**(7), 879-84.

Clements, J., Narayan, O., Griffin, D., and Johnson, R. (1979). The synthesis and structure of visna virus. *Virology* **93,** 377.

Clements, J., Pedersen, F., Narayan, O., and Haseltine, W. (1980). Genomic changes associated with antigenic variation of visna virus during persistent infection. *Proc. Natl. Acad. Sci. (USA)* **77,** 4454.

Clements, J., Wall, R., Narayan, O., and al., e. (1994). development of transgenic sheep that express the visna virus envelope gene. *Virology* **200,** 370.

Cocchi, F., DeVico, A., Garzino-Demo, A., Cara, A., Gallo, R., and Lusso, P. (1996). The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nat. Med.* **2,** 1244.

Coffin, J. (1990). Molecular mechanisms of nuclaic acid integration. *J. Med. Virol.* **31,** 43.

Coffin, J. (1990). Retroviridae and their replication. 2nd ed. *In* "Virology" (B. Fields, D. Knipe, and R. Chanock, Eds.), pp. 1437. Raven Press, New York.

Coffin, J. (1990). Genetic variation of retroviruses. *In* "Applied Virology Research" (E. Kurstak, R. Marusyk, F. Murphy, and M. Van Regenmortel, Eds.), pp. 11. Plenum Press, New York.

Connor, R., and Ho, D. (1994). Transmisson and pathogenesis of human immunodeficiency virus 1. *AIDS res. Hum. Retroviruses* **10,** 321.

Crane, S., Kanda, P., and Clements, J. (1991). Identification of the fusion domain in the visna virus transmembrane protein. *Virology* **185,** 488.

Cullen, B., and Malim, M. (1991). The HIV-1 Rev protein: prototype of a novel class of eukaryotic post-transcriptional regulators. *Trends Biochem. Sci.* **16,** 346.

Cullen, B. (1992). Mechanisms of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* **56,** 375.

Cupp, C., Taylor, J. P., Khalili, K., and Amini, S. (1993). Evidence for stimulation of the transforming growth factor beta 1 promoter by HIV-1 Tat in cells derived from CNS. *Oncogene* **8**(8), 2231-6.

Cutlip, R., and Laird, G. (1976). Isolation and characterisation of a virus associated with progressive pnumonia (maedi) of sheep. *Am. J. Vet. Res.* **37,** 1377.

Cutlip, R., Lehmkuhl, H., Wood, R., and Brogden, K. (1985). Arthritis associated with ovine progressive pneumonia. *Am. J. Vet. Res.* **46,** 65.

Dalgliesh, A., Beverley, P., Clapham, P., Crawford, D., Greaves, M., and Weiss, R. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312,** 763.

Dalziel, R., Hopkins, J., Watt, N., Dutia, B., Clarke, H., and McConnell, I. (1991). Identification of a putative cellular receptor for the lentivirus visna virus. *J. Gen. Virol.* **72,** 1905.

Damasky, C., and Werb, Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* **4,** 772.

Davis, J., and Clements, J. (1989). Characterisation of a cDNA clone encoding the visna virus transactivating protein. *Proc. Natl. Acad. Sci. (USA)* **86,** 414.

De Boer, G. (1970). Antibody formation in Zwoegerzieke, a slow infection in sheep. *J. Immunol.* **104,** 414.

DeJong, J., Goudsmit, J., Keulen, W., Klaver, B., Krone, W., Tersmette, M., and DeRonde, A. (1992). Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. *J. Virol.* **63,** 272.

Deng, H., Liu, R., Ellmeier, W., and et. al. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381,** 661.

Dereuddre-Bosquet, N., Clayette, P., Martin, M., Benveniste, O., Fretier, P., Jaccard, P., Vaslin, B., Lebeaut, A., and Dormont, D. (1997). Lack of interleukin 10 expression in monocyte-derived macrophages in response to in vitro infection by HIV type 1 isolates. *AIDS Res Hum Retroviruses* **13**(11), 961-6.

Dhib-Jalbut, S., Gogate, N., Jiang, H., Eisenberg, H., and Bergey, G. (1996). Human microglia activate lymphoproliferative responses to recall viral antigens. *J Neuroimmunol* **65**(1), 67-73.

Di Santo, E., Alonzi, T., Fattori, E., and al., e. Overexpression of IL-6 in the CNS of

transgenic mice increases central but not systemic pro-inflammatory cytokine production. *Brain Res.* **740,** 239.

Dickson, C., Eisenman, R., Fan, H., Hunter, E., and Teich, N. (1982). "Protein biosynthesis and assembly.". RNA Tumour Viruses (R. Weiss, N. Teich, H. Varmus, and J. Coffin, Eds.) Cold Spring Harbor, New York.

Dollard, S. C., James, H. J., Sharer, L. R., Epstein, L. G., Gelbard, H. A., and Dewhurst, S. (1995). Activation of nuclear factor kappa B in brains from children with HIV-1 encephalitis. *Neuropathol Appl Neurobiol* **21**(6), 518-28.

Doms, R., Lamb, R., Rose, J., and Helenius, A. (1993). Folding and assembly of viral membrane proteins. *Virology* **193,** 545.

Doolittle, R., Feng, D.-F., Johnson, M., and McClure, M. (1989). Origens and evolutionary relationship of retroviruses. *Q. Rev. Biol.* **64,** 1.

Dow, S., Poss, M., and Hoover, E. (1990). Feline immunodeficiency virus: a neurotropic lentivirus. *J. AIDS* **3,** 658.

Dragic, T., Litwin, V., Allaway, G., and et. al. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381,** 667.

Dubois-dalcq, M., Narayan, O., and Griffin, D. (1979). Cell surface changes associated with mutation of visna virus in antibody-traeted cell cultures. *Virology* **92,** 353.

Dutia, B., Hunt, P., Sargan, D., Dalziel, R., and Hopkins, J. (1994). Sequence of the sheep interleukin-10-encoding cDNA. *Gene* **149,** 393.

Earl, P., Moss, B., and Doms, R. (1991). Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J. Virol.* **65,** 2047.

Ebrahimi, B., Roy, D., Bird, P., and Sargan, D. (1995). Cloning, sequencing and expression of the ovine interleukin 6 gene. *Cytokine* **7,** 232.

Eigen, M., and Beibricher, C. (1988). Sequences and quaisispecies distribution. *In* "RNA Virus Genetics" (E. Domingo, J. Holland, and P. Ahlquist, Eds.), Vol. 3, pp. 211. CRC Press, Florida.

Elder, J., Lerner, D., Hasselkus-Light, C., Fontenot, D., Hunter, E., Luciw, P., Montelaro, R., and Philips, T. (1992). Distinct subsets of retroviruses encode dUTPase. *J. Virol.* **66,** 1791.

Elkabes, S., DiCicco-Bloom, E., and Black, I. (1996). Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. *J. Neurosci.* **16,** 2508.

Ensoli, B., Barillari, G., and gallo, R. (1991). Pathogenesis of AIDS-associated Kaposi's sarcoma. *Hematol. Oncol. Clin. North. Am.* **5,** 281.

Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R. A., Wingfield, P., and Gallo, R. C. (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J Virol* **67**(1), 277-87.

Epstein, L., Sharer, L., Cho, E., Myenhofer, M., Navia, B., and Price, R. (1984). HTLV-III/LAV-like retrovirus particles in the brains of patients with AIDS encephalopathy. *AIDS Res.* **1,** 447.

Erfle, V., Stoeckbauer, P., Kleinschmidt, A., Kohleinsen, B., Mellert, W., Stavrou, D., and Brack-Werner, R. (1991). Target cells for HIV in the central nervous system: macrophages or glial cells? *AIDS* **5,** 1301.

Esch, F., Baird, A., Ling, N., and al., a. (1985). Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine acidic FGF. *Proc. Natl. Acad. Sci. (USA)* **82,** 6507.

Everall, I., Luthert, P., and Lantos, P. (1991). Neuronal loss in the frontal cortex in HIV infection. *Lancet* **337,** 1119.

Fan, L., and Peden, K. (1992). Cell-free transmission of Vif mutants of HIV-1. *Virology* **190,** 19.

Farnet, C., and Haseltine, W. (1991). Determination of viral proteins present in human immunodeficiency virus type 1 preintegration complex. *J. Virol.* **65,** 1910.

Fattori, E., Lazzaro, D., Musiani, P., Modesti, A., Alonzi, T., and Ciliberto, G. (1995). IL-6 expression in neurons of transgenic mice causes reactive astrocytosis and increase in ramified microglial cells but no neuronal damage. *Eur J Neurosci* **7**(12), 2441-9.

Feng, Y., Broder, C., Kennedy, P., and Berger, E. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272,** 872.

Fitch, W., Leiter, J., Li, X., and Palese, P. (1991). Positive Darwinian evolution in human

influenza A viruses. *Proc. Natl. Acad. Sci. (USA)* **88,** 4270.

Flanagan, J., Krieg, A., Max, E., and Khan, A. (1989). Negative control region at the 5' end of murine leukemia virus long terminal repeats. *Mol. Cell Biol.* **9,** 739.

Flanagan, J., Becker, K., Ennist, D., Gleason, S., Driggers, P., Levi, B., Appella, E., and Ozato, K. (1992). Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. *Mol. Cell Biol.* **12,** 38.

Folks, T., Justement, J., Kinter, A., Dinarello, C., and fauci, A. (1987). Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238,** 800.

Ford, A., Goodsall, A., Hickey, W., and Sedgwick, J. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *J. Immunol.* **154,** 4309.

Fujiwara, J., and Mizuuchi, K. (1988). Retroviral DNA integration: Structure of an integration intermediate. *Cell* **54,** 497.

Fuxe, K., Tinner, B., Zoli, M., and al., e. (1996). Computer-assisted mapping of basic fibroblast growth factor immunoreactive nerve cell populations in the rat brain. *J. Chem. Neuroanat.* **11,** 13.

Gabuzda, D., Hess, J., Small, J., and Clements, J. (1989). Regulation of the visna virus long terminal repeat in macrophages involves cellular factors that bind sequences containing AP-1 sites. *Mol. Cell Biol.* **9,** 2728.

Gabuzda, D., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W., and Sodroski, J. (1992). Role of vif in replication of human immunodeficiency virus type 1 in CD4+ lymphocytes. *J. Virol.* **67,** 6322.

Gabuzda, D., Li, H., Lawrence, K., Vasir, B., Crawford, K., and Langhoff, E. (1994). Essential role of vif in establishing productive HIV-1 infection in peripheral blood T lymphocytes and monocyte/macrophages. *J. AIDS* **7,** 908.

Gallaher, W., Ball, J., Garry, R., Griffin, M., and Montelaro, R. (1989). A general model for the transmembrane proteins of HIV and other retrovirises. *AIDS Res. Hum. Retrovirises* **5,** 431.

Gdovin, S., and Clements, J. (1992). Molecular mechanisms of visna virus Tat: identification of the targets for transcriptional activation and evidence for a post-

transcriptional effect. *Virology* **188,** 438.

Gehrmann, J., Schoen, S., and Kreutzberg, G. (1991). Lesion of the rat entorhinal cortex leads to a rapid microglial reaction in the dedante gyrus. A light and electron microscopical study. *Acta Neuropathol.* **82,** 442.

Gehrmann, J., Bannati, R., and Kruetzberg, G. (1993). Microglia in the immune surveillance of the brain: human microglia constitutively express HLA-DR molecules. *J. Neuroimmunol.* **48,** 189.

Gelderblom, H. (1991). Assembly and morphology of HIV: potential effect of structure on viral function. *AIDS* **5,** 617.

Gendelman, H., Narayan, O., Kennedy-Stoskopf, S., Clements, J., and Pezeshkpour, G. (1984). Slow virus-macrophage interactions. Characterization of a transformed cell line of sheep alveolar macrophages that express a marker for susceptibility to ovine-caprine lentivirus infections. *Lab. Invest.* **51,** 547.

Gendelman, H., Narayan, O., Molineaux, S., Clements, J., and Ghoybi, Z. (1985). Slow, persistent replication of lentiviruses: a role for tissue macrophages and macrophage precursors in bone marrow. *Proc. Natl. Acad. Sci. (USA)* **82,** 7086.

Gendelman, H., Narayan, O., Kennedy-Stoskopf, S., Kennedy, P., Ghotbi, Z., Clements, J., Stanley, J., and Pezeshkpour, G. (1986). Tropism of sheep lentiviruses for monocytes: susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages. *J. Virol.* **58,** 67.

Genis, P., Jett, M., Bernton, E. W., Boyle, T., Gelbard, H. A., Dzenko, K., Keane, R. W., Resnick, L., Mizrachi, Y., Volsky, D. J., and et al. (1992). Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interactions: implications for the neuropathogenesis of HIV disease. *J Exp Med* **176**(6), 1703-18.

Georgsson, G., Palsson, P., Panitch, H., Nathanson, N., and Petrusson, G. (1977). The ultrastructure of early visna lesions. *Acta Neuropathol.* **37,** 127.

Georgsson, G., Martin, J. R., Klein, J., Palsson, P. A., Nathanson, N., and Petursson, G. (1982). Primary demyelination in visna. An ultrastructural study of Icelandic sheep with clinical signs following experimental infection. *Acta Neuropathol* **57**(2-3), 171-8.

Georgsson, G., Houwers, D., Palsson, P., and Petursson, G. (1989). Expression of viral

antigens in the central nervous system of visna-infected sheep: an immunohistochemical study on experimental visna induced bu virus strains of increased neurovirulence. *Acta Neuropathol.* **77,** 299.

Georgsson, G. (1994). Neuropathologic aspects of lentiviral infections. *Ann. N. Y. Acad. Sci.* **724,** 50.

Georgsson, G. (1997). *3rd European Workshop on Ovine and Caprine retroviruses, Jaca, Spain.*

Giangaspero, M., Gruner, L., Nishikawa, H., Tabbaa, D., and Vacirca, G. (1993). Lungworms, maedi visna and mixed infections with respiratory viruses in Syrian Awassi sheep. *Vet. Res.* **17,** 371.

Giulian, D., Woodward, J., Young, D. G., Krebs, J. F., and Lachman, L. B. (1988). Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. *J Neurosci* **8**(7), 2485-90.

Giulian, D., Vaca, K., and Noona, C. (1990). Secretion of neurotoxins by mononuclear phagocytes. *Science* **250,** 1593.

Giulian, D., Wendt, E., Vaca, K., and Noona, C. (1993). The envelope glycoprotein of HIV-1 stimulates release of neurotoxin from monocytes. *Proc. Natl. Acad. Sci.* **90,** 2769.

Giulian, D., Li, X., George, J., and Ruteck, P. (1994a). The impact of microglia-deived cytokines upon gliosis in the CNS. *Dev. Neurosci.* **16,** 128.

Giulian, D., Li, J., Leara, B., and Keenan, C. (1994b). Phagocytic microglia release cytokines and cytotoxins that regulate the survival of astrocytes and neurones in culture. *Neurochem. Int.* **25,** 227.

Giulian, D., Yu, J., Li, X., Tom, D., and al., e. (1996). Study of receptor mediated neurotoxins released by HIV-1 infected mononuclear phagocytes found in human brain. *J. Neurosci.* **16,** 3139.

Glabinski, A. R., Balasingam, V., Tani, M., Kunkel, S. L., Strieter, R. M., Yong, V. W., and Ransohoff, R. M. (1996). Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain. *J Immunol* **156**(11), 4363-8.

Glass, J., and Johnson, R. (1996). Human immunodeficiency virus and the brain. *Annu. Rev. Neurosci.* **19,** 1.

Goff, S. (1990). Retroviral reverse transcriptase: synthesis, structure, and function. *J.*

AIDS **3,** 817.

Gomez-Pinilla, F., Lee, J., and Cotman, C. (1992). Basic FGF in adult rat brain: cellular distribution and response to entrohinal lesion and fimbria-fornix transection. *J. Neurosci.* **12,** 345.

Gonda, M., Luther, D., Fong, S., and Tobin, G. (1994). Bovine immunodeficiency virus: molecular biology and virus-host interactions. *Virus Res.* **32,** 155.

Gorman, O., Bean, W., Kawaoka, Y., Donatelli, I., Guo, Y., and Webster, R. (1991). Evolution of influenza A virus nucleoprotein genes: Implications for the origens of H1N1 human and classical swine viruses. *J. Virol.* **65,** 3704.

Gospodarowicz, D., and Moran, J. (1974). Effect of a fibroblast growth factor, insulin, dexamethasone, and serum on the morphology of BALB/c 3T3 cells. *Proc Natl Acad Sci U S A* **71**(12), 4648-52.

Gospodarowicz, D. (1990). Fibroblast growth factor: Chemical structure and biologic function. *Clin. Orthop.* **257,** 231.

Gourdou, I., Mabrouk, K., Harkiss, G., Marchot, P., Watt, N., Hery, F., and Vigne, R. (1990). [Neurotoxicity in mice due to cysteine-rich parts of visna virus and HIV-1 Tat proteins]. *C R Acad Sci III* **311**(4), 149-55.

Gridley, T. (1991). Insertional versus targeted mutagenesis in mice. *New Biol.* **3,** 1025.

Griffin, D., Narayan, O., and Adams, R. (1978). Early immune responses in visna, a slow viral disease of sheep. *J. Infect. Dis.* **138,** 340.

Grimalia, R., Fuller, B., Rennert, P., Nelson, M., Hammarskjold, M., Potts, B., Murray, M., Putney, S., and Gray, G. (1992). Mutations in the principal neutralization domain of the human immunodeficiency virus type 1 affect syncytium formation, virus infectivity, growth kinetics, and neutralization. *J. Virol.* **66**(1875).

Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M., and Scherer, S. S. (1996). Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *J Neurosci* **16**(19), 6107-18.

Groux, H., Tropier, G., Monte, D., Mouton, Y., Capron, A., and Ameisen, J. (1992). Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. *J. Exp. Med.* **175,** 331.

Gruber, M., Weih, K., Boone, E., Smith, P., and Clouse, K. (1995). Endogenous

macrophage CSF production is associated with viral replication in HIV-1-infected human monocyte-derived macrophages. *J. Immunol.* **154,** 5528.

Gruters, R., Terpstra, F., De Jong, R., Van Noesel, C., Van Lier, R., and Miedema, F. (1990). Selective loss of T cell functions in different stages of HIV infection. Early loss of anti-CD3-induced T cell proliferation followed by decreased anti-CD3-induced cytotoxic T lymphocyte generation in AIDS-related complex and AIDS. *Eur. J. Immunol.* **20,** 1039.

Gundadottir, M., and Palsson, P. (1967). Transmission of maedi by inoculation of a virus grown in tissue culture from maedi-affected lungs. *J. Infect. Dis.* **117,** 1.

Haase, A., Stowring, L., Narayan, O., Griffin, D., and Price, D. (1977). Slow persistent infection caused by visna virus: role of host restriction. *Science* **195,** 175.

Haase, A., Stowring, L., Harris, J., Traynor, B., Ventura, P., Pleuso, R., and Brahic, M. (1982). Visna DNA synthesis and the tempo of infection in vitro. *Virology* **119,** 1982.

Haase, A., Gantz, D., Blum, H., Stworing, L., Ventura, P., geballe, A., Moyer, B., and Brahic, M. (1985). Combined macroscopic and microscopic detection of viral genes in tissues. *Virology* **140**.

Harkiss, G., Watt, N., King, T., Williams, J., and Hopkins, J. (1991). Retroviral arthritis: phenotypic analysis of cells in the synovial fluid of sheep with inflammatory synovitis associated with visna virus infection. *Clin Immunol. Immunopathol.* **60,** 106.

Harmache, A., Vitu, C., Russo, P., Bouyac, M., Hieblot, C., Peveri, P., Vigne, R., and Suzan, M. (1995). The caprine arthritis encephalitis virus tat gene is dispensible for efficient viral replication in vitro and in vivo. *J. Virol.* **69,** 5445.

Harris, J., Scott, J., Traynor, B., Brahic, M., Stowring, L., Ventura, P., Haase, A., and Peluso, R. (1981). Visna virus DNA: discovery of a novel gapped structure. *Virology* **113,** 573.

Havenith, C. E., Askew, D., and Walker, W. S. (1998). Mouse resident microglia: isolation and characterization of immunoregulatory properties with naive CD4+ and CD8+ T-cells. *Glia* **22**(4), 348-59.

Hayes, G., Woodroofe, M., and Cuzner, M. (1988). Microglia express MHC class II in normal and demyelinating human white matter. *Ann. N. Y. Acad. Sci.* **540,** 501.

Hayman, M., Arbuthnott, G., Harkiss, G., Brace, H., Filippi, P., Philippon, V., Thomson,

D., Vigne, R., and Wright, A. (1993). Neurotoxicity of peptide analogues of the transactivating protein tat from Maedi-Visna virus and human immunodeficiency virus. *Neuroscience* **53,** 1.

Heaphy, S., Dingwell, C., Ernberg, I., Gait, M., Green, S., Karn, J., Lowe, A., Singh, M., and Skinner, M. (1990). HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. *Cell* **60,** 685.

Hess, J., Clements, J., and Narayan, O. (1985). Cis- and trans-activating transcriptional regulation of visna virus. *Science* **229,** 482.

Hess, J., Small, J., and Clements, J. (1989). Sequences in the visna virus long terminal repeat that control transcriptional activity and respond to viral trans-activation: involvement of AP-1 sites in basal activity and trans-activation. *J. Virol.* **63,** 3001.

Hetier, E., Ayala, J., Bousseau, A., and Prochiantz, A. (1991). Modulation of interleukin-1 and tumor necrosis factor expression by beta-adrenergic agonists in mouse ameboid microglial cells. *Exp Brain Res* **86**(2), 407-13.

Hirano, T., Akira, S., Taga, T., and Kishimoto, T. (1990). Biological and clinical aspects of interleukin 6 [see comments]. *Immunol Today* **11**(12), 443-9.

Hirsch, V., Zack, P., Vogel, A., and Johnson, P. (1991). Simian immunodeficiency virus infection of macaques: end-stage disease is characterized by widespread distribution of proviral DNA in tissues. *J. Infect. Dis.* **163,** 976.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26,** 365.

Ho, D., Neumann, A., Perelson, A., Chen, W., Leonard, J., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373,** 123.

Hofmann, B., Jakobson, K., Odum, N., and al., e. (1989). HIV-induced immunodeficiency. Relatively preserved phytohemagglutinin as oposed to decreased pokeweed mitogen responses may be due to possibly preserved responses via CD2/phytohemagglutinin pathway. *J. Immunol.* **142,** 1874.

Hu, W., and Temin, H. (1990). Genetic consequences of packaging two RNA genomes in one retroviral particle: Pseudodiploidy and high rate of genetic recombination. *Proc. Natl. Acad. Sci. (USA)* **87,** 1556.

Hughes, E., Bell, J., and Simmonds, P. (1997). Investigation of population diversity of human immunodeficiency virus type 1 in vivo by nucleotide sequencing and length polymorphism analysis of the V1/V2 hypervariable region of env. *J. Gen. Virol.* **78,** 2871.

Hurwitz, A. A., Lyman, W. D., and Berman, J. W. (1995). Tumor necrosis factor alpha and transforming growth factor beta upregulate astrocyte expression of monocyte chemoattractant protein-1. *J Neuroimmunol* **57**(1-2), 193-8.

Huso, D., Narayan, O., and Hart, G. (1988). Sialic acid on the surface of caprine arthritis-encephalitis virus define biological properties of the virus. *J. Virol.* **62,** 1974.

Inouye, S., M-Y., H., Eagle, S., and Inouye, M. (1989). Reverse transcriptase associated with the biosynthesis of the branched RNA-linked msDNA myxococcus xanthus. *Cell* **56,** 709.

Jansson, M., Popovic, M., Karlsson, A., Cocchi, F., Rossi, P., Albert, J., and Wigzell, H. (1996). Sensitivity to inhibition by beta-chemokines correlates with biological phenotypes of primary HIV-1 isolates. *Proc. Natl. Acad. Sci. (USA)* **93,** 15382.

Jolly, P., and Narayan, O. (1989). Evidence for interferernce, co-infections and intertypic virus enhancement by ovine-caprine lentiviruses. *J. Virol.* **63,** 4682.

Kaaya, E. E., Castanos-Velez, E., Amir, H., Lema, L., Luande, J., Kitinya, J., Patarroyo, M., and Biberfeld, P. (1996). Expression of adhesion molecules in endemic and epidemic Kaposi's sarcoma. *Histopathology* **29**(4), 337-46.

Kang, C., Nara, P., Chamat, S., Caralli, V., Ryskamp, T., haigwood, N., Newman, R., and Kohler, H. (1991). Evidence from non-V3-specific neutralizing antibodies that interfer with gp120/CD4 binding in human immunodeficiency virus 1-infected humans. *Proc. Natl. Acad. Sci. (USA)* **88,** 6171.

Katz, R., and Skalka, A. (1990). Generation of diversity in retroviruses. *Annu. Rev. Genet.* **24,** 409.

Kennedy-Stoskopf, S., and Narayan, O. (1986). Neutralizing antibodies to visna lentivirus: Mechanisms of action and possible role in virus persistence. *J. Virol.* **59,** 37.

Kermode, A., Tofts, P., MacManus, D., Kendall, B., Kingsley, D., Moseley, I., du Bouly, E., and McDonald, W. (1988). Early lesion of multiple sclerosis. *Lancet* **2,** 1203.

Kimura, M. (1968). Evolutionary rate at the molecular level. *Nature* **217,** 624.

Klein, M., van Baalen, C., Holwerda, A., and al., e. (1995). Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* **181,** 1365.

Kliks, S., Shioda, T., Haigwood, N., and Levy, J. (1993). V3 variability can influence the ability of an antibody to neutralize or enhance infection by diverse strains of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. (USA)* **90,** 11518.

Kolson, D. L., Buchhalter, J., Collman, R., Hellmig, B., Farrell, C. F., Debouck, C., and Gonzalez-Scarano, F. (1993). HIV-1 Tat alters normal organization of neurons and astrocytes in primary rodent brain cell cultures: RGD sequence dependence. *AIDS Res Hum Retroviruses* **9**(7), 677-85.

Kolson, D., Collman, R., Hrin, R., Balliet, J., Laughlin, M., McGann, K., Debouck, C., and Gonzalez-Scarano, F. (1994). Human immunodeficiency virus type 1 Tat activity in human neuronal cells: uptake and transactivation. *J. Gen. Virol.* **75,** 1927.

Koot, M., Keet, I., Vos, A., and al., e. (1993). Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cells depletion and progression to AIDS. *Am. Intern. Med.* **118,** 681.

Korber, B., Kunstman, K., Patterson, B., Furtado, M., McEvilly, M., Levy, R., and Wolinsky, S. (1994). Genetic difference between blood- and brain-derived viral sequences from human immunodeficiency virus type 1-infected patients: evidence of conserved elements in the V3 regions of the envelope protein of brain-derived sequences. *J. Virol.* **68,** 7467.

Krajewski, S., James, H., Ross, J., Blumberg, B., Epstein, L., Gendelman, H., Gummuluru, S., Dewhurst, S., Sharer, L., Reed, J., and Gelbard, H. (1997). Expression of pro- and anti-apoptosis gene products in brains from paediatric patients with HIV-1 encephalitis. *Neuropathol. Appl. Neurobiol.* **23,** 242.

Kramer, G., and Argos, P. (1984). Primarily structural comparison of RNA-dependent polymerase from plant, animal, and bacterial viruses. *Nucleic Acid Res.* **12,** 7267.

Kure, K., Llena, J. F., Lyman, W. D., Soeiro, R., Weidenheim, K. M., Hirano, A., and Dickson, D. W. (1991). Human immunodeficiency virus-1 infection of the nervous system: an autopsy study of 268 adult, pediatric, and fetal brains. *Hum Pathol* **22**(7), 700-

10.

Lackner, A., Smith, M., Munn, R., Martfeld, D., Gardner, M., Marx, P., and Dandekar, S. (1991). Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *Am. J. Pathol.* **139,** 609.

Lafortune, L., Nalbantoglu, J., and Antel, J. P. (1996). Expression of tumor necrosis factor alpha (TNF alpha) and interleukin 6 (IL-6) mRNA in adult human astrocytes: comparison with adult microglia and fetal astrocytes. *J Neuropathol Exp Neurol* **55**(5), 515-21.

Larimore, M., Butera, S., and Callahan, G. (1988). Spontaneous interferon production by pulmonary leukocytes is associated with lentivirus-induced lymphoid interstitial pneumonia. *J. Immunol.* **140,** 779.

Lechner, F., Machado, J., Bertoni, G., Seow, H., Dobbelaere, D., and Peterhans, E. (1997). Caprine arthritis encephalitis virus dysregulates the expression of cytokines in macrophages. *J. Virol.* **71,** 7488.

Lee, Y., and Coffin, J. (1990). Efficient autointegration of avian retrovirus DNA in vitro. *J. Virol.* **64,** 5958.

Lee, W., McConnell, I., and Blacklaws, B. (1994). Cytotoxic activity against maedi-visna virus-infected macrophages. *J. Virol.* **68,** 8331.

Lee, W., McConnell, I., and Blacklaws, B. (1996). Electron microscope studies of the replication of a British isolate of maedi visna virus in macrophages and skin cell lines. *Vet. Microbiol.* **49,** 293.

Legastelois, I., Cottin, V., Mornex, J. F., and Cordier, G. (1997). Alveolar macrophages from sheep naturally infected by visna-maedi virus contribute to IL-8 production in the lung. *Vet Immunol Immunopathol* **59**(1-2), 131-9.

Letvin, N., and King, N. (1990). Immunologic and pathologic manifestations of the infection of rhesus monkeys with simian immunodeficiency virus of macaques. *J. AIDS* **3,** 1023.

Li, S., Kaaya, E., Feichtinger, H., Putkonen, P., Parravicini, C., Bottiger, D., Biberfeld, G., and Biberfeld, P. (1991). Monocyte/macrophage giant cell disease in SIV-infected cynomolgus monkeys. *Res. Virol.* **142,** 173.

Lillien, L., Sendtner, M., Rohrer, H., Hughes, S., and Raff, M. (1988). Type-2 astrocytes

development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. *Neurone* **1,** 485.

Linial, M., and Miller, A. (1990). Packaging: Sequence requirements and implications. *In* "Retroviruses: strategies of replication" (R. Swanstrom, and P. Vogt, Eds.), pp. 125. Springer-Verlag, Berlin.

Lipton, S. A. (1992). Models of neuronal injury in AIDS: another role for the NMDA receptor? [see comments]. *Trends Neurosci* **15**(3), 75-9.

Lujan, L., Begara, I., Collie, D., and Watt, N. (1993). Phenotypic analysis of cells in bronchoalveloar lavage fluid and peripheral blood of maedi visna-infected sheep. *Clin. Exp. Immunol.* **91,** 272.

Lutley, R., Petursson, G., Palsson, P., Georgsson, G., Klein, J., and Nathanson, N. (1983). Antigenic drift in visna: virus variation during long-term infection of Icelandic sheep. *J. Gen. Virol.* **64,** 1433.

Majors, J. (1990). The structure and function of retroviral long terminal repeats. *In* "Retrovirises: Strategies for replication" (R. Swanstrom, and P. Vogt, Eds.), pp. 49-92. Springer-Verlag, Berlin.

Mallat, M., Houlgatte, R., Brachet, P., and Prochiantz, A. (1989). Lipopolysaccharide-stimulated rat brain macrophages release NGF in vitro. *Deviol.. B* **133,** 309.

Masliah, E., Achim, C., Ge, N., DeTeresa, R., Terry, R., and Wiley, C. (1992). Spectrum of human immunodeficiency virus-associated neocortical damage. *Ann. Neurol.* **32,** 321.

Matsumoto, Y., Ohmori, K., and Fujiwara, M. (1992). Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo-mimicking conditions. *Immunology* **76,** 209.

Maury, W. (1994). Monocyte maturation controls expression of equine infectious anemia virus. *J. Virol.* **68,** 6270.

McChesney, M., Altman, A., and Oldstone, M. (1988). Suppression of T lymphocyte function by measles virus is due to cell cycle arrest in G1. *J. Immunol.* **140,** 1269.

McCloskey, T., Ott, M., Tribble, E., Khan, S., Teichberg, S., Paul, M., Pahwa, S., Verdin, E., and Chimule, N. (1997). Dual role of HIV Tat in regulation of apoptosis in T cells. *J. Immunol.* **158,** 1014.

McCune, J., Rabin, L., Feinberg, M., Liebermann, M., Kosek, J., Reyes, G., and

Weissman, I. (1988). Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* **53,** 55.

McGeoch, D. (1990). Protein sequence comparisons show that the "pseudoproteases" encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase activity. *Nucleic Acids Res.* **18,** 4105.

McGuire, T., Crawford, T., and Henson, J. (1971). Immunofluorescent localization of euqine infectious anemia virus in tissue. *Am. J. Pathol.* **61,** 283.

McIlwraith, C., and Kitchen, D. (1978). Neurologic signs and neuropathology associated with a case of equine infectious anemia virus. *Cornell Vet.* **68,** 238.

Meric, C., and Goff, S. (1989). Characterisation of Moloney murine leukemia virus mutants with single amino acid substitutions in Cys-His box of the nucleocapsid protein. *J. Virol.* **63,** 1558.

Merrill, J. (1992). Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system of AIDS. *J. Immunother.* **12,** 167.

Milich, L., Margolin, B., and Swanstrom, R. (1993). V3 loop of the human immnuodeficiency virus type 1 Env protein: interpreting sequence variability. *J. Virol.* **67,** 5623.

Miller, S., and Karpus. (1994). The immunopathogenesis and regulation of T-cell-mediated demyelinating diseases. *Immunology Today* **15,** 356.

Milner, R., and Ffrench_Constant, C. (1994). A developmental analysis of oligodendroglial integrins in primary cells: changes in alpha v-associated beta subunits during differentiation. *Development* **120,** 3497.

Moelling, K., Bolognesi, D., Bauer, H., Busen, W., Plassmann, H., and Hausen, P. (1971). Association of viral reverse transcriptase with an enzyme degrading the RNA-DNA hybrids. *Nature New Biol.* **234,** 240.

Molina, J. M., Scadden, D. T., Amirault, C., Woon, A., Vannier, E., Dinarello, C. A., and Groopman, J. E. (1990). Human immunodeficiency virus does not induce interleukin-1, interleukin- 6, or tumor necrosis factor in mononuclear cells. *J Virol* **64**(6), 2901-6.

Moore, J., and Nara, P. (1991). The role of the V3 loop of the gp120 in HIV infection. *AIDS* **5,** S21.

Moore, J., Cao, Y., Qing, L., Sattentau, Q., Pyati, J., Koduri, R., Robinson, J., Barbas, C.

r., Burton, D., and Ho, D. (1995). Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *J. Virol.* **69,** 101.

Moreno, B., Harkiss, G., Woodall, C., and Watt, N. (1997). *3rd European Workshop on Ovine and Caprine Retroviruses, Jaca, Spain.*

Morganti-Kossmann, M. C., Kossmann, T., and Wahl, S. M. (1992). Cytokines and neuropathology. *Trends Pharmacol Sci* **13**(7), 286-91.

Muesing, M., Smith, D., Cabradilla, C., Benton, C., Lasky, L., and Capon, D. (1985). Nucleic acid structure and expression of the human AIDS/lymphodenopathy retrovirus. *Nature* **313,** 450.

Munis, J., Kornbluth, R., Guatelli, J., and Richman, D. (1992). Ordered appearance of human immunodeficiency virus type 1 nucleic acids following high multiplicity infection in macrophages. *J. Gen. Virol.* **73,** 1899.

Munn, R., Marx, P., Yamamato, J., and Gardner, M. (1985). Ultrastructural comparison of the retrovirises associated with human and simian acquired immunodeficiency syndromes. *Lab. Invest.* **53,** 194.

Murphy, F., and Kingsbury, D. (1991). Virus Taxonomy. *In* "Fundemental Virology" (B. Fields, D. Knipe, and R. Chanock, Eds.), pp. 9-36. Raven Press, New York.

Myers, G., Rabson, A., Joseph, S., Berzofsky, J., and Wong-Staal, F. (1990). "Human retroviruses and AIDS." Los Alamos National Library, Los Alamos, N.M.

Myers, G., Berzofsky, J., Korber, B., Smith, R., and Pavlakis, G. (1993). "Human Retroviruses and AIDS 1993: A Compilation and Snalysis of Nucleic Acid and Amino Acid Sequences." Los Alamos National Library, Los Alamos, NM.

Nanduri, V., and Modak, M. (1990). Lysine-329 of murine leukemia virus reverse transcriptase: Possible involvement in the temple-primer binding function. *Biochemistry* **29,** 5258.

Nara, P., Hatch, W., Dunlop, N., Robey, W., Arthur, L., Gonda, M., and Fischinger, P. (1987). Simple, rapid, quantitaive, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. *AIDS Res. Hum. Retrovirises* **3,** 283.

Narayan, O., Sheffer, D., Clements, J., and Tennekoon, G. (1985). Rstricted replication of

lentiviruses. Visna viruses induce a unique interferon during interaction between lymphocytes and infected macrophages. *J. Exp. Med.* **162,** 1954.

Narayan, O., and Clements, J. (1989). Biology and pathogenesis of lentiviruses. *J. Gen. Virol.* **70,** 1617.

Narayan, O., Zink, M., Gorrell, M., Crane, S., Huso, D., Jolly, P., Salatarelli, M., Adams, R., and Clements, J. (1993). The lentiviruses of sheep and goats. *In* "The Retroviridae" (J. Levy, Ed.), Vol. 2, pp. 229-255. 4 vols. Plenum, New York, New York.

Nath, A., Psooy, K., Martin, C., Knudsen, B., Magnuson, D. S., Haughey, N., and Geiger, J. D. (1996). Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. *J Virol* **70**(3), 1475-80.

Nathanson, N., Panitch, H., Palsson, P., Petursson, G., and Georgsson, G. (1976). Pathogenesis of visna. II. Effect of immunosuppression upon early central nervous system lesions. *Lab. Invest.* **35,** 444.

Nathanson, N., Petursson, G., Georgsson, G., Palsson, P., Martin, J., and Miller, A. (1979). Pathogenesis of visna. IV. Spinal fluid studies. *J. Neuropathol. Exp. Neurol.* **38,** 197.

Nathanson, N., Martin, J., Georgsson, G., Palsson, P., Lutley, R., and Petursson, G. (1981). The effect of post-immunization on the severity of experimental visna. *J. Comp. Pathol.* **91,** 185.

Navia, M., and McKeever, B. (1990). A role for the aspartyl protease from the human immunodeficiency virus type 1 (HIV-1) in the orchestration of virus assembly. *Ann. N.Y. Acad. Sci.* **616,** 73.

Navikas, V., Link, J., Persson, C., Olsson, T., Hojeberg, B., Ljungdahl, A., Link, H., and Wahren, B. (1995). Increased mRNA expression of IL-6, IL-10, TNF-alpha, and perforin in blood mononuclear cells in human HIV infection. *J Acquir Immune Defic Syndr Hum Retrovirol* **9**(5), 484-9.

Neddermann, P., Graziani, R., Ciliberto, G., and Paonessa, G. (1996). Functional expression of soluble human interleukin-11 (IL-11) receptor alpha and stoichiometry of in vitro IL-11 receptor complexes with gp130. *J Biol Chem* **271**(48), 30986-91.

Njenga, M., Pavelko, K., Baisch, J., Lin, X., David, C., Leibowitz, J., and Rodreguez, M. (1996). Theiler's virus persistence and demyelination in major histocompatibility

complex class II-deficient mice. *J. Virol.* **70,** 1729.

Nottet, H., Jett, M., Flanagan, C., Zhai, Q., and al., e. (1995). A regulatory role for astrocytes in HIV-1 encephalitis. An overexpression of eicosanoids, PAF, and TNF-alpha by activated HIV-1-infected monocytes is attenuated by human astrocytes. *J. Immunol.* **154,** 3567.

Nottet, H., Flanagan, E., Flanagan, C., and al., a. (1996). The regulation of quinolinic acid in HIV-infected monocytes. *J. Neurovirology* **2,** 1996.

Nuovo, G., and Alfieri, M. (1996). AIDS dementia is associated with massive activated HIV-1 infection and concommitant expression of several cytokines. *Mol. Med.* **2,** 358.

O'Toole, C., Lowdell, M., Chargelegue, D., and Colvin, B. (1992). decline in CTL and antibody responses to HIV-1 p17 and p24 antigens in HIV-1-infected hemophiliacs irrespective of disease progression. A 5 year follow-up study. *AIDS Res. Hum. Retroviruses* **8,** 1361.

Oberste, M., and Gonda, M. (1992). Conservation of amino-acid motifs in lentivirus Vif proteins. *Virus Genes* **6,** 95.

Okamoto, M., Makino, M., Kitajima, I., Maruyama, I., and Baba, M. (1997). HIV-1-infected myelomonocytic cells are resisitant to Fas-mediated apoptosis: effect of tumour necrosis factor-alpha on their Fas expression and apoptosis. *Med. Microbiol. Immunol.* **186,** 11.

Onuma, M., Koomoto, E., Furuyama, H., Yasutomi, Y., Taniyama, H., Iwai, H., and Kawakami, Y. (1992). Infection and dysfunction of monocytes induced by experimental inoculation of calves with bovine immunodeficiency-like virus. *J. AIDS* **5,** 1009.

Ozel, M., Pauli, G., and Gelderblom, H. R. (1988). The organization of the envelope projections on the surface of HIV. *Arch Virol* **100**(3-4), 255-66.

Palsson, P. (1974). Slow virus diseases of animals and man (R. Kimberlin, Ed.). North-Holand/American Elsevier, New York.

Pantaleo, G., Graziosi, C., Demarest, J., and al., e. (1993). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362,** 355.

Pantaleo, G., Demarest, J., Soudeyns, H., Graziosi, C., and al., e. (1994). Major expression of CD8+ T cells with a predominant V beta usage during the primary immune

response to HIV. *Nature* **370,** 463.

Paxton, W., Dragic, T., Koup, R., and Moore, J. (1996). The beta-chemokines, HIV type 1 second receptors, and exposed uninfected persons. *AIDS Res. Hum. Retroviruses* **12,** 1203.

Paya, C. V., Leibson, P. J., Patick, A. K., and Rodriguez, M. (1990). Inhibition of Theiler's virus-induced demyelination in vivo by tumor necrosis factor alpha. *Int Immunol* **2**(9), 909-13.

Pedersen, N. (1993). The feline Immunodeficiency Virus. *In* "The Retroviridiae" (J. Levy, Ed.), Vol. 2, pp. 181-219. 4 vols. Plenum Press, New York, New York.

Peluso, R., Haase, A., Stowring, L., Edwards, M., and Ventura, P. (1985). A Trojan Horse mechanism for the spread of visna virus in monocytes. *Virology* **147,** 231.

Perry, V., and Gordon, S. (1988). Macrophages and microglia in the nervous system. *Trends Neurosci.* **11,** 273.

Perry, L., Wilkerson, M., Hullinger, G., and Cheevers, W. (1995). depressed CD4+ T lymphocyte proliferativetibody response and enhanced antibody response to viral antigen in chronic lentivirus-induced arthritis. *J. Infect. Dis.* **171,** 328.

Persidsky, Y., Limoges, J., McComb, R., and al., e. (1996). Human immunodeficiency virus encephalitis in SCID mice. *Am. J. Pathol.* **149,** 1027.

Petito, C. K., Vecchio, D., and Chen, Y. T. (1994). HIV antigen and DNA in AIDS spinal cords correlate with macrophage infiltration but not with vacuolar myelopathy. *J Neuropathol Exp Neurol* **53**(1), 86-94.

Petursson, G., Nathanson, N., Georgsson, G., Panitch, H., and Palsson, P. (1976). Pathogenesis of visna I. Sequential virologic, serolig, and pathologic studies. *Lab. Invest.* **35,** 402.

Petursson, G., Turelli, P., Matthiasdottir, S., Gerogsson, G., Andresson, O. S., Torsteinsdottir, S., Vigne, R., Andresdottir, V., Gunnarsson, E., Agnarsdottir, G., and Querat, G. (1998). Visna virus dUTPase is dispensible for neuropathology. *J. Virology* **72,** 1657.

Philippon, V., Vellutini, C., Gambarelli, D., Harkiss, G., Arbuthnott, G., Metzger, D., Roubin, R., and Filippi, P. (1994). The basic domain of the lentiviral Tat protein is responsible for damages in mouse brain: involvement of cytokines. *Virology* **205**(2), 519-

Poignard, P., Fouts, T., Naniche, D., Moore, J., and Sattentau, Q. (1996). Neutralizing antibodies to human immunodeficiency virus type-1 gp120 induce envelope glycoprotein subunit dissociation. *J. Exp. Med.* **183,** 473.

Poignard, P., Klasse, P., and Sattentau, Q. (1996). Antibody neutralization by HIV-1. *Immunology Today* **17,** 239.

Pomerantz, R., Trono, D., Feinberg, M., and Baltimore, D. (1990). Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model of latency. *Cell* **61,** 1271.

Porcelli, S. (1995). The CD1 family: a third lineage of antigen-presenting molecules. *Adv. Immunol.* **59,** 1.

Pousset, F. (1994). Developmental expression of cytokine genes in the cortex and hippocampus of the rat central nervous system. *Brain Res Dev Brain Res* **81**(1), 143-6.

Power, C., McArthur, J., Johnson, R., Griffin, D., Glass, J., Perryman, S., and Chesebro, B. (1994). Demented and nondemented patients with AIDS differ in brain-derived human immunodeficiency virus type 1 envelope sequences. *J. Virol.* **68,** 4643.

Preston, B., Sauvaget, I., Delaure, M., and Tordo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO* **8,** 3867.

Probert, L., Akassoglou, K., Kassiotis, G., Pasparakis, M., Alexopoulou, L., and Kollias, G. (1997). TNF-alpha transgenic and knockout models of CNS inflammation and degeneration. *J Neuroimmunol* **72**(2), 137-41.

Pulliam, L., Clarke, J., McGuire, D., and McGrath, M. (1994). Investigation of HIV-infected macrophages neurotoxin production from patients with AIDS dementia. *Adv. Neuroimmunol.* **4,** 195.

Pyrah, I., and Watt, N. (1996). Immunohistological study of the depressed cutaneous DTH response in sheep naturally infected with an ovine lentivirus (Maedi-Visna virus). *Clin. Exp. Immunol.* **104,** 32.

Querat, G., Audoly, G., Sonigo, P., and Vigne, R. (1990). Nucleotide sequence analysis of SA-OMVV, a visna-related ovine lentivirus: phylogenetic history of lentiviruses. *Virology* **175,** 434.

Rabson, A., Daugherty, D., Venkatesan, S., Boulukos, K., Benn, S., Folks, T., Feorino, P., and Martin, M. (1985). Transcription of novel open reading frames of AIDS retrovirus during infection of lymphocytes. *Science* **229,** 1388.

Raff, M., Miller, R., and Noble, M. (1983). A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303,** 390.

Raff, M. (1989). Glial cell diversification in the rat optic nerve. *Science* **243,** 1450.

Ransohoff, R. M., Hamilton, T. A., Tani, M., Stoler, M. H., Shick, H. E., Major, J. A., Estes, M. L., Thomas, D. M., and Tuohy, V. K. (1993). Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis. *Faseb J* **7**(6), 592-600.

Rappolee, D., Brenner, C., Schultz, R., Mark, D., and Werb, Z. (1988). Developmental expression of PDGF, TGF-alpha, and TGF-beta genes in preimplantation mouse embryos. *Science* **241,** 1823.

Rayburn, H. (1992). Edinburgh, Edinburgh.

Reed, L., and Muench, H. (1938). A simple method of estimating fifty percent end-points. *Am. J. Hygiene* **24,** 493.

Renno, T., Krakowski, M., Piccirillo, C., Lin, J., and Owens, T. (1995). TNFalpha expression by resident microglial and infiltrating leukocytes in the CNS of mice with EAE. Regulation by Th1 cytokines. *J. Immunol.* **154,** 944.

Rhind, S. (1996). Department of Veterinary Pathology, The University of Edinburgh, Scotland.

Ringheim, G. E., Burgher, K. L., and Heroux, J. A. (1995). Interleukin-6 mRNA expression by cortical neurons in culture: evidence for neuronal sources of interleukin-6 production in the brain. *J Neuroimmunol* **63**(2), 113-23.

Rivera, S., Gold, S. J., and Gall, C. M. (1994). Interleukin-1 beta increases basic fibroblast growth factor mRNA expression in adult rat brain and organotypic hippocampal cultures. *Brain Res Mol Brain Res* **27**(1), 12-26.

Robbins, D. S., Shirazi, Y., Drysdale, B. E., Lieberman, A., Shin, H. S., and Shin, M. L. (1987). Production of cytotoxic factor for oligodendrocytes by stimulated astrocytes. *J Immunol* **139**(8), 2593-7.

Roberts, J., Bebenek, K., and Kunkel, T. (1988). The acuracy of reverse transcriptase

from HIV-1. *Science* **242,** 11171.

Rodriguez, M., and Sriram, S. (1988). Successful therapy of Theiler's virus-induced demyelination (DA strain) with monoclonal anti-Lyt-2 antibody. *J. Immunol.* **140,** 2950.

Rossol, S., Gianni, G., Rossol_Voth, R., and al., e. (1992). Cytokine-mediated regulation of monocyte/macrophage cytotoxicity in HIV-1 infection. *Med. Microbiol. Immunol.* **181,** 267.

Rostad, S., Sumi, S., Shaw, C., Olson, K., and McDougal, J. (1987). Human immunodeficiency virus (HIV) infection in brains with AIDS-related leukoencephalopathy. *AIDS Res. Hum. Retroviruses* **3,** 363.

Rostworowski, M., Balasingam, V., Chabot, S., Owens, T., and Yong, V. W. (1997). Astrogliosis in the neonatal and adult murine brain post-trauma: elevation of inflammatory cytokines and the lack of requirement for endogenous interferon-gamma. *J Neurosci* **17**(10), 3664-74.

Ruddle, N. H., Bergman, C. M., McGrath, K. M., Lingenheld, E. G., Grunnet, M. L., Padula, S. J., and Clark, R. B. (1990). An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J Exp Med* **172**(4), 1193-200.

Ruegg, C., Clements, J., and Strand, M. (1990). inhibition of lymphoproliferation and protein kinase C by synthetic peptides with sequence identity to the transmembrane and Q proteins of visna virus. *J. Virol.* **64,** 2175.

Rwambo, P., Issel, C., Hussain, K., and Montelaro, R. (1990). In vitro isolation of neutralization escape mutant of equine infectious anemia virus (EIAV). *Arch. Virol.* **111,** 275.

Rychlik, W., Spencer, W., and Rhoads, R. (1990). Optimization of the annealing temperature for DNA amplification in vitro. *Nucleic Acids Res.* **18,** 6409.

Rychlik, W., Spencer, W., and Rhoads, R. (1991). Optomization of the annealing temerature (erratum). *Nucleic Acid Res.* **19,** 698.

Sabatier, J. M., Vives, E., Mabrouk, K., Benjouad, A., Rochat, H., Duval, A., Hue, B., and Bahraoui, E. (1991). Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *J Virol* **65**(2), 961-7.

Sadaie, M., Benter, T., and Wong-Staal, F. (1988). Site-directed mutagenesis of two

trans-regulatory genes (tat-III, trs) of HIV-1. *Science* **239,** 910.

Salinovich, O., Payne, S., Montelaro, R., Hussain, K., Issel, C., and Schnorr, K. (1986). Rapid emergence of novel antigenic and genetic variants of equine infectious anaemia virus during persistent infection. *J. Virol.* **57,** 1986.

Saltarelli, M., Querat, G., konings, D., Vigne, R., and Clements, J. (1990). Nucleotidesequence and transcriptional analysis of molecular clones of CAEV which generate infectious virus. *Virology* **179,** 347.

Saltarelli, M., Schoborg, R., Gdovin, S., and Clements, J. (1993). The CAEV tat gene trans-activates the viral LTR and is necessary for efficient viral replication. *Virology* **197,** 35.

Samaniego, F., Markham, P., Gallo, R., and Ensoli, B. (1995). Inflammatory cytokines induce AIDS-Kaposi's sarcoma-derived spindle cells to produce and release basic FGF and anhance Kaposi's sarcoma-like lesion formation in nude mice. *J. Immunol.* **154,** 3582.

Sargan, D., and Bennet, I. (1989). A transcriptional map of visna virus: definition of the second intron structure suggests a rev-like gene product. *J. Gen. Virol.* **70,** 1995.

Sargan, D., Bennet, I., Cousens, C., Roy, D., Blacklaws, B., Dalziel, R., Watt, N., and McConnell, I. (1991). Nucleotide sequence of EV1, a British isolate of maedi-visna virus. *J. Gen. Virol.* **72,** 1893.

Sasseville, V., Smith, M., mackay, C., and al., e. (1996). Chemokine expression in SIV-induced AIDS encephalitis. *Am. J. Pathol.* **149,** 1459.

Sawyer, R., Harada, F., and Dahlber, J. (1974). Virion-associated RNA primer for Rous sarcoma virus DNA synthesis: isolation from uninfected cells. *J. Virol.* **28,** 279.

Sceppler, J., Nicholson, J., Swan, D., Ahmed-Ansari, A., and McDougal, J. (1989). Down-modulation of MHC-I in a CD4+ T cell line, CEM-E5, after HIV-1 infection. *J. Immunol.* **143,** 2858.

Schall, T., and Bacon, K. (1994). Chemokines, leukocuye trafficking, and inflammation. *Curr. Opin. Immunol.* **6,** 865.

Schindelmeiser, J., and Gullotta, F. (1991). HIV-p24-antigen-bearing macrophages are only present in brains of HIV- seropositive patients with AIDS-encephalopathy. *Clin Neuropathol* **10**(3), 109-11.

Schuitemaker, H., Koot, M., Kootstra, N., and et al. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J. Virol.* **66,** 1354.

Schulz, T., Reeves, J., Hoad, J., Tailor, C., Stephens, P., Clements, G., Ortlepp, S., and al., e. (1993). Effect of mutations in the V3 loop on HIV-1 gp120 on infectivity and susceptibility to proteolytic cleavage. *AIDS Res. Hum. Retrovirises* **9,** 159.

Sciacca, F., Sturzl, M., Bussolino, F., and al., a. (1994). Expression of adhesion molecules, platelet-activating factor, and chemokines by Kapos's sarcoma cells. *J. Immunol.* **153,** 4816.

Sedgwick, J., Schwender, S., Imrich, H., Dorries, R., Butcher, R., and ter Meulen, V. (1991). Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc. Natl. Acad. Sci.* **88,** 7438.

Seimenis, A., Papadopolos, C., Mastroyanni, M., and Managana, O. (1985). .

Sellon, D., Perry, S., Coggins, L., and Fuller, F. (1992). Wild-type equine infectious anemia virus replicates in vivo predominantly i n tissue macrophages, not in peripheral blood monocytes. *J. Virol.* **62,** 5906.

Selmaj, K., and Raine, C. S. (1988). Tumor necrosis factor mediates myelin damage in organotypic cultures of nervous tissue. *Ann N Y Acad Sci* **540,** 568-70.

Selmaj, K. W., Farooq, M., Norton, W. T., Raine, C. S., and Brosnan, C. F. (1990). Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor. *J Immunol* **144**(1), 129-35.

Selmaj, K. W., and Raine, C. S. (1995). Experimental autoimmune encephalomyelitis: immunotherapy with anti- tumor necrosis factor antibodies and soluble tumor necrosis factor receptors. *Neurology* **45**(6 Suppl 6), S44-9.

Sharma, D., Zink, M., Anderson, M., Adams, R., Clements, J., Joag, S., and Narayan, O. (1992). Derivation of neurotropic simian immunodeficiency virus from exclusively lymphotropic parental virus: pathogenesis of infection in macaques. *J. Virol.* **66,** 3550.

Sharpless, N., Gilbert, D., Vandercam, B., Zhou, J., Verdin, E., Ronnett, G., Friedman, E., and Dubois-Dalcq, M. (1992). The restricted nature of HIV-1 tropism for cultured neuronal cells. *Virology* **191,** 813.

Shaw, G. M., Harper, M. E., Hahn, B. H., Epstein, L. G., Gajdusek, D. C., Price, R. W., Navia, B. A., Petito, C. K., O'Hara, C. J., Groopman, J. E., and et al. (1985). HTLV-III infection in brains of children and adults with AIDS encephalopathy. *Science* **227**(4683), 177-82.

Sheng, W. S., Hu, S., Kravitz, F. H., Peterson, P. K., and Chao, C. C. (1995). Tumor necrosis factor alpha upregulates human microglial cell production of interleukin-10 in vitro. *Clin Diagn Lab Immunol* **2**(5), 604-8.

Shi, B., De Girolami, U., He, J., Wang, S., Lorenzo, A., Busciglio, J., and Gabuzda, D. (1996). Apoptosis induced by HIV-1 infection of the central nervous system. *J. Clin. Invest.* **98,** 1979.

Shimojo, M., Nakajima, K., Takei, N., Hamanoue, M., and kohsaka, S. (1991). Production of basic fibroblast growth factor in cultured rat brain microglia. *Neurosci. Lett.* **123,** 229.

Shuhabuddin, M., Bentsman, G., Volsky, B., Rodriguez, I., and Volsky, D. (1996). A mechanism of restricted human immunodeficiency virus type 1 expression in human glial cells. *J. Virol.* **70,** 7992.

Sigurdadottir, B., and Thormar, H. (1964). Isolation of a viral agent from the lungs of sheep affected with maedi. *J. Infect. Dis.* **114,** 55.

Sigurdsson, B., Grimsson, H., and Palsson, P. (1952). Maedi, a chronic, progressive infection of sheep's lungs. *J. Infect. Dis.* **90,** 233.

Sigurdsson, B. (1954). Maedi, a slow progressive pnumonia of sheep: an epizoological and a pathological study. *Brit. Vet. J.* **110,** 255.

Sigurdsson, B., Thormar, H., and Palsson, P. (1960). Cultivation of visna virus in tissue culture. *Arch. Ges. Virusforschung* **10,** 368.

Skoff, R. (1990). Gliogenesis in rat optic nerve: astrocytes are generated in a single wave before oligodendrocytes. *Dev. Biol.* **139,** 149.

Smith, M. E., Somera, F. P., and Eng, L. F. (1983). Immunocytochemical staining for glial fibrillary acidic protein and the metabolism of cytoskeletal proteins in experimental allergic encephalomyelitis. *Brain Res* **264**(2), 241-53.

Soilu-Hanninen, M., Eralinna, J., Hukkanen, V., Royatta, M., Salmi, A., and Salonen, R. (1994). Semliki Forest virus infects mouse brain endothelial cells and causes blood-brain

barrier damage. *J. Virol.* **68,** 6291.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A., and Wain-Hobson, S. (1985). Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. *Cell* **42,** 369.

Sonigo, P., Barker, C., Hunter, E., and Wain-Hobson, S. (1986). Nucleotide sequence of Mason-Pfizer monkey virus: An immunosuppressive D-type retrovirus. *Cell* **45,** 375.

Sopper, S., Demuth, M., Stahl-Hennig, C., Hunsmann, G., Plesker, R., Coulibaly, C., Czub, S., Ceska, M., Koutsilieri, E., Riederer, P., Brinkmann, R., Katz, M., and ter Meulen, V. (1996). The effect of simian immunodeficiency virus infection in vitro and in vivo on the cytokine production of isolated microglia and peripheral macrophages from rhesus monkey. *Virology* **220**(2), 320-9.

Sparger, E., Shacklett, B., Renshaw-Gegg, L., Barry, P., Pedersen, N., Elder, J., and Luciw, P. (1992). Regulation of gene expression directed by the long terminal repeat of the feline immunodeficiency virus. *Virology* **187,** 165.

Spehar, T., and Strand, M. (1994). Cross-reactivity of anti-human immunodeficiency virus type 1 gp41 antibodies with human astrocytes and astrocytoma cell lines. *J. Virol.* **68,** 6262.

Sporn, M. B., Roberts, A. B., Wakefield, L. M., and de Crombrugghe, B. (1987). Some recent advances in the chemistry and biology of transforming growth factor-beta. *J Cell Biol* **105**(3), 1039-45.

Springer, T. (1990). Adhesion receptors of the immune system. *Nature* **346,** 425.

Stanley, J., Bhaduri, L., Narayan, O., and Clements, J. (1987). Topographical rearrangements of visna virus envelope glycoprotein during antigenic drift. *J. Virol.* **61,** 1019.

Staskus, J., Cyr, S., Rank, J., Weitgrefe, S., Haase, A., Cook, R., Fast, D., and al., e. (1991). Isolation of replication-competent clones of visna virus. *Virology* **181,** 228.

Steagall, W., Robek, M., Perry, S., Fuller, F., and Payne, S. (1995). Incorporation of uracil into viral DNA correlates with reduced replication of EIAV in macrophages. *Virology* **210,** 302.

Stephens, R., Casey, J., and Rice, N. (1986). Equine infectious anemia virus gag and pol genes: relatedness to visna and AIDS virus. *Science* **231,** 589.

Stephens, E., McClure, H., and Narayan, O. (1995). The proteins of lymphocyte- and macrophage-tropic strains of simian immunodeficiency virus are processed differently in macrophages. *Virology* **206,** 535.

Stephens, E., Liu, Z., Zhu, G., Adany, I., Joag, S., Foresman, L., Berman, N., and Narayan, O. (1995). Lymphocyte-tropic simian immunodeficency virus causes persistent infection in the brains of rhesus monkeys. *Virology* **21,** 600.

Stoiber, H., Thielens, N., Ebenbichler, C., Arlaud, G., and Dierich, M. (1994). The envelope glycoprotein of HIV-1 gp120 and human complement C1q bind to the same peptides derived from three different regions of gp41, the transmembrane glycoprotein of HIV-1, and share antigenic homology. *Eur. J. Immunol.* **24,** 294.

Stowring, L., Hasse, A., Petrusson, G., Georgsson, G., Palsson, P., R., L., Roos, R., and Szuchet, S. (1985). Detection of visna virus antigens and RNA in glial cells in foci of demyelination. *Virology* **141,** 311.

Stramer, S., Heller, J., Coombs, R., Parry, J., Ho, D., and Allain, J. (1989). Markers of HIV infection prior to IgG antibody seropositivity. *JAMA* **26,** 64.

Strijbos, P., Zamani, M., Rothwell, N., Arbuthnott, G., and Harkiss, G. (1995). Neurotoxic mechanisms of transactivating protein Tat of Maedi-Visna virus. *Neurosci. Lett.* **197,** 215.

Takahashi, K., Wesselingh, S. L., Griffin, D. E., McArthur, J. C., Johnson, R. T., and Glass, J. D. (1996). Localization of HIV-1 in human brain using polymerase chain reaction/in situ hybridization and immunocytochemistry. *Ann Neurol* **39**(6), 705-11.

Taupin, V., Renno, T., Bourbonniere, L., Peterson, A. C., Rodriguez, M., and Owens, T. (1997). Increased severity of experimental autoimmune encephalomyelitis, chronic macrophage/microglial reactivity, and demyelination in transgenic mice producing tumor necrosis factor-alpha in the central nervous system. *Eur J Immunol* **27**(4), 905-13.

Taylor, J., and Illmesee, R. (1975). Site on the RNA of an avian sarcoma virus at which primer is bound. *J. Virol.* **16,** 553.

Taylor, J. (1977). An analysis of the role of tRNA species as primers for the transcription into DNA of RNA tumor virus genomes. *Biochem. Biophys. Acta* **473,** 57.

Teich, H. (1982). Taxonomy of retroviruses. *In* "RNA tumor viruses" (R. Weiss, N. Teich, and H. Varmus, Eds.), pp. 25-207. Cold Spring Harbor, New York.

Teich, N. (1985). Taxonomy of retroviruses. *In* "RNA tumor Viruses - supplements and Appendices" (R. Weiss, H. Teich, H. Varmus, and J. Coffins, Eds.), pp. 1-16. Cold Spring Harbor, New York.

Temin, H. (1970). Malignant transformation of cells by viruses. *Prospect Biol. Med.* **14,** 11.

Temin, H., and Mizutani, S. (1970). RAN-directed DNA polymerase in virions of Rous sarcoma virus. *Nature* **226,** 1211.

Temin, H. (1976). The DNA provirus hypothesis. *Science* **192,** 1075.

Temin, H. (1989). Retrons in bacteria. *Nature* **339,** 254.

Teo, I., Veryard, C., Barnes, H., An, S., Jones, M., Lantos, P., Luthert, P., and Shaunak, S. (1997). Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. *J. Virol.* **71,** 2928.

Teodoro, J., and Branton, P. (1997). regulation of apoptosis by viral gene products. *J. Virol.* **71,** 1739.

Than, S., Hu, R., Oyaizu, N., Romano, J., Wang, X., Sheikh, S., and Pahwa, S. (1997). Cytokine pattern in relation to disease progression in human immunodeficiency virus-infected children. *J Infect Dis* **175**(1), 47-56.

Thiele, D. L., and Lipsky, P. E. (1985). Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes. *Proc Natl Acad Sci U S A* **82**(8), 2468-72.

Thielens, N., Bally, I., Ebenbichler, C., Dierich, M., and Arlaud, G. (1993). Further characterization of the interaction between the C1q subcomponent of human C1 and the transmembrane envelope glycoprotein gp41 of HIV-1. *J. Immunol* **151,** 6583.

Thomas, F., Chalk, C., Lalonde, R., Robitaille, Y., and Jolicoeur, P. (1994). Expression of human immunodeficiency virus type 1 in the nervous system of transgenic mice leads to neurological disease. *J. Virol.* **68,** 7099.

Thormar, H. (1961). An electron micrsocpe study of tissue cultures infected with visna virus. *Virology* **14,** 463.

Thormar, H. (1963). The growth cycle of visna virus in monolayer cultures of sheep cells.

Virology **19,** 273.

Thormar, H., and Hegadottir, H. (1965). A comparison of Visna and Maedi Viruses. *Res. Vet. Sci.* **6,** 456.

Thormar, H., Barshatzky, M., Amesen, K., and Kozlwski, H. (1983). The emergence of antigenic variants is a rare event in long-term visna virus infection in vivo. *J. Gen. Virol.* **64,** 1427.

Threadgill, D., Steagall, W., Flaherty, M., Fuller, F., Perry, S., Rushlow, K., Le Grice, S., and Payne, S. (1993). Characterisation of equine infectious anaemia virus dUTPase: growth properties of a dUTPase-deficient mutant. *J. Virol.* **67**(2592).

Tiley, L., Malim, M., and Cullen, B. (1991). Conserved functional organization of the human immunodeficiency virus type 1 and visna virus Rev proteins. *J. Virol.* **65,** 3877.

Tirumalai, R., and Modak, M. (1991). Photoaffinity labelling of the primer binding domain in murine leukemia virus reverse transcriptase. *Biochemistry* **30,** 6436.

Tornatore, C., Meyers, K., Atwood, W., Conant, K., and Major, E. (1994). Temporal patterens of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J. Virol.* **68,** 93.

Torsteinsdottir, S., Georgsson, G., Gisladottir, E., Rafnar, B., Palsson, P., and Petursson, G. (1992). Pathogenesis of central nervous system lesions in visna: cell-mediated immunity and lymphocyte sybsets in blood, brain and cerebrospinal fluid. *J. Neuroimmunol.* **41,** 149.

Torsteinsdottir, S., Georgsson, G., Gisladottir, E., Rafnar, B., Palsson, P., and Petursson, G. (1994). Pathogenesis of central nervous system lesions in visna cell-mediated immunity and lymphocyte subsets in blood, brain, and cerebrospinal fluid. *Ann. N. Y. Acad. Sci.* **724,** 159.

Torsteinsdottir, S., Agnarrsdottir, G., Matthiasdottir, S., Rafnar, B., Andresdottir, V., Andresson, O., Petursson, G., Palsson, P., and Georgsson, G. (1997). In vivo and in vitro infection with two different molecular clones of visna virus. *Virology* **229,** 370.

Tower, D. B. (1973). The role of astroglia as modulators of neuronal function in cerebral cortex: comparative data, and observations in vivo and in vitro in fluid, electrolyte and amino acid interrelationships. *Vopr Biokhim Mozga* **8,** 269-88.

Trotter, J., Crang, A., Schachner, M., and Blakemore, W. (1993). Lines of glial precursor

cells immortalised with a temperature-sensitive oncogene give rise to astrocytes and oligodendrocytes following transplantation into demyelinated lesions in the central nervous system. *Glia* **9,** 25.

Trujillo, J., McLane, M., Lee, T., and Essex, M. (1993). Molecular mimecry between the human immunodeficiency virus type 1 gp120 V3 loop and human brain proteins. *J. Virol.* **67,** 7711.

Turelli, P., Petursson, G., Guiguen, F., Mornex, J., Vigne, R., and Querat, G. (1996). Replication properties of dUTPase-deficient mutants of caprine and ovine lentiviruses. *J. Virol.* **70,** 1213.

Tyor, W. R., Glass, J. D., Griffin, J. W., Becker, P. S., McArthur, J. C., Bezman, L., and Griffin, D. E. (1992). Cytokine expression in the brain during the acquired immunodeficiency syndrome. *Ann Neurol* **31**(4), 349-60.

Tyor, W. R., Power, C., Gendelman, H. E., and Markham, R. B. (1993). A model of human immunodeficiency virus encephalitis in scid mice. *Proc Natl Acad Sci U S A* **90**(18), 8658-62.

Ulvestad, E., Williams, K., Mork, S., Antel, J., and Nyland, H. (1994). Phenotypic differences between human monocyte/macrophages and microglial cells studied in situ and in vitro. *J. Neuropathol. Exp. Neurol.* **53,** 492.

Van der Molen, E., Vecht, U., and Houwser, D. (1985). A chronic indurative mastitis in sheep, associated with maedi/visna virus infection. *Vet. Q.* **7,** 112.

Vandenabeele, P., Declercq, W., Vanhaesebroeck, B., Grooten, J., and Fiers, W. (1995). Both TNF receptors are required for TNF-mediated induction of apoptosis in PC60 cells. *J Immunol* **154**(6), 2904-13.

Varmus, H., and P., B. (1989). Retroviruses. *In* "Mobile DNA" (D. Berg, and M. Howe, Eds.), pp. 53-108. American Society for Microbiology, Washington, D.C.

Verma, I. (1977). The reverse transcriptase. *Biochem. Biophys. Acta* **473,** 1.

Vigne, R., Filippi, P., Querat, G., Sauze, N., Vitu, C., Russo, P., and Delori, P. (1982). Precursor polypeptides to structural proteins of visna virus. *J. Virol.* **42,** 1046.

Vigne, R., Barban, V., Querat, G., Mazarin, V., Gourdou, I., and Sauze, N. (1987). Transcription of visna virus during its lytic cycle: evidence for a sequential early and late gene expression. *Virology* **161,** 218.

Wahl, S. M., Allen, J. B., McCartney-Francis, N., Morganti-Kossmann, M. C., Kossmann, T., Ellingsworth, L., Mai, U. E., Mergenhagen, S. E., and Orenstein, J. M. (1991). Macrophage- and astrocyte-derived transforming growth factor beta as a mediator of central nervous system dysfunction in acquired immune deficiency syndrome. *J Exp Med* **173**(4), 981-91.

Watt, N., Roy, D., and McConnell, I. (1990). A case of visna in UK. *Vet. Record* **600,** 601.

Weiner, A., and Maizels, N. (1987). tRNA-like structures tag the 3' ends of genomic RNA molecules for replication: implications for the origin of protein synthesis. *Proc. Natl. Acad. Sci. (USA)* **84,** 7383.

Weiss, C., and White, J. (1993). Characterization of stable Chinese hamster ovary cells expressing wild-type, secreted, and glycosylphosphatidylinositol-anchored human immunodeficiency virus type 1 envelope glycoprotein. *J. Virol.* **67,** 7060.

Weiss, R. (1996). HIV receptors and the pathogenesis of AIDS. *Science* **272,** 1885.

Welsh, C., Tonks, P., Nash, A., and Blakemore, W. (1987). The effect of L3T4 T cell depletion on the pathogenesis of Theiler's murine encephalomyelitis virus infection in CBA mice. *J. Gen. Virol.* **68,** 1659.

Wiley, C., Schrier, R., Nelson, J., Lampert, P., and Oldstone, M. (1986). Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc. Natl. Acad. Sci.* **83,** 7089.

Wilkerson, M., Davis, W., and Cheervers, W. (1995). Peripheral blood and synovial fluid mononuclear cell phenotypes in lentivirus induced arthritis. *J. Rheumatol.* **22,** 8.

Wiskerchen, M., and Muesing, M. (1995). Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J. Virol.* **69,** 376.

Woodall, C., Mylne, J., McKelvie, W., and Watt, N. (1994). A technique for the sequential isolation of RNA and DNA from embryos for screening for viruses. *J. Virol. Methods* **46,** 263.

Wu, P., Price, P., Du, B., Hatch, W. C., and Terwilliger, E. F. (1996). Direct cytotoxicity of HIV-1 envelope protein gp120 on human NT neurons. *Neuroreport* **7**(5), 1045-9.

Xiong, Y., and Eickbush, T. (1988). Similarity of reverse transcriptase-like sequences of viruses, transposon elements, and mitochondrial introns. *Mol. Biol. Evol.* **5,** 675.

Yang, O., Kalams, S., Trocha, A., Cao, H., Luster, A., Johnson, R., and Walker, B. (1997). Suppression of human immunodeficiecy virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolic mechanisms. *J. Virol.* **71,** 3120.

Yong, V. W., Moumdjian, R., Yong, F. P., Ruijs, T. C., Freedman, M. S., Cashman, N., and Antel, J. P. (1991). Gamma-interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. *Proc Natl Acad Sci U S A* **88**(16), 7016-20.

Yoo, J., Chen, H., Kraus, T., Hirsch, D., Polyak, S., George, I., and Sperber, K. (1996). Altered cytokine production and accessory cell function after HIV-1 infection. *J Immunol* **157**(3), 1313-20.

Yu, G.-L., Bradley, J., Attardi, L., and Blackburn, E. (1990). In vitro alteration of telomere sequences and senscense caused by mutated tetrahymena telomerase RNAs. *Nature* **344,** 126.

Zapp, M., Hope, T., Parslow, T., and Green, M. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiecy virus Rev protein: a dual function for an arginine-rich binding motif. *Proc. Natl. Acad. Sci. (USA)* **88,** 7734.

Zapp, M., Stern, S., and Green, M. (1993). Small molecules that selectivity block RNA binding of HIV-1 Rev protein inhibits Rev function and viral production. *Cell* **74,** 969.

Zerlin, M., Levison, S., and Goldman, J. (1995). early patterns of migration, morphogenesis, and intermediate filament expression of subventricular zone cells in the postnatal rat forebrain. *J. Neurosci.* **15,** 7238.

Zhang, J., Martin, L., Watson, E., Montelaro, R., Weat, M., Epstein, L., and Murphey-Corb, M. (1988). Simian immunodeficiency virus/delta-induced immunodeficiency disease in rhesus monkeys: relation of antibody response and antigenemia. *J. Infect. Dis.* **158,** 1277.

Zheng, Z., and Zuker-Franklin, D. (1992). apparent ineffectiveness of natural killer cells vis-a-vis retrovirus-infected targets. *J. Immunol.* **148,** 3679.

Ziegler-Heitbrock, H., and Ulevitch, R. (1993). CD14: a cell surface receptor and

differentiation marker. *Immunology Today* **14,** 121.

Zink, M., Narayan, O., Kennedy, P., and Clements, J. (1987). Pathogenesis of visna/maedi and caprine arthritis-encephalitis: new leads on the mechanism of restricted virus replication and persistent inflammation. *Vet. Immunol. Immunopathol.* **15,** 167.

Zink, M., and Narayan, O. (1989). Lentivirus-induced interferon inhibits maturation and proliferation of monocytes and restricts the replication of caprine arthritis-encephalitis virus. *J. Virol.* **63,** 2578.

Zink, M. C., Yager, J. A., and Myers, J. D. (1990). Pathogenesis of caprine arthritis encephalitis virus. Cellular localization of viral transcripts in tissues of infected goats. *Am J Pathol* **136**(4)**,** 843-54.

Zink, M., and Johnson, L. (1994). Pathobiology of lentivirus infections in sheep and goats. *Virus Res.* **32,** 139.

Zinkernagel, R., and Doherty, P. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248,** 701.

APPENDIX

Table A1 Stock solutions

Common stock solutions, reagents and chemicals used in this thesis are listed in the table. Buffers were prepared with distilled water, whereas reagents used in enzyme assays, for example dNTP mix, was prepared with Sigama water. Reagents were stored at room temperature unless stated otherwise.

Buffer/reagent	Concentration	components
SDS-PAGE running buffer	10X	To make 500 ml: Tris-base (15g), Glycine (72g), and SDS (5g)
TAE buffer	50X	To make 500 ml: Tris-base (121g), o.5M EDTA (pH 8) (50 ml), Glacial acetic acid (28.55 ml)
SSC buffer	20X	To make one litre: NaCl (175.3g), $Na_3Citrate.2H_2O$ (88.2g), H_2O 800 ml, pH adjusted to 7.0 with 1 M HCL, and volume made up to one litre with H_2O
Denhardt's solution	50X	To make 100 ml: one g polyvinylpyrrolidone, one g BSA, and one g Ficoll 400. Once dissolved, the solution was kept at −20 °C aliquots
PBS buffer	1X	To make one litre: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 ml of distilled H_2O. The pH was adjusted to 7.4 with HCl, and the volume made up to one litre
Random hexamers	1 nM	Purchased solution is at 50 optical density units. This is diluted in 1.06 ml of Sigma water to give a stock solution of 1nmole/µl
dNTP mix	1 mM	dATP, dCTP, dCTP, and dGTP were purchased as 100 mM solutions (Bhringer). Stock solution was prepared by mixing one microlitre of each solution (4 µl in total), plus 96 µl of water
Salmon sperm DNA	5 mg/ml	Sperm DNA was shredded into small (1-2 mm) fragments and added to a beaker of heated SDW. Addition of large fragments of DNA results in formation of insoluble aggregates. The volume of dissolved DNA solution was adjusted to give a 5 mg/ml solution. The solution was stored as 5-ml aliquots at −20 °C. Prior to use, an aliquot was thawed and boiled for five minutes before use
NBT	2 mg/ml	NBT was dissolved in 0.1 M Tris (pH 9.5) at 2 mg/ml
BCIP/X-phosphate	10 mg/ml	BCIP/X-phosphate was dissolved in N-N´-dimethylformamide at 10 mg/ml
DNA loading bugger	5X	Dissolve 15% (W/V) of Ficol 400 (Sigma) in 1x TAE, then add small quantity of Orange G powder (~500 µg) to give colour to the solution

	ATPase PCR	Cytokine PCR	MVV PCR
Sigma water	325 µl	340 µl	345 µl
Primers (forward and reverse)	10 µl of each	10 µl of each	10 µl of each
£10 x dNTPs	50 µl	50 µl	50 µl
10 x PCR buffer	50 µl	50 µl	50 µl
$MgCl_2$ (50 mM)	35 µl (3.5 mM)	20 µl (2 mM)	15 µl (1.5 mM)

Table A2 Master PCR mixes

Master mixes were prepared and stored at –20 °C, and used repeatedly. Both the 10 x PCR buffer and magnesium chloride solutions are provided with the enzyme. Primers were used neat as supplied. This is usually at 100 pM. However, primers can be diluted and used at 30-50 pM. All PCR reactions were performed by a hot-start at 80 °C for five minutes prior to addition of *Taq pol*. After the addition of enzyme, amplification was performed by denaturing at 95 °C for one minute, annealing at 55 °C for one minute and extension at 72 °C for two minutes. After the required cycles, an extension step at 72 °C for five minutes preceded the end of PCR reactions

£ The dNTP stock solution is made up at 1mM. The final working concentration is 100 µM.

Interaction	ΔH (kcal/mol)	ΔS (cal/K)
AA/TT	9.1	24.0
AT/TA	8.6	23.9
TA/AT	6.0	16.9
CA/GT	5.8	12.9
GT/CA	6.5	17.3
CT/GA	7.8	20.8
GA/CT	5.6	13.5
CG/GC	11.9	27.8
GC/CG	11.1	26.7
GG/CC	11.0	26.6

Table A3 Thermodynamic values used for calculating Ta^{OPT} values

Thermodynamic values refer to the disruption of the interaction in an existing duplex at 1 M NaCl, pH 7, and 25 °C. The units are per mole of interaction between given combinations of bases (Rychlik *et al.*, 1990, 1991).

	Main gel	Stack gel
Bis (0.8%)/Acrylamide (30%)	3 ml	1 ml
$1 M Tris-HCl	1.5 ml	1.25 ml
Distilled water	1.5 ml	6.2 ml
10% SDS	60 µl	100 µl
10% Ammonium persulphate	19 µl	50 µl
TEMED	2.5 µl	10 µl

Table A4 SDS-PAGE mix

Gel ingredients were mixed afresh and poured into a Biorad mini-gel protein II apparatus to give two 15% gels. This was gently overlaid with SDW, and allowed to set for one hour at room temperature. The stack gel was made up just prior to being poured over the main gel. The gel was run in at 200 volts for 40 minutes in 1x running buffer.

$: In the main gel, the pH of 1M Tris-HCl is 8.7, whereas in the stack gel, the pH is 6.8.