Immune Responses To Maedi-Visna Virus

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Declaration

The experiments and composition of this thesis are, unless otherwise stated, my own work. No part of this work has been, or is being, submitted for any other degree, diploma or other qualification.

Hugh Reyburn (February, 1992)

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ABSTRACT OF THESIS (Regulation 3.5.10)

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The immune response to maedi-visna virus has been investigated, both in persistently infected sheep and in the acute phase of the primary immune response to infection with maedi-visna.

Maedi-visna infected sheep develop detectable levels of anti-viral antibody by 4-6 weeks after experimental infection. These antibodies are directed against the envelope and core structural proteins of the virus and initially are of the IgM isotype, but later switch to IgG. These IgG anti-visna antibodies are restricted to the IgG_1 subclass.

The functional significance of this isotypic restriction of the anti-visna antibody response was studied using *in vitro* assays of antibody activity against virus infected cells. It was found that visna specific antibodies were able to direct antibody-mediated complement-dependent cytotoxicity, but not antibody dependent cell mediated cytotoxicity against virus infected cells. These observations are consistent with the known properties of ruminant immunoglobulin G subclasses. These persistently infected sheep were also shown to have developed a $CD4^+$ T cell response to maedi-visna virus.

The acute phase of the immune response to maedi-visna infection was studied in a lymphatic cannulation model. Infection with maedi-visna induced both virus neutralising antibodies and virus specific T cells, but these failed to prevent the establishment of a persistent viral infection.

The generation of the data on immune responses to maedi-visna virus described above was facilitated by the production of recombinant p25 *gag* protein and p25 specific polyclonal and monoclonal antibodies.

Contents **Page**

Title page i
Acknowledgements ii
Declaration iii
Abstract iv
Contents v
List of abbreviations x

Chapter 1: Introduction **1**

1.1 Introduction 2
1.2 Classification of Retroviruses 2
1.3 Genome of the virus 3
1.4 Virus structure
1.4.1 Virion morphology 6
1.4.2 Viral antigens
1.4.2.1 Virion peptides
1.4.2.1.1 ***Gag*** gene products 6
1.4.2.1.2 ***Pol*** gene products 8
1.4.2.1.3 ***Env*** gene products 9
1.4.2.2 Regulatory proteins 10
1.5 Infection and virus replication
1.5.1 Infectious inocula 11
1.5.2 Transmission of virus 14
1.5.3 Cellular tropism of visna 14
1.5.4 Entry of virion into the cell (
1.5.4.1 Entry 16
1.5.4.2 Interference 17
1.5.5 Reverse transcription & Proviral DNA 18
1.5.6 Viral gene expression 19
1.5.7 Translation 23
1.5.8 Virion assembly and egress 24
1.5.9 Effect of infection on the host cell 25
1.6 Virus-host relationship
1.6.1 Introduction 26
1.6.2 Natural resistance mechanisms 27

Page

1.6.3 Humoral immune responses 31
1.6.4 T cell mediated immunity 35
1.6.5 Antigenic variation 38
1.6.6 Maedi-visna disease 39
1.7 Conclusion 41

Chapter 2: Materials and Methods **43**
2.1 Materials 44
2.2 Bacterial culture
2.2.1 Media 44
2.2.2 Bacterial strains and culture 44
2.2.3 Transformation of *E. coli* 45
2.3 Preparation of DNA and molecular cloning techniques
2.3.1 Vectors used in cloning, sequencing and expression 46
2.3.2 Polymerase Chain Reaction 46
2.3.3 DNA agarose gel electrophoresis 48
2.3.4 Preparation of DNA fragments for blunt-ended cloning 48
2.3.5 Restriction endonuclease digestion of DNA 48
2.3.6 Preparation of cut vector 49
2.3.7 Ligation of DNA 49
2.3.8 Miniprep of plasmid DNA 49
2.3.9 Purified plasmid preparations 50
2.3.10 Quantitation of DNA 51
2.4 Blot hybridisations
2.4.1 Southern blotting 51
2.4.2 Preparation of [^{32}P]-labelled DNA probes 52
2.4.3 Hybridisation of nitrocellulose filters 52
2.5 DNA sequencing
2.5.1 Preparation of single stranded DNA 53
2.5.2 Sequencing reactions 53
2.5.3 Double strand sequencing 54
2.5.4 Sequencing gel electrophoresis 55
2.5.5 Sequence analysis 55
2.6 Yeast culture
2.6.1 Media 55

Page

2.6.2 Yeast culture and strains 55
2.6.3 Yeast transformation 56
2.6.4 Preparation of protein extracts 57
2.7 Mammalian tissue culture
2.7.1 Cell lines and cell culture 57
2.7.2 Viruses 58
2.7.3 Monoclonal antibody production
2.7.3.1 Myeloma cell culture 58
2.7.3.2 Procedure of cell fusion 58
2.7.3.4 Hybridoma cell culture 59
2.8 Antigen preparation
2.8.1 Preparation of visna antigen for Western blotting 59
2.8.2 Purification of visna 60
2.8.3 Purification of Ty-virus-like particles (Ty-VLP's) from yeast 60
2.8.4 Purification of p25 from the Ty-p25 VLP's 61
2.9 Detection of antigen
2.9.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of proteins 62
2.9.2 Staining of SDS-PAGE
a) Coomassie Blue staining 62
b) Silver staining 62
2.9.3 Immunofluorescent staining of cell monolayers 63
2.9.4 Reverse transcriptase assay 63
2.10 Transmission electron microscopy
2.10.1 Yeast sections 64
2.10.2 Negative staining of purified particles 65
2.11 Detection of specific antibody
2.11.1 Enzyme linked immunosorbent assay (ELISA) 65
2.11.2 Immunoblotting 65
2.11.3 Virus neutralisation 66
2.11.4 Antibody dependent cell-mediated cytotoxicity (ADCC)
a) Anti-visna ADCC 67
b) Anti-SRBC ADCC 67
2.11.5 Antibody-complement-mediated lysis 68

2.12 Immunochemistry
2.12.1 Coupling of protein to cyanogen bromide activated Sepharose 4B 69
2.12.2 Affinity chromatography 69
2.12.3 Purification of IgG 70
2.12.4 Determination of immunoglobulin class and subclass of monoclonal antibodies 70
2.12.5 Conjugation of biotin to immunoglobulin 70
2.12.6 Conjugation of Horse Radish Peroxidase (HRP) to IgG 70
2.13 Cellular immunology
2.13.1 Preparation of sheep peripheral blood mononuclear cells (PBMC) 71
2.13.2 Antigen specific lymphocyte proliferation assay 71
2.13.3 Flow cytometry 71
2.13.4 Cell depletion 72
2.14 Procedures involving experimental animals
2.14.1 Mice 72
2.14.1.1 Immunisation of mice for monoclonal antibody production 72
2.14.1.2 Collection of mouse serum 73
2.14.1.3 Preparation of single cell suspension of mouse splenocytes 73
2.14.1.4 Production of ascitic fluid 73
2.14.2 Rabbits 73
2.14.2.1 Immunisation of rabbits 74
2.14.2.2 Collection of rabbit sera 74
2.14.3 Sheep 74
2.14.3.1 Experimental infection of sheep with maedi-visna 74
2.14.3.2 Field cases of visna infection 75
2.14.3.3 Lymphatic cannulation 75

Chapter 3: Immune responses to maedi-visna virus in persistently infected sheep **76**
A. Humoral immune responses
3.1 Introduction 77
3.2 Results
3.2.1 Development of the antibody response to maedi-visna virus 81
3.2.2 Antibody responses in persistently infected sheep 88
3.2.3 Action of anti-visna antibody against virus infected cells 95

Page

B. T cell responses

3.3 Introduction 106
3.4 Results
3.4.1 Lymphoproliferative response in persistently infected sheep 107
3.5 Summary of Chapter 3 results 115
3.6 Discussion 115

Chapter 4: The acute immune response to maedi-visna virus studied at the level of a single lymph node **123**
4.1 Introduction 124
4.2 Results
4.2.1 Maedi-visna specific antibody in efferent lymph 130
4.2.2 Visna specific T cell responses 145
4.2.3 Lymphocyte proliferation 147
4.3 Summary of Chapter 4 results 151
4.4 Discussion 151

Chapter 5: Cloning and expression of p25, the major core protein of maedi-visna virus **158**
5.1 Introduction 159
5.2 Results
5.2.1 Cloning and expression of the p25 structural gene 160
5.2.2 Antigenic authenticity of recombinant p25 protein 171
5.2.3 Monoclonal antibody production 176
5.2.4 An assay for detection of p25 antigen 181
5.3 Summary of Chapter 5 results 188
5.4 Discussion 188

Chapter 6: Discussion **193**
6.1 Introduction 194
6.2 Maedi-visna as a model system for lentiviral pathogenesis 194
6.3 A model of the acute immune response to lentiviral infection 195
6.4 Host-virus interactions in persistently infected animals 198
6.5 Future prospects 201

Page

References 203

Appendix 235

List of Abbreviations

APC	antigen-presenting cell
bp, kbp	base pair(s), kilobase pairs
BSA	bovine serum albumin
CAEV	caprine arthritis-encephalitis virus
CD	cluster of differentiation
Ci, mCi, μCi	Curie, milliCurie, microCurie
Con A	Conconavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DME	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCA	Freund's complete adjuvant
ICFA	Incomplete Freund's adjuvant
FcR	Fc receptor
FCS	foetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
g, mg, μg	gramme, milligramme, microgramme
ng, pg	nanogramme, picogramme
HAT	hypoxanthine, aminopterin, thymidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV	herpes simplex virus
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IM	intramuscularly
IP	intraperitoneally
IV	intravenously
kDa	kiloDaltons
KLH	keyhole limpet haemocyanin
LCA	leukocyte common antigen

LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
M, mM, μM	molar, millimolar, micromolar
mA	milliamps
MHC	major histocompatability complex
min	minutes
mm	millilmetre
μl, ml	microlitre, millillitre
mRNA	messenger RNA
MVV	maedi-visna virus
NK	natural killer
NMS	normal mouse serum
OPD	orthophenylenediamine
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PEG	polyethyleneglycol
RNA	ribonucleic acid
sc	subcutaneously
SRBC	sheep red blood cell(s)
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
U	enzyme units
V	Volts

Chapter 1

Introduction

1.1 Introduction

Maedi-visna virus was originally described in Iceland as an aetiologic agent of chronic interstitial pneumonia (maedi) and demyelinating leukoencephalomyelitis (visna) in sheep (Sigurdsson, 1954., Sigurdsson & Palsson, 1958., Sigurdsson *et al*, 1952., 1957). Maedi-visna is a relatively common infection of sheep in most parts of the world (Adams *et al*, 1984), but the incidence of clinical disease is relatively low. However in the last decade lentiviral infections of both man, the human immunodeficiency virus (HIV), and other animals have been described (reviewed in Narayan & Clements, 1990) and these viruses have been recognised as important pathogens. The advent of HIV has resulted in the development of intense interest in all aspects of lentiviral biology, and maedi-visna virus is now studied, not only as a pathogen in its own right, but also as a model for some aspects of the pathogenesis of HIV infection and disease (Haase, 1986., Narayan & Clements, 1990).

1.2 Classification of Retroviruses

Retroviruses are a family of viruses with positive-sense, single-stranded RNA genomes, but which replicate via a double-stranded DNA intermediate (Teich, 1984). Traditionally the Retroviridae have been divided into three subfamilies; the Oncovirinae, the Spumavirinae and the Lentivirinae (Teich, 1984). This classification is based primarily on pathogenicity and morphologic criteria, rather than genome relationships. When nucleotide sequence data and genome structure are used as criteria for classification seven groups of retroviruses, composed of all well analysed isolates, can be defined (Coffin, 1990).

In either classification, the Lentivirinae form a discrete subfamily of exogenous retroviruses which cause slow, progressive inflammatory disease *in vivo*, a highly lytic infection *in vitro*, but are not directly implicated in any malignancies (Teich, 1984., Haase, 1986., Coffin, 1990). Maedi-visna virus is the prototype of the Lentivirinae, although the Human Immunodeficiency Virus, type 1 (HIV-1) is now the best characterised member of this subfamily.

1.3 Genome of the virus

The genomic RNA of visna is a positive sense, linear, single stranded RNA (60S-70S) composed of two identical sub-units (30S-35S) (Beemon *et al*, 1976., Vigne *et al*, 1977). Each retroviral genomic RNA molecule is associated with a single tRNA molecule: the primer on which reverse transcriptase initiates DNA synthesis (Coffin, 1990). The specific tRNA molecule appropriated for this purpose by visna virus is $tRNA_{lys-1,2}$ (Sonigo *et al*, 1985).

The genomic RNA of retroviruses resembles messenger RNA's of eukaryotic cells in that its 5' end has a methylated cap structure and its 3' end is polyadenylated (Varmus, 1982., Coffin, 1990). Sequences at the 3' end of the viral RNA, in the U3 region contain the enhancer-promoter elements for initiation of RNA transcription (Sonigo *et al*, 1985., Hess *et al*, 1986). In addition, the 5' and 3' ends of the RNA also contain short repeated sequences ('R' sequences) that are important for viral replication (Varmus, 1982). During the synthesis of proviral DNA (reviewed in Varmus & Swanstrom, 1985) the 5' and 3' ends of genomic RNA molecules are duplicated and a copy of each transposed so that the DNA provirus has identical terminal regions; the long terminal repeats (Fig 1.1).

The visna genome encodes the three structural genes, organised 5' to 3' (***gag*, *pol*, *env***), typical of all retroviruses. However, visna also displays a genetic feature unique to the lentiviruses: the presence of a collection of short open reading frames (ORF's) located between the ***pol*** and ***env*** genes and at the 3' end of the ***env*** gene (Sonigo *et al*, 1985). The functions of proteins encoded by these open reading frames are best characterised for HIV-1, the genome of which codes for at least six accessory genes (reviewed in Cullen & Greene, 1990). Visna virus displays a less complex genetic organisation; it appears to express only three auxiliary gene products (Fig. 1.1) (Davis *et al*, 1987., Vigne *et al*, 1987., Sargan & Bennet, 1989).

Figure 1.1

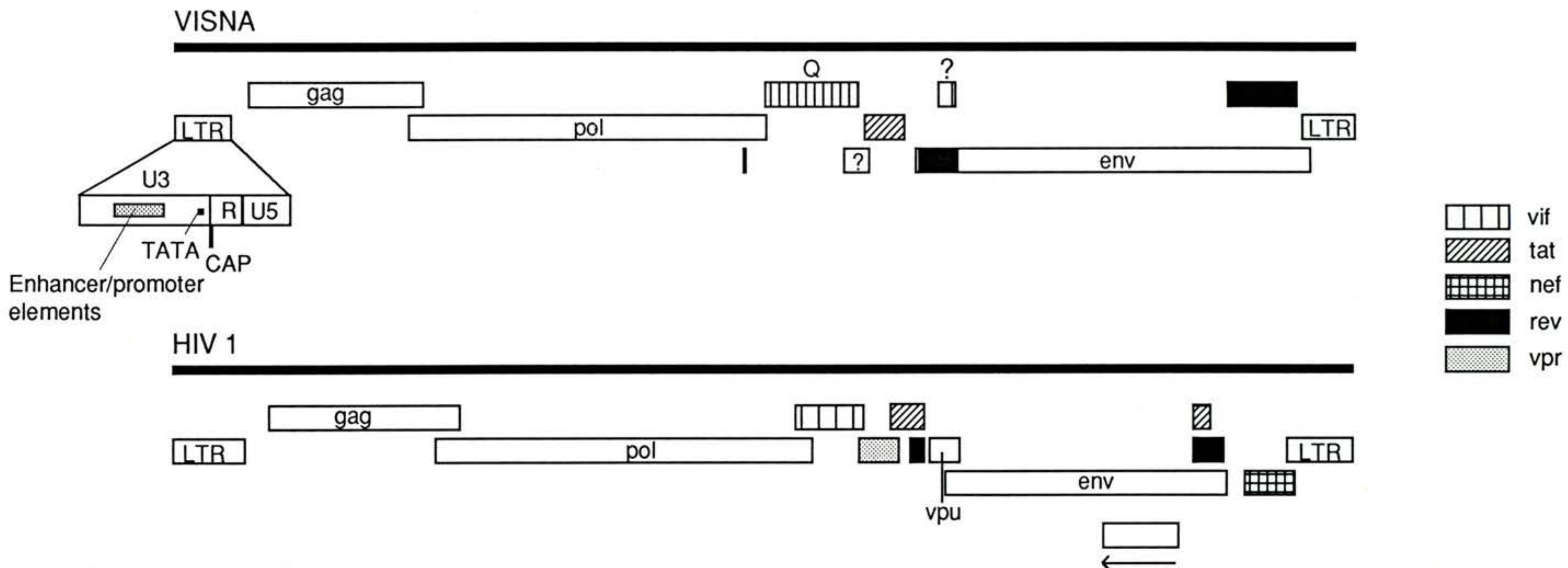
A comparison between the DNA genome of the ruminant lentivirus, Maedi-visna and HIV-1, the best characterised member of the Lentivirinae.

ORF Q, has been suggested, on the basis of its central position in the genome, to encode the visna equivalent of the *vif* protein of HIV-1 (Sonigo *et al*, 1985). However there is no significant amino-acid sequence homology between the protein product of ORF Q and HIV-1 vif (Sonigo *et al*, 1985).

The question marks indicate short ORF's in the visna genome whose codon usage is compatible with eventual translation (Sonigo *et al*, 1985). To date, neither RNA transcripts nor protein products of these ORF's have been reported.

The expanded view is a schematic diagram showing the organisation of the visna LTR. Important enhancer-promoter elements are found in the U3 region. The cap site, the 5' terminus of all the viral RNA's, is situated at the 5' end of the R region. The TATA box, located 5' of the cap site, is the recognition signal (promoter) for *pol II* RNA transcription (reviewed in Clements *et al*, 1990).

Fig. 1.1



1.4 Virus structure

1.4.1 Virion morphology

Virions of Maedi-visna virus (MVV) are enveloped particles 70-100nm in diameter (Thormar, 1961., Coward *et al*, 1970). Their surface is decorated with clusters of spikes which are believed to represent the envelope glycoprotein of visna virus (Dubois-Dalcq *et al*, 1976). The internal nucleocapsid, or core, has a distinctive bar-shape typical of lentiviruses (Gonda *et al*, 1985), and is 30-40nm in diameter (Thormar, 1961).

1.4.2 Viral Antigens

1.4.2.1 Virion peptides

About twenty polypeptides are incorporated into the visna virus particle (Haase & Baringer, 1974., Lin & Thormar, 1979). The major constituents of the virion are the envelope glycoprotein gp135, and the ***gag*** proteins p16, p25 and p14 (Haase & Baringer, 1974., Vigne *et al*, 1982), which form the nucleocapsid of visna virus.

The virion core also contains several proteins that have important roles during viral replication. These include the enzyme reverse transcriptase, p68 (Lin & Papini, 1979., Filippi *et al*, 1982). By analogy with other retroviruses (Varmus, 1982., Coffin, 1990), the core probably also contains a protease and integrase.

Other polypeptides present in the virion (Haase & Baringer, 1974., Lin & Thormar, 1979) are believed to be either the polypeptide precursors or intermediates of viral structural proteins, or cellular proteins incorporated in virus particles as they bud from the infected cell (Vigne *et al*, 1982).

1.4.2.1.1 ***Gag*** gene products

The three ***gag*** proteins of visna are produced by proteolytic cleavage of a major polyprotein precursor, pr55 (Vigne *et al*, 1982). The order of ***gag*** proteins within the

precursor is NH_2-p16-p25-p14-COOH (Sonigo *et al*, 1985). The ***gag*** polypeptides of lentiviruses form the capsid structure within the enveloped virion and are important in maintaining critical protein-nucleic acid contacts during viral assembly and reverse transcription (reviewed in Arnold & Arnold, 1991., Coffin, 1990). Assembly of virions is a relatively poorly understood aspect of retroviral replication (see section 1.5.8), but it is interesting to note that the ***gag*** precursor protein of HIV-1, p55, when expressed in eukaryotic cells is able to assemble into a virus-like particle and bud from the cell in a manner reminiscent of the morphogenesis of HIV (Gheysen *et al*, 1989., Hu *et al*, 1990).

In most retroviruses the matrix protein, p16 in visna, is the ***gag*** protein in closest association with the virus membrane (Coffin, 1990). Consistent with this observation, the NH_2 terminus of most retroviral matrix proteins are myristylated (Dickson *et al*, 1985., Mervis *et al*, 1988., Veronese *et al*, 1988), a modification characteristic of many proteins that lie on the internal face of the cell membrane. It has been suggested that some mammalian retroviruses may exploit this modification of the ***gag*** polyprotein as a targeting mechanism to localise viral budding (Rein *et al*, 1986., Rhee & Hunter, 1987). It is not known whether the visna p16 is myristylated, but myristylation of the matrix protein is not a feature of all retroviruses; the matrix proteins of neither Avian leukaemia-sarcoma virus (ALSV) nor Equine infectious anaemia virus (EIAV) are myristylated (Pepinsky & Vogt, 1984., Henderson *et al*, 1984).

The capsid protein, p25, is hydrophobic and forms the major internal structural feature of the virion - the core shell (Vigne *et al*, 1982., Sonigo *et al*, 1985). Little is known about capsid protein structure or mechanism of action in any retrovirus, but in Moloney murine leukaemia (MuLV) virus mutations in the capsid protein eliminate virus assembly altogether (Hsu *et al*, 1985).

The nucleocapsid protein, p14, of maedi-visna is a small basic protein containing the 'zinc-finger' motif, Cys-X_2-Cys-X_9-Cys, characteristic of retroviral nucleic acid binding proteins (Sonigo *et al*, 1985). Although the function of this protein in visna virus is not definitely known, this protein is found in association with the genomic RNA in the virion (Lin & Thormar,

1974., Filippi *et al*, 1982). It has been suggested that in Rous sarcoma virus the nucleocapsid protein may be involved in the process of retroviral virion RNA dimerisation (Bieth *et al*, 1990). For HIV-1, transfection of cells with viral genomes containing mutations in the cysteine motif of the nucleocapsid protein led to the production of virus particles normal in protein content, but lacking detectable viral RNA (Aldovini & Young, 1990), implying a role for the nucleocapsid protein in packaging of genomic RNA within virions.

The ***gag*** genes are highly conserved among lentiviruses of each host species. p25, the major core protein of visna, is the most highly conserved of any of the visna virus genes (Sargan *et al*, 1991). Moreover the ***gag*** proteins, especially p25, are immunogenic, eliciting a strong antibody response during visna infection (Houwers & Nauta, 1989). The antibodies are group specific, recognising a wide spectrum of visna isolates, but the significance of anti-***gag*** antibodies in the immune response to visna is unclear (see section 1.6.3). The ***gag*** proteins of lentiviruses are also major targets for T-cell responses (Miller *et al*, 1990., Nixon *et al*, 1988).

1.4.2.1.2 ***Pol*** gene products

The ***pol*** gene of visna encodes the RNA dependent DNA polymerase (reverse transcriptase) and putative endonuclease/integrase and protease of visna (Sonigo *et al*, 1985., Sargan *et al*, 1991). The ***pol*** gene products are believed to be produced as a ***gag/pol*** precursor, pr150, (Sonigo *et al*, 1985., Vigne *et al*, 1982) which the viral protease cleaves to liberate the ***pol*** gene products. By analogy with other retroviruses, it is thought that this precursor polyprotein is generated by ribosomal slippage in the ***gag-pol*** overlap leading to a frameshift during translation (Jacks *et al*, 1988., reviewed in Hatfield & Oroszlan, 1990). Of the three putative ***pol*** gene products only the reverse transcriptase of visna virus has been characterised (Lin *et al*, 1973., Lin & Papini, 1979). The virion DNA polymerase is associated with the viral core (Lin & Thormar, 1974) and, like all reverse transcriptases, the visna enzyme has been shown to have an absolute requirement for divalent cations for

polymerase activity *in vitro* (Haase *et al*, 1974., Coffin, 1990). In visna the preferred ion is Mg^{2+} at 1-4mM (Lin & Papini, 1979).

1.4.2.1.3 ***Env*** gene products

The ***env*** gene of MVV encodes a single large, 115kd, precursor polypeptide (Sonigo *et al*, 1985., Sargan *et al*, 1991) with approximately thirty potential glycosylation sites, many of which are utilised (Vigne *et al*, 1982). These carbohydrate moieties protect the virions from proteases and reduce the affinity of neutralising antibodies for the virus, in some cases sequestering neutralising epitopes from the immune system (Huso *et al*, 1988).

The ***env*** gene is translated to produce a single, glycosylated precursor polypeptide, gpr150, which is then thought to be proteolytically cleaved to produce the two major glycopeptide species associated with the virion, the outer membrane envelope glycoprotein gp135, and the transmembrane glycoprotein gp46 (Haase & Baringer, 1974., Vigne *et al*, 1982). It has been shown for HIV-1 that endoproteolytic cleavage of the ***env*** precursor gp160 is essential for virion infectivity and syncytium formation (McCune *et al*, 1988).

In lentiviruses the external envelope glycoprotein then associates with the transmembrane glycoprotein via noncovalent interactions (Kowalski *et al*, 1987). However the surface envelope glycoprotein spikes are easily lost from the virion surface (Gelderblom *et al*, 1985., Schneider *et al*, 1986). In HIV-1, loss of virion gp120 has been shown to result in reduced virus infectivity (McKeating *et al*, 1991), but the relevance of these *in vitro* observations to disease states *in vivo* in HIV infection is unclear as is whether a similar situation obtains during infection with maedi-visna.

The nature of the higher order structures of lentivirus envelope glycoproteins is unclear. The structure of MVV glycoproteins is not known: the envelope glycoproteins of HIV have been variously reported to exist in the virion as dimers, (Earl *et al*, 1990., Thomas *et al*, 1991), trimers (Einfield & Hunter, 1988., Weiss *et al*, 1990) and tetramers (Pinter *et al*, 1989., Schawaller *et al*, 1989) stabilised by interactions between their gp41 domains.

The envelope glycoproteins contain many biologically important determinants: the determinants of virus-cell receptor interaction and virus induced cell fusion (Crane *et al*, 1988., Lifson *et al*, 1986*a*., 1986*b*), and the major epitopes for virus neutralising antibody (Scott *et al*, 1979).

1.4.2.2 Regulatory proteins

The genome of visna virus includes several short ORF's, at least two of which have been shown to encode positive acting regulatory proteins(Davis & Clements, 1989., Gourdou *et al*, 1989., Tiley *et al*, 1990) (see section1.5.6.2). The protein products of these two genes have been named ***Tat*** and ***Rev***, after their functional equivalents in HIV-1, and both of these proteins have been reported to be recognised by sera from visna infected sheep (Davis & Clements, 1989), suggesting that these proteins are expressed *in vivo*.

The ***tat*** gene (ORF-S) of visna, present as a single coding exon, encodes a 10-11kd protein. This protein includes a basic domain and a cysteine cluster , both of which features are important for protein function (Davis & Clements, 1989., Gourdou *et al*, 1989). The visna ***tat*** protein has been shown to act as a post-transcriptional transactivator of visna virus LTR-dependent gene expression.

The other short ORF of visna virus, whose protein product has an identified function is the ***rev*** gene. This protein, which has an M_r of around 18kd, contains a basic domain and a leucine rich motif, both features which have been identified as being critical for ***rev*** function *in vitro* (Malim *et al*, 1989., Hope *et al*, 1990., Tiley *et al*, 1990). During *in vitro* infection ***rev*** protein is expressed both early, when it is a major viral product, and late in the course of visna replication (Mazarin *et al*, 1990). The ***rev*** protein of maedi-visna virus localises to the nuclei and particularly to the nucleoli of transfected cells (Tiley *et al*, 1990), like the ***rev*** proteins of primate lentiviruses (Felber *et al*, 1989., Malim *et al*, 1989). Visna ***rev*** protein has been shown to act as a post-transcriptional activator of viral structural gene expression (Tiley *et al*, 1990).

The gene product of the third ORF, ***Q***, is a hydrophilic and basic protein of 29kDa. The function of the protein is unknown, but it is expressed late in the virus life cycle and is located in the cytosol, although it does not accumulate at the cell membrane and is not found in virions (Audoly *et al*, 1991).

1.5 Infection and virus replication

1.5.1 Infectious inocula

Infectious inocula of visna virus are likely to comprise a population of related viral quasispecies. This situation is a reflection of the remarkable plasticity of the retroviral genome and its capacity for rapid evolution, a feature of retroviral/lentiviral biology thought to be of great importance in the pathogenesis of lentivirus-induced disease.

Replication of retroviral genomes proceeds by a series of enzymatic reactions initiated by the virus-encoded reverse transcriptase (RT), but also involving host cell-encoded enzymes such as DNA polymerases which are involved in DNA replication, and RNA polymerase II, which transcribes proviral DNA into both genomic RNA and viral mRNA's (Coffin, 1990., Varmus & Swanstrom, 1985) and a variety of mutational mechanisms act, during replication, to maintain the heterogeneity of retroviral populations.

Due to the lack of an exonucleolytic (proofreading) activity (Battula & Loeb, 1976), reverse transcriptase is prone to error. The error rate of the visna RT enzyme has not been characterised, but *in vitro* other retroviral RT's have been shown to have rates of base misincorporation ranging from 10^{-4}/base for the reverse transcriptases of avian retroviruses (Dougherty & Temin, 1988., Leider *et al*, 1988) to four to five-fold that rate for the RT of HIV-1 (Preston *et al*, 1988., Roberts *et al*, 1988). It is important to note that the rate of misincorporation is not likely to be uniform across the genome, but seems to be dependent on the specific sequence context (Roberts *et al*, 1988., 1989), some types of error being more common than others. Deletions, insertions, duplications and recombinational events

with viral and/or cellular sequences may also occur during reverse transcription (Dorner *et al*, 1985., O'Rear & Temin, 1982), although it should be noted that nucleic acid sequences related to visna virus are absent from uninfected sheep cells (Haase & Varmus, 1973., Weiss *et al*, 1975., Brahic *et al*, 1977).

Host cell DNA polymerases show an extremely low rate of misincorporation and are unlikely to contribute to retroviral mutation. However, if the error rate of RNA polymerase II approximates to that of other RNA-synthesising enzymes then errors in genomic RNA synthesis may also be important in generating sequence diversity within the viral population (reviewed in Coffin, 1990).

The above describes the mechanisms by which the heterogeneity of retroviral populations is generated and maintained, but it should be remembered that mutations in the viral genome gain significance only insofar as they confer a selective advantage to the variant virus, so favouring continued replication.

Evidence for visna virus variation *in vivo* is drawn mainly from studies documenting the co-existence of antigenic variants within individual sheep. This has been defined by biological assays such as neutralisation phenotype (Narayan *et al*, 1978., Lutley *et al*, 1983., Thormar *et al*, 1983), and epitope-mapping experiments (Stanley *et al*, 1987). Genetic change in the visna genome *in vivo* has also been demonstrated. Clements *et al*, (1980., 1982), using RNase T_1 fingerprint analysis of viral RNA's of serial visna isolates, demonstrated that visna variants emerge primarily through the accumulation of point mutations, mainly within two kilobases of the 3' terminus of the genome, ie. in the envelope glycoproteins. The limited amount of sequence information available on visna isolates confirms that regions of ***env***, especially those overlapping ***rev*** sequence, are the most variable between visna isolates (Querat *et al*, 1990., Sargan *et al*, 1991). It has been suggested that the extensive variation in ***env*** may be a result of ***env*** variant viruses being fixed in the population by immune selection (Sargan *et al*, 1991., see section 1.6.5).

The population of lentiviral species that exist within an individual may differ in biological properties other than antigenicity. In HIV-1 infection, variant genomes from a single patient

may show differential tropism for T-cell and myelomonocytic cell lines (Cheng-Mayer *et al*, 1990., Fisher *et al*, 1988., Sakai *et al*, 1989). In addition to host cell range, HIV isolates have been shown to be distinct with respect to other biological parameters such as replication rate & syncytium-inducing capacity (Tersmette *et al*, 1988., 1989*a*). The prevalence of particular types of HIV variants is different in early and late stages of infection and this may be related to disease progression (reviewed in Miedema *et al*, 1990).

A similarly detailed analysis of the biological phenotypes of visna isolates has not been reported. However, in preliminary studies, Querat *et al* (1984) classified ovine lentivirus isolates into two groups, on the basis of their replication in fibroblast cultures *in vitro*: Group I isolates replicate rapidly and cause complete lysis of the infected cultures, whereas group II isolates fail to induce complete lysis of the cells, but instead establish a persistent infection of the cell line. Querat *et al* could isolate only one type of virus, either lytic or persistent, from naturally infected sheep, however it is possible that highly lytic isolates may have been isolated preferentially in their culture system since virus infectivity was assessed by the development of cytopathic effects in a cell line co-cultivated with the material from which virus isolation was being attempted. Analysis of the protein and nucleic acid content of each of these types of isolate demonstrated that lytic and persistent isolates, although related, were genetically distinct (Querat *et al*, 1984).

Lairmore *et al* (1987) compared the replication phenotypes of ovine lentivirus isolates *in vitro* in synovial membrane cells and alveolar macrophages. These workers demonstrated that isolates which are highly lytic in cell culture *in vitro* (group I isolates) could be subdivided into two groups: isolates which are lytic in synovial cells, but grow only poorly in macrophages, and isolates which cause rapid fusion and lysis of both synovial cells and macrophages. Further it was demonstrated that this *in vitro* replication phenotype correlated with *in vivo* pathogenicity; isolates causing highly lytic infections of macrophages *in vitro* being the most pathogenic *in vivo* (Lairmore *et al*, 1987., 1988). Thus disease pathogenesis may be markedly influenced by biologic differences between visna isolates, which may co-exist within an infected individual.

1.5.2 Transmission of virus

Horizontal transmission is the primary means of viral dissemination between sheep, and virus infected colostrum and milk are probably the major vectors for transmission, although infection via the respiratory route by inhalation of infected droplets has also been implicated (De Boer *et al*, 1979., Gudnadottir, 1974., Narayan & Cork, 1985). Visna infection is probably transmitted by virus inside monocyte/macrophages in these secretions (Haase, 1986). This is likely since *in vivo* visna infection is characterised by minimal levels of cell-free infectious virus. Viraemia is almost exclusively cell-associated (Petursson *et al*, 1976), and monocyte/macrophages are the major cell types infected *in vivo* (Narayan *et al*, 1982., Gendelman *et al*, 1985).

Most of the published evidence suggests that germline and intrauterine transmission of visna do not occur (De Boer *et al*, 1979., Gudnadottir, 1974., Haase, 1986., Houwers *et al*, 1983), although some reports have suggested that there may be a small chance of intrauterine infection occurring (Cross *et al*, 1975., Cutlip *et al*, 1981).

1.5.3 Cellular tropism of visna

Both *in vitro* and *in vivo* the ruminant lentiviruses have a marked tropism for cells of the monocyte-macrophage lineage; *in vitro* visna replicates productively in macrophages cultivated from either the peripheral blood or the lungs of uninfected sheep (Narayan *et al*, 1982., Gendelman *et al*, 1986). *In vivo* the virus maintains this tropism for cells of the monocyte/macrophage lineage, although *in vivo* viral replication is restricted (Geballe *et al*, 1985., Gendelman *et al*, 1985).

The basis of the restricted cellular tropism exhibited by visna virus is not understood. Retroviruses have a specific requirement for interaction with a cell-surface receptor molecule for infection (reviewed in Weiss, 1985., Coffin, 1990) and this interaction is likely to be of crucial importance in determining the cellular tropism of the virus. However this only refers to infection of a cell by a free virus particle. In visna, as in other lentiviruses, infection may also

occur by direct fusion of an infected cell with uninfected cell(s) (Harter & Choppin, 1967., Thormar, 1963),thus bypassing any need for a specific receptor.

To date, a cell-surface molecule which acts as a specific receptor for visna virus has not been identified, thus the contribution of a putative receptor moiety to determining tropism cannot be defined. However, it has recently been shown that visna virus binds specifically to cell membrane proteins of 15, 30 and 50 kDA, and that antibodies to the 50kDA protein block the binding of visna virus to permissive cells (Crane *et al*, 1991*a*). It has been claimed that molecules of the ovine MHC class II complex may be components of a cellular receptor for maedi-visna virus (Dalziel *et al*, 1991), however monoclonal antibodies to sheep MHC class II molecules do not infection of permissive cells by visna virus. Moreover, cell susceptibility to visna infection does not correlate with expression of MHC class II molecules.

It has been reported that cell lines from a variety of species possess receptors which allow the attachment of visna/vesicular stomatitis virus pseudotypes, but not productive infection by visna virus itself (Gilden *et al*, 1981), suggesting that events after receptor-binding are important in the infective process. Moreover, it has also been suggested that group I and group II visna isolates (see section 1.4.1) form distinct interference groups which may differ in receptor specificity (Jolly & Narayan, 1989).

Apart from the CD4-gp120 interaction (reviewed in Capon & Ward, 1991), very little is known about how retroviruses interact with their receptors, and it is difficult to generalise from what is known. However, it is generally assumed that the retroviral protein involved in binding to the cellular receptor is the virion surface envelope glycoprotein (Weiss 1985., Coffin, 1990) and therefore that important determinants of cellular tropism reside in this protein.

It has also been reported that antibodies to visna enhance virion binding to macrophages *in vitro*; this process being mediated by antibody-Fc receptor interactions (Jolly *et al*, 1989) and this is another potential route of viral infection.

The cellular tropism of retroviruses may also be affected by later, biochemical, aspects of viral replication machinery. For example, it has been shown that the cellular tropism of some murine retroviruses is affected by specific alterations in their long terminal repeats (LTR's)

(Celander & Haseltine, 1984., Holland *et al*, 1985., Rosen *et al*, 1985). In contrast the cellular tropism of HIV-1 is believed to be determined by events early in viral replication, prior to provirus formation (Cann *et al*, 1990., Kim *et al*, 1990) and that the viral LTR is not a major determinant of cellular tropism (Pomerantz *et al*, 1991).

The role of the visna virus LTR in determining cellular tropism has been investigated in a transgenic mouse model (Small *et al*, 1989). In these experiments transgenic mice were constructed which expressed the bacterial gene chloramphenicol acetyl transferase (CAT) under the control of the visna LTR. Expression of CAT activity in these mice was mainly restricted to macrophages, or tissues expected to contain macrophage populations such as brain, lung and spleen. Activation of peritoneal macrophages resulted in enhanced induction of the visna LTR. Small *et al* interpreted these results as evidence of a central role for the visna LTR in determining macrophage tropism, possibly indicating that cellular factors required for visna virus LTR-dependent gene expression in this system are mainly restricted to macrophages. An interpretation supported by the finding that *in vivo* visna replication appears to be confined mainly to sheep monocytes/macrophages (Gendelman *et al*, 1986).

1.5.4 Entry of the virion into the cell

1.5.4.1 Entry

In vitro experiments indicate that visna attaches to cells quickly, within 5-15 minutes (August *et al*, 1977., Kennedy-Stoskopf & Narayan, 1986) and with high affinity: the rate of virus binding to cells is faster than the rate of virus neutralisation by immune serum (Kennedy-Stoskopf & Narayan, 1986).

Following binding of the virus to its receptor, entry of the virion into the cell occurs by fusion of the virion envelope and the cell membrane as a result of which process the virion core is released into the cytoplasm (reviewed in Marsh & Helenius, 1989). Membrane fusion by lentiviruses occurs at a pH typical of the cell surface (Stein *et al*, 1987., McClure *et al*,

1988) and neither viral endocytosis nor intracellular compartmentalisation of intact virions appear to be a feature of the infective process (Maddon *et al*, 1988., Stein *et al*, 1987).

The mechanisms by which fusion and viral entry occur are poorly understood. The visna transmembrane envelope glycoprotein, gp46, contains a conserved sequence of hydrophobic amino-acids at its NH_2 terminus (Sargan *et al*, 1991), which shows some homology to the fusion domains of other fusogenic viruses (Gallaher, 1987., Freed *et al*, 1990., White *et al*, 1983) and it may be that membrane fusion is mediated by this region of the visna transmembrane protein. Consistent with this, it has been reported that in the primate lentiviruses gp41 is likely to be the viral protein which mediates fusion (Bosch *et al*, 1989., Kowalski *et al*, 1987). Antisera against visna gp135 have been reported to block visna-induced cell fusion (Crane *et al*, 1988), but this report did not distinguish between a direct effect of gp135 in fusion and steric hindrance of the gp46/cell membrane interaction by antibodies bound to gp135. Subsequent studies by these workers (Crane *et al*, 1991*b*) demonstrated both, that a peptide derived from the hydrophobic domain of gp46 could induce cell-cell fusion and that antisera to this peptide could inhibit visna virus induced cell fusion.

It has also been reported that penetration, at least in HIV-1, is also dependent on an as yet unidentified cellular component (Maddon *et al*, 1986., Tersmette *et al*, 1989*b*), which may be a cell-surface protease capable of cleaving specific sites within the envelope glycoprotein leading to activation/exposure of the fusion domain (Hattori *et al*, 1989., Stephens *et al*, 1990).

1.5.4.2 Interference

In general, cells infected by retroviruses display a very strong resistance to superinfection by viruses that utilise the same receptor as the preinfecting virus, while remaining susceptible to infection by viruses that use a different receptor. This phenomenon, called interference, is believed to arise by interaction between the ***env*** protein of the preinfecting virus and its receptor. The level at which this interaction occurs is not known, but virion formation is not

necessary for interference. *In vitro* interference is virtually absolute (reviewed in Weiss, 1985).

A related phenomenon, known as 'early interference' occurs without viral infection of the cell and is believed to be due to interaction between the viral ***env*** protein and the cellular receptor at the cell surface, thus blocking access of other viruses to the receptor (Weiss, 1985). Early interference has been demonstrated to occur in visna infection *in vitro* (Jolly & Narayan, 1989), preincubation of cells with group I isolates causing reduced binding of other group I isolates, but not group II isolates and *vice versa*.

1.5.5 Reverse Transcription & Proviral DNA

After entry of the virion into the cytoplasm the retroviral RNA genome is reverse transcribed into double-stranded proviral DNA (reviewed in Varmus & Swanstrom, 1985., Coffin, 1990). This process is carried out in a structure derived from the virion core, using enzymatic activities that enter the cell in the virion, although it has been shown that cellular factors may be required for complete reverse transcription: infection of resting, quiescent $CD4^+$ cells by HIV-1 results in incomplete reverse transcription yielding a labile replicative intermediate and abortive infection (Zack *et al*, 1990). If the resting cell harbouring this structure is mitogenically stimulated then virus production can result, suggesting that cellular activation may allow completion of reverse transcription. Similarly, in a murine retrovirus system, defects affecting cellular DNA synthesis appear to alter reverse transcription (Richter *et al*, 1984). That said, visna replication during *in vitro* infection has been shown to be independent of cellular DNA synthesis and mitosis (Haase, 1975., Trowbridge *et al*, 1980).

Visna DNA replication occurs predominantly in the nucleus of the infected cell (Haase *et al*, 1982), rather than in the cytoplasm as occurs in oncovirus infections (Varmus & Swanstrom, 1985). *In vitro* visna proviral DNA is detectable within the first hour post infection (Clements *et al*, 1979., Haase *et al*, 1982), and the time course of virus production can be altered by manipulation of the extent of early proviral DNA synthesis (Haase *et al*, 1982).

In tissue culture visna virus DNA is predominantly present as extrachromosomal DNA molecules that are linear duplexes with a gap close to the centre of the plus strand (Harris *et al*, 1981). A minor population of viral DNA is composed of two circular forms differing by the length of one long terminal repeat (Clements & Narayan, 1981., Harris *et al*, 1981). In contrast to the oncoviruses whose expression is dependent on integrated provirus (Varmus, 1982), integration of visna proviral DNA appears to play a relatively insignificant role in viral gene expression (Harris *et al*, 1984), suggesting that, at least *in vitro*, unintegrated visna virus DNA can serve as a template for transcription. The significance of the apparently insignificant role of integration in the life cycle of visna is difficult to assess. Several studies attempting to correlate lentivirus DNA forms and growth *in vitro* have been described (Cheevers *et al*, 1982., Harris *et al*, 1984., Muesing *et al*, 1985). In general these studies suggest that persistent, non-cytopathic infections are associated with a predominance of integrated viral DNA, whereas cytopathic infections display a predominance of unintegrated proviral DNA.

Analyses of the state and structure of the DNA provirus *in vivo* have not been reported for visna, but this topic has been investigated in other lentiviral diseases. In SIV infected macaques the vast majority (85%-100%) of proviral DNA, in a variety of tissues, was found to be unintegrated (Hirsch *et al*, 1991). Similarly, analysis of lymph node tissue from an AIDS patient revealed that high levels of unintegrated proviral DNA were present (Shaw *et al*, 1984). To date, information on the significance of the relatively large amounts of linear, non-integrated provirus found in *in vivo*-derived material in the pathogenesis of lentiviral disease has not been reported.

1.5.6 Viral gene expression

Lentiviruses, like other retroviruses, utilise cellular transcription machinery for the efficient expression of the DNA provirus. Transcription of viral DNA into genomic RNA and mRNA is accomplished by cellular RNA polymerase II (reviewed in Coffin, 1990., Varmus &

Swanstrom, 1985). The U3 region of the viral DNA genome contains sequences homologous with regions of cellular genes known, or suspected, to be important for transcription (Chen & Barker, 1984) and transcription begins downstream of these elements. It is thought that *pol II* binds to the viral DNA at or near the TATA box. Transcription is then initiated at the U3-R junction (or cap site) and proceeds through the viral genome to the poly (A) sequence in the R region of the 3' LTR (Narayan & Clements, 1990). Efficient recognition of the 3' poly (A) site, rather than the 5' poly (A) tract, may be due to the close proximity of the latter to the cap site (Sanfacon & Hohn, 1990), alternatively, efficient recognition of the 3' poly (A) site may be mediated by transcribed sequences located 5' of the downstream, but not the upstream poly (A) signal (Brown *et al*, 1991).

The viral LTR thus acts as a promoter for transcription of proviral DNA and gene expression of visna virus is regulated through transcriptional control elements in the viral LTR (reviewed in Clements *et al*, 1990).

The basal activity of the visna virus LTR in the resting cell is poorly defined. It is known that viral gene expression is highly restricted in monocytes *in vivo*, and that differentiation and/or activation of these cells results in activation of viral gene expression (Gendelman *et al*, 1985., 1986), indicating that cellular transcriptional factors that activate macrophage specific genes may be necessary for activation of viral transcriptional control elements. In HIV, external signals which may induce cellular activation and viral replication include mitogens and cytokines such as TNFα and IL-6 (Rosenberg & Fauci, 1989). Many of these signals exert their effect via transcriptional activators such as NF-κB (reviewed in Steffy & Wong-Staal, 1991) and it is likely that a similar situation exists in visna infection.

A number of sequences within the visna virus LTR have been identified as being important both for basal transcriptional activity and for activation of the viral LTR (Hess *et al*, 1989., Gabudza *et al*, 1989).These include the TATA box and recognition sequences for the transcriptional factors AP-1 and AP-4. The AP-4 site is present as a single copy, whereas multiple degenerate copies of the AP-1 element are found in the visna LTR, although the AP-1 site most proximal to the TATA box is the most important for transcriptional activity.

After activation, lentiviral gene expression, at least *in vitro*, is characterised by a more complex pattern of transcription than other retroviruses: visna virus infected cells contain at least six species of mRNA (Davis *et al*, 1987., Vigne *et al*, 1987., Sargan & Bennet, 1989).

Early virus gene expression is marked by the accumulation of doubly spliced mRNA's which encode viral regulatory gene products including ***tat*** and ***rev*** (Davis & Clements, 1989., Gourdou *et al*, 1989). The visna ***tat*** gene product increases gene expression from the viral promoter by increasing the steady-state level of viral RNA (Hess *et al*, 1989). Whether ***tat*** acts at a transcriptional or post-transcriptional level is not fully understood, but unlike HIV-1 ***tat*** the primary targets for the visna transactivating protein are believed to lie upstream of the TATA box in the U3 region (Hess *et al*, 1989). These ***tat*** responsive sequences in the U3 are the same sequences as are required for basal transcriptional activity.

The other regulatory gene product of visna virus, ***rev***, localises to the nucleoli of infected cells (Tiley *et al*, 1990., Audoly *et al*, 1991) and is required for the expression, late in the virus life cycle, of the unspliced and singly spliced mRNA's encoding ***gag***, ***pol***, ***env*** & ***Q*** ORF gene products. The mechanism of action of ***rev*** protein is uncertain but appears to involve activating nuclear export of incompletely spliced viral mRNA species which would otherwise be sequestered within the nucleus of infected cells. This effect is dependent on *cis* -acting ***rev*** -response elements (RRE) located within the ***env*** mRNA (Tiley *et al*, 1990). It has been proposed, in HIV, that ***rev*** acts to regulate viral RNA expression by selectively packaging viral transcripts, carrying the RRE, into rod-like nucleoprotein complexes blocking the access of cellular splicing machinery to the viral mRNA (Heaphy *et al*, 1991).

Thus early in infection doubly spliced 1.4kb and 1.7kb mRNA transcripts are the predominant species of viral RNA present in infected cells. Later however, around 72 hours post-infection, larger unspliced and singly-spliced mRNA transcripts which encode the viral structural proteins are predominant (Davis *et al*, 1987., Vigne *et al*, 1987).

The pattern of transcription described above is that observed during lytic replication *in vitro*. However *in vivo*, viral gene expression is highly restricted. Relatively few cells are infected: 1 in 10^6/1 in 10^7 leukocytes in peripheral blood, (Petursson *et al*, 1976.,

Gendelman *et al*, 1985., 1986). Moreover, expression of proviral DNA is blocked at the level of transcription (Haase *et al*, 1977., Brahic *et al*, 1981). The viral RNA content of infected cells *in vivo* is about two orders of magnitude less than during lytic replication *in vitro* and only a very few of the infected cells contain viral structural proteins (Haase *et al*, 1977., Brahic *et al*, 1981., Gendelman *et al*, 1985., 1986).

The basis of this restricted replication *in vivo* is not well understood, but a variety of factors could influence viral replication.

It is not known whether true latency, ie. persistence of viral DNA in the absence of transcription, occurs during lentiviral infection *in vivo*. The peripheral blood of HIV-infected individuals contains 1 in 10^4 to 1 in 10^5 cells expressing viral mRNA (Harper *et al*, 1986), whereas the frequency of cells containing proviral DNA, as assayed by PCR and limiting dilution, is around 100 times greater (Schnittman *et al*, 1989). This has been interpreted as suggesting that the majority of infected cells are transcriptionally inactive (McCune, 1991), an interpretation supported by experiments quantitating infectious virus in PBMC's and plasma (Ho *et al*, 1989). However, quantitative *in-situ* hybridisation studies on the choroid plexus of visna infected sheep have revealed that the proportions of cells expressing viral DNA or RNA *in vivo* are similar and that this number agrees well with the total number of infected cells in the tissue as determined in endpoint dilution assays (Haase *et al*, 1977., Brahic *et al*, 1981),

If latency does not occur during visna infection *in vivo* it could be hypothesised that the low (basal ?) level of transcription detected *in vivo* may produce regulatory mRNA's that act to suppress viral replication. To date, however, the nature of the RNA transcripts detected in *in vivo* remains completely uncharacterised for both visna and HIV. A candidate negative regulatory protein, in HIV, is that encoded by the ***nef*** (negative factor) gene. Some studies have suggested that ***nef*** acts to inhibit LTR-dependent gene expression, however others have observed no effect of ***nef*** on either viral replication or gene expression (reviewed in Cullen & Greene, 1990). The existence of neither a discrete ***nef*** ORF nor a ***nef***-like protein have been reported in visna or any of the other non-primate lentiviruses.

Another potential constraint on visna replication is the seeming requirement for macrophage activation for efficient viral replication (Narayan *et al*, 1983., Gendelman *et al*, 1986). *In vivo* cellular activation is likely to be transient, thus viral replication will not be favoured. Moreover, at least for HIV-1, it has been reported that a protein, rpt-1, selectively expressed in resting cells can down-regulate viral gene expression (Patarca *et al*, 1988).

1.5.7 Translation

Translation of retroviral mRNA's is believed to follow the standard 'scanning' model, in which ribosomal subunits initially bind to the 5' capping group and then move down the RNA until an AUG initiation codon is recognised (Petersen & Hackett, 1985).

In all retroviruses analysed to date the ***gag*** and ***pol*** genes can be thought of as a single translational unit which is expressed to yield either ***gag*** or ***gag/pol*** polyproteins, in a ratio of around 20:1, ***gag:gag/pol*** (reviewed Coffin, 1990). In lentiviruses it is thought that the ***gag/pol*** precursor is generated by ribosomal slippage in the overlap between the ***gag*** and ***pol*** ORF's (Jacks *et al*, 1988., Hatfield & Oroszlan, 1990). A potential ribosomal slippage sequence (AGGGAAA) has been identified in this location in the visna genome (Querat *et al*, 1990., Sargan *et al*, 1991).

Unlike the translation of ***gag*** and ***pol***, which are synthesised from full length RNA species by free polyribosomes in the cytosol, ***env*** protein is synthesised, from a singly-spliced subgenomic mRNA transcript, by polyribosomes in the rough endoplasmic reticulum. ***Env*** proteins are first glycosylated shortly after translation and later, after removal of a signal peptide, ***env*** proteins undergo further post-translational modifications, including further glycosylation and cleavage into outer and trans-membrane subunits, in the Golgi as they move to the cell surface (reviewed in Dickson *et al*, 1982., 1985).

Analysis of the translation of ***tat*** and ***rev*** proteins has not been reported.

1.5.8 Virion assembly & egress

Virion assembly is a poorly understood aspect of retroviral replication, but electron microscopic studies have revealed that in most retrovirus groups capsid assembly and budding of virions occur almost simultaneously.

A generally accepted model of retrovirus virion assembly (Bolognesi *et al*, 1978), hypothesises that virus assembly is modulated by interactions between the plasma membrane, virion RNA and the three domains of the ***gag*** precursor protein: the C-terminal (nucleocapsid) protein interacts with the genomic RNA to cause RNA dimerisation and ensure its incorporation into the assembling virion (Aldovini & Young, 1990., Bieth *et al*, 1990). Interactions between the central capsid domains determine the shape of the virion, while the myristylated NH_2-terminal matrix domain targets this complex to the cell membrane (Rein *et al*, 1986., Rhee & Hunter, 1987) and may also interact with ***env*** proteins to ensure envelopment of the virus (Gebhardt *et al*, 1984., Satake & Luftig, 1983).

Extensive mutational studies (reviewed in Coffin, 1990), have shown that only the matrix and capsid ***gag*** proteins are absolutely required for formation of a retroviral virus-like particle.

Assembling visna virions are first seen as a crescent-shaped patch on the cell membrane which gradually bulges from the membrane until the the ends of the crescent meet to form a complete envelope and the virus buds from the cell (Thormar, 1961., Dubois-Dalcq *et al*, 1976). This protrusion of the cell membrane, characteristic of the budding process, appears to be initiated by attachment of a capsid to a region of the cell membrane slightly modified in the appearance by the formation of clusters of globular units, suggested to be viral ***env*** proteins (Dubois-Dalcq *et al*, 1976). Interestingly, visna virus differs in its pattern of budding between infected macrophages and fibroblasts. During infection of monocyte/macrophages virus particles accumulate intracellularly in intra-cytoplasmic vacuoles (Narayan *et al*, 1982), whereas infection of fibroblastic cells is marked by extensive virus budding from the plasma membrane (Thormar, 1961., Dubois-Dalcq *et al*, 1976). The basis of this different behaviour is unclear, but a similar situation pertains in HIV, infected macrophages accumulate virus

particles intracellularly, while infected T cells release large numbers of virions into the extracellular environment (reviewed in Meltzer *et al*, 1990). The HIV particles which accumulate inside macrophages are infectious when mechanically released from the cell, but do appear to differ from 'T-cell grown' HIV in being relatively lacking in gp120, the outer membrane envelope glycoprotein. Whether this is true for 'macrophage-grown' visna virus is at present unknown.

Following assembly and budding the retroviral protease, incorporated into the virion as a ***gag-pol*** fusion protein is somehow activated, possibly the protease domain of the ***gag/pol*** precursor is only slightly active and requires a high local concentration of precursor such as occurs in the virion before it can cleave the ***gag/pol*** precursor to liberate the protease moiety. Alternatively protease activity may be dependent on dimer formation and this may not occur until encapsidation is complete. Whatever the mechanism, once cleavage occurs the free protease may exert its full activity to cleave the ***gag*** precursor into the individual ***gag*** proteins (Coffin, 1990) and this structural rearrangement of the capsid results in a visible condensation of the virion core shortly after release of the virus from the infected cell (Thormar, 1961). HIV mutants lacking protease activity synthesise non-infectious virions which have an 'immature' appearance, but are still capable of budding from the infected cell (Peng *et al*, 1989).

1.5.9 Effect of infection on the host cell

Typical retrovirus infections *in vitro* produce relatively low copy numbers of viral mRNA's and use only 1-2% of host cell protein synthesis (reviewed in Varmus & Swanstrom, 1982., Dickson *et al*, 1982), although high levels of HIV-1 RNA (300,000 to 2,500,000 copies) and correspondingly high levels of protein synthesis have been reported in cytocidal infections *in vitro* (Somasundaran & Robinson, 1988). Choroid plexus cells infected *in vitro* with visna virus contain, on average, only 5,000 copies of viral RNA per infected cell (Haase *et al*, 1982).

In both monocyte/macrophages and fibroblasts *in vitro* infection with visna virus leads to the development of a cytopathic effect characterised by the appearance of stellate cells, extensive syncytium formation and degeneration of infected cells (Sigurdsson, 1960., Narayan *et al*, 1982), although the rate of development of these changes is slower in macrophage cultures. In HIV, cell fusion and syncytium formation are mediated by the viral envelope glycoproteins (Lifson *et al*, 1986*a*., 1986*b*), although molecules important in cellular adhesion may also play a role in this process (Pantaleo *et al*, 1991). It is known that visna virus proteins, including gp135 & gp46, are involved in cell fusion (Crane *et al*, 1988., 1991*b*., Harter & Choppin, 1967) but the role of host cell proteins in visna virus-induced fusion is unknown.

1.6 Virus - Host Relationship

1.6.1 Introduction

It is thought that the major routes for transmission of maedi-visna infection are via colostrum and respiratory secretions (Narayan & Cork, 1985., Narayan & Clements, 1990), and since viraemia is almost exclusively cell-associated (Petursson *et al*, 1976), it is believed that infected cells, in these fluids, are the major vehicle for dissemination of visna infection. As the major cell type infected *in vivo* is the monocyte/macrophage (Gendelman *et al*, 1985., 1986), it is likely that the cell spreading visna virus infection is a macrophage.

In vivo the majority of infected cells are expressing viral RNA, but not viral structural proteins (Haase *et al*, 1977., Brahic *et al*, 1981., Gendelman *et al*, 1985). It is unknown whether these cells contain viral regulatory proteins such as ***rev*** and ***tat***. Thus the dissemination of visna both between and within individuals while sequestered within an infected macrophage, not expressing viral antigens, may effectively be invisible to the immune system. This has been termed the 'Trojan-Horse' hypothesis for spread of lentiviral infection (Peluso *et al*, 1985., Haase, 1986).

From the preceeding it can be seen that there are two major stages in the virus life cycle at which lentiviruses and the host defence mechanisms may interact: the virus infected cell which is induced/activated to synthesise viral antigens, and cell-free virus released perhaps by lysis of infected cells. The former is primarily a target for cell-mediated immune responses, whereas the latter is mainly a target for humoral immune mechanisms, although this division is not absolute.

1.6.2 Natural resistance mechanisms

Natural resistance mechanisms of the host require no previous exposure to foreign antigen for induction and so are immediately available against infection. Effector mechanisms of this type include macrophages, natural killer (NK) cells and interferon. These are all relatively non-specific in their action.

To date no information on antiviral effects mediated by monocyte/macrophages in visna infected sheep has been reported. In HIV infection purified monocytes were unable to lyse HIV infected target cells, even after activation with gamma interferon (IFN-γ) (Ruscetti *et al*, 1986). Moreover monokines such as IL-6 and TNF-α have been implicated both in upregulating HIV replication and in the pathogenesis of disease symptoms during HIV infection (Rosenberg & Fauci, 1990., Poli *et al*, 1990). In contrast Wong *et al* (1988) reported that the combination of IFN-γ and TNF-α could not only protect cells from HIV infection, but also kill HIV infected cells.

Monocytes and macrophages are the major cell types infected with visna *in vivo* (Gendelman *et al*, 1985., 1986), but the effect of lentiviral infection on monocyte/macrophage function is uncertain. The effect of visna virus infection on accessory cell antigen-presenting function has not been studied directly, but alveolar macrophages from visna infected sheep have been reported to be activated *in vivo*, as assessed by spontaneous release of chemotactic factors and fibronectin (Cordier *et al*, 1990). In contrast, others have reported that alveolar macrophages infected with visna *in vitro*, show reduced responses to

chemotactic factors (Myer *et al*, 1988). It has been reported that HIV infection of mononuclear phagocytes results in a reduced ability to mediate extra- and intra-cellular killing (Baldwin *et al*, 1990). Some workers have reported that antigen-presenting functions of monocytes infected with HIV *in vitro* is decreased (Petit *et al*, 1988., Macatonia *et al*, 1989., Ennen *et al*, 1990), whereas others have reported no effect (Mann *et al*, 1990). Defects were not observed in accessory cell function of PBMC's from HIV infected individuals (Clerici *et al*, 1990*b*). This is probably a reflection of the very low frequency of circulating infected monocyte/macrophages *in vivo* in HIV infected individuals (Massari *et al*, 1990., Schnittman *et al*, 1989), an observation similar to that reported for visna infected sheep (Petursson *et al*, 1976., Gendelman *et al*, 1986). The function of tissue monocyte/macrophages in lentivirus infection has received very little study, but it has been reported that SIV infection of bone-marrow macrophages in macaques correlated with disease progression *in vivo* (Kitagawa *et al*, 1991).

To date there are no reports investigating a role for NK cells in the immune response to visna infection. However several studies have examined the role of this cell type in the immune response to HIV infection. Non-MHC (Major histocompatability complex) restricted cytotoxicity against HIV infected target cells has been reported in HIV infected patients (Ruscetti *et al*, 1986), the highest levels occurring in asymptomatic seropositive individuals (Weinhold *et al*, 1988). It has been suggested that much of this activity may be mediated by circulating non-T, $CD16^+$ large granular lymphocytes (LGL's) armed *in vivo* with cytophilic antibody (Tyler *et al*, 1990). However NK cell mediated cytotoxicity against HIV infected targets has also been observed in unfractionated cells from both HIV-infected and uninfected individuals in the presence of IL-2 (Rook *et al*, 1985).

The major target antigen for non-MHC restricted cytotoxicity, in HIV infection, is thought to be gp120 since uninfected target cells coated with purified gp120 become susceptible to lysis by NK cells (Weinhold *et al*, 1988). Moreover, in both HIV and SIV systems, it has been shown that target cells infected with recombinant vaccinia-***env***, but not vaccinia-***gag***,

viruses are recognised and lysed by $CD16^+$, MHC class I unrestricted cells (Riviere *et al*, 1989., Yamamoto *et al*, 1990).

Much of the literature published on the role of interferons in the immune response to visna is contradictory, consequently interpretation of the data is difficult.

In vitro co-cultivation of peripheral blood mononuclear cells (PBMC's) with visna infected macrophages results in the production of a unique interferon (Narayan *et al*, 1985), termed lentivirus-induced interferon, LV-IFN (Zink *et al*, 1987). LV-IFN is a non-glycosylated protein which is heat and acid stable (Lairmore *et al*, 1988., Narayan *et al*, 1985), and is reported to have an M_r of either 54-64 kDa (Narayan *et al*, 1985), or 25-34 kDa (Lairmore *et al*, 1988). Induction of LV-IFN production appears to require the interaction of T-cells with infected macrophages and this process can be blocked by antibodies to MHC class II (Narayan *et al*, 1985., cited in Zink & Narayan, 1989). LV-IFN thus appears to have properties of both IFN-α and IFN-γ.

In vitro LV-IFN has been reported to inhibit the replication of visna virus in macrophages (Kennedy *et al*, 1985). Lentiviral replication in infected monocyte/macrophages is closely tied to cellular activation and differentiation (Narayan *et al*, 1983., Gendelman *et al*, 1986) and it has been proposed that LV-IFN indirectly restricts the replication of visna virus *in vitro* by inhibiting proliferation and differentiation of monocytes (Zink & Narayan, 1989). These workers also reported that LV-IFN had a direct inhibitory effect on virus gene expression in infected macrophages, blocking the viral life cycle at the level of transcription. In contrast, other workers reported that *in vivo* levels of spontaneous IFN production, by pulmonary leukocytes from visna infected sheep, are positively correlated with lentiviral infection of alveolar macrophages (Lairmore *et al*, 1988). Moreover Lairmore *et al* also reported that ovine lentivirus replication in fibroblastic cells was inhibited only slightly by IFN produced by cultured pulmonary lymph node leukocytes from visna/maedi infected sheep, either with or without lentivirus-infected macrophages in the cultures. Carroll *et al* (1978) also reported that the replication of visna virus in sheep choroid plexus cells was not affected by natural, *in vivo* prepared, ovine IFN.

LV-IFN has also been reported to induce the expression of MHC class II antigens on macrophages *in vitro* (Kennedy *et al*, 1985), a finding which may be reflected *in vivo*: a persistent and high level of MHC II antigen expression is seen in macrophage like cells in inflammatory lesions in the lung. Moreover alveolar macrophages isolated from visna infected sheep show increased levels of MHC II expression (Cordier *et al*, 1990), as do macrophage/dendritic cells isolated from the carpal joints of visna infected sheep with inflammatory synovitis (Harkiss *et al*, 1991). It has been suggested that both *in vitro* and *in vivo* LV-IFN may cause other changes in macrophages associated with macrophage activation eg. the induction of prostaglandin E_2 (PGE_2) (Zink & Narayan, 1989) and increased levels of spontaneous release of neutrophil chemotactic activity and fibronectin. However Lairmore *et al*, (1988), reported that there were no significant differences in the levels of either IL-2 and PGE_2 production or mitogen responsiveness between pulmonary lymph node leukocytes from visna-infected sheep and uninfected controls.

Thus local IFN production, associated with visna infection, may have either beneficial and/or detrimental effects on the infected host. Inhibition of monocyte/macrophage maturation, and possibly viral gene expression, may slow viral replication and postpone the onset of disease. Alternatively local IFN production in infected tissues, by induction of MHC class II antigen expression and possibly activation of macrophage populations, may serve to accelerate the entry of leukocytes into virus-induced lesions thus promoting inflammatory changes and cell-mediated tissue damage.

In summary, at the moment the role of interferons in visna infection *in vivo* is unclear. The discrepancies in results described above may be due to differences between groups in the preparation of the IFN containing material and in the cell types in which the influence of IFN on viral replication was assessed. HIV replication has been shown to be susceptible to IFN control, but again the mechanisms by which these effects are achieved are unclear (reviewed in Kornbluth *et al*, 1990).

1.6.3 Humoral immune responses

Sheep infected with maedi-visna virus develop a virus specific serum antibody response in the first few months post infection (Petursson *et al*, 1976). Visna virus-specific antibodies are first detected 1-2 months after infection using complement fixation and indirect immunofluorescence assays (Gudnadottir & Kristinsdottir, 1967., De Boer, 1970., Petursson *et al*, 1976). A longer period, 3-6 months, is required before the development of virus neutralising antibodies (Gudnadottir & Palsson, 1966., De Boer, 1970., Petursson *et al*, 1976), although these then remain at high levels throughout the course of the disease (Gudnadottir & Palsson, 1966).

Visna virus infected sheep have been shown to make antibodies reactive with virtually all the viral proteins (Lin & Thormar, 1979), but the pattern of development of these antibodies after infection and their persistence through the course of disease are unclear. Using agar gel precipitation techniques Dawson, (1985), and Klein *et al*, (1985), suggested that antibodies to gp135 were the first to appear after infection and that not all infected sheep made antibodies to the ***gag*** proteins. In contrast, other workers have reported that antibodies to p25 appear early in the course of infection and that they persist and predominate (Houwers & Schaake, 1987., Houwers & Nauta, 1989). In HIV infection, antibody responses are directed to both structural and non-structural viral proteins, but the major antibody responses are against the ***env*** and ***gag*** gene products (Pan *et al*, 1987).

It is believed, for both visna virus and HIV, that virus neutralising antibodies are reactive predominantly with the outer membrane envelope glycoprotein, gp135 (Scott *et al*, 1979., Matthews *et al*, 1986). Fine mapping of the epitopes in visna gp135 which elicit neutralising antibodies has not been carried out, but in HIV-1 infection the majority of virus neutralising antibodies are directed against a single epitope in the third variable domain of gp120 (the so-called V3 loop) (Matsushita *et al*, 1988., Palker *et al*, 1988., Rusche *et al*, 1988). However this region is not generally accepted to be a potent neutralisation epitope for either HIV-2 or SIV (Moore & Weiss, 1991).

Although the external envelope glycoproteins of lentiviruses are the major antigens which elicit neutralising antibodies other viral proteins such as the transmembrane envelope glycoprotein, gp41, and the matrix protein, p17, may also be targets for virus neutralising antibodies, at least in HIV infection (Schrier *et al*, 1988., Sarin *et al*, 1986., Papsidero *et al*, 1989).

The ***gag*** proteins of maedi-visna, especially p25, elicit a strong antibody response during infection. These antibodies are group specific, probably reflecting the highly conserved nature of the ***gag*** genes between visna isolates (Sargan *et al*, 1991), and are often used as an index for infection (Houwers & Nauta, 1987). In general the functional significance of anti-***gag*** antibodies in lentivirus disease pathogenesis is unclear, but decline or loss of antibody titre to the viral core proteins in HIV infection is strongly associated with progression to AIDS (Kalyanaraman *et al*, 1984., Weber *et al*, 1987). Although it has been suggested, in HIV, that a decline in anti-p24 antibody titres may simply reflect an increase in p24 antigenaemia with consequent immune complex formation and clearance (Lange & Goudsmit, 1987., Von Sydow *et al*, 1988). Loss of antibody responses to viral core proteins may correlate with the development and extent of pathological lesions in visna infection also (Houwers & Nauta, 1989), but the basis of this association between anti-core protein antibody reactivity and disease progression is unknown. The major core proteins of neither visna nor HIV are targets for virus neutralising antibody (Scott *et al*, 1979., Weber *et al*, 1987). However HIV p24 is expressed on the surface of virus-infected cells, at least *in vitro*, (Laurent *et al*, 1989) and thus may be a target for antibody mediated cytotoxicity.

The role of virus neutralising antibody in the protection from lentiviral disease *in vivo* is unclear. In Equine Infectious Anaemia Virus (EIAV) infection, virus infection and replication results in acute disease which ceases after induction of type-specific neutralising antibodies (reviewed in Clements *et al*, 1988). However, in both visna and HIV, it is unclear whether there is any correlation between neutralising antibody titres and disease progression (Narayan & Clements, 1990., Rosenberg & Fauci, 1989). In kinetic experiments it has been shown that the rate at which neutralising antibody binds to free visna virus is significantly

slower than that of virus binding to cells (Kennedy-Stoskopf & Narayan, 1986), suggesting a possible mechanism for virus spread *in vivo* in the presence of high levels of serum neutralising antibodies. Moreover antigenic variation in the envelope proteins of visna virus (see section 1.6.5) may also allow the virus to evade immune surveillance: the presence of neutralising antibody has been shown to select for neutralisation-resistant visna variants both *in vitro* (Narayan *et al*, 1977), and *in vivo* (Narayan *et al*, 1981).

As well as virus neutralisation, virus-specific antibody may also direct the destruction of virus-infected cells (reviewed in Sissons & Oldstone, 1980). This may occur by either, or both, of two processes: antibody dependent cell-mediated cytotoxicity (ADCC) and/or antibody-mediated complement-dependent cytotoxicity (ACC).

ADCC has been suggested to play an important role in the control of several viral infections; herpesvirus (IBR) and paramyxovirus (PI3) infection of cattle (Rouse *et al*, 1976., Stott *et al*, 1975), cytomegalovirus infections of man (Quinnan *et al*, 1982) and retroviral diseases of cats (de Noronha *et al*, 1978). To date, no studies on this aspect of immunity in relation to MVV infection have been reported, but several groups have reported that sera from HIV-infected individuals can mediate ADCC activity *in vitro* (Rook *et al*, 1987., Ojo-Amaize *et al*, 1987).

Antibodies mediating ADCC in HIV infection are believed to react predominantly with the outer membrane envelope glycoprotein (Lyerly *et al*, 1987., Shepp *et al*, 1988), although the transmembrane glycoprotein may also be a target for ADCC (Blumberg *et al*, 1987). However there is no correlation between titres of virus-neutralising antibody and ADCC antibody titres (Bottiger *et al*, 1988), and ADCC reactivity is mainly group specific (Lyerly *et al*, 1987). Interestingly, several investigators have reported that there may be a correlation between ADCC antibody titre and clinical stage of disease, in that AIDS patients tend to have lower titres of ADCC activity than healthy seropositive individuals (Rook *et al*, 1987., Ljunggren *et al*, 1987).

Antibody-mediated complement-dependent cytotoxicity (ACC) has been shown to be an important component of the immune response to feline and murine retroviruses (Grant *et al*,

1978., Fischinger *et al*, 1982). It has also been demonstrated that ACC is involved in the immune response of ruminants to herpesvirus infection (Babiuk *et al*, 1975), moreover antibody-mediated fixation of complement has also been shown to enhance ADCC in a ruminant system (Rouse *et al*, 1977*a*., 1977*b*).

Visna-specific complement fixing antibodies are first detected 1-2 months post infection and persist thereafter (Gudnadottir & Kristinsdottir, 1967., De Boer, 1970., Petursson *et al*, 1976), but results of direct tests of the ability of these antibodies to lyse visna virus or visna infected cells have not been reported. Group-specific ACC has been observed in chimpanzees experimentally infected with HIV (Nara *et al*, 1987*a*), but even high titre anti-gp120 human sera have been reported not to mediate HIV-specific ACC (Lyerly *et al*, 1987., Nara *et al*, 1987*b*). It has been suggested that the occurrence of anti-HIV ACC in infected chimpanzees may be related to the ability of these animals to remain relatively healthy despite infection with HIV (Rosenberg & Fauci, 1989).

It has been reported that non-neutralising antibodies to lentiviruses may enhance viral infection of macrophages by allowing the uptake of virus, as immune complexes, via Fc receptors (Takeda *et al*, 1988) and/or complement receptors (Robinson *et al*, 1989). This mechanism of virus infection has been suggested to occur in visna infection (Jolly *et al*, 1989). However these studies were all carried out in established cell lines and/or cultured blood mononuclear cells. Cell types which may not be representative of the situation *in vivo*.

Antibody to HIV, in either the presence or absence of complement, has been reported to have no enhancing effect on virus replication in freshly isolated normal, primary blood monocytes and peritoneal macrophages (Shadduck *et al*, 1991).

The antibody isotypes that are produced in the humoral immune response to visna virus infection have not been well characterised. Mehta & Thormar (1974), reported that virus neutralising activity was associated with both IgM and IgG isotypes, whereas Petursson *et al* (1983), suggested that no anti-visna reactivity was associated with antibodies of the IgM isotype. Both groups reported that anti-visna antibody activity was readily demonstrated in the

IgG_1 subclass, while the occurrence and significance of IgG_2 antibodies to visna virus were unclear (Petursson *et al*, 1983).

The significance of virus-specific antibodies in protection from lentivirus infection *in vivo* is not well understood. Recently it has been reported that passive transfer of plasma, containing high titres of anti-HIV antibodies, from healthy HIV-infected individuals to AIDS or ARC patients resulted in a sustained decrease in p24 antigenaemia and an increase in anti-viral antibody titre to levels higher than those expected from the passive immunisation alone, however this did not appear to alter disease progression as assessed by $CD4^+$ lymphocyte depletion (Jackson *et al*, 1988., Karpas *et al*, 1988). More persuasive evidence of a protective role for antibody has been obtained in an SIV system in which passive immunisation of macaques with high titre anti-SIV serum from vaccinated, immune macaques protected the recipients from subsequent viral challenge (Putkonen *et al*, 1991). However this study did not define the mechanism of antibody protection: whether the challenge virus was neutralised by antibody or subsequently eliminated by antibody-mediated cytotoxicity. A recent report (Stott *et al*, 1991) has suggested that protection of monkeys from HIV-2/SIV challenge may be correlated with antibody reactivity to components of the human cell lines in which these virusesare routinely grown, rather than with antiviral antibody titres. Thus at the moment data obtained from monkey-protection experiments should be interpreted only cautiously.

1.6.4 T cell mediated immunity

Cell-mediated immune responses in visna infection have not been well characterised, but in the majority of viral infections studied T cell mediated immune responses are believed to be of prime importance both for clearance of virus infected cells and in regulating aspects of the humoral immune response (Mims & White, 1984., Vitetta *et al*, 1991).

Griffin *et al* (1978), reported that sheep experimentally infected with maedi-visna made only a transient cell-mediated immune response to visna virus: lymphocyte proliferation

against MVV being undetectable after 6 weeks post-infection. This study however was terminated only three months after infection. Larsen *et al* (1982*b*), and Sihvonen (1984) reported that intermittent and irregular cellular responses to visna could be detected in a three year period following experimental visna infection. These studies however, were poorly controlled and consequently the data presented is difficult to interpret. Moreover none of these studies made any attempt to characterise either the viral antigens eliciting a response, or the lymphocyte populations responding in these assays.

That visna virus does elicit T-cell mediated immune responses is suggested by the observation that the basic lesion of visna-induced pathology is infiltration and proliferation of mononuclear cells in an active-chronic inflammatory process (Oliver *et al*, 1981., Narayan & Cork, 1985), both $CD4^+$ and $CD8^+$ lymphocytes comprise a proportion of these infiltrating cells (Lairmore *et al*, 1986., Harkiss *et al*, 1991). Moreover inflammatory foci in the brain correspond to areas containing cells that are actively expressing viral RNA and antigen (Stowring *et al*, 1985). It has also been shown that the development of these lesions is due, at least in part, to an immunopathological response to viral antigens; vigorous immunosuppression, commenced shortly prior to infection, markedly reduced the level of inflammatory lesions seen in experimentally infected sheep (Nathanson *et al*, 1976).

Cellular immune responses to lentivirus infection have, however, been well studied in HIV and SIV infected individuals. These studies have shown that there is a complex and vigorous T-lymphocyte response during lentivirus infection *in vivo*. Virus-specific, MHC restricted cytotoxic T-lymphocytes (CTL) can be detected in peripheral blood (Walker *et al*, 1987., Riviere *et al*, 1989), cerebrospinal fluid (Sethi *et al*, 1988) and in inflammatory lung lesions (Plata *et al*, 1987). Viral antigens recognised by circulating CTL's of HIV seropositive individuals include the structural proteins ***gag*, *env*** and ***pol*** as well as the viral regulatory proteins ***nef*** and ***vif*** (Walker *et al*, 1987., 1988., Riviere *et al*, 1989., Nixon *et al*, 1988., Langlade-Demoyen *et al*, 1988., Chenciner *et al*, 1989). The effector cells mediating these effects are mainly $CD8^+$, MHC class I restricted T cells although some anti-***env*** cytotoxicity has been found to be non-MHC restricted (see section 1.6.2).

It has been reported that CD4+, MHC class II restricted T-cells reactive with gp120 can be derived from the peripheral blood of HIV seronegative individuals after primary immunisation either *in vitro* (Lanzavecchia *et al*, 1988., Siliciano *et al*, 1988) or *in vivo* (Orentas *et al*, 1990). These CD4+ T cells can exert cytotoxic activity *in vitro*, but CTL's with this phenotype have not been detected *in vivo*, in HIV infected patients.

HIV specific CTL's appear to be present in unusually high frequencies in infected individuals (Hoffenbach *et al*, 1989., Gotch *et al*, 1990). HIV specific CTL activity can be demonstrated in freshly isolated PBMC's without *in vitro* restimulation (Walker *et al*, 1987., Koup *et al*, 1989). With the possible exception of acute infectious mononucleosis, where there is a high level of 'HLA-unrestricted' cell mediated lysis (Strang & Rickinson, 1987), such a response has not been observed in other viral infections.

It has been demonstrated that virus-specific CD8+ lymphocytes can suppress both initial and ongoing HIV replication in autologous PBMC's *in vitro* (Walker *et al*, 1986., Tsubota *et al*, 1989). These depletion and reconstitution experiments showed that CD8+ T cells could reduce reverse transcriptase activity in infected cultures in an antigen specific, MHC-restricted manner. Similar results have been obtained in an SIV system (Kannagi *et al*, 1988). The mechanisms by which CD8+ T cells mediate these effects have not been well defined. Cytotoxic activity may be of importance for control of virus growth (Tsubota *et al*, 1989), although other workers have suggested that HIV replication is inhibited via non-cytotoxic mechanisms (Brinchmann *et al*, 1990., Walker *et al*, 1991).

The significance of CTL in the pathogenesis of HIV disease is unclear. In various model systems virus-specific T-cell mediated lysis of infected cells has been shown to be necessary and sufficient for clearance of viral infection (Larsen *et al*, 1983., Moskophidis *et al*, 1987., Taylor & Askonas, 1986). That HIV infection invariably proceeds to AIDS and death indicates that the occurrence of a strong CTL response in the initial phase of infection is somehow insufficient for protection. In longitudinal studies of HIV infected individuals it has been observed that there is a decline in frequency of virus-specific CTL and CTLp as the patient progresses to ARC and AIDS (Hoffenbach *et al*, 1989). This may be due to a reduction in the

growth potential of $CD8^+$ cells in infected persons (Pantaleo *et al*, 1990), a defect which may specifically affect memory/precursor T cells (Gotch *et al*, 1990). Alternatively this may reflect the development of suppressor T cells in later stages of HIV disease (Joly *et al*, 1989).

In this connexion it is interesting to note that protective retroviral cellular immunity has been shown to require both $CD4^+$ and $CD8^+$ immune T-cells (Hom *et al*, 1991). In natural HIV infection virus-specific $CD4^+$ 'T-helper' cell responses appear to be specifically deficient in both prevalence and strength: lymphocyte proliferation in response to inactivated HIV antigen is weak or absent in HIV seropositive persons (Reddy *et al*, 1987., Wahren *et al*, 1987), whereas the proliferative responses of the same individuals to cytomegalovirus and herpes simplex virus are similar to normal controls (Wahren *et al*, 1987). This has been interpreted as evidence for HIV antigen specific anergy (McChesney & Oldstone, 1989), however PBMC's from HIV seropositive individuals respond to culture with synthetic peptides corresponding to regions of HIV ***gag***, ***pol*** and ***env*** genes, by proliferation and/or production of IL-2 (Schrier *et al*, 1989., Clerici *et al*, 1990*a*). The significance of these observations is unclear, but chimpanzees and gibbon apes chronically infected with HIV have strong group-specific lymphoproliferative responses to HIV antigens (Eichberg *et al*, 1987., Lusso *et al*, 1988) and it has been suggested that the occurrence of these responses may contribute to the prevention of the development of AIDS in these species.

1.6.5 Antigenic variation

As described previously (section 1.5.1) lentiviruses mutate at a high rate, ensuring genetic heterogeneity in the viral population. Visna virus undergoes antigenic variation, as assessed serologically, during infection in individual hosts (reviewed in Narayan *et al*, 1987., Clements *et al*, 1988). Narayan *et al* (1977), demonstrated that antigenically distinct strains of visna could be isolated from sheep persistently infected with visna virus. The presence of neutralising antibody selecting for neutralisation-resistant variants both *in vitro* (Narayan *et al*, 1977) and *in vivo* (Narayan *et al*, 1981). These variants arise mainly by point mutations within the ***env*** gene (Scott *et al*, 1979., Clements *et al*, 1980., 1982), which result in

conformational alterations of the outer membrane envelope protein (Stanley *et al*, 1987). In visna infection *in vivo*, however, these variants do not replace the infecting serotype and, in most long-term infections, the inoculum virus strain persists and spreads without the emergence of antigenic variants (Lutley *et al*, 1983., Thormar *et al*, 1983). Antigenic variation has also been described during HIV infection (Nara *et al*, 1990), but again the functional significance, if any, of these observations is unclear.

Antigenic variation in T cell epitopes is theoretically possible also. This mechanism has been formally demonstrated as operative during LCMV infection of mice transgenic for an LCMV-specific T cell receptor molecule (Pircher *et al*, 1990). Evidence for mutation in CTL epitopes of HIV has recently been obtained (Phillips *et al*, 1991), however these mutations did not result in escape from CTL surveillance, but rather a change in the CTL epitope recognised. In contrast, the persistence of a CTL epitope *in vivo* over a 14 month period, despite the presence of specific CTL activity has recently been reported in an HIV-infected individual (Meyerhans *et al*, 1991). Given these contrasting observations, the significance of such mutantions in an outbred population is unclear. In HIV infection heterogeneity of CTL activity, both with respect to HLA restriction and recognition of specific epitopes, has been observed for both ***gag***-specific and reverse transcriptase-specific CTL clones (Johnson *et al*, 1991., Walker *et al*, 1989).

1.6.6 Maedi-visna disease

The major clinical presentation of the disease complex caused by maedi-visna virus infection of sheep is dyspnoea and wasting (maedi) (Sigurdsson, 1954). Visna, a neurological manifestation of MVV infection, is characterised by afebrile posterior ataxia which progresses over a course of weeks to months leading to recumbent hind limb paralysis (Sigurdsson *et al*, 1957). Other diseases associated with MVV infection include arthritis and mastitis (Oliver *et al*, 1981., Narayan & Cork, 1985).

Clinical maedi-visna disease is due to chronic inflammation in particular organs or tissues. Histologically, the lesions are characterised by the infiltration and proliferation of mononuclear cells in an active-chronic inflammatory process in organ systems such as the brain, lung, joints and mammary gland (Narayan & Cork, 1985). As disease progresses this inflammatory infiltrate destroys normal tissue architecture and compromises function. The basis of these inflammatory changes is unknown, but could include virus antigen-specific responses and chemotactic responses to abnormal cytokine release.

Unlike HIV infection, secondary disease resulting from the gross immune deficiency associated with $CD4^{+}$ lymphocyte dysfunction and destruction in HIV infection (reviewed in Rosenberg & Fauci, 1989), is not a major feature of MVV disease (Narayan & Clements, 1990). Some workers have suggested that infection with MVV may cause a mild immunosuppression (Myer *et al,* 1988), however most studies have found no convincing evidence for immunosuppression associated with MVV infection (Griffin *et al,* 1978., Larsen *et al,* 1982*a*., Dr. P. Bird, *personal communication*). It has been reported that there is a reduction in the CD4/CD8 ratio in the peripheral blood of sheep in late stages of MVV disease (Kennedy-Stoskopf *et al,* 1989). However this has not been observed by other groups, studying both experimentally infected sheep and field cases of maedi, (P. Bird, *personal communication,* G. Petursson, *personal communication*)

The progression of madi-visna-induced disease may be enhanced by the presence of other viruses. This has not been demonstrated formally, but strong circumstantial evidence, from Iceland and Holland, indicates that visna virus is disseminated more readily, and that disease is more severe, in flocks where sheep affected with sheep pulmonary adenomatosis (SPA) coexist (Palsson, 1976., Houwers & Terpstra, 1984). Moreover it has been shown that experimental co-infection of sheep with SPA and visna leads to an extremely rapid progression of disease, which has been suggested to result from a 'pathogenetic synergism' between the two agents (DeMartini *et al,* 1987), but the basis of this phenomenon is unknown.

For HIV-1 it has been shown that gene products from a number of viruses can *trans*-activate the HIV LTR *in vitro* (reviewed in Nelson *et al*, 1990., Tevethia & Spector, 1989), and it has also been shown that prior infection of cells with another virus may induce susceptibility to HIV infection in previously non-permissive cells (Lusso *et al*, 1991., McKeating *et al*, 1990). It is possible that similar phenomena may play a role in the pathogenesis of visna-induced disease.

1.7 Conclusion

Lentiviral disease, with its long incubation period, gradual onset and protracted progressive course requires continuous virus replication. Viral persistence, in the face of a vigorous host immune response, is thus the central feature of lentiviral pathogenesis (Haase, 1986).

It is likely that sequestration of visna virus within infected macrophages is a major mechanism of viral persistence and yet mechanisms of immunity against virus infected cells have received very little study in maedi-visna infection. The other major mechanism of lentiviral persistence *in vivo* is the ability of the virus to maintain itself in a state of restricted replication within infected cells. However, neither the molecular basis of this replication state nor the reasons why it occurs are understood. A plausible hypothesis as to why viral replication *in vivo* is restricted, rather than lytic, is that this is, at least in part, a response by the virus to the development of an active immune response in the initial period post-infection. Yet despite the vast amount of work which has been done on the immunology of HIV infection in persistently infected individuals, our understanding of the immediate, acute immune response to lentiviral infection of lymphoid tissue is poor and thus our knowledge of factors affecting viral replication *in vivo* incomplete.

Thus the aims of this project were two-fold. Firstly, to investigate more closely the immune responses of sheep persistently infected with maedi-visna virus, in particular mechanisms of immunity to virus infected cells. Secondly to attempt to analyse the acute

immune responses of sheep to visna infection. This work was to be based on experiments involving lymphatic cannulation (Hall & Morris, 1962), a technique which allows direct access to the output of a peripheral lymph node responding to acute viral challenge.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were supplied by SIGMA Chemical Co., Poole, Dorset, England or BDH Chemicals Ltd., Poole, England unless otherwise stated. Radiolabelled compounds were supplied by Amersham International plc., Amersham, Bucks, England. Unless otherwise stated all enzymes used in molecular biological techniques were supplied by Boehringer Mannheim, BCL, Lewes, England.

2.2 Bacterial culture

2.2.1 Media

The basic bacterial growth medium was LB (Luria-Bertani) medium (1% tryptone, 0.5% yeast extract, 1% NaCl made to pH 7 with NaOH). Ampicillin was added to 150µg/ml. All the plasmids used in this project conferred ampicillin resistance. LB agar plates were made with LB medium and 1% Bacto-agar (Difco, Molesley, Surrey). For "blue-white" selection (Sambrook *et al*, 1989) of bacteria transformed with pTZ derived plasmids, 40µl of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) (20mg/ml in dimethylformamide) and 20µl of isopropylthio-ß-D-galactoside (IPTG) (40mg/ml in SDW) were spread on the surface of premade LB agar plates and allowed to dry before plating out bacteria.

2.2.2 Bacterial strains and culture

E. coli strain JM101 (*supE, thi,* Δ*(lac-proAB),* [F', *traD* 36, *proAB, lacI* ^{q} Z ΔM15] (Yanisch-Perron *et al*, 1985) was used for bacterial transformations involving pTZ based plasmids, while the *E. coli* strain JM83 (*ara,* Δ*(lac-proAB), rpsL(=strA),* ϕ80, *lac* Z ΔM15) (Yanisch-Perron *et al*, 1985) was used for transformations with non-pTZ-based plasmids (see section 2.3.1). Single colonies of untransformed *E. coli* were grown by streaking an inoculum from a frozen stock on 1% agar plates made with minimal medium (42mM Na_2HPO_4, 22mM KH_2PO_4, 19mM NH_4Cl, 9mM NaCl, 100µM $CaCl_2$, 0.2% glucose).

Transformed bacteria were grown on LB agar supplemented with 150μg/ml ampicillin. Bacterial stocks were frozen in 15% glycerol and stored at -70°C (Maniatis *et al,* 1982).

2.2.3 Transformation of *E.coli*

E. coli cells competent for transformation by plasmid DNA were prepared by a modification of the method of Hanahan (1983). 0.2ml of a fresh overnight culture of *E. coli* in LB medium was diluted 1:100 in *psi* broth (2% tryptone, 0.5% yeast extract, 20mM $MgSO_4$, 10mM NaCl, 5mM KCl) and grown, with shaking at 37°C, until the OD_{550}=0.3. 5ml of this culture were diluted 1:20 in *psi* broth and grown until the OD_{550}=0.48. This culture was chilled briefly on ice, and then centrifuged (2000x **g,** 4°C, 5min). The cell pellet was resuspended in 33ml ice-cold TfBI (35mM sodium acetate, 100mM $CaCl_2$, 100mM RbCl, 50mM $MnCl_2$, 15% glycerol, made to pH 5.9 with acetic acid), held on ice for a further 10-15 min. and then centrifuged (2000x **g**, 4°C, 5min). This cell pellet was resuspended in 4ml of TfBII (10mM morpholinopropane sulfonic acid, 10mM RbCl, 75mM $CaCl_2$, 15% glycerol, made to pH 6.8 with KOH) and kept on ice for 20min. Aliquots (0.2ml) were then snap-frozen in liquid N_2 and stored at -70°C. Competent *E. coli* prepared in this manner routinely yielded 5×10^6-10^7 transformants per μg of intact pTZ 19R.

For bacterial transformation, aliquots of competent *E. coli* were thawed on ice and DNA (2-5μl) added. The suspension was then chilled on ice for 30min prior to heat-shocking (42°C, 60-90s), to increase the uptake of DNA. Cells were cooled briefly and then each aliquot was made to 1ml with LB (no antibiotics) and incubated, with vigorous shaking (230rpm), at 37°C for 1hr. 200μl of these bacteria were plated out onto LB agar plates supplemented with 150μg/ml ampicillin.

Ampicillin resistant colonies were picked and checked for the required plasmid by restriction enzyme analysis.

2.3 Preparation of DNA and molecular cloning techniques

Many of the methods used in handling and cloning DNA were based on methods given in 'Molecular Cloning. A laboratory manual.' (Sambrook *et al*, 1989). Routine techniques, such as phenol-chloroform extraction, butanol extraction and ethanol precipitation were performed as described in this manual.

2.3.1 Vectors used in cloning, sequencing and expression

Some of the characteristics of the plasmids used in this project are summarised in Table 2.1. pTZ 18R, pTZ 19R and M13KO7 were purchased from Pharmacia. Plasmids pMA5620, pOGS 40 and pUG 41S were gifts from Dr. S.E. Adams, British Bio-technology Ltd., Oxford.

The *LEU2* gene in pMA5620 and pOGS 40 lacks a promoter and is referred to as *LEU2-d*. It is thought that the poor expression of this gene creates a selective advantage for cells that have a higher copy number of the plasmid (Kingsman *et al*, 1990).

2.3.2 Polymerase Chain Reaction (PCR)

Low molecular weight DNA, prepared from EV1 (Sargan *et al*, 1991) infected fibroblasts by the method of Clements *et al*, (1979), (a gift from Dr. D.J. Roy, Dept. Vet Pathology, University of Edinburgh) was used as the template in a PCR. This material contains less than 0.1% proviral DNA (Dr. D.J. Roy, *personal communication*). 1μg of low molecular weight DNA was mixed with 0.1nmol of each primer (Oswel DNA, Edinburgh) and this was made to 10μl with sterile distilled water (SDW). Remaining PCR reagents were then added. Amplifications were done,under liquid paraffin, in 90μl of 50mM KCl, 25mM Tris-HCl pH 8.8, 6mM $MgCl_2$, 3mM dithiothreitol, 170μg/ml bovine serum albumin (BSA), 300μM (each) dNTP's and 5 units of Taq polymerase.

Thirty cycles of PCR were carried out: denaturation at 95°C, 0.6min; annealing at 45°C, 0.5min; extension at 71°C, 2.5min; final extension, 9min. The aqueous phase was removed

Table 2.1

Vector	***E. coli* replicon**	**Yeast replicon**	**Selectable markers in *E. coli***	**Selectable markers in yeast**	**Reference**
pTZ 18R	pMB1	N/A	Amp^r, ΔlacZ	N/A	Pouwels *et al*, 1985
pTZ 19R	pMB1	N/A	Amp^r, ΔlacZ	N/A	Pouwels *et al*, 1985
M13KO7	p15A	N/A	Kan^r	N/A	Vieira & Messing, 1987
pMA5620	pAT153	2μm	Amp^r	Leu^+	Adams *et al*, 1987*a*
pOGS 40	pAT153	2μm	Amp^r	Leu^+	Gilmour *et al*, 1989
pUG 41S	pMB1	*ARS1*	Amp^r	Ura^+	Lue *et al*, 1987

N/A - none available

from under paraffin, extracted once with phenol-chloroform, once with chloroform and ethanol precipitated. The PCR product was then gel purified for use in subsequent cloning steps.

2.3.3 DNA agarose gel electrophoresis

Agarose gels, 0.8-1% agarose, were made using TAE buffer (40mM Tris-HCl, 8mM sodium acetate, 0.4mM EDTA, pH 7.85), Ethidium bromide was added to the agarose, when cool, to a final concentration of 0.1µg/ml. DNA was loaded into wells in 5% glycerol, 12.5mM EDTA pH 7.9, 0.01% SDS, with bromophenol blue. Preparative gels were run in the dark, and minimally exposed to ultraviolet light. Gels were examined on an ultra-violet (UV) transilluminator (UV emission λ=304nm).

DNA was recovered from agarose gels using a Geneclean II kit (BIO 101 Inc., La Jolla, California, USA.) as described by the manufacturerer.

2.3.4 Preparation of DNA fragments for blunt-ended cloning

Conversion of protruding ends to blunt ends was done, using the Klenow fragment of *E. coli* DNA polymerase 1 (Jacobsen *et al*, 1974), according to Sambrook *et al*, (1989). Fragment ends were phosphorylated by treatment with T4 polynucleotide kinase (Sambrook *et al*, 1989).

2.3.5 Restriction endonuclease digestion of DNA

Digestions were carried out, in the incubation buffer supplied with the enzyme, in 20-50µl total volumes with 1-25U enzyme/µg purified DNA. DNA was digested for 2-3hrs at 37°C unless otherwise stated. Miniprep DNA (2µl) was digested with 5-10U of enzyme for 2-3hrs at 37°C.

2.3.6 Preparation of cut vector

Purified plasmid DNA was digested with the required restriction endonuclease (10U/μg DNA) for 3hrs at 37°C. To remove the 5' phosphate of the linearised plasmid, and so reduce the chances of plasmid religating to itself (Sambrook *et al*, 1989), calf intestinal phosphatase, in excess, was added for the last 30min of the digestion. Digested plasmid DNA was then separated on a preparative agarose gel and linearised plasmid recovered from the agarose using a Geneclean II kit. This DNA , in SDW, was stored at -20°C.

2.3.7 Ligation of DNA

For ligation of DNA fragments with cohesive termini, 5U of T4 DNA ligase were used, 10U of enzyme were used for ligation of fragments with blunt, phosphatased ends. Ligation reactions were carried out, in the incubation buffer supplied with the ligase, in a final volume of 10μl. 50-100ng of vector DNA were used in each ligation, at a molar ratio of insert: vector of 5: 1.

In ligation reactions where the DNA fragments had cohesive termini, insert and vector DNA were mixed and heated to 65°C. The mix was allowed to cool to room temperature and then ligase and ligase buffer were added. Ligation reactions were incubated, at room temperature, for 16-20hrs.

For blunt-ended ligations, insert and vector DNA were treated as described above except that the reaction was incubated for 16-20hrs at 15°C.

Ligation mixtures were used to transform *E. coli* without purification.

2.3.8 Miniprep of plasmid DNA

Transformed *E. coli* were grown overnight in LB broth supplemented with ampicillin (150μg/ml) and 1.5ml of this culture were used for preparation of plasmid DNA. Plasmid DNA was isolated by a modification of the 'alkaline lysis' method of Ish-Horowicz & Burke (1981). Briefly, the bacteria were pelleted, digested with lysozyme and then lysed in alkaline SDS. Chromosomal DNA was precipitated by addition of high salt (3M Na acetate, pH 4.8), spun

out and plasmid DNA recovered from the supernatant by ethanol precipitation. The phenol/chloroform extraction and RNase treatments described in the original protocol were omitted. The final DNA pellet was resuspended in 20µl of SDW & 2µl of miniprep DNA were used for each restriction enzyme digestion.

2.3.9 Purified plasmid preparations

Large scale preparations of plasmid DNA were based on the alkaline lysis methods of Birnboim & Doly, (1979) and Ish-Horowicz & Burke (1981). *E coli* were grown, with shaking (230rpm) at 37°C, for 24hrs in 500ml of LB broth with ampicillin (150µg/ml). Bacteria were pelleted by centrifugation (7500x **g**, 4°C, 5min) and the supernatant removed. Cells were resuspended in 36mls of GTE (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA) and 4ml of freshly made lysozyme (40mg/ml in GTE) were added, mixed in thoroughly and incubated at room temperature for 10min. 80ml of freshly prepared alkaline SDS (0.2M NaOH, 1% SDS) were then added, swirled gently to mix fully, and the suspension incubated on ice for a further 10min. To this, 40ml of ice-cold 5M potassium acetate, pH 4.8 was added, mixed well and incubated on ice for 1hr with occasional gentle stirring to break up lumps of precipitate. 10ml of SDW were then added and the suspension centrifuged (15000x **g**, 4°C, 5min) to pellet chromosomal DNA and bacterial debris. Avoiding any floating precipitate, the supernatant was transferred to a fresh centrifuge bottle and 0.6 volumes of cold (-20°C) isopropanol added. This was mixed well and allowed to stand at room temperature for 15min. Nucleic acid was recovered by centrifugation (9500x **g**, 4°C, 5min) and the pellet resuspended in 30ml TE, pH 8 (10mM Tris-HCl pH 8, 1mM EDTA). 30g of solid caesium chloride were dissolved in this suspension, ethidium bromide (10mg/ml in SDW) was added to a final concentration of 800µg/ml and allowed to intercalate to the DNA. The caesium chloride gradient was formed by centrifugation at 40000rpm for 18hrs at 20°C in a Beckman VTi 50 rotor (Beckman L8-60M ultracentrifuge). After centrifugation the lower, closed circular, plasmid DNA band was collected and butanol extracted 3-5x to remove the ethidium bromide. Plasmid DNA was then precipitated with 2½ volumes of 70% ethanol and washed with 70%

ethanol. This was repeated twice. The purified preparation of DNA was then taken up in SDW and the concentration measured by spectrophotometry. Plasmid DNA, in SDW, was stored at -70°C.

2.3.10 Quantitation of DNA

The absorbance of purified DNA solutions was measured spectrophotometically at 260nm and 280nm. Pure DNA gives an approximate OD_{260}/OD_{280} absorption ratio of 1.8. An OD_{260} of 1 corresponds to approximately 50µg/ml of double stranded DNA. The concentration of DNA in less highly purified solutions, such as miniprep DNA, was estimated by running an aliquot on ethidium bromide containing agarose gels and comparing the fluorescence of the sample with that of a series of standards of known DNA concentration (Maniatis *et al,* 1982).

2.4 Blot hybridisations

2.4.1 Southern blotting

Restriction endonuclease digested DNA was electrophoresed through 0.8% horizontal agarose gels for 16-20hrs at 20mA. The gel was examined under UV, photographed, and the DNA was then transferred to a nitrocellulose filter (Hybond-C, Amersham) according to Southern (1975). After electrophoresis, the DNA in the gel was denatured by soaking the gel in two changes (500ml) of 1.5M NaCl, 0.5M NaOH over 1hr, with gentle shaking. The gel was then neutralised by soaking in three changes (500ml) of 1M Tris HCl, pH 8, 1.5M NaCl over 1hr. Transfer of DNA was carried out by blotting 20x SSC (0.3M tri-sodium citrate, 3M NaCl, pH 7) through the gel, through the nitrocellulose membrane and into Whatman 3MM paper, overlaid with a stack of paper towels, overnight. After blotting the agarose gel was examined under UV to check the efficiency of transfer of DNA from the gel to the filter. The nitrocellulose was washed in 6x SSC, drained and allowed to dry for 30min at room

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temperature. The DNA was baked onto the filter for 2hrs at 80°C in a vacuum oven. Filters were used immediately in hybridisation experiments.

2.4.2 Preparation of [^{32}P]-labelled DNA probes

DNA fragments to be used as probes were prepared by restriction enzyme digestion from plasmids and purification from agarose gels. Probe fragments were labelled *in vitro* with [α-^{32}P]-dCTP using the Klenow fragment of *E. coli* DNA polymerase 1 and random oligonucleotide primers (Feinberg & Vogelstein, 1983, 1984). The reaction mix was incubated for 3-4hrs at 37°C, and the reaction stopped by the addition of oligo-stop buffer (50mM Tris HCl pH 7.5, 50mM NaCl, 5mM EDTA pH 8, 0.5% SDS). Incorporation of radioactive precursors was monitored by acid precipitation (12% trichloracetic acid) (Sambrook *et al*, 1989). Probes with a specific activity of 10^8-10^9cpm/µg DNA were used in hybridisations.

Immediately before use, probe DNA was denatured by boiling for 5min and then quenching on ice.

2.4.3 Hybridisation of nitrocellulose filters

Nitrocellulose filters were floated on the surface of 6x SSC until wet and were then prehybridised for 16-18hrs at 68°C in 6x SSC, 0.5% SDS, 5x Denhardt's solution (Denhardt's solution is 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA; Denhardt, 1966) & 100µg/ml sheared, denatured salmon sperm DNA (approximately 200µl prehybridisation fluid/cm^2 of filter). Hybridisation reactions were performed at 68°C for 16-18hrs in 6xSSC, 0.01M EDTA, 5x Denhardt's solution, 0.5% SDS & 100µg/ml sheared, denatured salmon sperm DNA with ^{32}P-labelled, denatured probe (approximately 50µl hybridisation mix/cm^2 filter). After hybridisation, filters were submerged in 2x SSC, 0.5% SDS for 5 min and then transferred to a fresh tray containing 2x SSC, 0.1% SDS and incubated at room temperature for 15min. The blots were then washed in 0.1x SSC, 0.5% SDS at 68°C with gentle agitation, after 2hrs the buffer was changed and the incubation continued for a further 30min. The filter was air-dried

at room temperature and then exposed to Kodak XS-1 film, with phospho-tungstate intensifying screens, at -70°C.

2.5 DNA Sequencing

2.5.1 Preparation of single stranded DNA

Single stranded DNA, for use as template in sequencing reactions, was prepared by a modification of the method of the protocol obtained from the supplier of the 'phagemid (Pharmacia literature). JM101 cells transformed with the recombinant pTZ were grown in 2x YT (1.6% tryptone, 1% yeast extract, 86mM NaCl, made to pH 7.4 with K_2HPO_4) supplemented with ampicillin (150µg/ml) till OD_{660}= 0.5. 400µl of this culture (approximately 1.6×10^8 cells) were infected with helper phage M13KO7, at a multiplicity of infection of 10, and shaken vigorously for 1hr at 37°C. The culture was then made to 10ml with 2x YT supplemented with ampicillin (150µg/ml). Kanamycin was added to 70µg/ml and the culture was grown at 37°C for 16-18hrs with vigorous shaking. The supernatant was then centrifuged (4000x **g**, 4°C, 15min) to remove bacterial cells and 'phage was precipitated from the supernatant by the addition of of polyethylene glycol/ NaCl (to final concentrations of 4% PEG, 700mM NaCl). After 30min on ice the precipitated phage was recovered by centrifugation (11000x **g**, 4°C, 40min), resuspended in 400µl of TEN buffer (20mM Tris HCl pH 7.5, 20mM NaCl, 1mM EDTA) and extracted four times with phenol/chloroform pH 8, three times with chloroform and ethanol precipitated. The DNA pellet was resuspended in 22µl of SDW and 2µl of this suspension were electrophoresed through 1% agarose and examined under UV to determine the yield of DNA.

2.5.2 Sequencing reactions

DNA was sequenced using the dideoxynucleotide chain termination method (Sanger *et al,* 1977) and [α-^{35}S]-dATP (Biggin *et al,* 1983) with a Sequenase kit (USB corporation) and

M13 reverse sequencing primer (Pharmacia). Sequencing reactions were done according to the protocol supplied with the Sequenase kit.

2.5.3 Double strand sequencing

Double-strand sequencing of supercoiled plasmid DNA was performed as follows; plasmid DNA, purified on caesium chloride/ethidium bromide gradients, was used as template for the sequencing reaction. 3-5µg of purified plasmid DNA were boiled for 3min with primer (20:1 molar excess, primer:template) in Sequenase reaction buffer and snap cooled on ice. This procedure denatured the DNA and allowed the primer to anneal. Sequencing reactions were performed as for single-strand sequencing except that the labelling reaction was done in the presence of 8% dimethyl sulphoxide (DMSO).

2.5.4 Sequencing gel electrophoresis

DNA sequencing samples were electrophoresed through 6% acrylamide (19:1 acrylamide/bisacrylamide, 7.67M urea in 45mM Tris HCl, 45mM Boric acid, 5mM Na_2EDTA, pH 8.3) slab gels cast in a Bio-Rad Sequi-Gen nucleic acid sequencing cell (38 x 50cm). Prior to casting the gel, acrylamide solutions were de-gassed under vacuum and filtered through a 0.45µm filter. 5mm wide loading wells were formed using a 'sharksteeth' comb. The buffer in the electrode tanks was TBE (45mM Tris HCl, 45mM H_3BO_4, 5mM EDTA, pH 8.3). Prior to loading samples gels were pre-run at 50W until the gel temperature reached 50°C.

Gels were loaded with 3µl volume of sequencing samples, in adjacent tracks, in the order ATGC. Immediately before loading onto the gel sequencing samples were denatured by heating to 80°C for 3min and then quenching on ice. Sequencing gels were run for 2-6hrs at 50°C (40-50W).

Gels were dried under vacuum at 80°C and exposed to Kodak XAR-5 film for 1-3 days at room temperature.

2.5.5 Sequence analysis

Sequence analysis was done using version 6.2 of the University of Wisconsin Genetics Computing Group package (Devereux *et al,* 1984).

2.6 Yeast culture

2.6.1 Media

Non-transformed yeast were grown in YEPD medium (2% peptone, 1%yeast extract, 2% glucose). Yeast transformants were grown in selective medium; either Sc-glc medium (0.67% yeast nitrogen base (YNB) without amino-acids (Difco Ltd.), 1% glucose) or Sc-gal medium (0.67% YNB, 0.3% glucose, 1% galactose). Agar plates were made with either YEPD or SC-glc medium and 2% bacto-agar. Where indicated, tryptophan and uracil were added to a final concentration of 20µg/ml.

2.6.2 Yeast culture and strains

Yeast culture conditions and induction for protein expression were based on the protocols described by Kingsman *et al,* (1990).

The protease deficient *Saccharomyces cerevisiae* strain BJ 2168 (**a**, *leu2-3, leu2-112, trp1, ura3-52, prb1-1122, pep4-3, pcr1-407, gal2)* (Jones, 1991) (a gift from Dr. S.E. Adams, British Bio-technology Ltd., Oxford) was used for all yeast expression work.

Single colonies of BJ 2168 were grown by streaking an innoculum from a frozen stock on YEPD plates. Transformed yeast were grown on Sc-glc media, with appropriate supplements for the auxotrophic requirements of the yeast transformant.

Yeast stocks, for long term storage, were prepared from cultures in late-log/ early stationary phase (OD_{600}=1). Equal volumes of yeast culture and sterile 40% glycerol were thoroughly mixed, placed at -20°C for 3-4hrs and then transferred to -70°C for long term storage.

For large scale culture yeast were grown, from glycerol stocks, in sterile conical flasks (culture fluid no more than a quarter of the flask volume), on an orbital shaker (New Brunswick Scientific) operating at 260rpm. Typically, 100ml of Sc-glc media (plus supplements) were innoculated with a single glycerol stock of yeast transformant and incubated, with vigorous shaking, till the cell density was 2-4 x 10^7 cells/ml. Each of 2 x 500ml of Sc-glc media was then innoculated with 50ml of the pre-culture and grown to 4-6 x 10^7 cells/ml. This litre of culture fluid was used to innoculate 8 x 500ml of either Sc-glc media (for constitutively expressing transformants) or Sc-gal media (to induce protein expression in pOGS transformants) and cultured for a further 24-48hrs.

2.6.3 Yeast transformation

For yeast transformation, DNA was purified by centrifugation on caesium-chloride/ ethidium bromide gradients. Transformants were selected by their ability to grow on minimal media without leucine and/or uracil supplementation. Yeast were transformed using the method of Hinnen *et al,* (1978). 100ml of BJ 2168 were grown in YEPD, with shaking, to a cell density of 1-2 x 10^7cells/ml. Yeast were harvested by centrifugation (200x **g**, 5min, room temperature), washed in 1M sorbitol pH 5.6 and treated with glusulase (NEN Research Products, Stevenage, Herts.), to digest the yeast cell wall, till approximately 90% of the yeast cells had been spheroplasted, as monitored by phase contrast microscopy. Spheroplasts were washed twice in 1M sorbitol, once with STC (1M sorbitol, 10mM $CaCl_2$, 10mM Tris HCl pH 7.5) and resuspended in 1ml STC. DNA (2-3µg, in less than 10µl final volume) was added to 100µl of competent cells, the suspension was mixed gently and incubated at room temperature for 15min. The yeast cells were then osmotically shocked, to increase the uptake of DNA, by addition of 1ml 44% PEG for 10min. Spheroplasts were spun down gently (200x **g**, 5min), the supernatant decanted and the pellet resuspended in 1ml 1M sorbitol. Aliquots of this suspension were added to 20ml of warm regeneration agar (1M sorbitol, 0.67% YNB, 1% glucose, 3% Bacto-agar), mixed gently and poured into a petri dish. Agar plates were allowed to set, inverted and incubated at 30°C.

2.6.4 Preparation of protein extracts

5 x 10^8 cells were harvested, after galactose induction where necessary, and washed once in ice-cold SDW. All subsequent procedures were carried out at 4°C. Cells were resuspended in 1ml of TEN buffer (100mM Tris pH 7.4, 140mM NaCl, 2mM EDTA) and vortexed, for three periods of 1min, with 1min on ice between vortexing, with 1g of acid-washed and baked glass beads (BDH, 40 mesh). The whole mixture was collected after allowing the glass beads to settle. This is a total cell extract (Kingsman *et al*, 1990).

2.7 Mammalian tissue culture

2.7.1 Cell lines and cell culture

Sheep epidermal fibroblast cultures were grown in Dulbecco's modified Eagle's medium (DME) (Cat No. 074-02100, Gibco Biocult, Uxbridge) supplemented with 2mM L-glutamine, 25mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) and 10% foetal calf serum (FCS) (Azo-Tek, Ltd., Herts.).

Cell lines were passed using 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 27mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.2) and were grown at 37°C with 5% CO_2 in either 75, 175 or 225cm^2 tissue culture flasks.

Stocks of cells were cryopreserved, at 2 x 10^6 cells/ml, in an ice-cold mixture of 90% FCS, 10% DMSO. 2 x 10^6 cells, in freezing mix, were cooled to -70°C using a programmed cell freezer (Planar Products, London) and stored under liquid nitrogen. To resuscitate frozen cell lines, an aliquot was thawed quickly at 37°C, diluted 1:1 with warm DME/ 10% FCS and then spun through 20ml of DME/ 10% FCS (200x **g**, 5min) before being layered on the bottom of a 25cm^2 tissue culture flask in DME/ 10%FCS. Cells were incubated overnight and the medium replaced with fresh DME/ 10% FCS.

2.7.2 Viruses

Visna isolates EV1 (Sargan *et al,* 1991) and K1514 (Petursson *et al,* 1976) were grown in sheep fibroblast cultures maintained in DME/ 2% FCS. Cultures were infected with visna at a low multiplicity of infection (approximately 0.05 tissue culture infectious doses($TCID_{50}$/cell) and virus containing supernatant was harvested when the cultures displayed extensive cytopathic effect. Visna virus was titred on epidermal fibroblasts in a quantal assay. Virus titres were calculated by the method of Reed and Muench (1938).

2.7.3 Monoclonal antibody production

2.7.3.1 Myeloma cell culture

Myeloma cells, NSO/1 (Galfrè & Milstein, 1981) were grown in RPMI 1640 (Cat. No. 074-1800, Gibco Biocult, Uxbridge) supplemented with 2mM L-glutamine and 10% foetal calf serum. Cells were maintained at 37°C in a humid atmosphere of 5% CO_2. Prior to a fusion NSO/1 were revived from frozen stocks into RPMI/10% FCS and maintained in logarithmic growth for 6-7 days by passing the culture daily at 10^5cells/ml.

2.7.3.2 Procedure of cell fusion

Cell fusion was done according to Eshhar (1985). Spleen lymphocytes (no more than 10^8cells) and myeloma cells were mixed at a ratio of 5:1 and spun down gently (200x **g**, 5min). The pellet was gently resuspended in 2ml of 40% PEG 1500 (Boehringer Mannheim), which had been prewarmed to 37°C, and incubated at 37°C for 2min. The PEG was then diluted by slow addition of prewarmed RPMI (supplemented with 2mM glutamine and 1mM sodium pyruvate, without serum). The cells were washed once and then plated out at (1-5) x 10^5 cells/ml in RPMI/ HAT (RPMI 1640, 15% FCS, 2mM glutamine, 1mM pyruvate, 100μM hypoxanthine, 0.4μM aminopterin, 16μM thymidine). Cultures were incubated at 37°C in 5% CO_2 in a humid incubator.

2.7.3.3 Cloning of hybridoma cells

Hybridoma cell lines were cloned by either of two methods:

a) Soft agar cloning: RPMI (with glutamine and pyruvate)/ 20% FCS containing 0.5% Bacto-agar (Difco Ltd.) was prepared and held at 46°C. 15ml of this mixture were put into 90mm petri dishes and left at room temperature to set. Cell dilutions, 10^5-10^2cells/ml, were prepared in RPMI/20%FCS, mixed 1:1 with agar-RPMI and spread out on the solidified agar plates. Plates were left 10min to allow the agar to set and were then incubated at 37°C, 5% CO_2 for 10-12 days. Single colonies were picked using a pasteur pipette and transferred to 24 well plates, for expansion and testing.

b) Limiting Dilution cloning: Irradiated (2000 Rads), syngeneic spleen cells were prepared as feeder cells (for preparation of spleen cell suspension see section 2.14.1.3), plated out in 96 well flat-bottomed tissue culture plates and incubated overnight at 37°C/5% CO_2. Hybridoma cells to be cloned were prepared at cell dilutions of 50, 10 and 1 cell(s)/ml and plated out at 100µl/well. Cultures were incubated for 5 days at 37°C, 5% CO_2. Wells containing single colonies were identified by visual inspection, expanded, tested and then recloned.

2.7.3.4 Hybridoma culture

Hybridoma cell lines were maintained in RPMI 1640 supplemented with 2mM L-glutamine, 1mM sodium pyruvate and 10% FCS. Cells were cryopreserved and resuscitated as described previously (section 2.7.1) except that cell lines were revived into RPMI/10% FCS.

2.8 Antigen preparation

2.8.1 Preparation of visna antigen for Western blotting

Fibroblast cultures were infected with visna at 0.05 $TCID_{50}$/ cell and maintained in DME/ 2% FCS until extensive cytopathic effect was observed. Culture supernatant and infected

cells were collected and pelleted by centrifugation (10000x **g**, 4°C, 16hrs). The pelleted material was resuspended in PBS and then centrifuged (120000x **g**, 4°C, 3hrs) to concentrate and disrupt the virus/ cell mix. The pellet was resuspended in PBS and this material was stored, in aliquots, at -70°C for use in immunoblotting experiments.

2.8.2 Purification of visna

Visna virus was purified from the supernatant of infected cell cultures by centrifugation on sucrose gradients. Briefly, the supernatant of cultures showing extensive cytopathic effect was harvested and clarified by centrifugation (10000x **g**, 4°C, 30min). Virus was pelleted from this material by centrifugation (10000x **g**, 4°C, 16-18hrs) and resuspended in a minimal volume of PBS. 25%-60% sucrose gradients were prepared by layering 3ml of each of 25%, 35%, 45%, and 60% sucrose solutions in a Beckman centrifuge tube (14mm x 95mm, ultraclear). These were held at 4°C overnight to diffuse and form the sucrose gradient. The clarified, pelleted visna was layered onto the sucrose gradients and centrifuged (100000x **g**, 4°C, 16hrs). Gradients were fractionated into 0.5ml aliquots and the virus containing fractions identified by analysis on SDS-PAGE and reverse transcriptase assay. Peak fractions were pooled, diluted in PBS and visna recovered by centrifugation (100000x **g**, 4°C, 16hrs). Purified virus was stored in aliquots at -70°C. This material contained all the major structural proteins of visna, as judged by immunoblot analysis with sera from infected sheep (data not shown).

2.8.3 Purification of Ty-virus-like particles (Ty-VLP's) from yeast

Ty-VLP's were purified from yeast by a modification of the method of Adams *et al,* (1987*a*) All steps in the purification protocol were performed at 4°C using pre-chilled buffers. Yeast cells were harvested by centrifugation (2500x **g**, 4°C, 10min) and, washed four times in ice-cold SDW. Approximately 5ml of packed cells were obtained per litre of culture fluid. Cell pellets were resuspended in 4ml of TEN buffer (100mM Tris HCl pH 7.4, 2mM EDTA, 140mM NaCl) plus a cocktail of protease inhibitors (chymostatin, antipain, leupeptin and pepstatin A

in DMSO, aprotonin in SDW (all at 625ng/ml) and 5μM phenyl methylsulfonyl fluoride in ethanol). 5ml of acid-washed and baked glass beads were added and the yeast cells broken open by three rounds of vortexing with glass beads. After each round of vortexing the suspension was centrifuged (2000x **g**, 4°C, 5min), the supernatant removed and kept on ice, and the cells resuspended in fresh TEN plus protease inhibitors. 90-95% breakage of yeast cells was achieved, as judged by phase contrast microscopy. The VLP-containing supernatants (approx. 12ml) obtained after breaking open the yeast were pooled, clarified by centrifugation (13000x **g**, 4°C, 20min) and then concentrated by centrifugation onto a 2ml 60% sucrose cushion (100000x **g**, 4°C, 1hr). Material at the interface and in the cushion was collected and dialysed overnight against TEN, with protease inhibitors. Ty-VLP's were purified from the dialysate by centrifugation through 15%-60% sucrose gradients (53000x **g**, 4°C, 3hrs). After centrifugation the gradient was fractionated into 2ml aliquots and VLP containing fractions identified by analysis on SDS-PAGE. Fractions containing VLP's were pooled and dialysed against TEN.

Protein concentrations were determined using a dye-binding assay (Bradford, 1976) obtained from Bio-Rad laboratories, using purified IgG as a standard. Purity of the antigen preparation was assessed by densitometric examination of Coomassie stained gels using a Shimadzu densitometer.

2.8.4 Purification of p25 from the Ty-p25 VLP's

Hybrid Ty-p25 VLP's were dialysed against several changes of 100mM Tris HCl pH 7.4, 10mM $CaCl_2$. The dialysate was then made to 0.05% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate) and incubated with *Factor Xa* protease (BCL) at a ratio of 100:1 (w/w) for 25hrs at 25°C. An aliquot of this digest was analysed by SDS-PAGE to check that digestion was complete. The suspension was then centrifuged (100000x **g**, 4°C, 1hr) to pellet the Ty-VLP's from the released, soluble p25. The p25 containing supernatant was dialysed against several changes of PBS, to remove detergent, and the protein concentration measured using the dye-binding assay. p25 protein was stored in aliquots at

-70°C. Purity of the antigen preparation was assessed by silver-staining of the p25 preparation separated on SDS-PAGE.

2.9 Detection of antigen

2.9.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of proteins

Protein separation was by discontinuous polyacrylamide gel electrophoresis with a Tris-glycine buffer containing SDS (384mM glycine, 0.1% SDS, 50mM Tris HCl), (Laemmli, 1970). Resolving gels were either 10% acrylamide slab gels or 5%-20% linear gradient acrylamide gels (37.5: 1 acrylamide: bisacrylamide) made with 375mM Tris HCl pH 8.7 and 0.1% SDS. Stacking gels were 3% acrylamide, 0.15% N,N'-methylenebisacrylamide made with 125mM Tris HCl pH 6.8 and 0.1% SDS. Samples, mixed with an equal volume of sample buffer (10% (v/v) ß-Me, 10% (w/v) SDS, 125mM Tris HCl pH 6.8, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue), were boiled for 3min before electrophoresis through vertical slab gels (Bio-Rad Mini-protean II Slab cells). Gels were run at 150V for 1-2hrs.

2.9.2 Staining of SDS-PAGE

Gels were stained by either of two methods:

a) Coomassie Blue staining: Coomassie Brilliant Blue G-250 was dissolved in methanol and then made to 20% methanol, 5% acetic acid and filtered through Whatman No. 1 filter paper. Gels were stained in Coomassie blue for 15min at room temperature and then destained in several changes of 20% methanol, 5% acetic acid.

b) Silver staining: Gels were fixed in 50% methanol, 10% acetic acid for 15min, 5% methanol, 7% acetic acid for 30min and then 10% glutaraldehyde for 30min. Gels were then washed thoroughly in several changes of distilled water over several hours. After washing, gels were stained in 0.1% silver nitrate solution for 15min, rinsed briefly in water and then developed with Na_2CO_3/formaldehyde solution (3% Na_2CO_3, 0.02% formaldehyde). Development was

halted by addition of solid citric acid and the gels were washed in several changes of distilled water. Gels were then fixed in 10% 'Ilfofix' for 1min and washed in three changes of water.

Gels were dried at 80°C under vacuum.

2.9.3 Immunofluorescent staining of cell monolayers

Cells were grown to confluence on glass coverslips (10mm diameter, Chance Propper Ltd., Smethwick, Warley, England) and infected with visna, at various multiplicities of infection, mock-infected controls were prepared also. At various times after infection cells were washed once in Hanks buffered salt solution (HBSS) (1mM $CaCl_2$, 5.4mM KCl, 0.4mM KH_2PO_4, 0.4mM $MgCl_2$, 0.4mM $MgSO_4$, 140mM NaCl, 0.7mM Na_2HPO_4, 0.1% glucose, 0.001% phenol red, 25mM HEPES) and fixed in 80% acetone/ 0.15M NaCl for 5min at room temperature. Coverslips were air dried and stored, dessicated, at -20°C till required.

Cells were first blocked in blocking buffer (PBS, 0.01 % Tween 80, 2% normal sheep serum) for 30min at room temperature. Blocking buffer was removed and primary antibody, diluted in blocking buffer, was added to the coverslip for 1hr at room temperature with gentle shaking. Cells were washed 5 times in PBS/ 0.01% Tween 80 (PBST) and incubated for 1hr, with shaking at room temperature, in a biotinylated anti-immunoglobulin antibody diluted in blocking buffer. Cells were again washed 5 times in PBST and then incubated for 1hr, with shaking, in avidin-FITC (Serotec) diluted 1:5000 in blocking buffer. Cells were washed 5 times in PBST and the coverslips were then mounted in Citifluor (Citifluor Ltd., London UK.) without allowing them to dry. Cells were viewed with a Leitz Ortholux II microscope using a water immersion lens at x625 magnification, and photographed under ultraviolet light with a Wild MPS 51 photographic attachment (Heerbrugg, Switzerland) and either Fujichrome ISO 1600 colour slide film or Kodak T-Max ISO 3200 monochrome print film.

2.9.4 Reverse transcriptase assay

This assay was based on an unpublished protocol of Dr. D.R. Sargan (Dept. Vet. Path. University of Edinburgh). Samples for assay (15µl), in triplicate, were placed in the wells of 96

well round-bottomed microtitre plates on ice. 10μl of 2.5x reaction mix (375mM KCl, 25mM $MgCl_2$, 25mM Tris HCl pH 8, 5mM DTT, 1,25mM EDTA, 0.25% Triton X-100, 62.5μg/ml BSA, 75μg/ml poly (rA). oligo($dT_{(12-18)}$), 5% deionised ethane diol, 100μCi/ml ^{3}H-TTP (tritiated thymidine tri-phosphate) (sp. act. 20Ci/mMol)) were added and the reaction incubated for 1hr at 37°C. The assay was stopped by the addition of 100μl of 10% (w/v) trichloroacetic acid (TCA) containing 50μg/ml yeast RNA to each well and the plate left on ice for 15min. Samples were harvested onto glass filter paper using a semi-automated harvester (Titertek) and the filters washed three times with 5% (w/v) TCA, twice with 70% ethanol and dried. ^{3}H-thymidine incorporation was assessed by liquid scintillation counting.

2.10 Transmission electron microscopy

2.10.1 Yeast sections

Yeast strains were prepared for transmission electron microscopy by a modification of the method of Byers & Goetsch (1975). Late log phase cultures (OD_{600nm}: 1-1.5) of yeast were fixed (0.1M cacodylate pH 6.8, 3% glutaraldehyde (EM grade), 5mM $CaCl_2$) for 30min at room temperature and then overnight, in fresh fixative, at 4°C with gentle mixing. Samples were then washed twice in pretreatment buffer (0.02M EDTA, 0.2M Tris HCl pH 8.1, 0.1 M ß-mercaptoethanol)and resuspended in 0.2M citrate phosphate buffer, pH 5.8 before treatment with 200μl of glusulase for 1hr at 30°C (to remove the cell wall). After spheroplasting, yeast cells were washed twice in citrate-phosphate buffer and post-fixed in 1% osmium tetroxide. Samples were then dehydrated through graded alcohol solutions and embedded in araldite. Ultrathin sections were cut, stained with 50% uranyl acetate (in ethanol) and Reynold's lead citrate and examined in a Philips TEM 400 operating at 100kV.

2.10.2 Negative staining of purified particles

One drop of purified particles, 1mg/ml in TEN, was placed on a Formvar coated grid and allowed to adhere for 15s. Excess fluid was drained off using filter paper and the grid washed three times with drops of distilled water. One drop of negative stain, 2% uranyl acetate in water, was then applied to the grid and the liquid drained off till only a thin film of stain was left. Grids were air-dried and examined in a Philips TEM 400 operating at 100kV.

2.11 Detection of specific antibody

2.11.1 Enzyme linked immunosorbent assay (ELISA)

Antigen was coated onto wells of 96 well, flat-bottomed Elisa plates by overnight incubation at 4°C. Plates were washed six times in borate buffered saline (100mM H_3BO_3, 25mM Na_2HBO_4, 75mM NaCl, pH 8.2) (BBS)/ 0.05% Tween 20 (BBST) and then blocked in BBS/ 1% BSA (BBA) for 1hr at 37°C. Sera, diluted in BBS/ 1% BSA/ 0.05% Tween 20 (BBTA), were added to the wells for 1hr at 37°C and the plates were again washed in BBST. Affinity purified anti-immunoglobulin, of appropriate specificity, conjugated to horse-radish peroxidase (HRP) (Dako), diluted in BBTA, was added to each well for 1hr at 37°C, the plates were washed in BBST and then developed with OPD substrate (ortho-phenylenediamine 0.4mg/ml in 50mM citric acid, 100mM Na_2HPO_4, pH 5 with 0.006% H_2O_2) for 30min at room temperature. The reaction was stopped by addition of 12.5% H_2SO_4 and the plates were read, at 492nm, in a Titertek plate reader.

2.11.2 Immunoblotting

After electrophoretic separation on SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond C, Amersham) using a semi-dry electroblotter (Ancos, Denmark) according to Kyhse-Andersen (1984). After blotting, the nitrocellulose membranes were 'blocked' using 5% non-fat dried milk (Sainsbury's) in PBS before overnight incubation in

primary antibody, also diluted in PBS/5% non-fat dried milk. Blots were washed in several changes of PBS, 0.05% Tween 20 (Sigma) and then incubated for 1hr in alkaline phosphatase conjugated secondary antibody of appropriate specificity. Blots were again washed in PBS/0.05% Tween 20 and then developed with nitro-blue tetrazolium (Sigma) & 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 0.1M Tris HCl pH 9.5 and $MgCl_2$ (Pluzek & Ramlau, 1988).

Development of blots was halted by washing in water.

2.11.3 Virus Neutralisation

Serial dilutions of heat inactivated antisera/lymph plasma (56°C, 30min) were incubated overnight at 4°C with 500 $TCID_{50}$ of EV1. The serum/virus mixture was then aliquoted into the first row of wells of a 96 well flat bottomed microtiter plate, seeded with 10^4 fibroblast cells (in DME/10% FCS) the previous day, and titrated, in doubling dilutions, across the plate. The plates were incubated for 2 hours at 37°C, 5% CO_2, after which the cells were washed once and refed with DME/2% FCS. After incubation for 7-10 days at 37°C/5% CO_2 the media was thrown off the culture and the cell monolayers were fixed for 5-10min in ice-cold acetone/methanol (1:1 mix). Fixed monolayers were then stained for 30min with 1% $K_2Cr_2O_7$ and destained for 5min with acetone/methanol. Counterstaining was for 5min with freshly made 50% Giemsa stain. Plates were destained for 1min with tapwater, dried and examined microscopically for the presence of cytopathic effect. Neutralisation of virus by serum was determined as a reduction in virus titre, as calculated by a quantal method (Reed & Muench, 1938). Antibody titres were defined as the greatest dilution of antisera which gave a 50% reduction in virus titre compared to controls (virus incubated with non-immune sera, or virus incubated without sera).

2.11.4 Antibody dependent cell-mediated cytotoxicity (ADCC)

a) Anti-visna ADCC

Prior to use in this assay test sera were heat-inactivated. 2×10^4 sheep epidermal fibroblasts were plated out in 96 well flat bottomed microtiter plates and incubated overnight at 37°C/5%CO_2. Cells were either mock infected or infected with visna at a multiplicity of infection of 1-2 $TCID_{50}$ per cell and incubated for 48hrs. Each well was then labelled by addition of 3µCi of $Na_2{}^{51}CrO_4$ (sp. act. 18-30mCi/mg ^{51}Cr) and overnight incubation. Monolayers were washed four times with RPMI/ 2%FCS, incubated with 100µl of dilutions of antisera, in RPMI/10% FCS and effector cells, PBMC, prepared as described in section 2.13.1, were added, in 100µl. Plates were incubated for varying times at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the cultures were centrifuged (200x **g**, 2min) and 100µl of supernatant were taken and counted in a gamma counter (LKB-Wallac 1274 Riagamma) for one minute. Assays were performed in triplicate for each dilution of antisera tested. Controls consisted of uninfected target cells, infected target cells incubated with medium alone, antibody with no effector cells, and effector cells alone.

Minimum ^{51}Cr release was taken from wells which had been incubated with effector cells, but no antibody. The cpm max. (total releasible ^{51}Cr) was determined by measuring the amount of radioactivity released in wells to which 1% Triton X-100 had been added in place of effector cells.

Results were expressed as the % specific lysis, calculated as below:

$$\% \text{ specific lysis} = \frac{\text{mean cpm test - mean cpm minimum}}{\text{mean cpm max. - mean cpm minimum}}$$

b) Anti-SRBC ADCC

Sheep red blood cells (SRBC) were prepared from freshly collected heparinised peripheral blood by washing twice with HBSS. For labelling, 5×10^6 or 10^7 SRBC in 0.2ml RPMI/ 10% FCS were incubated with 100µCi of $Na_2{}^{51}CrO_4$ for 1hr at 37°C. Target cells were then washed four times in RPMI/2% FCS before being resuspended in RPMI/10% FCS supplemented with 1mM sodium pyruvate, to minimise spontaneous ^{51}Cr release (Colsky &

Peacock, 1990). Assays were carried out in 96 well round-bottom microtiter plates. 50µl each of target cell (10^4 cells) and effector cell (10^6 cells) suspensions were added to each well and then 100µl of antiserum, diluted in RPMI/10% FCS, were added. The antiserum used was a polyclonal rabbit anti-SRBC antiserum (gift of Dr. J. Hopkins, Dept. Vet. Path., University of Edinburgh). This antiserum has been shown to mediate rosetting between antibody-coated SRBC and sheep macrophages (Gonzalez, 1989). Plates were incubated for 6-8hrs at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the cultures were centrifuged (200x **g**, 2min) and 100µl of supernatant were taken and counted in a gamma counter (LKB-Wallac 1274 Riagamma) for one minute. Assays were performed in triplicate for each dilution of antisera tested. Controls consisted of target cells incubated with medium alone, antibody with no effector cells, and effector cells alone.

Results were calculated as described in (a).

2.11.5 Antibody-Complement-mediated lysis

Target cells, both infected and uninfected were prepared as described in section 2.11.4. Cells were washed and incubated with dilutions of heat inactivated serum (100µl) and baby rabbit serum, 1:5 dilution (100µl), as a source of complement. After 1-3hrs incubation at 37°C/5%CO_2, 100µl of supernatant were taken and counted as above. Assays were performed in triplicate for each serum dilution tested.

Controls included cells incubated with medium alone; antibody, but no complement, and complement alone. Minimal release was taken from wells incubated with antibody, but no complement. Maximal release was taken from wells to which 1% Triton X-100 had been added. Results were calculated as described in 2.11.4.

2.12 Immunochemistry

Many of the methods used in the purification and preparation of immunoglobulins were based on protocols given in 'Immunochemistry in Practice' (Johnstone & Thorpe, 1987).

2.12.1 Coupling of protein to cyanogen bromide activated sepharose 4B

Ligands were coupled to CNBr activated Sepharose 4B (Pharmacia (Great Britain Ltd.), Bucks, U.K.) according to the protocol described in the Pharmacia guide to affinity chromatography (Pharmacia literature, 1983). Briefly, the required amount of CNBr-activated Sepharose 4B was washed and swollen on a sintered glass filter with 1mM HCl. Protein, in 0.1M $NaHCO_3$ pH 8.3 containing 0.5M NaCl, was added to the swollen Sepharose 4B and mixed for 2hrs at room temperature. To block remaining active groups the gel was washed into 1M ethanolamine pH 8 and mixed for 16hrs at 4°C. After blocking the gel was washed into BBS and stored at 4°C.

Before use the column was pre-eluted with 0.1M glycine pH 2.5 containing 0.5M NaCl and re-equilibrated in BBS.

2.12.2 Affinity chromatography

Sample was applied to the column and allowed to flow through slowly (approximately 20ml/hr). This was repeated and the column was then washed extensively with BBS (till no protein was detectable in the flow-through). The bound material was eluted by washing the column with 0.1M glycine-HCl pH 2.5, collected in 0.5ml fractions into 2M Tris HCl pH 9 (to neutralise the acid). Protein containing fractions were identified by spectrophotometry at 280nm. Peak fractions were pooled, dialysed against BBS, and stored at -20°C. The column was regenerated by extensive washing with BBS.

2.12.3 Purification of IgG

Rabbit, mouse and sheep IgG were purified from heat inactivated whole serum or ascites by affinity chromatography on either Protein-G Sepharose (Pilcher *et al*, 1991) or antigen-Sepharose columns.

2.12.4 Determination of immunoglobulin class and subclass of monoclonal antibodies

Immunoglobulin class and IgG subclass of mouse monoclonal antibodies were determined by double immunodiffusion analysis using class and subclass specific antisera (Serotec).

2.12.5 Conjugation of biotin to immunoglobulin

Purified immunoglobulin was biotinylated according to Bujdoso *et al*, (1991). To purified immunoglobulin, 1mg/ml in 0.1M $NaHCO_3$ pH 8.3, biotin-o-succinimide ester, in DMSO, was added in a ratio of 75μg biotin: 1mg immunoglobulin. This suspension was mixed immediately and incubated, with mixing, for 4hrs at room temperature. The biotinylated Ig was then dialysed extensively against BBS and stored, in aliquots, at -20°C.

2.12.6 Conjugation of Horse Radish Peroxidase (HRP) to IgG

Affinity purified IgG was conjugated to HRP by a modification of the method of Wilson & Nakane (1978). 4mg of HRP was dissolved in 1ml of distilled water, 0.2ml of freshly prepared 0.1M $NaIO_4$ was added and the solution stirred for 20min at room temperature. It was then dialysed overnight at 4°C against several changes of 1mM sodium acetate pH 4.4. The pH was raised to pH 9.5 by addition of 20μl of 0.2M sodium carbonate pH 9.5 and 8mg of immunoglobulin, in 1ml 0.01M sodium carbonate buffer pH 9.5. This was incubated for 2hrs at room temperature. 0.1ml of fresh $NaBH_4$ (4mg/ml in water) was added, to reduce free enzyme, and the mix left at 4°C for two hours. The peroxidase conjugated immunoglobulin was dialysed against several changes of BBS and stored at 4°C.

2.13 Cellular immunology

2.13.1 Preparation of sheep peripheral blood mononuclear cells (PBMC)

Sheep PBMC were prepared from peripheral venous blood taken into preservative free heparin (10U heparin/ ml blood). Buffy coat cells were obtained by centrifugation of heparinised whole blood (800x **g**, 20min) at room temperature. PBMC were prepared by centrifugation of the buffy coat, diluted in sterile PBS, over Lymphoprep (Nyegaard, Oslo, Norway) (800 x **g** applied to the interface for 20min). In some experiments PBMC were prepared by centrifugation of defibrinated blood over Lymphoprep. Sheep plasma, obtained from the buffy-coating step, was aliquoted and stored at -20°C.

2.13.2 Antigen specific lymphocyte proliferation assay

Freshly isolated PBMC were cultured in RPMI 1640 supplemented with 2mM glutamine, 100U/ml benzyl penicillin, 100U/ml streptomycin, 5×10^{-5}M 2-Mercaptoethanol and 10% FCS. 1×10^{5} PBMC were cultured in 96 well flat-bottomed micro-culture plates, in a final volume of 200µl, with the indicated concentrations of antigen. After 3-5 days, cultures were pulsed with 1µCi of tritiated methyl thymidine (^{3}H-TdR), specific activity 2µCi/mM, for five hours and then collected onto glass filter paper using a semi-automated cell harvester. ^{3}H-TdR incorporation was assessed by liquid scintillation counting in an LKB-Wallac 1218 Rackbeta. Cultures were set up in quadruplicate for each antigen concentration tested. Data is expressed as the geometric mean for the quadruplicate cultures (± one standard deviation).

2.13.3 Flow cytometry

Cell surface phenotype was assessed using aliquots of 10^{6} cells washed into ice-cold PBS containing 1mg/ml BSA, 0.1% sodium azide (PBA). Cells were incubated with 25µl of monoclonal antibody supernatant, or ascites (diluted in PBA) for 40min on ice. After three washes, 25µl of FITC-conjugated rabbit anti-mouse IgG were added for 20min on ice. Cells

were washed three times and resuspended in PBA. Staining was analysed by flow cytometry using a Becton-Dickinson FACScan.

2.13.4 Cell depletion

To deplete lymphocyte populations the method of magnetic cell sorting (MACS) was used (Miltenyi *et al*, 1990). Briefly, sheep PBMC were stained with the biotinylated monoclonal antibodies: SBU-T4 (anti CD4), SBU-T8 (anti-CD8) (Maddox *et al*, 1983) or an irrelevant, isotype-matched, control antibody (Sigma). The cells were washed once in PBS/5mM EDTA, incubated with streptavidin (Boehringer) for 15 min., washed again and then incubated with superparamagnetic biotinylated microbeads (Becton-Dickinson). Antibody stained cells were removed using a MACS cell sorter (Becton-Dickinson). Efficiency of cell depletion was monitored by flow cytometry.

2.14 Procedures involving experimental animals

All procedures involving experimental animals were carried out so as to comply with the regulations laid down in the Animals (Scientific Procedures) Act, 1986.

2.14.1 Mice

Balb/c mice were bred in the Department of Veterinary Pathology Animal House, University of Edinburgh.

2.14.1.1 Immunisation of mice for monoclonal antibody production

Mice were primed by subcutaneous injection of antigen, emulsified in Freund's complete adjuvant, in several sites. Subsequent immunisations were given by intraperitoneal injection of 10 - 20µg antigen in PBS at 2 - 3 week intervals. Mice were test bled 7 -8 days after each

booster immunisation. Three days prior to fusion mice were immunised by intravenous injection of 10μg of antigen in PBS.

2.14.1.2 Collection of mouse serum

Live mice were warmed under a heat lamp and then bled by removing a portion of the tail and collecting 100 - 200μl of blood. The blood was left at room temperature for 1hr to clot. The sample was then microfuged for 5min, the serum removed and stored, in aliquots, at -70°C.

2.14.1.3 Preparation of single cell suspension of mouse splenocytes

Mice were killed by cervical dislocation. The spleen was removed aseptically and teased apart in RPMI medium. Single cell suspensions were prepared by sedimentation of debris at 1x **g** for 5min. The cell suspension was then washed in RPMI and resuspended in medium.

2.14.1.4 Production of ascitic fluid

Six week old Balb/c mice were injected intraperitoneally with 0.5ml of pristane (tetramethylpentadecane). Seven to ten days later 10^7 hybridoma cells, in PBS, were injected intraperitoneally into each animal. Mice were inspected daily for signs of ascites formation. Ascitic fluid was tapped from the abdomen, using a wide gauge needle, centrifuged to remove cells and stored at -70°C. Mice were tapped twice only and then killed.

2.14.2 Rabbits

Rabbits (New Zealand White x Dutch) were bred in the Department of Veterinary Pathology Animal House, University of Edinburgh.

2.14.2.1 Immunisation of Rabbits

Rabbits were primed by deep intramuscular injection of 100µg antigen, emulsified in Freund's complete adjuvant, in two sites. Secondary immunisation was 100µg of antigen, in Freund's incomplete adjuvant, injected intramuscularly in two sites. Subsequent booster injections were administered by intravenous injection of antigen in PBS.

2.14.2.2 Collection of rabbit sera

Rabbits were bled from the lateral ear veins. Briefly, the ear over the vein was shaved and a spot of xylol applied to the ear tip. A single transverse incision was made through the lateral ear vein and blood collected into a sterile glass universal. The sample was left at room temperature to clot. After 1hr the clot was wrung and then left overnight at 4°C to allow the clot to contract. Clot free liquid was removed and the clot centrifuged (2500x **g**, 15min, 4°C). The expressed liquid was collected and pooled with the clot free serum. The pooled liquid was then centrifuged (1500x **g**, 15min, 4°C), the supernatant serum removed and stored, in aliquots, at -20°C.

2.14.3 Sheep

Finnish Landrace sheep (1-3yrs of age) were purchased from the Moredun Research Institute, Edinburgh. Infected and uninfected sheep were held, separately, in covered pens at the Moredun Research Institute.

2.14.3.1 Experimental infection of sheep with maedi-visna

Sheep were infected by intradermal and subcutaneous injection of clarified tissue culture medium containing 10^6 $TCID_{50}$ maedi-visna virus. The virus stock used for infection of sheep was minimally passaged *in vitro*.

2.14.3.2 Field cases of visna infection

Field cases of visna infected sheep were purchased from a farm where visna infection was endemic in the sheep flock.

2.14.3.3 Lymphatic cannulation

Lymphatic cannulations were performed by either Professor Ian McConnell or by Dr. John Hopkins (Dept. Veterinary Pathology, University of Edinburgh). Popliteal efferent lymphatics were cannulated as described by Hall & Morris (1962), prefemoral efferent lymphatics were cannulated according to Hall (1967). While cannulated, sheep were kept in standard pattern metabolism cages and fed hay and water *ad libitum*. Lymph was collected into sterile 250ml plastic bottles; containing 25000U each of penicillin and streptomycin, and 2500U of sodium heparin.

Chapter 3

Immune responses to maedi-visna virus in persistently infected sheep

A. Humoral immune responses

3.1 Introduction

Serum antibody is the classical marker of exposure to a virus and is believed to play an important role in the control of viral infections *in vivo.* Anti-viral antibody is thought to be of major importance in preventing the spread of viral infection by virtue of its ability to neutralise free virus in extracellular fluids (reviewed Daniels, 1975), however antibodies may also act against virus infected cells (reviewed in Sisson & Oldstone, 1980). Specific antibody may 'coat' the target cell expressing viral antigens at the cell surface allowing the attachment of cytotoxic cells, such as natural killer cells, monocyte/macrophages and neutrophils, via antibody-Fc receptor (FcR) interactions. Alternatively virus infected cells coated with IgM and/or IgG antibody may be rendered susceptible to complement-mediated lysis.

The ability of antibodies to mediate these effects; to interact with FcR-positive cells, or to activate complement, is dependent on the class and subclass of immunoglobulin produced in response to infection. The differing amino acid sequences of the constant region domains of the heavy chains of each Ig class and subclass imparts each subclass with distinct properties, most importantly in relation to the activation of biological effector mechanisms. This has been best studied for human and murine immunoglobulin G molecules (Burton *et al*, 1986., Burton, 1990), and is of particular interest when it is realised that antibody activity is not necessarily distributed evenly over the IgG subclasses: in humans, for example, antibodies to polysaccharide antigens, in bacterial infections, are usually of the IgG_2 isotype (Hammarstrom & Smith, 1986), whereas anti-viral antibodies are predominantly of the IgG_1 and IgG_3 subclasses (Skvaril, 1986). In mice viral infections mainly elicit specific antiviral, and polyclonal, antibodies of the IgG_{2a} and to a lesser extent IgG_{2b} subclasses (Coutelier *et al*, 1987., 1988., 1991).

In ruminants three major classes of immunoglobulin (Ig); IgM, IgA and IgG have been isolated and characterised (reviewed in Butler, 1983., 1986). Information on the physicochemical and biological properties of ruminant IgE is not available, although the gene encoding the IgE heavy chain constant region has been cloned (Knight *et al*, 1988). A ruminant IgD subclass has not yet been identified, but there is no reason to suppose that this molecule does not occur in ruminants.

Ruminant IgG molecules are comprised of two major subclasses, IgG_1 and IgG_2 (Butler, 1983), although there may be additional heterogeneity within the IgG_2 subclass (reviewed in Butler *et al*, 1987). $C\gamma$ genes encoding bovine IgG_1 and IgG_2 have been cloned (Knight *et al*, 1988) and two other $C\gamma$ genes identified, although it is not known whether these are expressed *in vivo*. Allotypic variants of both IgG_1 and IgG_2 have been described (reviewed Butler, 1986) and Capparelli *et al* (1982) have described an age-dependent allotype, present in both sheep and cattle, which they describe as associated with a third IgG subclass they designated IgG_3.

Some of the biological properties of ruminant immunoglobulins are summarised in Table 3.1. IgG_1 is the predominant immunoglobulin in serum, lymph, colostrum (it is responsible for the passive transfer of systemic immunity to the young ruminant) and at mucosal surfaces (reviewed in Butler, 1983., 1986). In general IgG_2 is found at lower concentrations, but, unlike IgG_1, considerable differences in IgG_2 concentration may occur between animals.

Extensive studies on the roles of the different IgG subclasses in immune responses of ruminants have not been carried out. It has been reported that immunisation of sheep with dinitrophenol conjugated to human γ-globulin elicits specific antibodies in both the IgG_1 and IgG_2 subclasses (Margni *et al*, 1973). Similarly rotavirus and coronavirus infections of calves elicit both IgG_1 and IgG_2 antiviral antibodies (Saif, 1987). Immunisation of sheep with pneumococcal polysaccharides or killed *Staphylococcus aureus* elicits predominantly IgM and IgG_1 antibodies (Emery *et al*, 1990., Kennedy & Watson, 1982), whereas live *S. aureus* vaccines induce mainly IgG_2 anti-staphyloccal antibodies and these are associated with protection from disease (Watson, 1984). Interestingly, it has been reported that a selective

Table 3.1

	IgM	IgG_1	IgG_2
Complement fixation			
Heterologous complement	+	+	-
Homologous complement	+	+	+
Heterologous PCA	ND	+	-
Homologous PCA	ND	+	+
FcR binding			
neutrophils	+[a]	-	+
fresh monocytes	+[a]	+/-	+
cultured monocytes	+[a]	+	+
alveolar macrophages	ND	+/-	+/-

a, in presence of added complement

PCA - passive cutaneous anaphylaxis

The information in the above table has been summarised from the following sources: Feinstein & Hobart (1969), Watson (1975), Micusan & Borduas (1977), Rossi & Kiesel (1977), McGuire *et al* (1979), Howard *et al* (1980), Yasmeen (1981) and Fleit *et al* (1986).

deficiency of IgG_2 in cattle is associated with an increased frequency of pyogenic bacterial infections (Nansen, 1972). The protective effects of IgG_2 antibodies against staphylococcal infections have been ascribed to their efficiency as opsonins promoting phagocytosis (Watson, 1987). It has also been reported that IgG_2 antibodies are 100 times more efficient than IgG_1 immunoglobulins at mediating antibody-dependent cytotoxicity (cited in Butler, 1983), but in general the functional significance of particular antibody classes and subclasses in immune responses of ruminants is unclear.

The antibody response to visna has mainly been studied in terms of virus-neutralising and complement-fixing antibodies (Gudnadottir & Palsson, 1966., Gudnadottir & Kristinsdottir, 1967., De Boer, 1970., Petursson *et al*, 1976). Sheep persistently infected with maedi-visna virus have been reported to make antibodies reactive with virtually all visna proteins (Lin & Thormar, 1979), but the pattern of development of these antibodies after infection and their persistence through the course of disease are unclear. Moreover knowledge of the antibody isotypes produced in response to infection with maedi-visna virus is confused. Petursson *et al* (1983), reported that antibodies to visna were mainly associated with the IgG_1 subclass and that they could not detect IgM or IgG_2 anti-MVV antibodies. In contrast, Mehta & Thormar (1974) could detect IgM visna-specific virus neutralising antibodies. CAEV infection of goats has been reported to elicit virus-neutralising antibodies in both IgG_1 and IgG_2 subclasses (McGuire *et al*, 1988).

In this chapter an analysis of the serum humoral immune response to visna virus is presented. In particular, the isotypes of the antiviral antibodies produced and the viral antigens recognised were analysed in detail. Subsequently experiments to assess some of the biological activities of anti-visna antibodies were carried out: in particular, the ability of anti-visna antibodies to direct lysis of virus-infected cells was investigated.

3.2 Results

3.2.1 Development of the antibody response to maedi-visna virus

A group of four sheep (1-2 years old) were infected with maedi-visna and bled at various intervals after infection. The anti-visna antibody titre was determined in ELISA assays using visna virus, purified on sucrose gradients, as antigen (Figure 3.1). By day 43 post-infection all the infected sheep had detectable anti-visna antibody titres, which increased gradually over the next 100 days.

Immunoblot analysis, against lysates of visna infected cells (Figure 3.2), showed that at the earliest time points at which anti-visna antibodies were detectable, both ***gag*** (p17 & p25) and ***env*** (gp135) specific antibodies appear to be present. At later time points antibodies to p25, p17 and gp135 still predominated, but the antibody response had broadened to include other viral proteins of approximate M_r 35, 38, 41 and 55kD. These reactivities probably include ***gag*** precursor proteins and processing intermediates and/or ***pol*** gene products, but the protein polypeptides of visna virus are insufficiently well characterised to allow definite identification of these bands as particular viral antigens.

The isotype of the anti-visna antibodies in plasma at these time points was then investigated in ELISA assays using mouse monoclonal antibodies specific for sheep IgG_1, IgG_2 and IgM (Beh, 1987., 1988) (Figure 3.3). From these results it can be seen that anti-visna antibody activity can be detected in both IgM and IgG_1 isotypes, but that IgG_2 anti-visna antibody is undetectable. A similar pattern of results was seen when the isotype of the anti-visna antibody response was investigated in immunoblot assays (data not shown., see Figure 3.4). It is not possible to compare the quantity of antibody between the different Ig classes because of inherent differences in assay sensitivity using MAbs with unique affinities.

Figure 3.1 Development of the antibody response to maedi-visna virus

Four Finnish-landrace sheep, 1-2yrs old, were infected with maedi-visna by intradermal and subcutaneous injection of 10^6 $TCID_{50}$ of virus. Sheep were bled, by jugular venipuncture, at the times indicated and plasma was collected and stored at -20°C until assayed for anti-visna antibody.

For assay of anti-visna antibody in plasma samples, purified visna virus (200ng/ml in PBS) was coated onto flexible Falcon PVC plates by overnight incubation at 4°C. After washing in BBS/0.05% Tween 20, the wells were blocked by incubation with BBS/1% BSA (200µl/well) for 1hr at 37°C. Plasma samples, diluted in BBS/BSA/ 0.05% Tween 20, were titrated in doubling dilutions starting from a 1/10 dilution. After 1hr incubation at 37°C, the plates were washed in BBS/Tween and then incubated for a further 1hr with a 1/4000 dilution of rabbit anti-sheep Ig conjugated to HRP (Dako) in BBS/Tween/BSA before washing and developing the plates with OPD as substrate.

Antibody titre is expressed as the reciprocal of the highest dilution of plasma which gave an OD_{492} twice that of plasma collected from each sheep prior to infection.

Figure 3.1

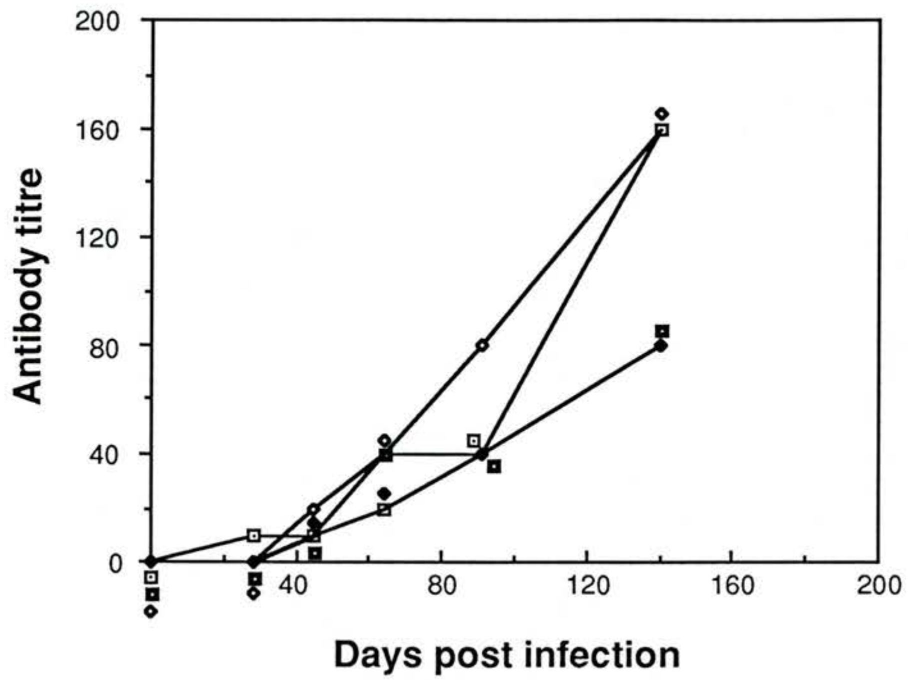


Figure 3.2 Specificity of anti-visna antibody in developing immune response

See Figure 3.1 for experimental details. Visna antigen, prepared as described in section 2.8.1 was separated on 5-20% linear gradient SDS-PAGE, run under reducing conditions, and electroblotted onto a nitrocellulose membrane. Strips, cut from these blots, were incubated overnight with pre- and post-infection plasma (days post infection are shown below strips), diluted 1:10 in PBS/5% non-fat dried milk. Blots were developed using affinity-purified anti-sheep immunoglobulin conjugated to alkaline-phosphatase (Sigma). Positions of molecular weight standards are indicated.

The results shown, obtained by probing blots with sera from sheep 1540H and 848A, are representative of the results seen with all four sheep. The bands seen at 17kD and 28kD are probably the ***gag*** proteins p17 and p25. The bands seen at around 135kD are probably the outer membrane envelope glycoprotein gp135.

The smearing seen at around 70kd is not visna specific since it is seen when blots are probed with sera from non-visna infected sheep (data not shown).

Figure 3.2

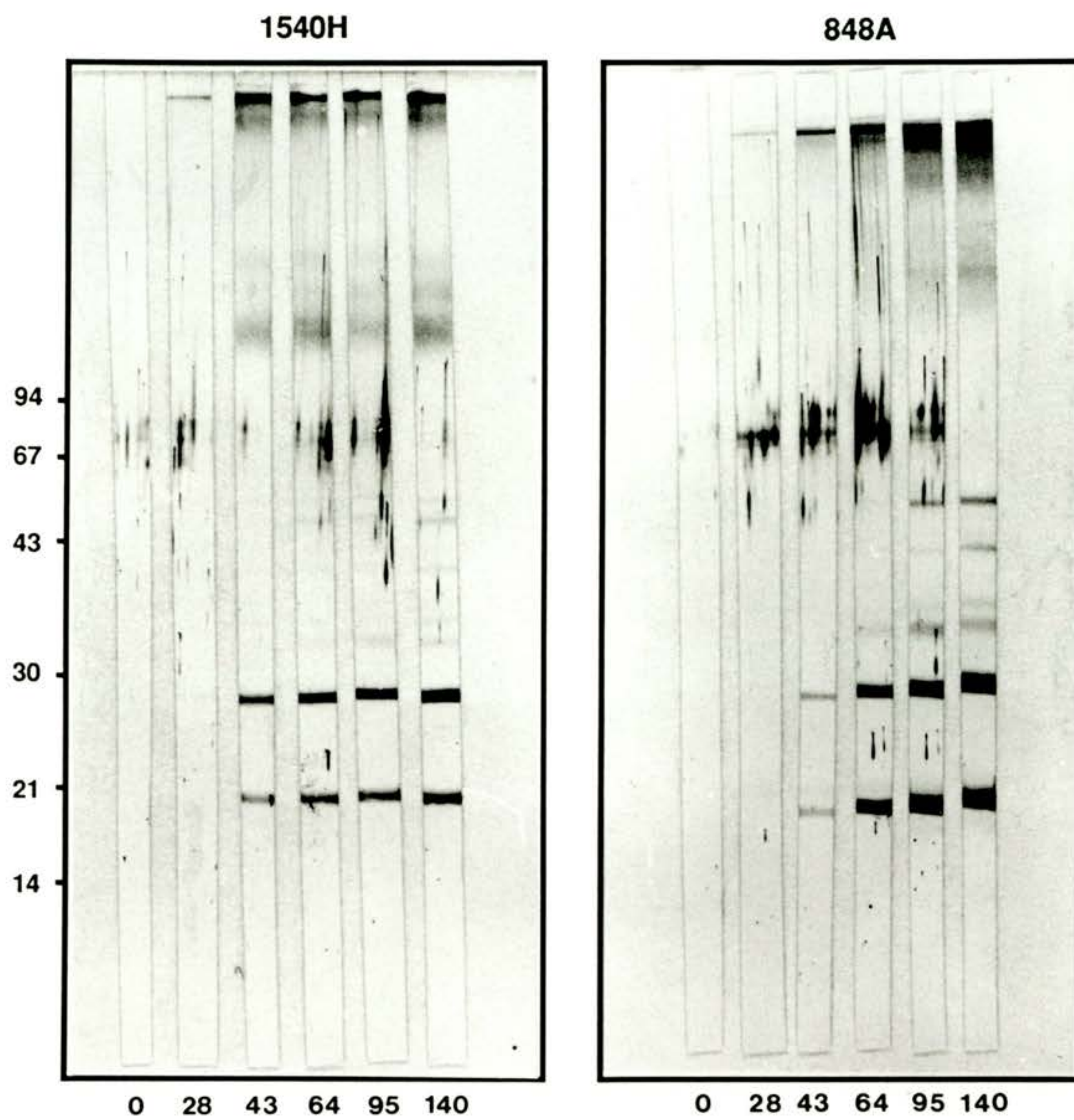
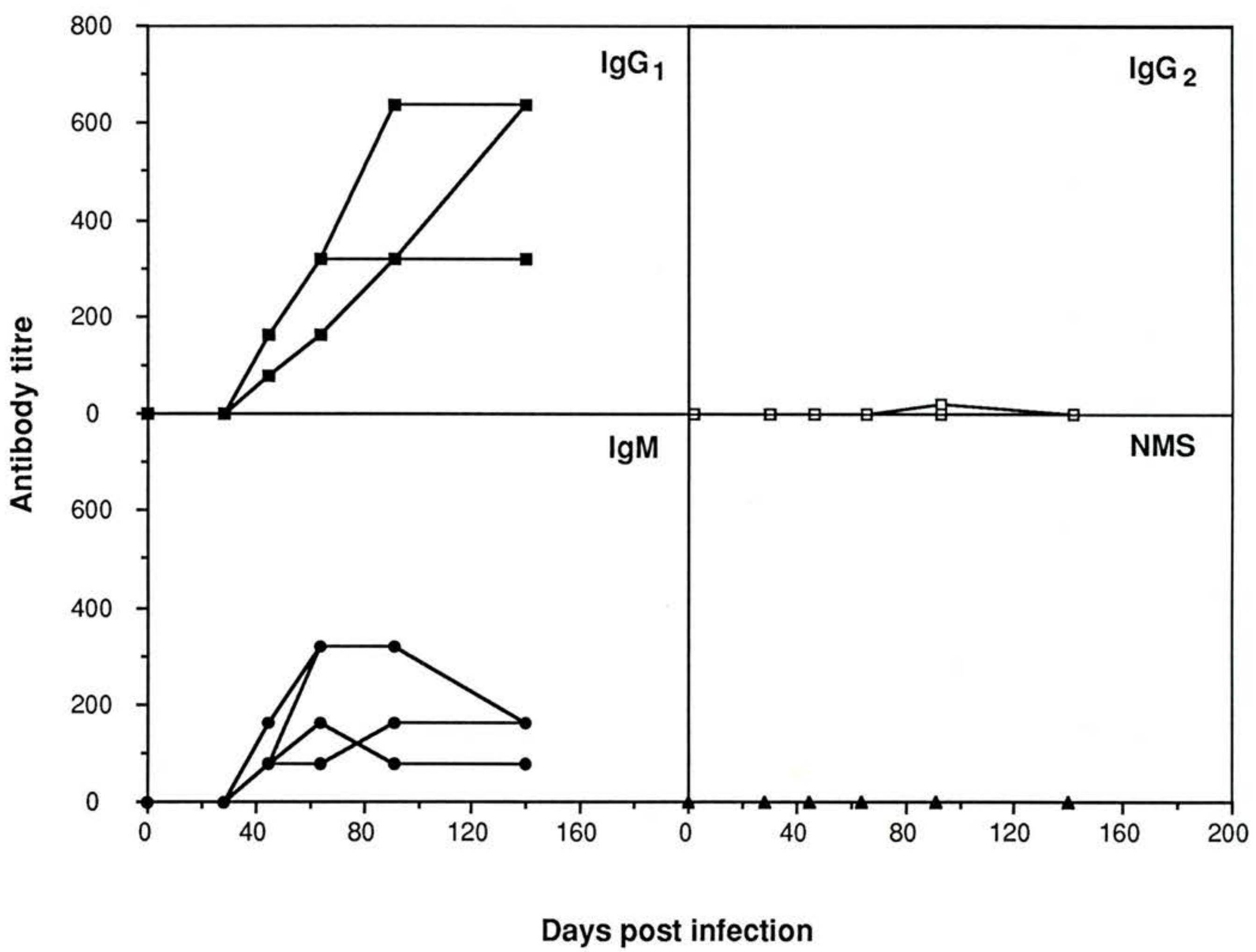


Figure 3.3 Analysis of the isotypes of anti-visna antibody in the developing immune response

For assay of individual Ig class and subclass the plasma samples were incubated on ELISA plates coated with sucrose-purified visna as in Figure 3.1. After incubation with sheep plasma, the plates were washed and incubated with mouse monoclonal antibodies specific for the various sheep Ig class and subclasses (gifts from Dr. K.J. Beh, CSIRO, Sydney). These monoclonal antibodies were used as ascites fluids diluted 1/500 - 1/2000 in BBS/BSA: McM1 (anti-$\gamma 1$ - 1/2000), McM3 (anti-$\gamma 2$ - 1/500), McM9 (anti-μ - 1/1000). The negative control was normal mouse serum diluted 1/500 in BBS/BSA. After washing, the plates were incubated with a 1/4000 dilution of rabbit anti-mouse IgG conjugated to HRP, preabsorbed against sheep Ig, in BBS/BSA for 1hr at 37°C, and developed with OPD as substrate.

Antibody titres are expressed as the reciprocal of the highest dilution of plasma which gave an OD_{492} reading twice that of plasma collected pre-infection.

Figure 3.3



3.2.2 Antibody responses in persistently infected sheep

The failure to detect anti-visna antibody activity in the IgG_2 subclass was an unexpected result and so this aspect of the immune response was investigated further. It has been reported that, in sheep, antigen-specific IgG_2 antibodies develop later in immune responses than antibodies of the IgG_1 subclass (English *et al*, 1976). Thus the pattern of anti-visna antibody isotypes was investigated in persistently infected sheep. These were either sheep which had been experimentally infected with maedi-visna for periods of 1-3 years or naturally infected animals. The results obtained from both these groups were similar and so the data from both groups have been pooled.

The isotypic restriction of anti-visna antibodies was analysed in immunoblotting experiments using virus-infected cells as antigen. Representative sets of immunoblots from six sheep are shown (Figure 3.4). These blots confirm that antibodies to visna virus antigens, including envelope glycoproteins, are not present in the IgG_2 subclass. Interestingly, these data also suggest that IgM antibodies to the viral envelope glycoproteins may not be present. In HIV infection IgM anti-HIV has been reported to be restricted to anti-p24 and anti-p17 activity (Bedarida *et al*, 1986., Joller-Jemelka *et al*, 1987), although others have suggested that there may be a weak IgM anti-***env*** response (Gaines *et al*, 1988). Analysis of a larger number of sera in ELISA assay against purified visna confirms that antibodies to visna virus are detectable only in the IgG_1 subclass (Figure 3.5).

To show that these mAb's could detect IgG_2 antibodies in immunoblotting assays, the antibody isotypes produced in response to Orf virus, a parapox virus infection of sheep, were investigated (Figure 3.6). It can be seen that sheep infected with this virus do make IgG_2 antibodies to Orf antigens, although the IgG_1 response is predominant. This experiment also demonstrates that restriction of the IgG antibody response to the IgG_1 subclass is not a general feature of ovine antiviral immune responses.

Figure 3.4 Immunoblot analysis of the isotypic restriction of the anti-visna antibody response

Immunoblots of visna antigen were prepared as described in Figure 3.2. Blots were incubated with sheep serum (1/10) in quadrupicate, after washing each strip was then probed with either McM1 (1/2000), McM3 (1/500), McM9 (1/1000) or normal mouse serum (1/500) and developed using affinity purified anti-mouse immunoglobulin conjugated to alkaline phosphatase.

Representative blots from six sera; GO30, GO27, GO94, YT44, YT41 and GO90, are shown. These are all naturally infected animals. Serum samples from experimentally infected animals showed a similar pattern of reactivity (data not shown).

Figure 3.4

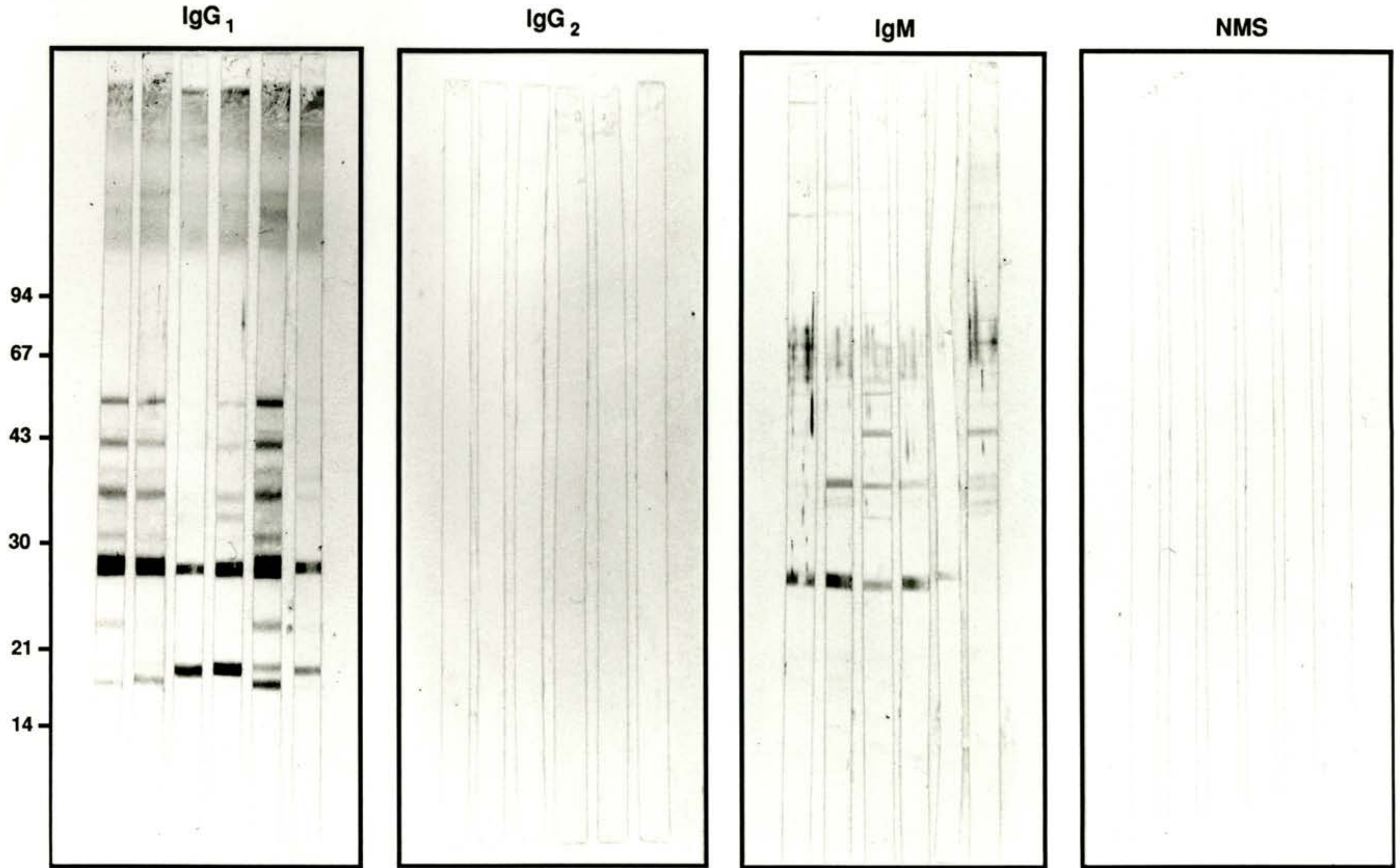


Figure 3.5 Isotypic restriction of the anti-visna antibody response in sheep persistently infected with maedi-visna virus

Sera from sheep infected with visna virus for periods of at least 1yr, were assayed for anti-visna antibody of either IgG_1, IgG_2 or IgM isotypes by ELISA assay as described in the legend to Figure 3.3.

Figure 3.5

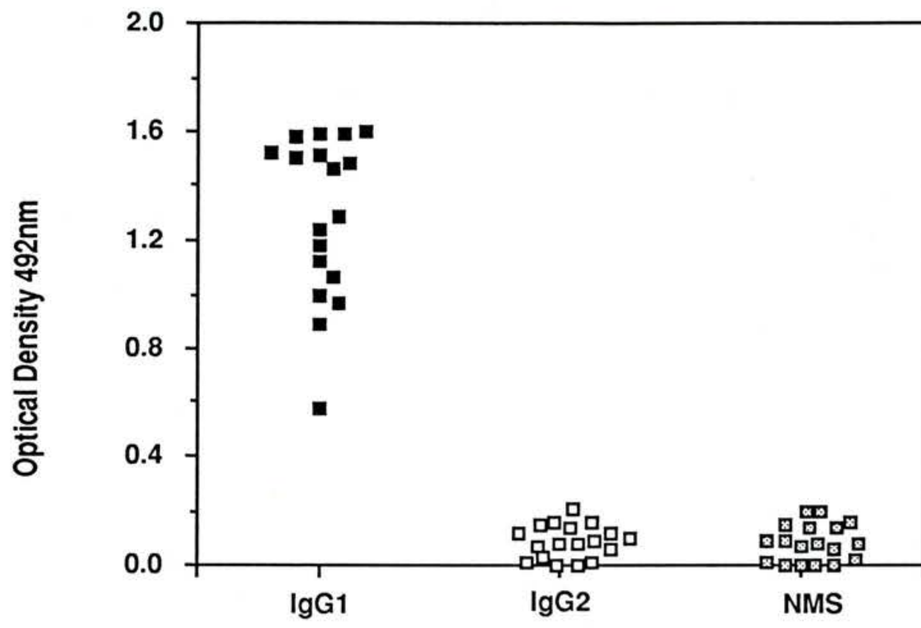


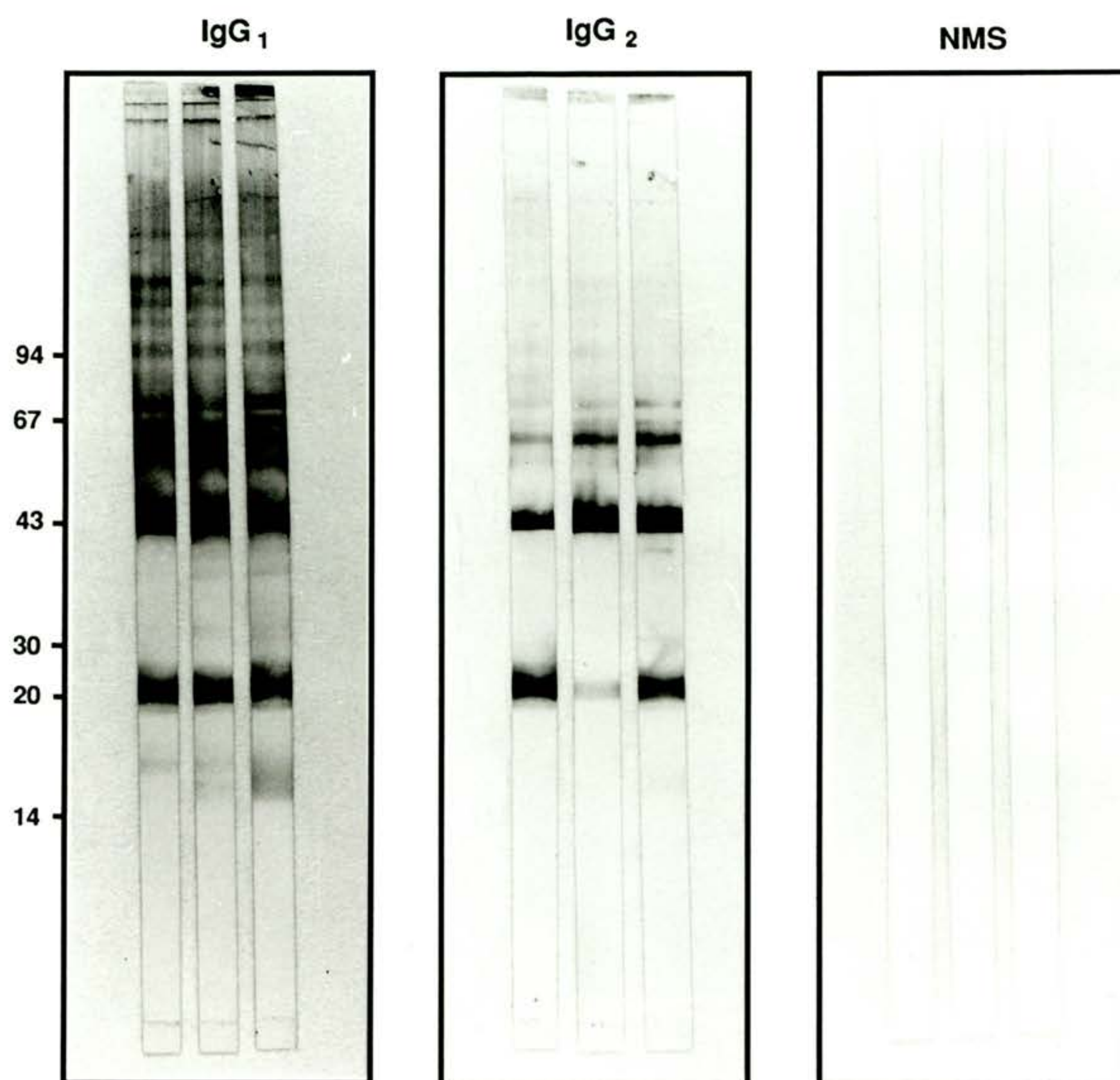
Figure 3.6 Analysis of the antibody isotypes produced in response to Orf virus infection

Orf virus antigen, purified from scabs from infected sheep by density gradient centrifugation, was separated on 5 - 20% SDS-PAGE and immunoblotted onto nitrocellulose membranes. Strips, cut from these blots, were probed with sera collected from three Orf infected sheep (diluted 1/50, from left to right: 1195P, 1196P & D573) and then the isotype specific monoclonal antibodies. Blots were developed as described previously.

Purified virions of Orf virus have been shown to contain at least 30-40 polypeptides (Thomas *et al*, 1980., Buddle *et al*, 1984) and at least sixteen viral antigens are recognised by sera from experimentally infected animals (McKeever *et al*, 1987). The major Orf virus protein recognised by sera from infected sheep is a polypeptide of 40-45kD (McKeever *et al*, 1987). This protein is believed to be a major component of the surface tubule-like structures on the virion (Thomas *et al*, 1980., Buddle *et al*, 1984., McKeever *et al*, 1987). Other viral proteins, of M_r 16.5, 22.5, 25.5, and 64kD are also believed to be components of the virion surface (Thomas *et al*, 1980), but the functions and/or structural roles of these proteins are unknown.

Both the Orf antigen and anti-Orf sera were gifts of Drs. Hugh Reid & D. Yirrell, Moredun Research Institute, Edinburgh & Dept. Medical Microbiology, University of Edinburgh.

Figure 3.6



3.2.3 Action of anti-visna antibody against virus infected cells

Plasma from visna-infected sheep was examined for the ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (Figure 3.7). Plasma from visna infected sheep (n=32) did not exhibit ADCC in this assay. A number of variables, including effector:target cell ratio, amount of anti-visna antibody and assay incubation time were modified to try and optimise the assay, but no ADCC activity could be demonstrated. Similar results were seen whether the PBMC effector cells were obtained from either visna infected or uninfected sheep. Culture of PBMC with IL-2 prior to use in assay, an attempt to promote NK/LAK cell activity (reviewed in Trinchieri, 1989), resulted in increased levels of lysis, but this was not virus specific (data not shown). The data shown in Figure 3.7A are representative of several assays performed over a 6-8hr incubation period. In some experiments target cells, antibody and effector cells were incubated together for 18-20 hrs, but again no visna-specific ADCC activity was detected (Figure 3.7B). However, levels of spontaneous ^{51}Cr release by target cells were high (30%) in these 20hr assays and it is possible that this could have masked low levels of activity.

There were a number of possible explanations for the failure to detect anti-visna ADCC activity in these assays and so a series of control experiments were carried out.

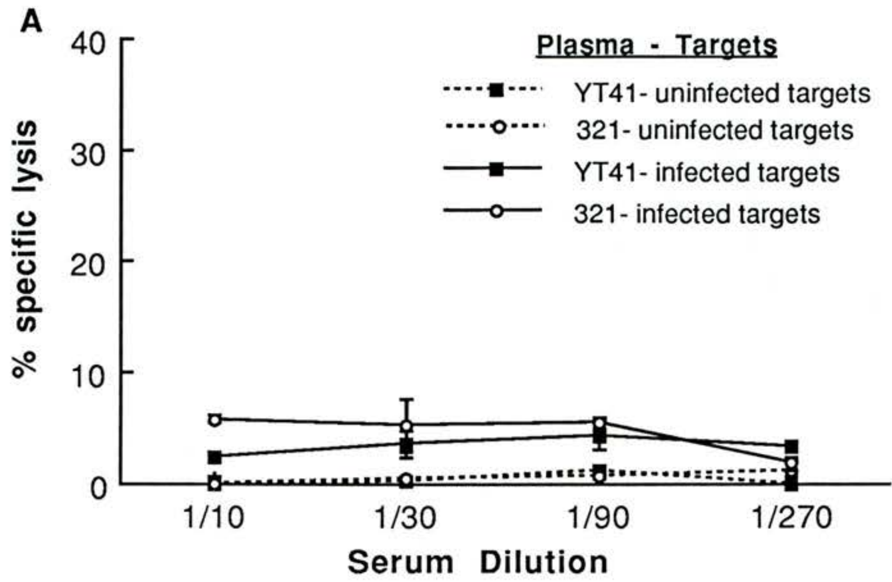
The target cells were confirmed as infected and producing viral antigens by immunofluorescence assays (Figure 3.8). Cell-surface expression of viral antigens and specific binding of anti-visna antibody to the target cells was demonstrated in antibody-dependent complement-mediated cytotoxicity (ACC) assays. In these experiments visna-infected and control, uninfected target cells were prepared and incubated with antibody as in the ADCC assays,however a source of complement, baby rabbit serum, was added instead of sheep PBMC. Results from such an experiment are shown in Figure 3.9.

These experiments show that viral antigens are expressed at the surface of the infected cell and that antibody in plasma from visna infected sheep is capable of binding to these antigens. That the cells can then be lysed by addition of complement

Figure 3.7 Plasma from visna-infected sheep do not mediate visna-specific ADCC

Plasma from visna infected sheep (YT41 and GO30) and uninfected sheep (321) were tested for activity in ADCC assays at the indicated dilutions. Effector cells were PBMC freshly isolated from uninfected sheep, used at an effector:target cell ratio of 100:1. Target cells were a skin cell line, infected with visna 72hrs previously at a multiplicity of infection of 2 $TCID_{50}$/cell, and radioactively labelled by overnight incubation with ^{51}Cr . Assays were incubated for 6hrs (A) or 18hrs (B) and the ^{51}Cr release determined. Minimum ^{51}Cr release into the supernatant was 8% (uninfected cells) and 11.2% (visna infected cells) in A; 20% (uninfected) and 30% (visna infected) in B. Incubation of target cells with PBMC, but without antibody gave levels of lysis comparable with target cells incubated with medium alone. Error bars show one standard deviation from the mean experimental values.

Each graph shows representative results from one experiment.


A
Plasma - Targets
YT41- uninfected targets
321- uninfected targets
YT41- infected targets
321- infected targets
% specific lysis
40
30
20
10
0
1/10
1/30
1/90
1/270
Serum Dilution

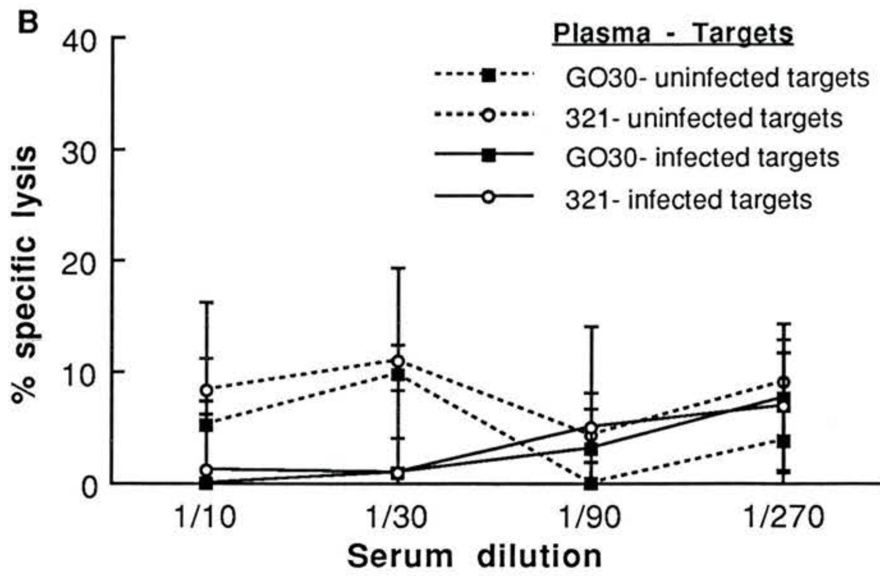
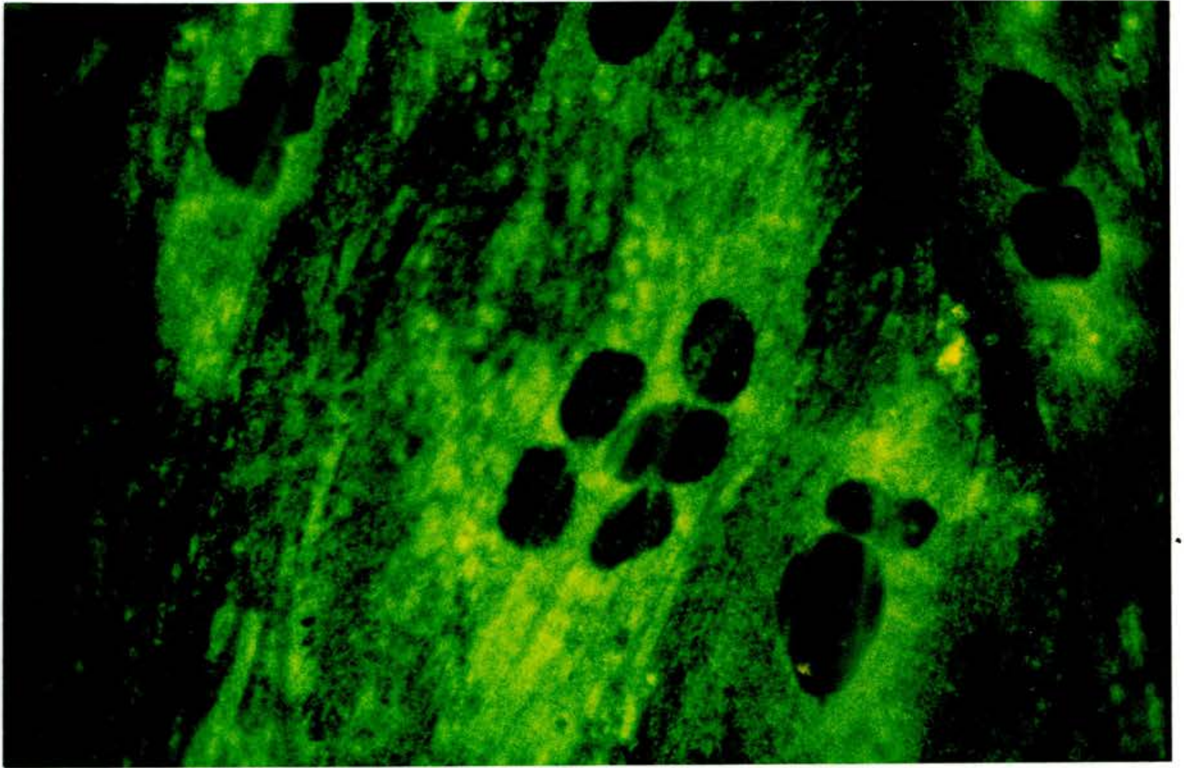

B
Plasma - Targets
GO30- uninfected targets
321- uninfected targets
GO30- infected targets
321- infected targets
% specific lysis
40
30
20
10
0
1/10
1/30
1/90
1/270
Serum dilution

Figure 3.8 Immunofluorescence analysis of visna-infected fibroblasts 72hr post-infection

Fibroblasts, mock-infected and infected with visna virus at a multiplicity of infection of $2TCID_{50}$/cell (as in Figure 3.7), were fixed in 80% acetone/0.15M NaCl and stained as in the methods. A shows the staining pattern observed when infected cells were stained with a rabbit antiserum to p25, the major core protein of maedi-visna virus (for characterisation of antiserum see chapter 5). B shows the pattern observed when infected cells were stained with non-immune rabbit antiserum. Both prints were exposed for the same length of time (30s). Mock-infected cells stained with the anti-p25 antiserum showed similar fluorescence to B. Magnification for both photographs is **x**1,714.

Figure 3.8

A



B

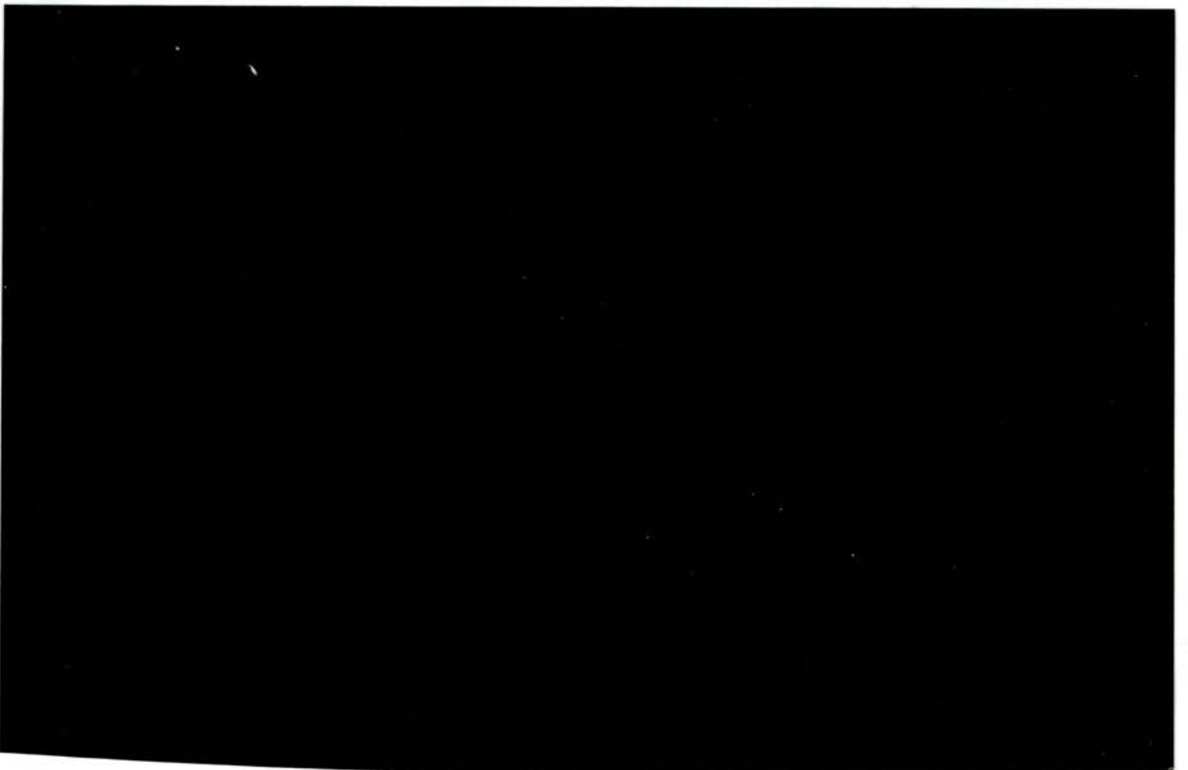
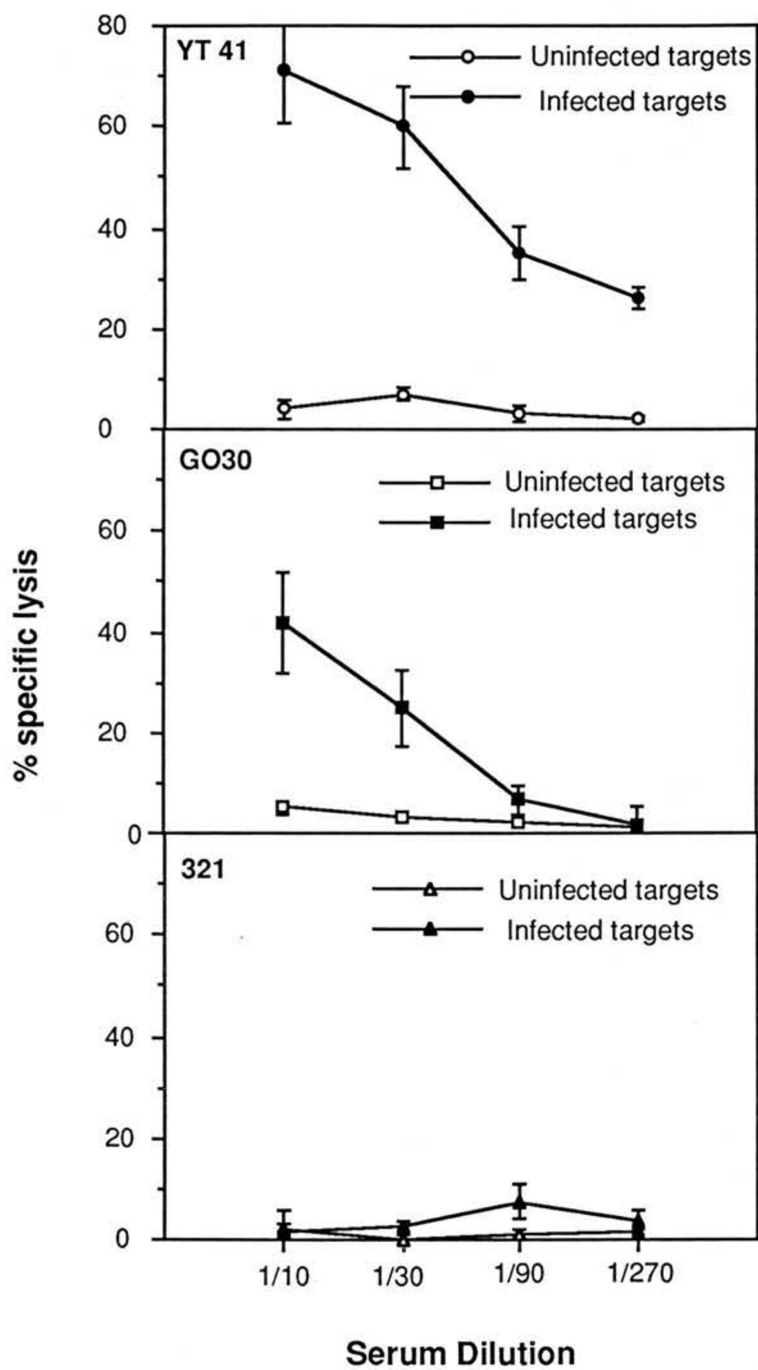


Figure 3.9 Plasma from visna infected sheep mediate ACC

Target cells prepared as described in Figure 3.7 were incubated with serial dilutions of plasma from visna infected sheep, YT41 & GO30, and uninfected sheep 321 in a final volume of 100μl. Rabbit complement, 100μl of a 1/5 dilution of rabbit serum, was then added. Assays were incubated for 3hrs and the ^{51}Cr release determined. Spontaneous release of ^{51}Cr into the supernatant was 6.4% (uninfected cells) and 12.4% (visna infected cells). Incubation of target cells with antibody alone or complement alone gave levels of lysis similar to those seen when targets were incubated with medium alone. Error bars show one standard deviation from mean experimental values.

A and B show results obtained when plasma from visna seropositive sheep were used in these assays, C shows the levels of lysis observed when control, visna-seronegative plasma was used to sensitise target cells.

Figure 3.9



suggests that capping of viral antigens subsequent to antibody binding (reviewed in Sissons & Oldstone, 1980) is not occurring in these assays. This experiment also suggests that target antigen density on visna infected cells is unlikely to be a major problem in these assays since in measles virus systems complement-dependent lysis requires approximately 10-fold more antibody to bind to the target cell than is needed to confer susceptibility to ADCC attack (Sissons & Oldstone, 1980).

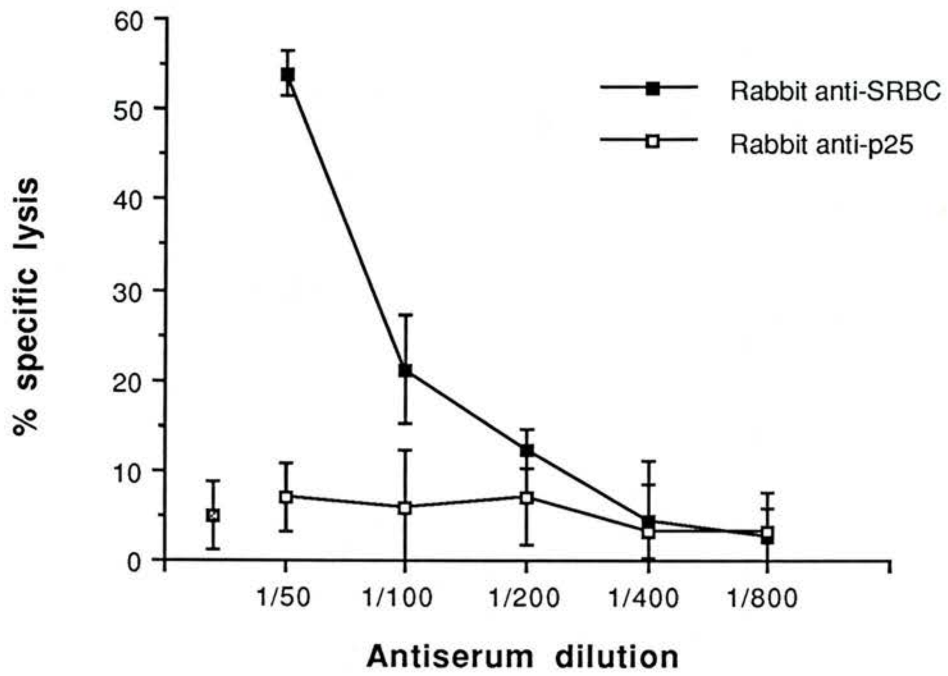
Another possible explanation for the failure to detect visna-specific ADCC in these assays was that the PBMC preparation did not contain cells capable of mediating ADCC. To test the killing ability of the PBMC population usd here, the efficiency of PBMC in ADCC assays against antibody-coated erythrocytes was investigated (Figure 3.10). This experiment demonstrates that ovine PBMC are capable of mediating ADCC, at least against RBC. Other workers have reported that mononuclear cells isolated from bovine peripheral blood are capable of lysing bovine herpesvirus infected cells (Rouse *et al*, 1976). Sheep PBMC have been shown to be capable of mediating antibody-dependent lysis of murine mastocytoma cells (Grant *et al*, 1975).

It has been shown that, in ruminants, neutrophils and monocyte-macrophages are the major effector cell populations in ADCC assays against either erythrocytes or virus-infected cells (Rouse *et al*, 1976., Grewal *et al*, 1977., Grewal & Rouse, 1979). Flow cytometric analysis of the PBMC preparations used in these experiments was carried out in order to obtain some evidence of the proportions of monocyte/macrophages and myeloid cells in these populations (representative data is shown in Table 3.2). Monoclonal antibody D9F has been suggested to recognise the ovine analogue of FcγRIII (Gonzalez, 1988). In humans expression of this molecule in the periphery is restricted to macrophages, PMN's and natural killer cells (reviewed Ravetch & Kinet, 1991). As yet, no biochemical characterisation of the antigen recognised by MAb M175 has been reported, but its expression has been reported to be restricted to myeloid and erythroid cells (Miyasaka *et al*, 1986), however recently this antibody has been shown to stain a small proportion of $CD4^+$ and $CD8^+$ lymphocytes

Figure 3.10 ADCC activity of sheep PBMC against antibody coated erythrocytes

10^4 sheep RBC, labelled with ^{51}Cr, were incubated with the indicated dilutions of either rabbit anti-SRBC or rabbit anti-p25 antiserum and 10^6 autologous PBMC. Assays were incubated for 6hrs and the ^{51}Cr release determined. Spontaneous release of ^{51}Cr into the supernatant was 3.3%.

Figure 3.10



SRBC incubated with Rabbit anti-SRBC (1/50), but without effector cells

Table 3.2

MAb (Ref)	Antigen	% positive cells
SBU-T4 (1)	CD4	27.6
SBU-T8 (1)	CD8	27.7
VPM 13 (2)	μ heavy chain	17.7
VPM 8 (2)	sIg (light chain)	34.1
M 175 (3)	myeloid/erythroid cells	39.7
D9F (4)	FcγRIII	26.2
VPM 19 (5)	MHC Class I	100

The above percentages are determined by reference to cells stained with normal mouse serum. The percentage of positively stained cells using this control was less than 1%.

Cells were analysed by FACS using a Becton-Dickinson & Co. FACScan system. 20,000 cells were analysed per sample with dead cells excluded on the basis of forward light scatter.

1. Maddox *et al*, 1985
2. Bird *et al*, *in preparation*
3. Miyasaka *et al*, 1986
4. Gonzalez, 1988
5. Hopkins & Dutia, 1990

(Dr. P. Bird, *personal communication*). This small amount of lymphocyte staining probably accounts for much of the difference between the percentages of D9F and M175 staining cells. These data indicate that the PBMC's used in these assays contained around 20-25% monocytes. No specific antibody reagents defining polymorphonuclear neutrophils (PMN's) are available for the sheep, however it has been reported that PBMC's prepared by gradient centrifugation commonly contain 2-4% PMN's (Grewal & Rouse, 1979).

B. T cell responses

3.3 Introduction

In the majority of viral infections studied T cell mediated immune responses are believed to be of prime importance for the clearance of viral infection (Mims & White, 1984), however the significance of T cell immunity in lentiviral infection is unclear. Individuals infected with HIV develop strong antibody and CTL responses to the virus (Rosenberg & Fauci, 1989) and yet invariably HIV infection progresses to AIDS and death, indicating that these responses are in in some way insufficient for clearance. It has been demonstrated in a murine retrovirus system that both $CD4^{+}$ and $CD8^{+}$ immune T cells are required to confer immunity to retroviral infection (Hom *et al*, 1991), yet in natural HIV infection virus-specific $CD4^{+}$ T-helper cell responses appear to be specifically deficient in both prevalence and strength (Wahren *et al*, 1987., McChesney & Oldstone, 1989).

Cell-mediated immune responses to visna virus in infected sheep have not been well characterised. Griffin *et al* (1978) reported that sheep experimentally infected with visna virus made only a transient cell-mediated immune response to visna virus. However this study was terminated after only three months. Other workers reported that cellular responses to visna virus antigen could be detected intermittently over a three year period following experimental infection (Larsen *et al*, 1982*b*., Sihvonen , 1984). The data of Larsen *et al* (1982) should be interpreted only cautiously since not all of the challenged animals

seroconverted after exposure to virus and virus could be isolated from only a few of the 'infected' animals. None of the experimental sheep developed any clinical evidence of disease during the experiment and post-mortem examination for evidence of development of pathological lesions was not carried out. Interpretation of the study reported by Sihvonen (1984) is also complicated by the fact that not all the sheep challenged with visna virus subsequently seroconverted, however a visna-like virus could be reisolated from these animals. None of these studies made any attempt to characterise either the viral antigens eliciting proliferative responses or the lymphocyte populations responding in these assays.

The first experiment described here is an investigation of whether sheep persistently infected with visna virus mount lymphoproliferative responses to purified virus. Following this, recombinant viral core protein (see Chapter 5) was tested as antigen in lymphoproliferation assays, and the phenotype of the the lymphocytes responding in these assays investigated.

3.4 Results

3.4.1 Lymphoproliferative responses in persistently infected sheep

The occurrence of T-cell immune responses in sheep persistently infected with maedi-visna for periods of 1-3 years was investigated using *in vitro* lymphocyte proliferation assays. PBMC from visna infected and control, uninfected sheep were cultured in either the presence or absence of visna virus purified by centrifugation through sucrose gradients and lymphoproliferative responses measured by tritiated thymidine (^{3}H-Tdr) incorporation. Representative results of such an experiment are shown in Table 3.3.

These data document the occurrence of virus-specific lymphocyte proliferative responses in sheep persistently infected with visna virus. Material prepared from uninfected skin cell cultures did not stimulate proliferation of lymphocytes from either visna infected or uninfected sheep (data not shown). Levels of antigen-specific proliferation fluctuated slightly between assays, but were always detectable. The observed variation in responsiveness between individual sheep is to be expected in an outbred population.

Table 3.3

Visna status	Sheep No.	Response (cpm [^{3}H]-Tdr ± SD) to Medium	Visna (S.I.)
Infected	90	4660 ± 537	13679 ± 1854 (3.4)
	91	3910 ± 126	22472 ± 2967 (5.7)
	92	1180 ± 332	6676 ± 554 (5.6)
	93	2317 ± 134	38764 ± 2760 (16.7)
Uninfected	738	1003 ± 361	566 ± 363 (0.56)
	739	873 ± 219	843 ± 366 (0.97)

PBMC's from both visna infected and uninfected sheep, prepared by differential centrifugation over 'Lymphoprep', were cultured with either medium alone or sucrose-gradient purified EV1 antigen (5μg/ml) for 5 days.

Lymphocyte proliferation was assessed by measuring ^{3}H-thymidine (^{3}H-Tdr) incorporation over the last five hours of culture. Data is expressed as cpm. ^{3}H-Tdr incorporated ± one standard deviation. S.I. = (*mean cpm. antigen stimulated cultures/mean cpm. unstimulated culture*).

To begin to define the antigens recognised by these proliferating lymphocytes the major core protein of maedi-visna virus, p25, was expressed (see Chapter 5) and the antigenicity of the recombinant protein tested in lymphoproliferation assays. p25 protein induced *in vitro* lymphocyte proliferation of PBMC from visna infected sheep in a dose dependent fashion (Figure 3.11). A control antigen preparation, the supernatant from ultracentrifuged, 'mock-digested' Ty-p25 (see Chapter 5) did not stimulate proliferation (Figure 3.11), nor did an irrelevant protein antigen such as ovalbumin (data not shown).

Proliferative responses to p25 (Stimulation Index > 2) could be detected in visna infected sheep (n=12), but not in uninfected controls (Figure 3.12). Several individuals proliferated only weakly to p25, but these responses were consistently detected in these sheep (at least three separate experiments). Statistical analysis, Wilcoxon's Rank Sum test for unpaired data, indicated that the level of proliferation of even the weakest responder is highly significantly different from uninfected controls ($P < 0.01$). No simple correlation was observed between the level of proliferation of an individual sheep to whole virus and to p25, probably reflecting the fact that in an outbred population differences exist between individuals in the number of T cells specific for individual viral antigens.

To define the lymphocyte subpopulations responding to viral antigens in these assays, lymphocyte depletion experiments were carried out. Using mouse monoclonal antibodies to the ovine homologues of the CD4 and CD8 molecules (Maddox *et al*, 1985), PBMC from visna infected sheep were selectively depleted of either $CD4^+$, $CD8^+$, or $CD4^+$ and $CD8^+$ T cells using a magnetic activated cell-sorter system (Miltenyi *et al*, 1990). Efficiency of depletion was over 95%, as monitored by flow cytometry. The anti-CD4 and anti-CD8 monoclonal antibodies are of the γ_{2a} isotype and so as a control PBMC were mock-depleted using an irrelevant γ_{2a} mouse monoclonal antibody (UPC 10). The various depleted and mock-depleted populations were then assayed for responsiveness to viral antigen, either purified virus or recombinant p25. A similar pattern of results was observed when either purified visna virus or recombinant p25 protein was used as antigen and so only the p25 response data are shown (Table 3.4).

Figure 3.11 Lymphocytes from visna infected sheep proliferate in response to p25 antigen in a dose dependent fashion.

PBMC's from an infected sheep were cultured with the indicated concentrations of either p25 or a control antigen preparation for five days. The control antigen preparation shown here is the supernatant obtained after ultracentrifugation of 'mock Factor Xa-digested' Ty-p25 VLP's.

Lymphocyte proliferation was assessed by measuring ^{3}H-thymidine (^{3}H-Tdr) incorporation over the last five hours of culture. Data is expressed as cpm. ^{3}H-Tdr incorporated ± one standard deviation.

Figure 3.11

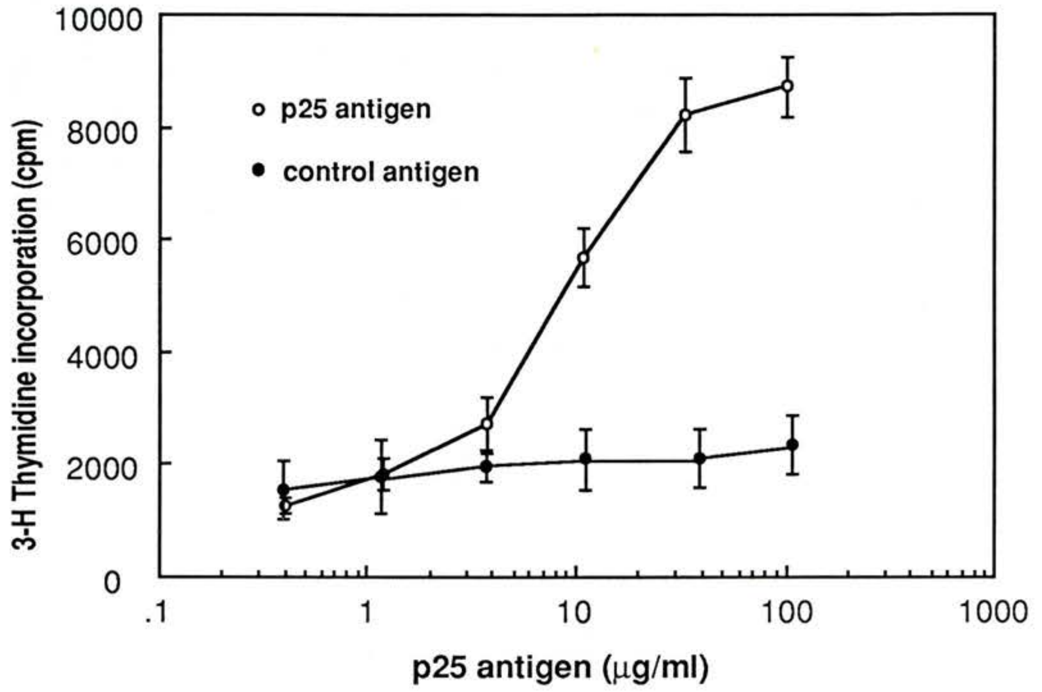


Figure 3.12 Proliferative responses of sheep PBMC's to recombinant p25

PBMC's from uninfected or maedi-visna infected sheep were cultured for 5 days with an optimal dose of purified recombinant p25 (15µg/ml). Cultures were then pulsed with ^{3}H-Tdr for 5 hours and harvested. Data is expressed as stimulation indices: S.I. = (*mean cpm. antigen stimulated cultures/mean cpm. unstimulated culture*).

Figure 3.12

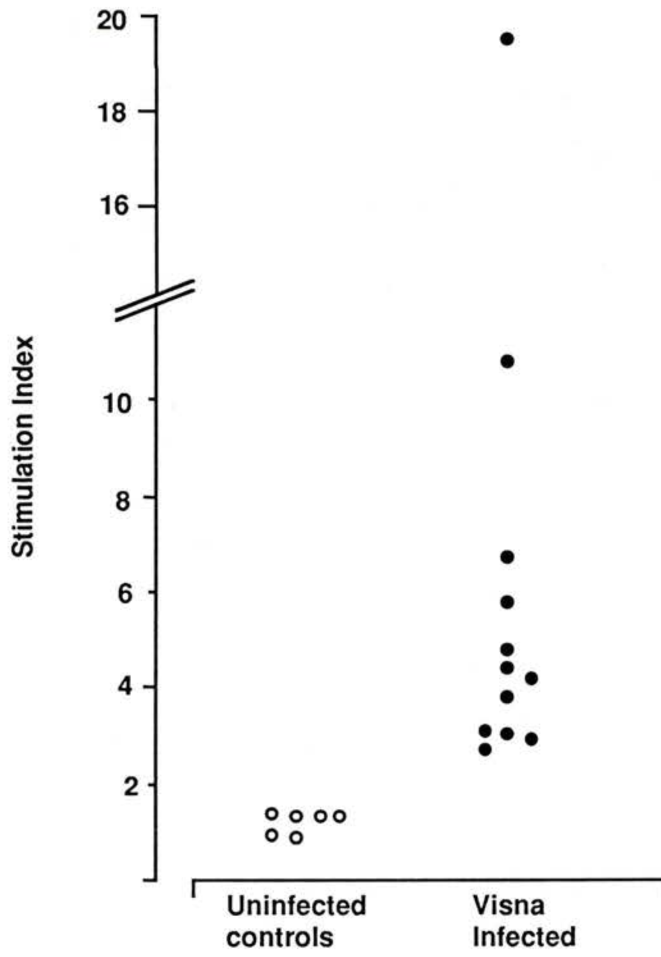


Table 3.4

	Response (cpm [^{3}H]-Tdr ± SD) to							
	Undepleted [1]		CD4 depleted [2]		CD8 depleted [3]		CD4 & CD8 depleted [4]	
Sheep	Medium	p25 (S.I.)	Medium	p25 (S.I.)	Medium	p25 (S.I.)	Medium	p25 (S.I.)
90	1060 ± 112	4257 ± 362 (4)	1283 ± 126	1367 ± 199 (1.1)	ND	ND	764 ± 156	557 ± 114 (0.73)
91	3528 ± 836	14694 ± 651 (4.2)	2144 ± 168	4909 ± 479 (2.3)	ND	ND	1293 ± 100	1247 ± 168 (0.96)
92	1276 ± 143	6871 ± 663 (5.4)	3151 ± 197	3529 ± 495 (1.1)	1644 ± 267	7127 ± 964 (4.4)	4123 ± 73	4112 ± 147 (1.0)
93	1491 ± 400	37073 ± 3586 (25)	720 ± 212	7054 ± 189 (9.8)	1055 ± 341	32689 ± 706 (31)	781 ± 126	1816 ± 329 (2.3)

1. Cells incubated with isotype matched irrelevant mAb and then processed for MACS depletion. This did not cause changes in the phenotype or proportions of lymphocyte populations.

2. %CD4 remaining after CD4 depletion (sheep no. - % CD4): 90 - 2%, 91 - 2.5%, 92 - 5%, 93 - 2%.

3. % CD8 remaining after CD8 depletion (sheep no. - % CD8): 92 - 2.7%, 93 - 3.2%.

4. %CD4 + CD8 remaining after CD4/CD8 depletion (sheep no. % CD4 + CD8): 90 - 3%, 91 - 3%, 92 - 2.7%, 93 - 3.2%.

These data demonstrate that for most visna infected sheep $CD4^+$ T cells are the major lymphocyte subset responding to viral antigen in these assays. However in some infected individuals proliferation to visna antigen could only be completely abrogated by depletion of both $CD4^+$ and $CD8^+$ T cells, depletion of $CD8^+$ T cells alone did not significantly affect the level of proliferation detected.

3.5 Summary of Chapter 3 results

1. After experimental infection of sheep with maedi-visna virus, the first antiviral antibodies detectable are specific for either ***gag*** p25 or ***env*** gp135. With time the antibody response broadens, but these reactivities predominate still.
2. The IgG antibody response to visna is restricted to the IgG_1 subclass.
3. Anti-visna antibodies can mediate antibody-mediated complement-dependent cytotoxicity (ACC), but not antibody-dependent cell-mediated cytotoxicity (ADCC), activity.
4. Visna infected sheep contain circulating virus-specific $CD4^+$ T cells, some of which are specific for p25.

3.6 Discussion

Previous studies on the development of the humoral immune response to maedi-visna virus have reported that visna virus specific antibodies are first detected 1-2 months after experimental infection, but that a longer period, 3-6 months is required before virus neutralising antibodies can be detected (Gudnadottir & Palsson, 1966., Gudnadottir & Kristinsdottir, 1967., De Boer, 1970., Petursson *et al*, 1976). The data presented here (Figures 3.1 & 3.2) are in broad agreement with these earlier studies with regard to the kinetics of the development of the antiviral antibody response and extend these earlier

reports by examining both the pattern of viral antigens recognised, and the antibody isotypes produced, in the developing immune response.

The experiments described here (Figures 3.2 & 3.4) suggest that antibodies to both ***gag*** and ***env*** antigens are produced early in infection and that these antibody reactivities predominate in the antibody response to maedi-visna virus. Houwers & Nauta (1989) reported that antibodies to p25 appeared early after infection, but that antibody to gp135 could be detected only infrequently. There are several points to be made regarding this study; firstly the data on the development of the anti-visna antibody response was based on experimental infection of only two sheep, each animal was infected by a different inoculation route and in neither case was the infectivity titre of the visna inoculum determined. Further, the immunoblot technique described was based on the use of viral antigen purified by differential centrifugation on sucrose gradients, a procedure which has been reported to result in the loss of external envelope glycoproteins from the purified virions (Bruns & Frenzel, 1979). Thus the failure to detect anti-gp135 antibodies in this paper is not entirely unexpected. The immunoblotting experiments presented here (Figures. 3.2 & 3.4) are based on the use of virus infected cells as antigen, so maximising the probability of all virally-encoded antigens being present. Moreover this data set is based on the experimental infection of four sheep by the same route with a standard, known, amount of maedi-visna virus, 10^6 $TCID_{50}$.

No relationship between tissue culture infectious doses and animal infectious doses has been described for visna, but in an SIV system the dose of virus required to infect rhesus monkeys was 100 times less than that needed to establish an infection *in vitro* (Murphey-Corb *et al*, 1989). If a similar ratio between virus infectious dose *in vivo* and *in vitro* holds for visna then the virus inocula used in these experiments, although less than those used by other workers (De Boer, 1970., Griffin *et al*, 1978., Larsen *et al*, 1982*b*., Sihvonen, 1984), are likely to be far in excess of that required to establish infection *in vivo*. Any significance of this on the pathogenesis of visna infection and/or the host immune

response is not known. No data is available on the dose of virus involved in natural transmission of infection.

Primates experimentally infected with either HIV or SIV produce anti-viral antibodies first detectable around 1month post-infection. At these early time points antibodies to both p24 and gp120/gp160 are present (Kannagi *et al*, 1986., Nara *et al*, 1987*a*). Similarly in HIV infection both anti-***gag*** and anti-***env*** antibodies are among the first anti-viral antibodies detected at or around seroconversion (Cooper *et al*, 1987). Thus the patterns of antibody responses to lentivirus infection are similar between visna infection of sheep and immunodeficiency virus infection of man and monkeys.

Analysis of the antibody isotypes produced in response to visna infection revealed that anti-viral antibodies were present in both IgM and IgG_1 isotypes, but that IgG_2 anti-visna antibodies were not detectable (Figures 3.3, 3.4, 3.5). Previous reports had also suggested that no significant anti-visna antibody activity was present in either the IgM class (Petursson *et al*, 1983) or the IgG_2 subclass (Mehta & Thormar, 1974., Petursson *et al*, 1983), but these studies were subject to several major criticisms; firstly, both these studies were based on analysis of serum immunoglobulin from only one sheep. Secondly, anti-visna antibody activity was assessed using techniques such as virus neutralisation and complement fixation, yet a heterologous source of complement was used in these assays (Petursson *et al*, 1983). Ovine IgG_2 does not activate heterologous complement (see Table 3.1). Last, antibody activity in the various immunoglobulin isotypes was assessed by fractionation of serum Ig on the basis of physico-chemical properties. Since some cross-contamination of IgG_1 with IgG_2 and vice-versa is difficult to exclude using these techniques (Butler, 1986., Dr. P. Bird, *personal communication*), these studies were unable to state definitively whether or not IgG_2 anti-visna antibodies were present in infected sheep. In the experiments presented here antibody activity was assessed directly in antigen binding assays, and analysis of antibody isotypes was carried out directly using mouse monoclonal antibodies specific for various isotypes of sheep immunoglobulin. Moreover assay of a number of sheep, at known times after infection has clearly demonstrated the occurrence of IgM anti-visna antibodies.

Isotypic restriction is not a feature of the immune response to HIV. Anti-HIV antibody activity is detectable in all four human IgG subclasses (Sundquist *et al*, 1986., Klasse & Blomberg, 1987., Khalife *et al*, 1988), although the IgG_1 and IgG_3 responses predominate, as they do in the majority of human antibody responses to viral infection (Skvaril, 1986). It is not valid to compare Ig isotypes across species, since speciation is thought to have occurred before the genetic events which gave rise to the development of the individual IgG subclasses (Callard & Turner, 1990).

The basis of the restriction of the IgG anti-visna antibody response to the IgG_1 subclass is not known. A selective deficiency of serum IgG_2 has been observed in some HIV infected individuals (Aucouturier *et al*, 1986., Parkin *et al*, 1989), but a specific defect of IgG_2 antibody production in visna infected sheep is unlikely. Visna infected sheep make both IgG_1 and IgG_2 antibodies when challenged with antigens such as ovalbumin and KLH (Dr. P. Bird, *personal communication*) and in a similar ratio to uninfected sheep.

There is evidence in the mouse that different lymphokines influence the production of different IgG subclasses *in vitro* (Snapper & Paul, 1987., Coffman *et al*, 1988) and that distinct types of murine helper T cell clones preferentially produce different lymphokines, (reviewed in Mosmann & Coffman, 1989) although the significance of these observations *in vivo* is unclear (reviewed in Finkelman *et al*, 1990).

Although a similar division of T helper cells may not hold true for other species, T cells with differing lymphokine production have been reported in man (Dohlstein *et al*, 1988., Umetsu *et al*, 1988) and the presence of particular subsets of T cells may correlate with resistance or susceptibility to certain infections (Salgame *et al*, 1991). In man, however, it is not clearly defined to what extent IgG subclass regulation is lymphokine dependent. Virtually no information on lymphokine regulation of IgG synthesis is available in the sheep, but it is a plausible hypothesis that the isotypic restriction seen in the humoral immune response to visna may reflect a bias in the nature of 'T-cell help' supplied by visna-specific T cells and/or that visna infection *in vivo* results in production of a particular set of cytokines. Consistent with this hypothesis, it has recently been reported that IFN-α preferentially enhances IgG_{2a}

secretion in the mouse (Finkelman *et al*, 1991). Virus infections tend to induce the production of IFN-α/β (reviewed in Joklik, 1990) and the predominant anti-viral isotype, in mice, is IgG_{2a} (Coutelier *et al*, 1987). Circulating visna specific $CD4^{+}$ 'T-helper cells' are present in infected sheep (Tables 3.3 & 3.4., Figure 3.12) and when (if ?) reagents allowing accurate quantitation of ovine cytokines become available then these cells could be examined for any bias in cytokine production.

Experiments to investigate the function of anti-visna antibody against visna virus infected cells yielded surprising results. Plasma from visna infected sheep contains antibodies capable of binding specifically to visna infected cells and mediating complement-dependent lysis of these cells (Figure 3.9). However these same plasma samples do not appear to mediate ADCC activity (Figure 3.7).

The major effector cells mediating ADCC in ruminant systems have been reported to be neutrophils and monocyte/macrophages (Rouse *et al*, 1976., Grewal *et al*, 1977., Grewal & Rouse, 1979). The PBMC's used as effector cells in these assays contained around 20%-25% monocyte/macrophages (Table 3.2), moreover they were capable of lysing antibody-coated erythrocytes (Figure 3.10). In human systems, monocyte/macrophages have been reported to be the major cell type capable of mediating lysis of antibody-coated erythrocytes (Poplack *et al*, 1984). The ability of sheep sera to direct ADCC in another virus system was not investigated here, although on reflection this would have been a better positive control system. Other workers, however, have demonstrated that bovine herpesvirus infected cells can be lysed in ADCC assays using PBMC and unfractionated sera (Rouse *et al*, 1976., Grewal *et al*, 1977., Grewal & Rouse, 1979).

That anti-visna antibodies mediate ACC, but not ADCC is in contrast to the situation observed in human infected with HIV. Anti-HIV antibodies have been reported to mediate ADCC (Rook *et al*, 1987., Ojo-Amaize *et al*, 1987), but not ACC (Nara *et al*, 1987*b*). Interestingly, chimpanzees infected with HIV or goats immunised with gp120 produce

antibodies capable of mediating both ADCC and ACC against HIV-infected cells (Nara *et al*, 1987*a*., 1987*b*).

Given the biological properties of ruminant immunoglobulins (Table 3.1) it is tempting to speculate that the failure to detect visna-specific ADCC is related to the isotypic restriction of the IgG anti-visna antibody response. IgG_2 is cytophilic for both neutrophils and fresh monocytes, whereas IgG_1 is only capable of adhering to peripheral blood monocytes which have been cultured *in vitro*. *In vitro* culture of monocytes is often equated with activation and/or differentiation of monocyte/macrophages and it would be interesting to assess the efficacy of cultured monocytes in anti-visna ADCC assays.

A proviso to the hypothesis that it is the absence of IgG_2 antibody in the humoral immune response to visna that results in the lack of detectable ADCC activity is that there is a report in the literature that IgG_1 antibody can mediate ADCC against murine mastocyoma cells (Grant *et al*, 1975). However this study was based on the fractionation of immunoglobulins into IgG_1 and IgG_2 by ion-exchange chromatography, a procedure which will not result in complete separation of IgG_1 from IgG_2 without further purification (Butler, 1986). Grant *et al* did not present data on the purity of their fractionated immunoglobulins. It is interesting to note that the data reported by Grant *et al* indicates that the 'IgG_1' fraction caused lower levels of specific lysis and that the kinetics of the lytic reaction were slower. This may indicate that a proportion, at least, of the ADCC activity measured was due to IgG_2 contamination of the IgG_1 fraction. However it is difficult to compare the relative activities of the IgG fractions assayed in this paper in directing ADCC, as no data is presented on the protein concentrations of the different IgG fractions used in these assays. Alternatively, Grant *et al* used high titre hyperimmune antiserum in their experiments and the apparent discrepancy between that data and the results presented here could be simply explained if IgG_1 antibodies are much less efficient at mediating ADCC than IgG_2 antibodies, but that their IgG_1 fractions contained more specific antibody than their IgG_2 fractions. The anti-visna antibody titres of the serum used in these experiments was relatively low.

It is unclear whether this lack of detectable anti-visna ADCC activity *in vitro* is of any significance *in vivo.* Antibody-mediated fixation of complement has been shown to enhance ADCC of target cells infected with a bovine herpes virus (Rouse *et al,* 1977*a*., 1977*b*). It is thus possible that IgG1 bound to visna infected target cells could fix complement and then indirectly activate ADCC by cells expressing complement receptors.

The role of different T cell subsets during immune responses to viral infection has been studied in a number of model systems (Cobbold *et al,* 1984., Leist *et al,* 1987., Taylor & Askonas, 1986) and while $CD8^+$ cytotoxic T lymphocytes are capable of clearing viral infections optimal antiviral responses require the presence of both $CD4^+$ and $CD8^+$ immune T cells. This has been directly shown in a murine retrovirus system where in adoptive transfer experiments both T cell subsets were required for protection from virus-induced disease (Hom *et al,* 1991). The data described here indicates that sheep persistently infected with the lentivirus maedi-visna mount a T cell immune response to the virus which includes $CD4^+$ T cells. In this connection it is interesting to note that although the majority of the visna reactive T cells in this assay were $CD4^+$, in two individuals $CD8^+$ lymphocytes appeared to make some contribution to this *in vitro* lymphoproliferation (Table 3.4). Lymphocyte proliferation to exogenous protein antigen *in vitro* is generally thought to result from MHC Class II restricted presentation of antigen to $CD4^+$ T cells (reviewed in Moller, 1988., Brodsky & Guagliardi, 1991).

It is interesting to note that in HIV infection, lymphocyte proliferation in response to HIV antigen is weak or absent in infected individuals (Wahren *et al,* 1987). Failure to detect a secondary T cell response to HIV antigen could result from either the failure of infected accessory cells to recruit HIV specific T cells into the immune response or as a direct consequence of viral infection of $CD4^+$ T cells. Since the sheep lentivirus is restricted in its tropism to cells of the monocyte/macrophage lineage, the data presented here suggest that the failure of the T cell response to HIV antigens is more likely to be due to lymphocyte

infection and dysfunction rather than a failure of antigen presenting cells to recruit HIV specific T cells.

That said, the significance of this $CD4^+$ T cell response in visna is unclear since visna infected sheep do not clear the viral infection. Quantative statements on the extent and significance significance of the T-helper response in visna infected sheep, requiring limiting dilution analysis of the T cells responding to visna and assay of the cytokines secreted by these cells may help to clarify this issue.

Chapter 4

The acute immune response to maedi-visna virus studied at the level of a single lymph node

4.1 Introduction

Lentiviral infections of man and animals result in disease characterised by a long asymptomatic period and a slow progression of clinical signs (Narayan & Clements, 1990). The pathogenesis of lentiviral disease *in vivo* can be thought of as occurring in several stages: acute infection (primary exposure to virus),

establishment of infection,

slow progression of disease (virus generally in restricted replication state), with development of pathological changes indirectly mediated by immune and inflammatory response of the host,

death, due to either primary or secondary disease.

Little is known about the progression of any of these stages *in vivo*. The pathophysiologic events occurring immediately post infection and in the interval between infection and development of clinical disease remain obscure; yet it is at these stages of the infective process that any immune mechanisms induced by prior vaccination must act to prevent, or clear, viral infection.

Moreover, it is possible that immune mechanisms activated in the acute response to lentiviral infection are important in driving the virus to adopt the 'restricted replication' state which is thought to be a major mechanism underlying both the persistence and spread of lentiviruses and the slow evolution of lentiviral disease (Haase, 1986). However, since early immune responses are likely to occur in lymphoid organs and may not even be apparent in peripheral blood (Westermann & Pabst, 1990), relatively little is known about the nature of the acute immunologic and virologic events which occur in the acute immune response to lentiviral infection.

Reimann *et al* (1991) studied the acute changes seen in lymph nodes of macaques in response to intravenous challenge with SIV. The major finding reported by these workers was a marked increase in numbers of $CD8^{+}$ T cells within the node early after infection with SIV. No consistent change in $CD8^{+}$ PBMC was noted during this time. The $CD8^{+}$ cells within the node appeared activated, in that they exhibited increased levels of MHC class II expression

and decreased expression of leukocyte adhesion molecule-1, however these same cells did not express CD25 (IL-2R) and were not proliferating. This phenotype resembles the 'chronic state of activation' of $CD8^+$ T cells seen in AIDS patients (Pantaleo *et al*, 1990). The basis of this 'semi-activated' phenotype of the $CD8^+$ lymph node cells from these macaques was not clear, but there was no correlation between the presence of $CD8^+$, MHC class II positive T cells and the detection of SIV-specific cytotoxic responses. These findings were interpreted as suggesting that this numerically important $CD8^+$ population may serve a regulatory function and may represent a suppressor population analogous to that detected in HIV infected individuals (Joly *et al*, 1989).

A model system which allows the study of acute (and chronic) immune responses by lymphoid tissue following viral challenge is lymphatic cannulation (Lascelles & Morris, 1961., Hall & Morris, 1962). The peripheral lymph nodes of sheep are easily accessible and, under general anaesthesia, it is possible to cannulate the efferent lymphatic vessel exiting a lymph node. Thereafter, lymph can be collected, in a quantitative manner, for periods of hours to months. The output of lymphocytes from a single resting lymph node of approximately 1g weight averages around 3-5 x 10^7 lymphocytes per hour (Hall & Morris, 1962., Hay & Hobbs, 1977). Of the small lymphocytes exiting the node, about 90% come from the blood, some 2%-4% are produced within the node and 5%-10% are derived from peripheral lymphatic input to the node (Hall & Morris, 1965*a*., Schoefl, 1972). Peripheral lymph is lymph that has not passed through a lymph node and differs from efferent lymph in that it contains some 10%-20% macrophage/dendritic cells and fewer B cells (MacKay *et al*, 1988., Bujdoso *et al*, 1989*a*). Afferent lymph T cells have been shown to be predominantly of a 'memory' phenotype, as defined by their level of expression of 'memory' T cell markers such as LFA-3 and CD2 (Mackay *et al*, 1990). In contrast, efferent lymph contains few, if any, macrophage/dendritic cells, 20%-30% B cells and approximately 70% T cells. Efferent lymph, from a resting node, is enriched for $CD4^+$ T cells (Mackay *et al*, 1988., Bujdoso *et al*, 1989*b*) and these T cells are predominantly of a 'naive' phenotype (Mackay *et al*, 1990).

Injection of antigen(s) into the drainage area of the cannulated node results in alterations in the efferent lymph output from the challenged node. Primary challenge of a lymph node with soluble protein antigens such as Ovalbumen (OvA) and PPD causes only small changes in lymph flow and cell outputs (Hopkins *et al, submitted for publication*) thus the bulk of the published data on responses to antigen, in this system, deal with secondary immune responses.

Antigenic challenge of the lymph node in primed animals results in marked alterations in the efferent lymphatic output of that node. From 6-24hrs after antigen challenge there is a striking reduction in the output of cells from the node, but no reduction in the flow of lymph fluid; a phenomenon known as cell shutdown (Cahill *et al*, 1976., Hall & Morris, 1965*b*). The cell shutdown response is believed to be a result of prostaglandin E_2 synthesis within the node, secondary to complement activation (Hopkins *et al*, 1981*a*., McConnell & Hopkins, 1981).

Following shutdown there is a biphasic increase in cell output from the node. About 48hrs after antigen challenge there is an initial peak in cell output which is thought to be due to the release of cells which had accumulated within the node during cell shutdown. This recruitment of cells to the antigenically stimulated node is not antigen-specific, but instead results from changes in lymphocyte traffic through the stimulated node. These changes are due to increases in both vascular permeability and lymphocyte input (Cahill *et al*, 1976., Hay & Hobbs, 1977). The second peak in cell ouput occurs 4-6 days after antigen challenge and is associated with firstly, an increased migration of cells from blood to the node, and secondly with antigen-driven lymphoproliferation within the node.

These kinetic changes in cell output are accompanied by changes in the lymphocyte subset composition of efferent lymph T cells. After antigenic stimulation of the node the composition of efferent lymph is altered initially by an increase in the proportion of $CD4^+$ T cells, followed by an increase in the proportion of $CD8^+$ T cells, this wave of $CD8^+$ T cells tends to occur when the cell output of the node is at its maximum (Bujdoso *et al*, 1989*b*).

Efferent lymphocytes are responsible for the establishment of immunological memory and the dissemination of the immune response to other lymphoid organs. If cells leaving an antigen stimulated node are removed by chronic lymphatic cannulation there is no priming for a secondary immune response (Hall & Morris, 1967., Smith *et al*, 1974); intravenous infusion of cells collected during the response, but free of antigen, can reverse this effect.

Thus using the model system described above injection of maedi-visna virus into the drainage area of a cannulated peripheral node allows continuous monitoring of the immunological and virological events occurring in the acute and sub-acute phases of the immune response of lymphoid tissue to lentiviral infection. The protocol used for these experiments is shown in Figure 4.1.

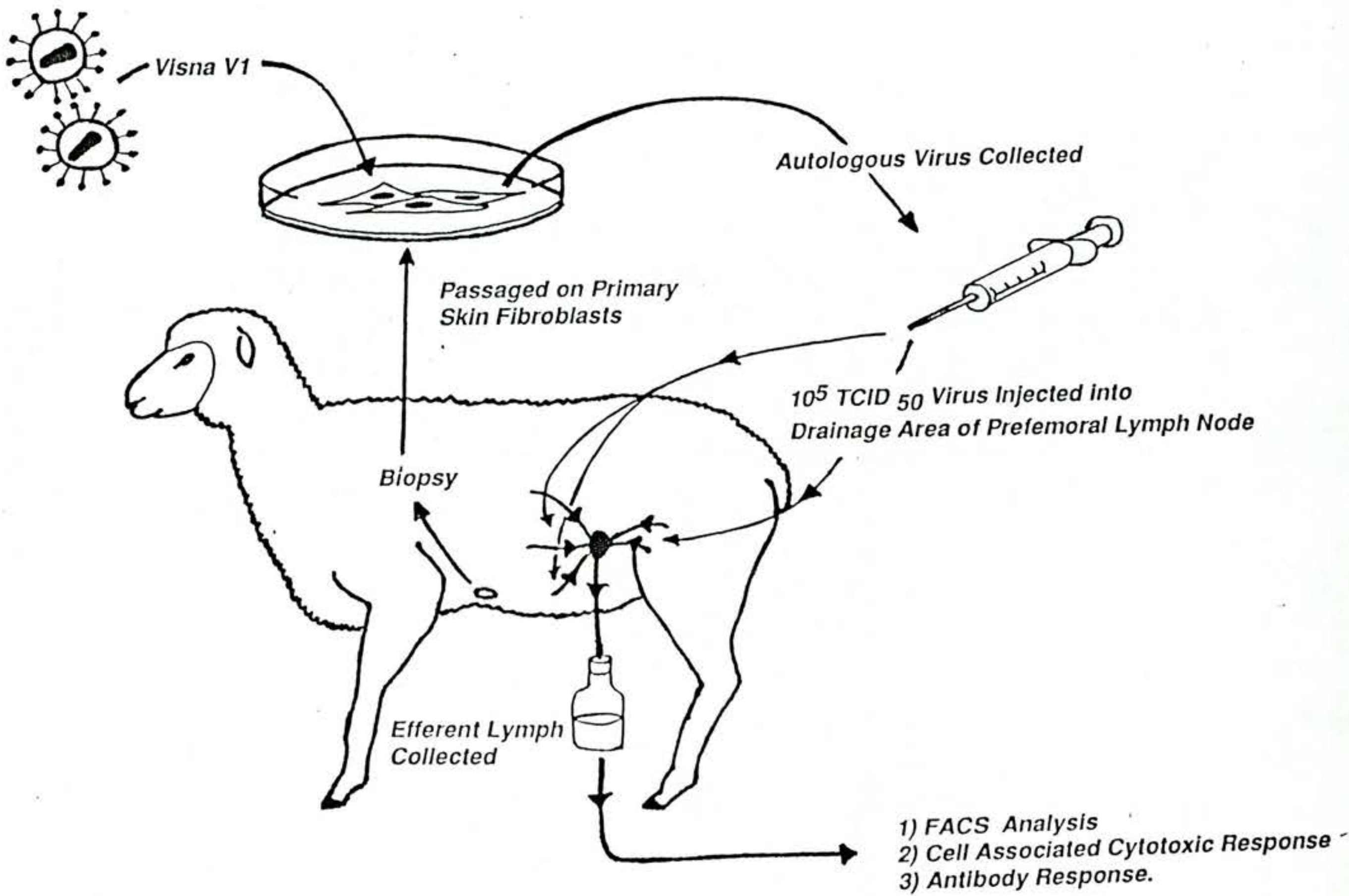
This chapter describes the antibody response to maedi-visna virus, as analysed in this system, and the visna-specific T cell response, as measured using *in vitro* lymphocyte proliferation assays. These data are my own work. These experiments, however, were carried out as part of a larger group of workers utilising this sytem to study a number of aspects of lentiviral pathogenesis and where appropriate, and with due acknowledgement, their results will also be discussed.

Figure 4.1 Experimental protocol

The efferent lymphatic vessel for the prefemoral lymph node of 1-3yr old randomly bred Finnish-Landrace sheep was cannulated as described by Hall (1967). The cannulation was allowed to stabilise following surgery and, about one week after cannulation, approximately 5×10^5 $TCID_{50}$ of maedi-visna virus (strain EV1 (Sargan *et al*, 1991)), grown in autologous skin cells was injected intradermally and subcutaneously into the drainage area of the node. Control sheep were challenged with autologous skin cell culture supernatant alone.

Within the efferent lymph exiting the node changes in cell number and phenotype (*Dr. P. Bird & D.J. Allen*), the development of visna-specific antibody (*HTR*), lymphocyte proliferation (*HTR*), cell-mediated cytotoxicity (*Dr. B.A. Blacklaws*) and the presence of infectious virus (*Dr. P. Bird & D.J. Allen*) was assessed.

Figure 4.1



4.2 Results

4.2.1 Maedi-visna virus specific antibody in efferent lymph

In total, five sheep with prefemoral efferent cannulations were studied in these experiments: three of these, sheep 657R, 649R and 683R were infected with 'autologous visna' (see Figure 4.1), sheep 1060P and 663R were mock-infected with autologous cell supernatant.

Maedi-visna virus specific antibody was first detected 3-4 days post infection (Figures 4.2 & 4.3). Immunoblot analysis (Figure 4.2, left-hand panel) demonstrated weak antibody reactivity to bands of apparent M_r of 24kDa and 28kDa (p25) around days 4-12 post-infection. Antibody to the viral envelope glycoprotein (gp135) was undetectable at this time, but later, from day 12 onwards anti-***env*** antibody could be detected (Figure 4.2, left-hand panel). Antibody to p25 was also detected later in the infection, apparently peaking at around days 26-30 post-infection. These bands were not seen when blots were probed with lymph plasma from a control sheep, 1060P (Figure 4.2, right-hand panel). Antibody reactivity to bands of 50-70kDa was also seen on immunoblots. This was not visna-specific since it was detected using lymph plasma from sheep 649R collected prior to visna challenge and with lymph samples from control sheep 1060P. The nature of the non-visna specific reactivity is unclear. It may represent an antibody response to cellular proteins or components of the tissue culture medium, however the challenge virus is grown in autologous cells and weak reactivity is seen prior to challenge in both sheep. This reactivity to bands of 50-70 kDa titrates at least as far as the anti-visna reactivity and is not inhibited by high salt concentrations (0.5M) or the presence of detergent (0.05% Tween 20).

Quantitative data on the anti-visna antibody response (Figure 4.3) was obtained by ELISA against recombinant p25 protein (see Chapter 5). There was some variation in the time at which anti-visna antibody was first detected after visna infection. Sheep 683R produced two discrete peaks of anti-p25 antibody; days 4-6 and days 12-18, which were of

Figure 4.2 Immunoblot analysis of the antibody response to maedi-visna virus in efferent lymph

Lymph was collected as described in section 2.14.3.2 and centrifuged to separate cells from lymph plasma. The cell-free plasma was stored at -20°C until used.

Visna antigen, prepared as described in section 2.8.1 was separated on 5-20% linear gradient SDS-PAGE, run under reducing conditions, and electroblotted onto a nitrocellulose membrane. Strips, cut from these blots, were incubated overnight with pre- and post-infection samples of lymph, diluted 1:10 in PBS/5% non-fat dried milk. Blots were developed using affinity-purified anti-sheep immunoglobulin conjugated to alkaline-phosphatase (Sigma). Positions of molecular weight standards are indicated. The arrows indicate the anti-p25 and anti-***env*** antibody reactivities. The bands on the blots probed with 1060P lymph are slightly higher as these strips were cut from a separate blot to that labelled 649R. Both blots, when probed with anti-visna sheep sera showed similar patterns of reactivity.

The left hand panel shows a set of blots obtained using lymph plasma samples from sheep 649R (visna challenged), from left to right the strips were probed with plasma samples collected every second day from 2 days pre-infection ie. day -2, day 0 etc. till day 32. For comparison with visna specific bands seen on blots probed with sheep serum see Figures 3.2 & 3.4. The right hand panel shows a set of blots probed with plasma from sheep 1060P (mock-challenged). In this panel the strips are probed with samples taken every second day from day 0 to day 18.

Figure 4.2

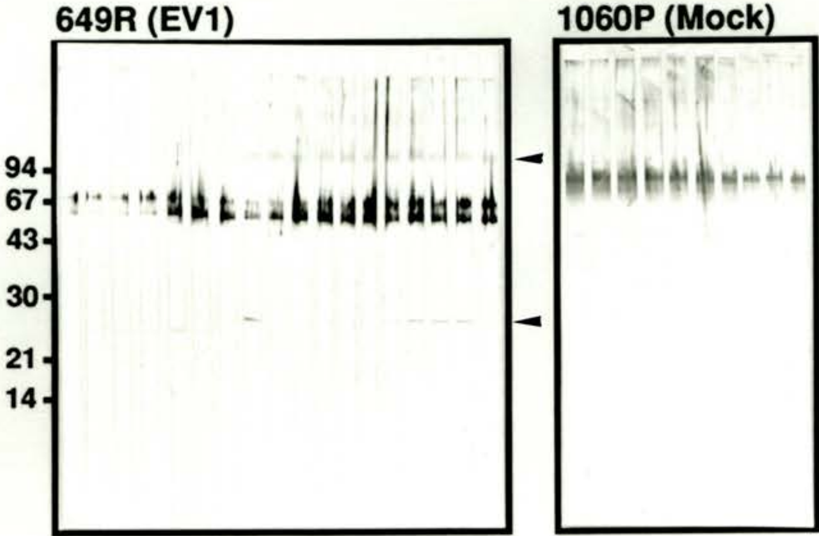


Figure 4.3 Anti-p25 antibody in efferent lymph plasma

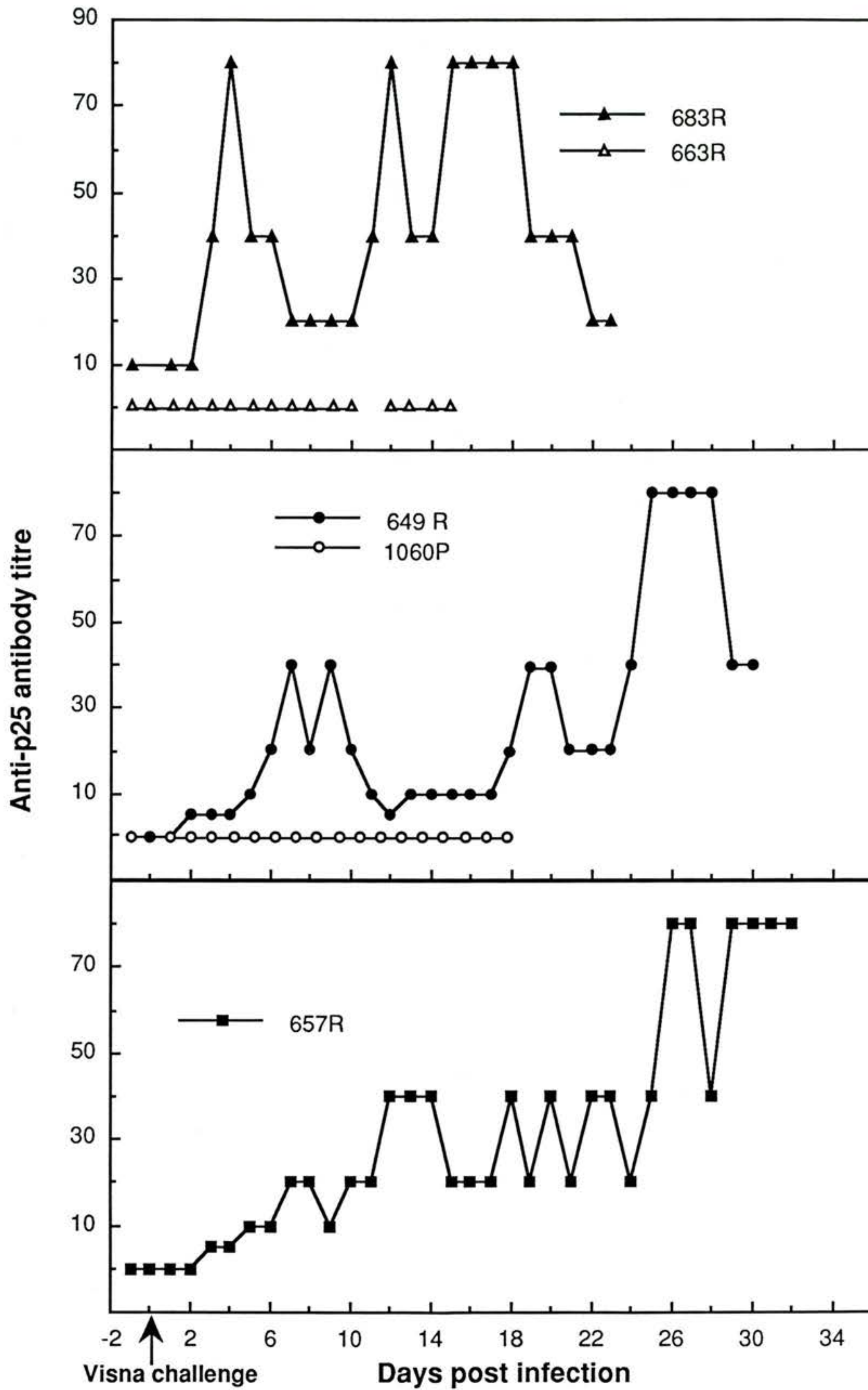
Samples of lymph plasma collected as described previously were assayed for anti-p25 antibody by ELISA against recombinant p25 protein (see Chapter 5).

Purified recombinant p25 (5µg/ml in 0.1M $NaHCO_3$, pH 9.6) was coated onto Falcon PVC plates by overnight incubation at 4°C. After washing in BBS/0.05% Tween 20, the wells were blocked by incubation with BBS/1% BSA (200µl/well) for 1hr at 37°C. Samples of lymph, diluted in BBS/BSA/ 0.05% Tween 20, were titrated in doubling dilutions starting from a 1/5 dilution. After 1hr incubation at 37°C, the plates were washed in BBS/Tween and then incubated for a further 1hr with a 1/4000 dilution of rabbit anti-sheep Ig conjugated to HRP (Dako) in BBS/Tween/BSA before washing and developing the plates with OPD as substrate.

Antibody titre is expressed as endpoint titre, taken as the reciprocal of the highest dilution of plasma which gave an OD_{492} twice that of background values (no lymph).

Lymph samples from control sheep 1060P and 663R contained no detectable anti-p25 antibody.

Figure 4.3



roughly equal titre. This cannulation stopped on day 23. For sheep 649R and 657R antibody was first detectable at around days 4-6 and the first peaks of anti-p25 antibody were at days 6-9 and 12-14 respectively, after this antibody titres declined slightly and then rose gradually to a peak at day 22. These kinetics are in good agreement with those seen on immunoblot analysis (Figure 4.2 & 4.4). When these cannulations were stopped antibody titres had not yet begun to decline. No anti-p25 antibody was detected in lymph from control sheep (Figure 4.3).

Analysis of the isotypes of the anti-visna antibody responses (Figure 4.4) suggests that the initial peak of antibody is of the IgM isotype whereas later in the infection IgG_1 anti-visna antibodies predominate. Again (see Chapter 3), no IgG_2 anti-visna antibodies were detected. The data shown was obtained by probing blots of visna antigen with lymph samples from sheep 657R. Blots probed with lymph from 649R and 683R also showed that the initial anti-visna antibody response is IgM while antibody produced later in the response is of the IgG_1 isotype (data not shown). These changes were parallelled by changes in the isotype of the sIg expressed on efferent lymph B cells. The anti-***env*** response in these blots is obscured by the non-visna specific smearing at the top of the blots. A number of approaches to eliminate this problem were tried, but none of these were successful.

Some of the observed variation in the anti-p25 antibody response between these sheep probably reflects the expected variation in immune responses between individuals in an outbred population. However, it is also possible that some of the variation in anti-p25 antibody titre seen using these assays may be due to failure to detect anti-p25 antibody because it is complexed with p25 antigen. In HIV infection, p24/anti-p24 immune complex formation *in vivo* has been shown to result in a decline in anti-p24 antibody titres (Von Sydow *et al*, 1988). This possibility was tested using a p25 antigen capture ELISA (see Chapter 5) developed with either anti-p25 antibody (Figure 4.5A) or antibody to sheep immunoglobulin (Figure 4.5B). These data demonstrate that a proportion, at least, of the anti-p25 antibody produced after visna infection may not be detectable in the ELISA used in Figure 4.3. This assay, however, does not distinguish between immune complexes formed

Figure 4.4 Analysis of the antibody isotypes detected in the primary anti-visna antibody response of the prefemoral lymph node

Immunoblots of visna antigen were prepared as described in Figure 4.2. Blots were incubated with lymph plasma (1/10) overnight at room temperature. Four immunoblots were used in this experiment; a single blot for all the lymph samples developed with each isotype specific reagent (Each blot contained similar amounts of all the visna antigens, as judged by probing each blot with pooled sera from visna infected sheep). After washing the strips were then probed with either McM1 (anti-γ1, 1/2000), McM3 (anti-γ2, 1/500), McM9 (anti-μ, 1/1000) or normal mouse serum (NMS) (1/500) and developed using affinity-purified anti-mouse-immunoglobulin conjugated to alkaline phosphatase. Positions of molecular weight standards are indicated.

In each panel the strips were probed with plasma samples collected, from sheep 657R, every second day of the experiment from 2 days pre-infection ie., from left to right, day -2, day 0 etc. till the end of the experiment.

Figure 4.4

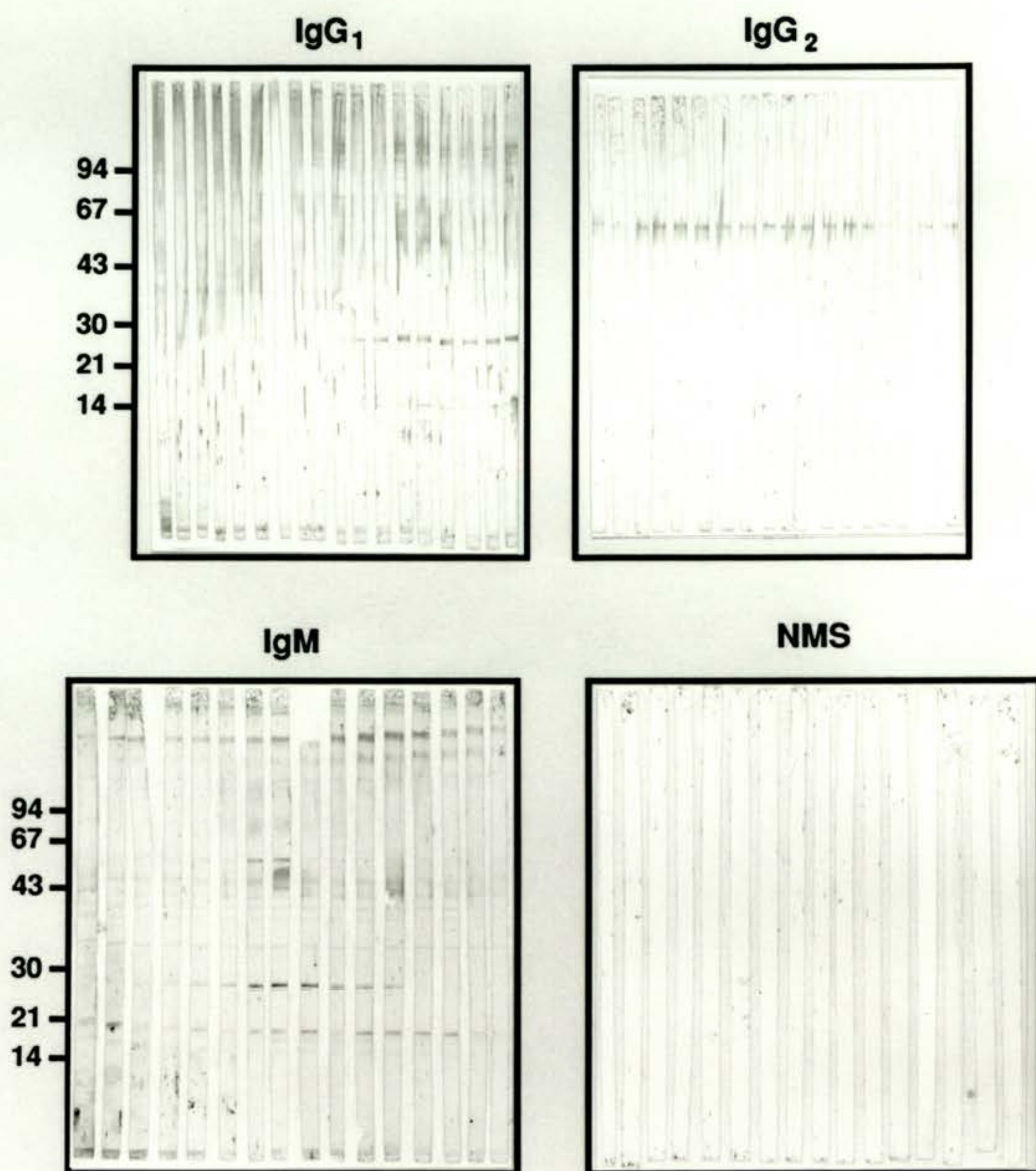
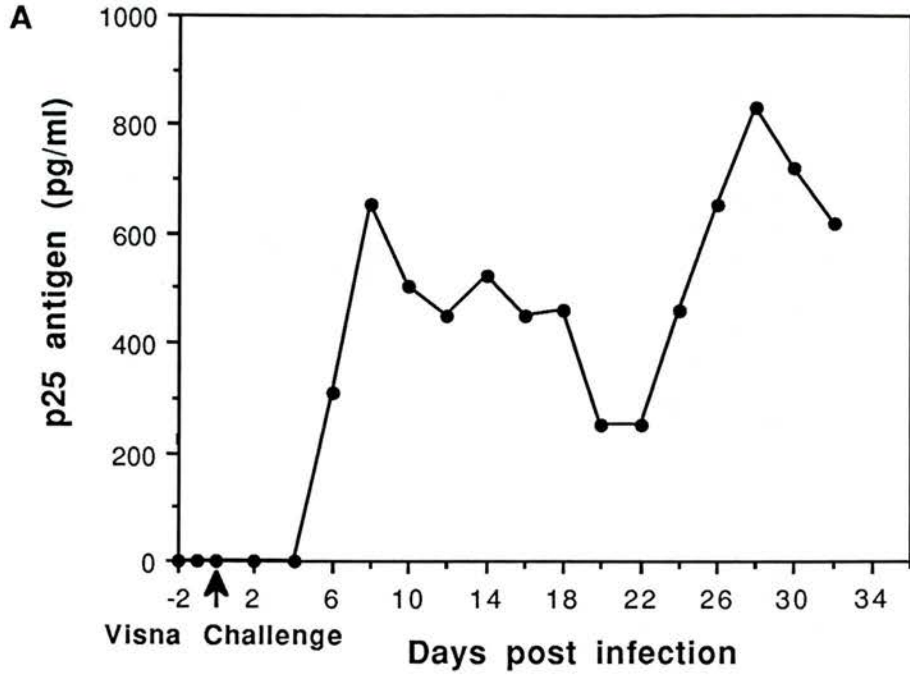


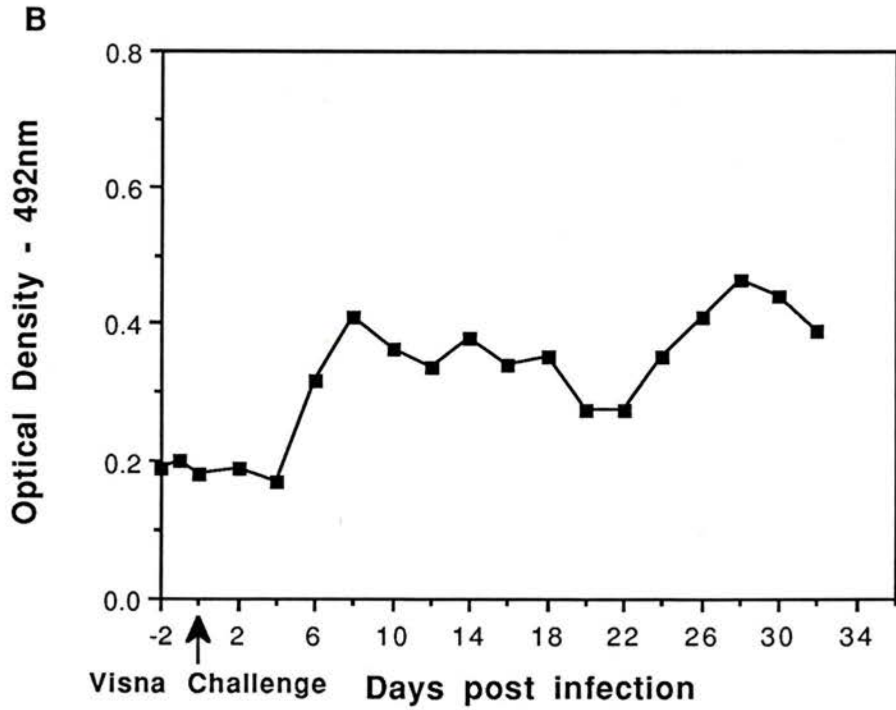
Figure 4.5 Detection of p25 antigenaemia and p25/anti-p25 immune complexes in sheep 649R

Affinity-purified rabbit anti-p25 antibody (10µg/ml in BBS) was coated onto flexible Falcon PVC plates by overnight incubation at 4°C. After washing in BBS/0.05% Tween 20, the wells were blocked by incubation with BBS/1% BSA/1% rabbit serum (BBR) (200µl/well) for 1hr at 37°C. After washing samples of undiluted lymph plasma (200µl), collected on the days indicated and stored at -20°C until use, were added to the wells . After 1hr incubation at 37°C, the plates were washed in BBS/Tween and then incubated for a further 1hr with either (A) biotinylated rabbit anti-p25 (20µg/ml in BBR), washed again and then incubated with extravidin-HRP (1/4000 in PBS/1% BSA) or (B) a 1/4000 dilution of rabbit anti-sheep Ig conjugated to HRP (Dako) in BBS/Tween/BSA . After a final wash plates were developed with OPD and absorbance at 492nm read using a Titertek ELISA reader.

In A, p25 antigen concentrations were derived by reference to a standard curve obtained by titrating known concentrations of recombinant p25 in the assay as described in Figure 5.11. In B, the data is presented as the mean OD_{492} of duplicate wells. The p25 antigen detection assay is sensitive down to around 150-300pg/ml (see Chapter 5), thus the levels of p25 antigen indicated for days 6, 20 and 22 should be interpreted only cautiously.

Figure 4.5





in vivo and soluble p25 antigen in the lymph competing, with p25 antigen bound to the ELISA plate, to bind efferent lymph anti-p25 antibody during the assay.

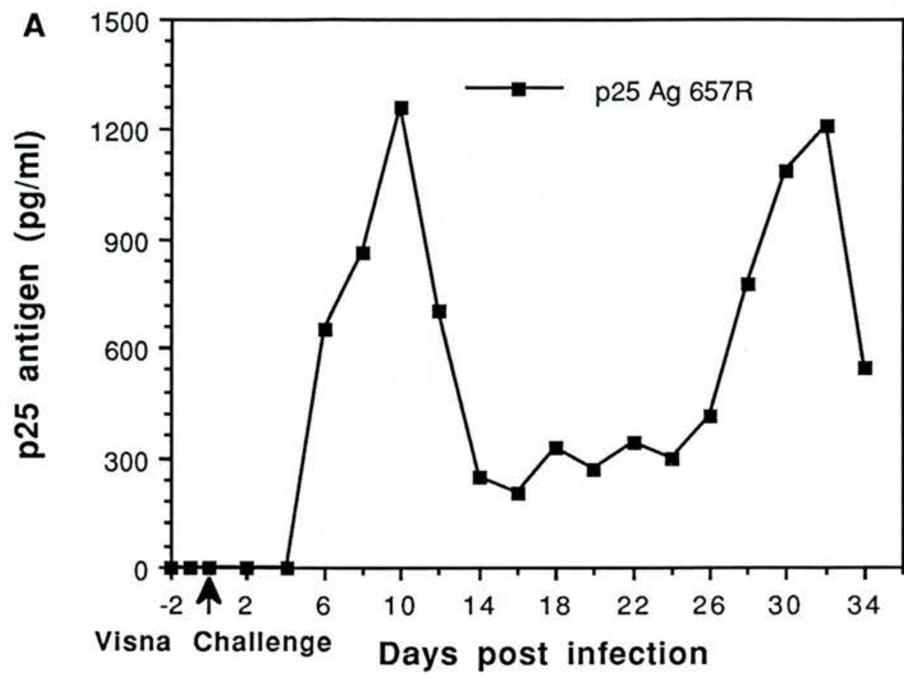
For sheep 657R and 683R, the levels of the initial peak of free p25 antigen in efferent lymph plasma (Figure 4.6) could be correlated with the exit of visna virus from the node, as judged by co-cultivation experiments (Appendix A, Figure A.1). It should, however, be pointed out that p25 antigen levels do not accurately reflect levels of infectious virus (see Chapter 5, section 5.5). Experiments using inert antigens, such as PPD and λ 'phage indicate that 80%-90% of an injected antigen exits the node within 6hrs post injection (Professor Ian McConnell, *personal communication*). It is thus unlikely that this p25 antigen in efferent lymph, first detected 8-10 days post infection, is merely antigen passively carried through the node, but rather reflects active viral replication. This is consistent with the detection, by PCR, of proviral DNA sequences within efferent lymph cells (Dr. D. R. Sargan, *personal communication*). Interestingly, the second peak of p25 antigen, which may be complexed with anti-p25 antibody (Figure 4.5A), was not parallelled by a second peak of infectious virus. This may be a reflection of a difference in sensitivity between the two assays, although it could also be interpreted as suggesting that viral replication (and hence p25 antigen production) is continuing, but that infectious virus is either neutralised by antibody or not released from the infected cell(s).

The development of virus neutralising antibody after visna challenge was therefore investigated (Figure 4.7). These experiments demonstrate that visna virus neutralising activity could first be detected 12-14 days post infection and that this activity gradually increased in titre with time. This correlates well with the development of antibody to visna ***env***, the major target antigen for virus neutralising antibodies (Scott *et al*, 1978), (Figure 4.2), and with the development of IgG_1 anti-visna antibody (Figure 4.4). The titres of neutralising antibody detected here (1/16) appear low when compared to other reports (Gudnadottir & Palsson, 1966., Petursson *et al*, 1976), however it should be remembered firstly, that the assays used are not directly comparable since the assay described here used 500 $TCID_{50}$ of visna, rather than the 100 $TCID_{50}$ of virus used by these workers Secondly

Figure 4.6 Detection of p25 antigen in efferent lymph of visna infected sheep

Lymph plasma (undiluted) was assayed for the presence of p25 antigen as described in Figure 4.5A. p25 antigen concentrations were derived by reference to a standard curve obtained by titrating purified recombinant p25 in the assay.

Figure 4.6



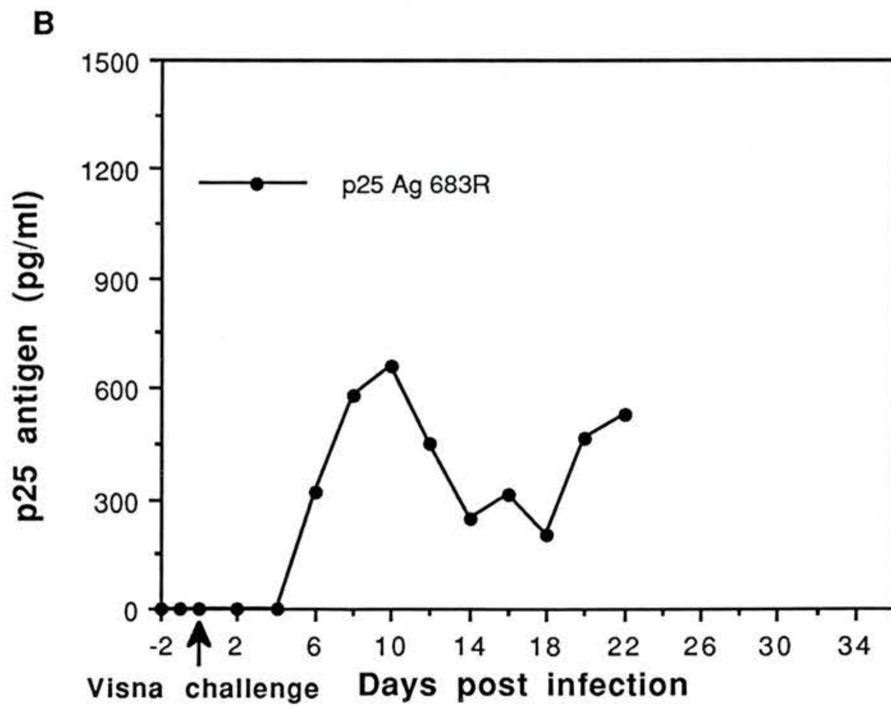


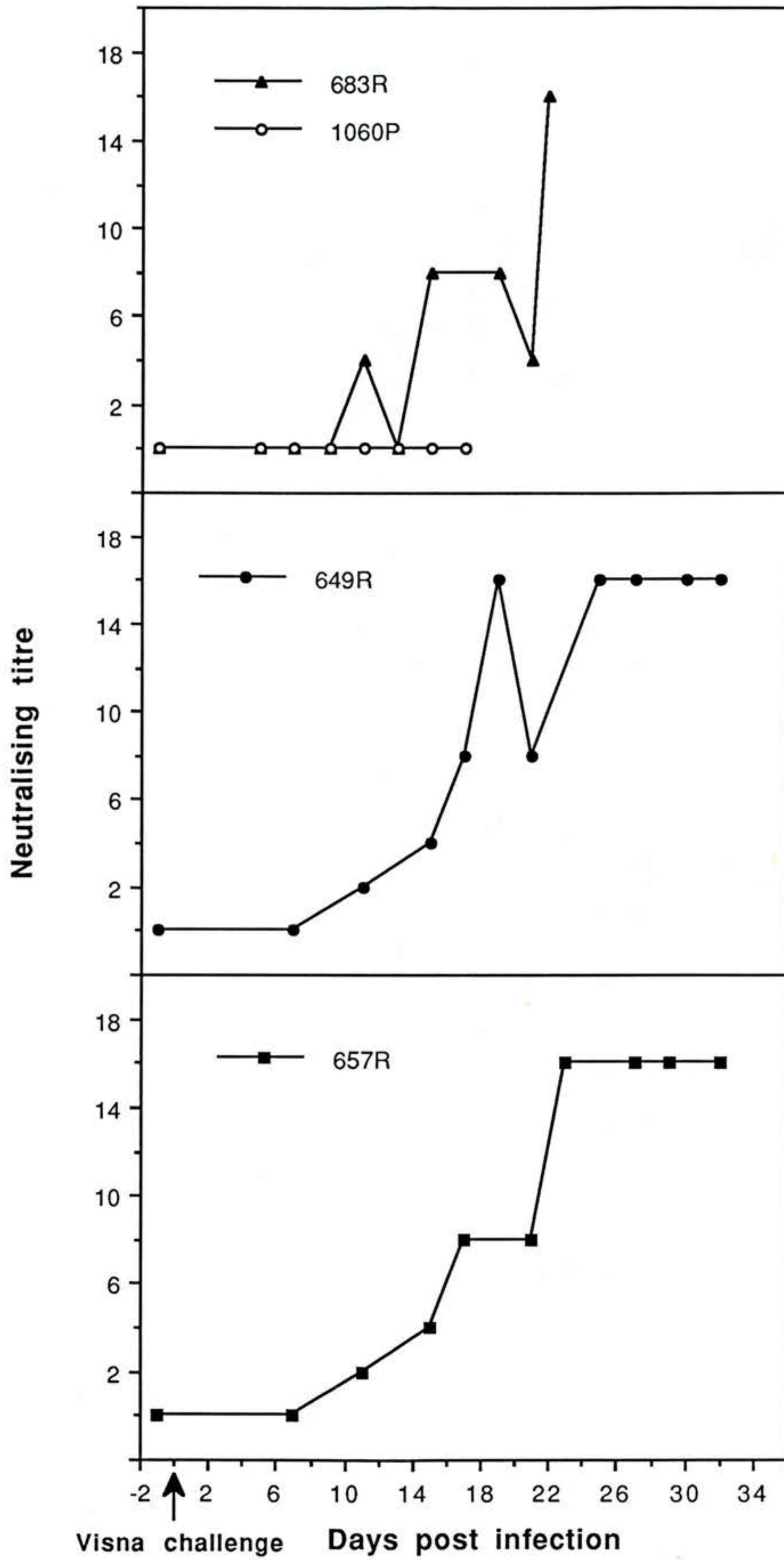
Figure 4.7 The development of maedi-visna virus neutralising activity after infection

Serial dilutions (from 1/2, in DME/2% FCS) of heat inactivated lymph plasma samples, collected on the days indicated, were mixed with 500 $TCID_{50}$ of EV1 virus and incubated for 24 hrs at 4°C. Infectious virus remaining was then assayed on skin cells (see section 2.11.3) and virus titres calculated by a quantal method (Reed & Muench, 1938).

Antibody titres are expressed as the reciprocal of the highest dilution of lymph which gave a 50% reduction in virus titre compared to controls (virus incubated with non-immune plasma, or virus incubated with medium alone).

Sheep 683R, 649R and 657R were challenged with maedi-visna virus. Sheep 1060P was 'mock-infected' with autologous cell supernatant.

Figure 4.7



this neutralising antibody is being detected 10-14 days post infection rather than 30-40 days into the response.

In these experiments lymph plasma collected from control sheep (challenged with autologous cell supernatant) did not neutralise visna virus (Figure 4.7). However, it has been reported that a non-immunoglobulin inhibitor of visna virus replication can be found in normal sheep serum (Thormar *et al*, 1979). Thus experiments were carried out to directly test whether the virus neutralisation observed in Figure 4.7 was antibody mediated. Samples of lymph, previously shown to have high titre neutralising activity were treated with either antiserum to sheep immunoglobulin or control, non-immune antiserum and then re-tested invirus neutralisation assays. The results of such an experiment are shown in Table 4.1. this demonstrates that at least the high titre neutralisation of visna virus observed in Figure 4.7 is antibody mediated.

4.2.2 Visna specific T cell responses

Maedi-visna virus infection results in a significant ($P<0.05$), almost four-fold increase in the percentage of lymphoblasts in efferent lymph compared to controls (Appendix A, Figure A.2). The peak of this response varied from day 7-16 between sheep and the maximum output of blast cells from the node exceeded 2 x 10^7 lymphoblasts/hour (*Blacklaws et al, manuscript in preparation*).

The most striking feature of this lymphoblast response was a dramatic increase in the percentage of $CD8^+$ blasts, peaking between days 6-10 (Appendix A, Figure A.2). At the peak of this response 20%-50% of the cells exiting the node were blast cells and 60%-80% of these lymphoblasts were $CD8^+$; on day 0, 2%-5% of the efferent lymphocytes were blast cells and only 10%-20% of these cells were $CD8^+$ (*Blacklaws et al, manuscript in preparation*). Visna specific cell-mediated cytotoxic activity was detected within the lymph cells coincident with the CD8 lymphoblast peak in 1/3 sheep (Dr. B.A. Blacklaws, *personal communication*).

Table 4.1

	Treatment	Neutralising titre	
		649R (day 31)	683R (day 22)
A	medium	32	16
B	control antiserum	32	16
C	anti-sheep Ig	2	0

Neutralisation assays were performed as for Figure 4.6, except that lymph plasma was diluted in medium (A), medium plus 200µl of high titre donkey anti-sheep IgG antiserum (C) or medium plus 200µl of control non-immune serum (B). The volume of medium added to each mixture was adjusted so that the final volume of each was equivalent. These mixtures were incubated overnight at 4°C and the remaining virus assayed as before. Samples of lymph plasma were not titrated beyond 1/32.

4.2.3 Lymphocyte proliferation

The ability of efferent lymphocytes to proliferate in response to visna antigen was tested, in *in vitro* lymphocyte proliferation assays, on the indicated days pre & post infection. Efferent lymph is mainly composed of T & B cells (around 70% and 30% of total cell numbers respectively) and contains few, if any, macrophage/dendritic cells. It has previously been reported that lymphocytes, including efferent lymphocytes, are poor stimulators of T cell proliferation in primary immune responses (Bujdoso *et al*, 1989*c*., Inaba & Steinmann, 1984). Thus a possible explanation for any failure to detect antigen-specific lymphoproliferation could be the absence of 'professional' antigen-presenting cells. To control for that possibility these assays were set up in duplicate; ie. in the presence, and absence, of autologous PBMC, collected prior to infection and cryopreserved, as a source of monocyte/macrophages.

In 2/2 visna infected sheep tested (649R & 683R) *in vitro* lymphocyte proliferation (S.I. > 2) to visna antigen (either sucrose-purified EV1 (Figure 4.8A) or p25 (data not shown)) was detected from day 15 post infection onwards. Significant proliferation to antigen was observed only when autologous PBMC were added to the cultures. Control antigens such as ovalbumin did not stimulate lymphoproliferation.

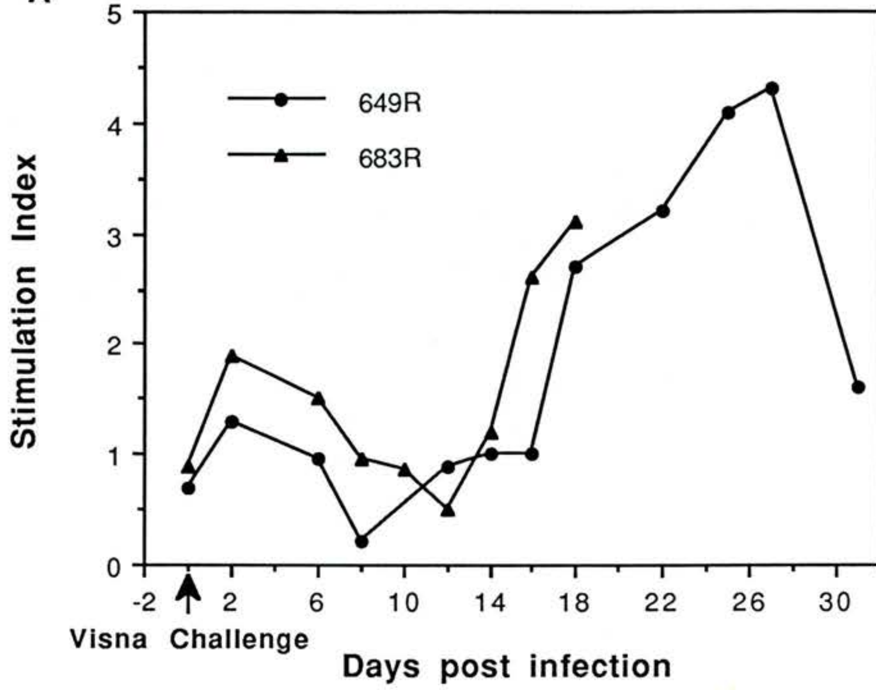
During the period of the CD8$^+$ lymphoblast response markedly decreased stimulation indices were obtained when efferent lymphocytes were cultured with the mitogen Conconavalin A, as can be seen in Figure 4.8B. The data in Table 4.2 indicates that this phenomenon is primarily due to a decrease in lymphocyte proliferation, although there is a slight contribution of an increased background. It is thus possible that antigen-specific T-cell responses may have occurred earlier than day 15, but not have been detected in this *in vitro* assay system since the phenomenon described above effectively results in a decreased sensitivity of the assay during the peak of the blast response.

The available data does not allow elucidation of the basis of this effect, but it is clear that its appearance coincides with the CD8$^+$ lymphoblast peak. One possible explanation could be that after five days culture *in vitro* the rapidly dividing blast cells have terminally

Figure 4.8 Lymphocyte proliferation by efferent lymphocytes in response to visna antigen

On the days indicated, freshly collected efferent lymphocytes were washed once and then cultured, at 5×10^5/ml, for 5 days with either an optimal dose of sucrose purified visna (4µg/ml) and 3×10^5 autologous PBMC (A), or Con A at 5µg/ml (B). Cultures were then pulsed with ^{3}H-Tdr for 5 hours and harvested. Data is expressed as stimulation indices: S.I. = (*mean cpm. antigen stimulated cultures/mean cpm. unstimulated culture*). Controls included cells cultured without antigen, cells cultured with an equal concentration of an irrelevant protein antigen and cells cultured with material prepared from mock-infected skin cell cultures. Autologous PBMC, collected prior to visna challenge and cryopreserved, proliferated in response to the mitogen Con A (5µg/ml), but did not proliferate in response to either sucrose purified visna or recombinant p25 protein.

Figure 4.8



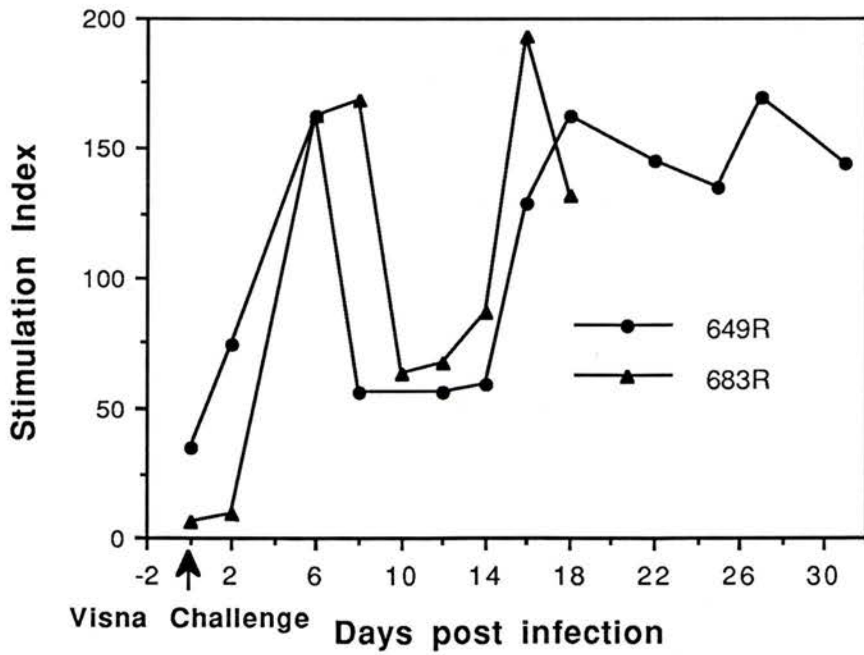


Table 4.2

	Response (cpm[^{3}H]-Tdr ± SD) to			
	649R		683R	
Days post infection	Medium	Con A (S.I.)	Medium	Con A (S.I.)
0	88 ± 15	3091 ± 200 (35)	130 ± 12	734 ± 161 (5.6)
2	1534 ± 369	114112 ± 4293 (74)	3363 ± 209	30197 ± 331 (9)
6	754 ± 251	122236 ± 9273 (162)	624 ± 212	101088 ± 4020 (162)
8	1577 ± 634	88818 ± 5423 (56)	770 ± 192	129593 ± 2059 (168)
10	N/A[a]	N/A	1730 ± 60	109759 ± 3000 (63)
12	274 ± 12	15315 ± 500 (55)	270 ± 42	18259 ± 7864 (67)
14	1263 ± 126	75297 ± 4621 (59)	1293 ± 250	112649 ± 1212 (87)
16	821 ± 44	105766 ± 5460 (128)	350 ± 23	67494 ± 627 (193)
18	1376 ± 821	223483 ± 7844 (162)	773 ± 50	112649 ± 1212 (131)
22	1400 ± 400	203000 ± 2102 (145)	ND[b]	ND
25	1908 ± 743	257352 ± 1481 (134)	ND	ND
27	758 ± 123	127344 ± 1428 (168)	ND	ND
31	1700 ± 259	244470 ± 4000 (143)	ND	ND

a. These cultures were lost due to bacterial contamination.

b. This cannulation stopped on day 23 post-infection.

differentiated and ceased to divide, thus this phenomenon may be an artefact of a five day culture period. Alternatively, and perhaps more interestingly, this phenomenon could be explained by suppression of lymphocyte proliferation by the activated $CD8^+$ cells which predominate in the lymph at these times. This latter possibility could be tested by titrating purified $CD8^+$ cells, collected at this time, into autologous antigen and mitogen stimulated cultures and assaying for inhibition of lymphocyte proliferation.

4.3 Summary of Chapter 4 results

1. Acute maedi-visna infection results in the production of anti-viral antibody within 3-4 days post-infection. Initially this antibody is of the IgM isotype, but later IgG_1 anti-visna antibody was detected.
2. Virus neutralising antibody was first detected in efferent lymph plasma around 12 days after infection and increased in titre with time.
3. *In vitro* lymphocyte proliferation to visna antigen could be detected about 15 days post-infection.

4.4 Discussion

The acute immunologic events following lentiviral infection are likely to be crucial in determining the course of disease. These early events in the pathogenesis of lentiviral infection are likely to occur in lymphoid organs, however, apart from the work of Reimann *et al* (1991), relatively little is known about acute immune responses to lentiviruses in these sites.

The model system described here: experimental infection of sheep with maedi-visna virus, via acute challenge of a cannulated lymph node, has the advantage that this system allows continuous monitoring of the acute events occurring in regional lymphoid tissue after lentiviral infection.

A striking feature of the early immune response to visna is the increased cellularity of the efferent lymphatic output from the challenged node (see Appendix A, Figure A.2). Seven to sixteen days after infection with maedi-visna virus there is an approximate four-fold increase in the percentage of lymphoblasts exiting the node. Continuing antigenic exposure is one obvious reason why these changes, in a primary response to visna virus, are much more dramatic than those seen in a primary response to soluble protein antigen, where only small changes in lymphocyte and blast cell output are seen (Hopkins *et al*, *submitted for publication*).

The majority of the lymphoblasts within this increase in cell output are $CD8^+$ (Appendix A. Figure A.2. Blacklaws *et al, in preparation*). This finding parallels the acute changes seen in the lymph nodes of macaques challenged with SIV (Reimann *et al*, 1991) and those of mice infected with LCMV, where again $CD8^+$ T cells predominate in the response (Lynch *et al*, 1989). The work of Reimann *et al* provides an intriguing parallel with one aspect of the immune response to visna seen in this system; visna infection results in a striking $CD8^+$ lymphoblast response, but, in the majority of sheep tested this CD8 lymphoblastosis was not associated with detectable levels of visna-specific cytotoxic activity. By analogy to HIV infection (Joly *et al*, 1989) and the SIV system described by Reimann *et al*, this could be interpreted as suggesting the presence of both virus-specific effector cells and 'suppressor cells' within this CD8 population. The balance between these two populations determining whether or not a CTL response can be detected *in vitro*. Certainly the data presented in Figure 4.8B and Table 4.2 could be interpreted as suggesting the existence of a population of 'suppressor cells', although there are other hypotheses to explain those observations.

In contrast to the failure to detect CTL activity, *in vitro* lymphocyte proliferation to visna antigen was detected in 2/2 infected sheep tested (Figure 4.8A). This proliferation was detected relatively late in the response, day 15 post infection onwards, but there is some evidence; the switch in isotype of the anti-visna antibody response, from IgM to IgG_1 (Figure 4.4), for 'T-helper', presumably $CD4^+$, activity within the node earlier in the response. The late detection of antigen-specific *in vitro* lymphocyte proliferation may be related to the

delayed kinetics of the lymphoblast response: lymphoblasts were detected between days 5-18. Previously it has been shown that specific antigen reactive T cells can be detected exiting a challenged node only when the blast response is declining (Hay *et al*, 1974., Cahill *et al*, 1979). This has been interpreted to mean that antigen causes the selective retention of antigen-specific lymphocytes within the node and that this is followed by the proliferation and differentiation of large numbers of antigen-sensitised cells which then leave the node as small lymphocytes (Trnka & Cahill, 1980). Another explanation for the failure to detect antigen-specific *in vitro* lymphoproliferation before day 15, which does not exclude the above, is that the relatively weak antigen-specific responses are masked by a decrease in the sensitivity of the assay during the period when blast cells are in the cultures (Figure 4.8B).

A third factor which could affect the T cell responses seen after infection with visna virus is that *in vivo* the major target cells for visna are those of the monocyte-macrophage lineage; the 'professional' antigen-presenting cell. In peripheral blood and afferent lymph the number of visna infected cells is low, approximately 1 in 10^6 cells infected as judged by *in situ* hybridisation analysis (Peluso *et al*, 1985., Gendelman *et al*, 1986., Dr. D.J. Roy, *personal communication*), and it is difficult to reconcile such a low frequency of infected cells with any direct, major effect of visna infection of macrophages on immune function or recognition. The possibility exists, however, that visna infection of macrophages may result in disordered secretion of monokines such as IL-1 and TNF-α and that this could influence the pattern of T cell responses observed. However no formal demonstration of such an effect has been reported.

In contrast to the late detection of antigen-specific T cells in efferent lymph, visna specific antibody was first detected 3-4 days post-infection (Figures 4.2 & 4.3) and antibody titres were then maintained or increased with time. Although there was some variation in the kinetics at which the antibody response developed, a reasonably similar pattern of antibody response could be discerned between sheep. There was an initial peak of anti-p25 antibody, which was mainly of the IgM isotype, followed by a second peak of IgG anti-p25 antibody. The anti-visna antibody titres detected are comparable to those observed in efferent lymph

following infection with Orf virus (Yirrell *et al*, 1991), the differences in the kinetics of the two reponses are probably due to the fact that Yirrell *et al* were studying secondary responses to Orf virus.

Some of the variation in anti-visna antibody response between sheep may reflect the fact that outbred sheep were used in these experiments, but it is interesting to note that sheep 683R, which produced more antibody and earlier than the other two sheep, had a low titre anti-p25 antibody response prior to visna infection. A similar phenomenon has been reported in an SIV system (Gotch *et al*, 1991), and it has been suggested that this may represent cross-reactivity between anti-SIV antibodies and antibodies to endogenous retroviruses. If a similar situation obtained for sheep 683R, then such antibodies may have influenced the development of the anti-visna p25 antibody response. It is important to note that visna virus could not be isolated from this sheep prior to infection with visna virus (Appendix A, Figure A.1), that this sheep was seronegative for antibodies to other visna proteins, as judged by western blot analysis (data not shown) and did not develop neutralising antibodies to visna virus until a time after infection similar to sheep 649R and 657R (Figure 4.7).

The detection of virus-specific antibody in efferent lymph coincided with both increased levels of total immunoglobulin in lymph and with increased levels of Ig synthesis by efferent lymph B cells cultured *in vitro* (Appendix A, Figure A.3a & b). However visna specific antibody in efferent lymph is of relatively low titre (Figure 4.3) and it was not possible to determine whether this antigen specific immunoglobulin is secreted by cells within the node or by free-floating, lymph borne B cells.

In response to challenge with salmonella lipopolysaccharide (LPS), specific antibody was produced by both lymph-borne B cells and cells within the node (English *et al*, 1976), but interpretation of this data is complicated by the fact that LPS is a potent B cell mitogen. In these visna challenge experiments 10^6 efferent lymph cells (20%-30% B cells), collected at various times through the cannulation, and cultured *in vitro* for seven days did not secrete detectable levels of anti-p25 antibody (data not shown). However, given that the virus specific antibody in efferent lymph is of relatively low titre, this may reflect a low frequency of visna

specific B cells exiting the node, a lack of sensitivity of the assay or both. In contrast to this failure to detect *in vitro* production of anti-visna antibody by efferent lymphocytes, Yirrell *et al* (1991) detected peak titres of anti-Orf antibody ranging from 1/40 to 1/320 in *in vitro* cultures of efferent lymphocytes collected during a secondary response to the virus. Other workers, studying secondary immune responses to protein antigens, have given estimates of the frequency of antigen-specific B cells exiting a challenged node ranging from 0.5% (Flynn, 1988) to 6% (Hay *et al*, 1972). Staining, with biotinylated p25 (followed by avidin-FITC), of efferent lymph cells exiting a node challenged with visna virus revealed very few p25 specific B cells (Dr. P. Bird, *personal communication*). Although it is possible that the presence of antigen-specific B cells may have been masked by binding of free p25 to surface immunoglobulin, these data imply that it is perhaps more likely that most of the anti-visna antibody is secreted by B cells within the node. This suggestion is supported by the observation that significant levels of visna-antigen specific lymphoproliferation were seen only when the cultures were supplemented by autologous PBMC. Visna specific B cells exiting the node would have been expected to act as antigen-presenting cells (reviewed in Lanzavecchia, 1990). No such effect was observed. The PBMC did not proliferate when cultured with visna antigen, thus it is likely that addition of these cells enhanced the proliferative response by supplying monocyte/macrophages to act as APC's.

Antibodies to p25, while a useful marker of the immune response to visna infection, are not thought to be of major importance in protection from disease. In contrast, dissemination of virus by extracellular routes has been reported to cease after the appearance of virus neutralising antibodies even though infectious virus. as detected by co-cultivation, is almost exclusively cell associated (Petursson *et al*, 1976). The basis of this correlation is unclear: immunosuppression does not relieve the restriction in virus gene expression (Nathanson *et al*, 1976., Narayan *et al*, 1977), but these data do not exclude the possibility that this restriction is initially imposed by host immune responses and then maintained by other mechanisms. This hypothesis is consistent with the data presented here: a reduction in viral replication *in vivo*, as measured by either infectious centre assay (Appendix A, Figure A.4) or

p25 antigen assay (Figures 4.5 A & 4.6), coincides with the appearance of virus neutralising antibody in efferent lymph (Figure 4.7). Direct evidence of a causal relationship is lacking, but these data provide strong circumstantial evidence for an important role in restriction of the dissemination of virus *in vivo* by neutralising antibody to maedi-visna virus produced shortly after infection.

However this suggestion is in contrast to the observations reported by Geballe *et al* (1985). Using quantitative *in situ* hybridisation these workers demonstrated that restriction in viral nucleic acid synthesis became evident 7-8 days after infection of sheep by intra-pulmonic inoculation of visna virus. In these experiments virus neutralising antibody was not detected until 3-4 weeks after infection, but these workers were assaying blood plasma for neutralising antibody, rather than the efferent lymphatic output of the regional lymphoid tissue draining the site of virus infection. It is therefore possible that virus neutralising antibody was produced early after infection and influenced viral replication, but that initially this antibody was diluted, when the lymph entered the circulation, below detectable levels in blood plasma. In one of these cannulation experiments (657R), serum antibody responses were analysed in parallel with the efferent lymph antibody response. Interestingly, no visna-specific antibody could be detected in the serum of this sheep until approximately one month after the end of the cannulation (in agreement with Figure 3.1), whereas visna specific antibody could be detected in efferent lymph from 3-4 days after infection (data not shown). This data is in agreement with the above hypothesis, but it is possible that the removal of antigen-specific efferent lymphocytes via the cannulated lymphatic may also have contributed to this delayed systemic response (McConnell *et al*, 1974., Hopkins *et al*, 1981).

As described above there appears to be an inverse relationship between detection of cell associated virus and the appearance of virus neutralising antibody, but the significance of virus-specific T cell responses in this system is unclear. Cellular immune responses are believed to be critical determinants of protection and clearance in most viral infections (Mims & White, 1984., Reddehase *et al*, 1989) and yet there is no obvious temporal correlation between either the CD8 lymphoblast response (Appendix A, Figures A.2) or virus specific *in*

specific *in vitro* lymphoproliferative responses (Figure 4.8A) and a decline in virus replication. Further, virus-specific CTL activity was not detected in the majority of sheep tested, although that said, inhibition of HIV replication in acutely infected $CD4^+$ T cells has been shown to involve a non-cytotoxic mechanism mediated by $CD8^+$ T cells (Brinchmann *et al*, 1990., Walker *et al*, 1991). These studies have demonstrated a role for soluble factor(s) in suppression of virus growth, although cell:cell contact appears to be required for optimal antiviral activity (Walker *et al*, 1991). Thus although *in vitro* neutralisation of visna virus appears to be mediated entirely by antibody (Table 4.1); *in vivo* the products of activated T cells eg. IFN-γ, and tumour necrosis factor (Yamamoto *et al*, 1986., Paliard *et al*, 1988) may contribute to the observed reduction in virus replication. Further investigation of this point would require assay of both efferent lymph, and the supernatant of cultures of efferent lymphocytes proliferating *in vitro* in response to visna antigen, for cytokines suspected to be important in regulating lentiviral gene expression *in vivo*.

Whatever the basis of the observed restriction in virus dissemination *in vivo*, viral replication is not completely eliminated. The data in Figures 4.5A and 4.6 indicate that while the first peak of p25 antigen production initially declines, a second wave of antigenaemia can be detected later in the course of the cannulation. Thus although viral replication, as measured by infectious centre assay, cannot be detected, these data imply that viral replication, albeit at a low level, is continuing in the face of the host immune response.

Chapter 5

Cloning and expression of p25, the major core protein of maedi-visna virus

5.1 Introduction

Maedi-visna virus infection of sheep results in a spectrum of disease pathology: encephalomyelitis, interstitial pneumonitis, lymphadenopathy and wasting, reminiscent of that seen in HIV infection of man (Georgsson *et al*, 1990). However, in contrast to HIV, the major cell types productively infected with visna *in vivo* are of the monocyte-macrophage lineage (Gendelman *et al*, 1985., 1986). Maedi-visna virus infection of sheep may thus prove to be a useful model for certain aspects of HIV pathogenesis, since both the effects of lentiviral infection on the immune system and the immune response to lentiviral infection can be studied in a situation where viral infection of lymphocytes is not a recognised feature of disease.

The utility of this system is, however, limited by the paucity of visna virus specific reagents. In order to generate large quantities of an individual viral protein for use in immunological assays (see Chapters 3 & 4) and the generation of specific antisera the gene encoding p25 was cloned via PCR (Saiki *et al*, 1988) and expressed using the yeast Ty-VLP system (Adams *et al*, 1987*a*).

In this system p25 is expressed as a Ty-p25 fusion protein which self-assembles to form virus-like particles. Exploitation of the physical properties of these properties allows relatively easy purification of the Ty-p25 VLP's from yeast. This is the first report on the cloning and expression of a structural protein of maedi-visna virus.

Recombinant p25 protein was then used to generate p25 specific antisera, both polyclonal and monoclonal antibodies, which have been used to develop a sensitive detection assay for the major core protein of maedi-visna virus.

5.2 Results

5.2.1 Cloning and expression of the p25 structural gene

The p25 gene was cloned by polymerase chain reaction (PCR) performed on extrachromosomal DNA isolated from visna infected cells. Using PCR allowed precise cloning of p25 coding sequence only, without the necessity for extensive *in vitro* manipulation of DNA. Further, the PCR primers were designed to create restriction sites (*Bam HI*) flanking the p25 gene and to insert a stop codon immediately following the p25 coding sequence (Figure 5.1A), thus facilitating subsequent steps in cloning. Sequence coding for a *Factor Xa* protease cleavage site (Nagai & Thorgerson, 1984) was also inserted into the 5' PCR primer, thus facilitating subsequent purification of p25 from the hybrid Ty-p25 particle. In order to allow efficient cleavage of p25 from the fusion protein using *Factor Xa* enzyme, nucleotides coding for the first amino acid of p25, a proline residue (CCT), were omitted from the 5' primer (Gilmour *et al*, 1989).

PCR generated a product of the predicted size (approximately 650bp) for the p25 gene. This PCR product, when cloned into pTZ 19R and sequenced, proved to be a full length p25 gene with 98% homology to the EV1 nucleotide sequence (Sargan *et al*, 1991) (Figure 5.1B).

The p25 gene was then excised from pTZ 19R by *Bam HI* digestion and ligated into the unique *Bam HI* site of the yeast/ *E. coli* shuttle vector pOGS 40 (Fig. 5.2) (Gilmour et al, 1989). A gene inserted into the *Bam HI* site of this plasmid is expressed as a TyA fusion protein, under the control of a hybrid PGK-GAL (PAL) promoter. This is the strong yeast phosphoglycerate kinase (PGK) gene promoter (Dobson *et al*, 1982) which has been modified to become galactose inducible (Kingsman *et al*, 1990).

A number of recombinant plasmids containing a single copy of the p25 gene in the correct orientation for expression were identified by Southern blot analysis of *Pvu II* digested plasmids (Fig. 5.3). Double strand sequencing through the 5' vector-insert junction of one of these plasmids (track 7), using a primer derived from the TYA gene sequence (Mellor *et al*,

Figure 5.1

A. Sequences of the sense and antisense primers used in a PCR to clone the visna p25 gene, from a Hirt DNA template, for expression in yeast.

B. The PCR product was cloned into pTZ 19R. Single stranded DNA was prepared (section 2.5.1) from recombinant plasmids containing the p25 gene, identified by restriction enzyme digest of DNA extracted from overnight cultures grown from white colonies, and sequencing reactions carried out as described (section 2.5.2). Sequence analysis and amino-acid translation was done on a Vax system using version 6.2 of the University of Wisconsin Genetics Computing Group package (Devereux *et al*, 1984).

Figure 5.1A

5' primer (sense)

Bam HI *first 20bp of p25 (-CCT)*
TCCCGGGATCCATAGAAGGTAGAATTGTAAATCTGCAAGCAGG
Factor Xa

3' primer (antisense)

Bam HI *last 20bp of p25*
ACACCCGGGATCCTTCTACCCTTCTGATCCTACATCTC
Stop

Figure 5.1B

```
       ATTGTAAATCTGCAAGCAGGGGGAGAAGTTGGAAGGCGGTAGATTCAGTAGTCTTCCAG
   1 ---------+---------+---------+---------+---------+---------+ 60
       TAACATTTAGACGTTCGTCCCCCTCTTCAACCTTCCGCCATCTAAGTCATCAGAAGGTC

       IleValAsnLeuGlnAlaGlyGlyArgSerTrpLysAlaValAspSerValValPheGln

       CAATTGCAAACTGTGGCTATGCAGCATGGCCTTGTGTCCGAGGATTTTGAAAGACAGCTG
  61 ---------+---------+---------+---------+---------+---------+120
       GTTAACGTTTGACACCGATACGTCGTACCGGAACACAGGCTCCTAAAACTTTCTGTCGAC

       GlnLeuGlnThrValAlaMetGlnHisGlyLeuValSerGluAspPheGluArgGlnLeu

       GCGTATTATGCTACTACATGGACAAGCAAGGATATATTAGAAGTATTGGCCATGATGCCT
 121 ---------+---------+---------+---------+---------+---------+180
       CGCATAATACGATGATGTACCTGTTCGTTCCTATATAATCTTCATAACCGGTACTACGGA

       AlaTyrTyrAlaThrThrTrpThrSerLysAspIleLeuGluValLeuAlaMetMetPro

       GGGAACAGAGCACAGAAAGAGCTGATTCAGGGAAAATTAAATGAAGAAGCAGAAAGATGG
 181 ---------+---------+---------+---------+---------+---------+240
       CCCTTGTCTCGTGTCTTTCTCGACTAAGTCCCTTTTAATTTACTTCTTCGTCTTTCTACC

       GlyAsnArgAlaGlnLysGluLeuIleGlnGlyLysLeuAsnGluGluAlaGluArgTrp

       GTGAGGCAGAACCCGCCAGGGCCAAATGTCCTCACGGTGGATCAAATCATGGGAGTAGGA
 241 ---------+---------+---------+---------+---------+---------+300
       CACTCCGTCTTGGGCGGTCCCGGTTTACAGGAGTGCCACCTAGTTTAGTACCCTCATCCT

       ValArgGlnAsnProProGlyProAsnValLeuThrValAspGlnIleMetGlyValGly

       CAAACAAATCAACAGGCATCACAGGCTAATATGGATCAACGAAGGCAACTGTGCTTGCAG
 301 ---------+---------+---------+---------+---------+---------+360
       GTTTGTTTAGTTGTCCGTAGTGTCCGATTATACCTAGTTGCTTCCGTTGACACGAACGTC

       GlnThrAsnGlnGlnAlaSerGlnAlaAsnMetAspGlnArgArgGlnLeuCysLeuGln

       TGGGTCATAACAGCCTTGAGAGCGGTAAGGCATATGTCGCATAGGCCAGGTAACCCAATG
 361 ---------+---------+---------+---------+---------+---------+420
       ACCCAGTATTGTCGGAACTCTCGCCATTCCGTATACAGCGTATCCGGTCCATTGGGTTAC

       TrpValIleThrAlaLeuArgAlaValArgHisMetSerHisArgProGlyAsnProMet

       CTGGTAAAGCAGAAGAATACTGAGAGTTATGAAGATTTCATAGCGAGGTTGCTGGAAGCA
 421 ---------+---------+---------+---------+---------+---------+480
       GACCATTTCGTCTTCTTATGACTCTCAATACTTCTAAAGTATCGCTCCAACGACCTTCGT

       LeuValLysGlnLysAsnThrGluSerTyrGluAspPheIleAlaArgLeuLeuGluAla

       ATTGATGCAGAACCAGTCACCGATCCTATAAAAACATATTTCAAAAGTGACTCTGCATAC
 481 ---------+---------+---------+---------+---------+---------+540
       TAACTACGTCTTGGTCAGTGGCTAGGATATTTTTGTATAAAGTTTTCACTGAGACGTATG

       IleAspAlaGluProValThrAspProIleLysThrTyrPheLysSerAspSerAlaTyr

       ACGAATGCTAGTACAGATTGTCAAAAGCAAATGGACAGAGTCTTGGGAACTAGG
 541 ---------+---------+---------+---------+---------+---- 594
       TGCTTACGATCATGTCTAACAGTTTTCGTTTACCTGTCTCAGAACCCTTGATCC

       ThrAsnAlaSerThrAspCysGlnLysGlnMetAspArgValLeuGlyThrArg
```

Figure 5.2 Plasmid map of expression vector pOGS 40

The plasmid pOGS 40 is a derivative of the TYA plasmid pMA 5620 (Adams *et al*, 1987*a*), in which the constitutive PGK promoter (Dobson *et al*, 1982) has been modified by deletion of the UAS-PGK gene and the insertion of an upstream activating sequence from the *GAL1-10* gene (UAS-GAL) (West *et al*, 1984). This hybrid PGK-GAL (PAL) promoter is repressed by culture in glucose and induced by galactose (Kingsman *et al*, 1990). Signals for termination of transcription (PGK terminator) are located 3' of the unique *Bam HI* site (Kingsman *et al*, 1990). For replication in yeast the plasmid contains sequences from the endogenous multi-copy yeast plasmid, the 2µm circle and the *LEU2-d* gene as a selectable marker (Kingsman *et al*, 1990). For replication and selection in *E. coli* the plasmid contains sequences derived from pAT 153 (Twigg & Sherratt, 1980).

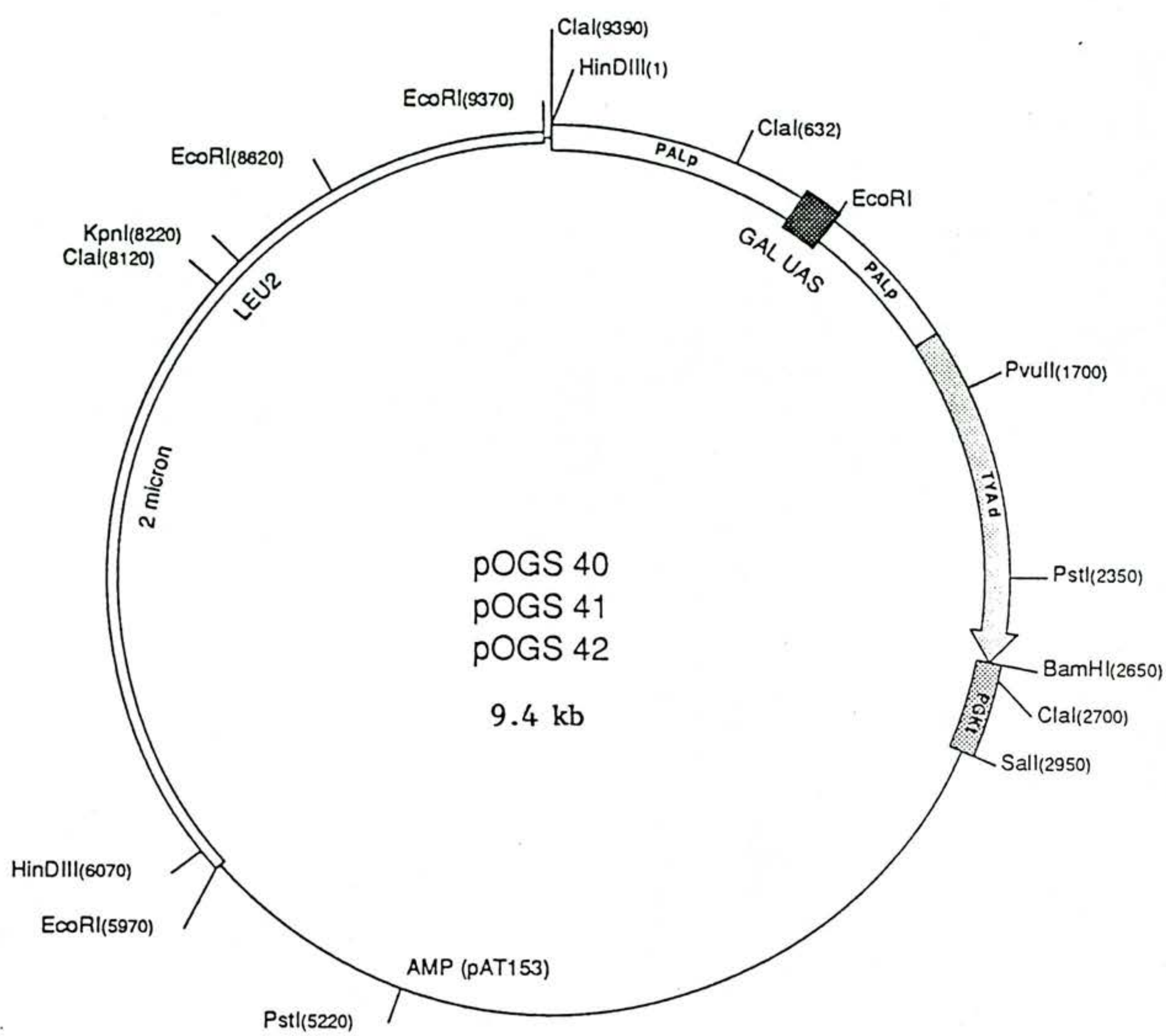

ClaI(9390)
HinDIII(1)
EcoRI(9370)
ClaI(632)
PALp
EcoRI
GAL UAS
PALp
EcoRI(8620)
KpnI(8220)
ClaI(8120)
LEU2
PvuII(1700)
2 micron
TYAd
pOGS 40
pOGS 41
pOGS 42
9.4 kb
PstI(2350)
BamHI(2650)
PGK1
ClaI(2700)
SalI(2950)
HinDIII(6070)
EcoRI(5970)
AMP (pAT153)
PstI(5220)

Figure 5.3 Southern blots of plasmid DNA to establish orientation of p25 gene in p25/pOGS 40 recombinants

A. Linearised plasmid pEV1P25.O (10kbp)

Positions of restriction enzyme sites are shown: B - *Bam HI* and P - *Pvu II.*

B. Southern blot

Plasmid DNA was extracted from transformed *E. coli* by lysis in alkaline-SDS and digested with *Pvu II.* Electrophoresis and Southern blotting procedures were carried out as described in Chapter 2. The blot was probed with the p25 gene PCR product which had been cloned into pTZ and sequenced (120ng DNA, 2.3 x 10^8cpm/µg). The p25 gene was excised from pTZ by *Eco RI/Hind III* digestion, gel purified and radiolabelled according to Feinberg & Vogelstein (1983., 1984). The blot was exposed to Kodak X-Omat S film for 20 minutes.

Marker DNA fragments were *Eco RI/Hind III* digestion products of λ DNA, marker sizes are indicated on left.

Predicted sizes of *Pvu II* fragments:

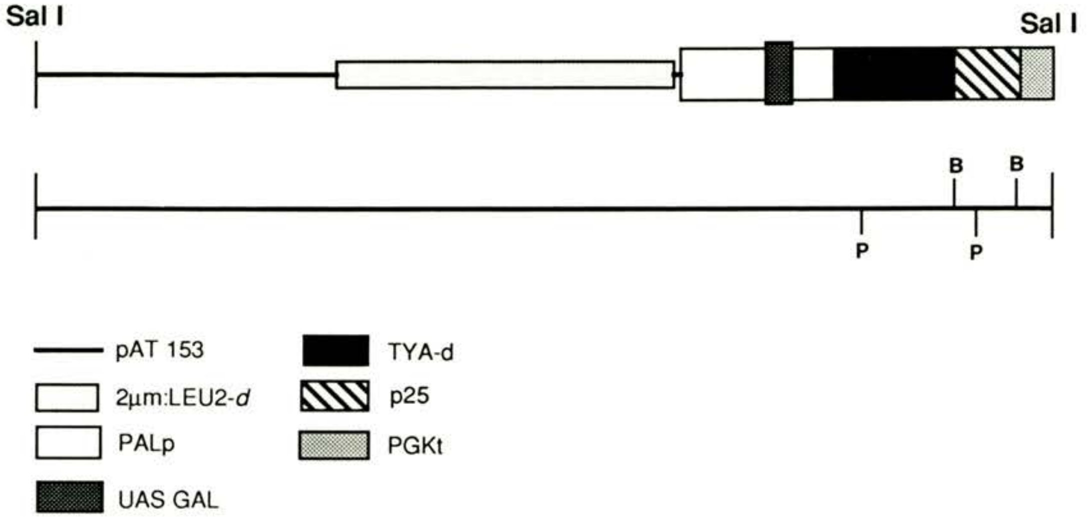
Correct orientation: 2 fragments - 8833bp, 1217bp

Wrong orientation: 2 fragments - 8583bp, 1467bp

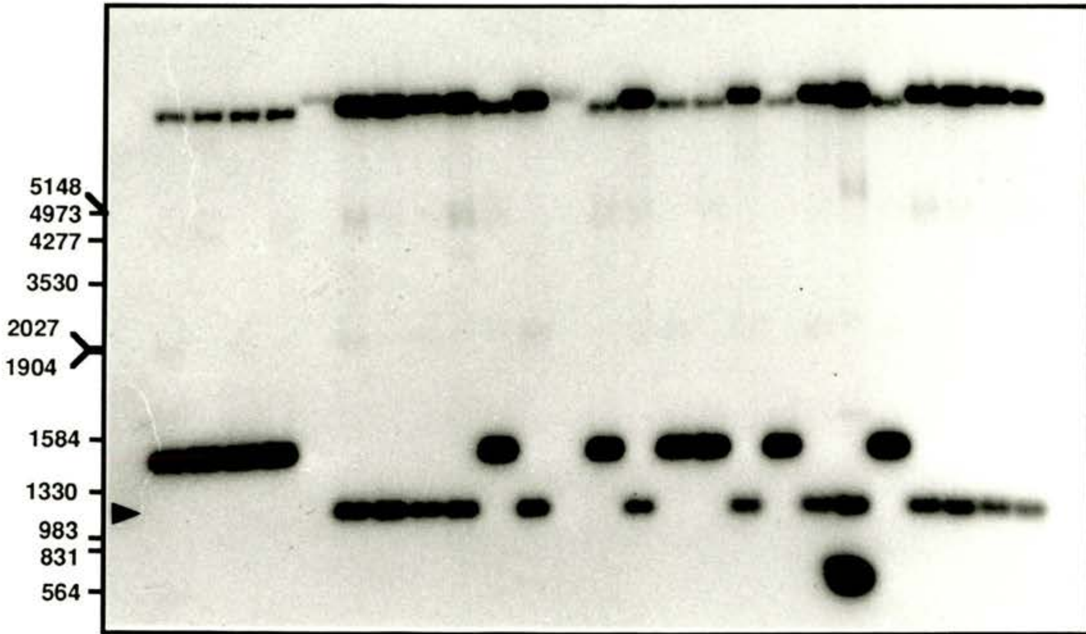
The arrow indicates the 1217bp band in those plasmids where the p25 gene is in the correct orientation for expression. Tracks 5 and 12 contain plasmids with no p25 insert. The plasmid in track 20 contains two copies of the p25 gene, both in the correct orientation for expression.

Figure 5.3

A



B



1985), confirmed that the gene was in the correct reading frame for expression. This plasmid was designated pEV1P25.O.

The protease deficient *Saccharomyces cerevisiae* strain BJ 2168 was simultaneously transformed to leucine and uracil independence with plasmids pEV1P25.O and pUG 41S. Plasmid pUG 41S overexpresses GAL4 protein under the control of a galactose inducible promoter (Lue *et al*, 1987). Co-transformation of yeast with this vector and a PAL-promoter containing VLP plasmid (Kingsman *et al*, 1990) results in increased expression levels since a major constraint on the level of protein expression in galactose inducible systems is the low level of GAL4 gene expression (Johnston & Hopper, 1982), especially if the cell contains multiple copies of the expression plasmid (Baker *et al*, 1987). Regulated overproduction of GAL4 increases protein expression levels and retains the inducibility of the system (Schultz *et al*, 1987).

Preliminary analysis of protein extracts of a number of yeast transformants containing pEV1P25.O and pUG 41S indicated that they all expressed similar levels of Ty-p25 fusion protein. One of these transformants was chosen for more detailed analysis and this strain was designated TEV1P25.U.

TEV1P25.U, either induced or uninduced, has a doubling time and growth curve similar to that of untransformed BJ 2168, indicating that high level expression of Ty-p25 protein is not toxic for yeast cells. As a result of this observation, the standard protocol for induction of protein expression in this system (24hrs in galactose medium, Burns *et al*, 1991), was modified so that p25 expression was induced by culture in galactose for 36-48hrs. This resulted in a 4-5 fold increase in yield of p25 fusion protein, per volume of culture fluid, over cultures induced for 24hrs.

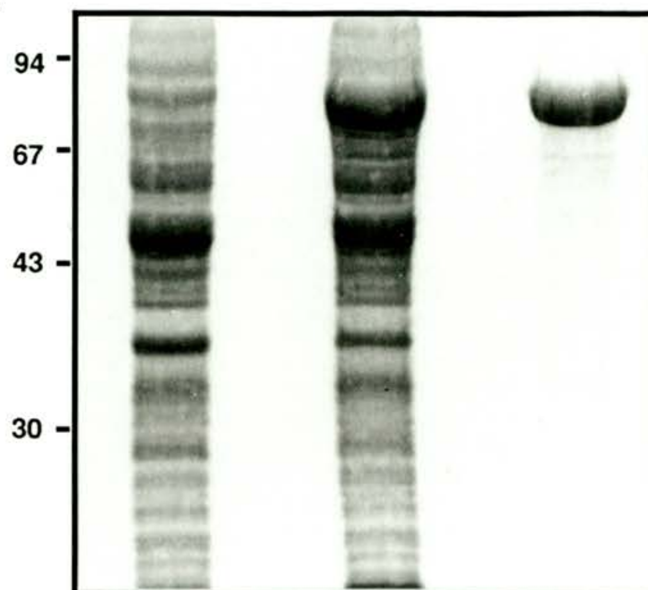
On Coomassie stained SDS-PAGE a prominent band of M_r. 75kd. could be seen in extracts of TEV1P25.U, but not untransformed yeast (Figure 5.4A). This is the predicted size of a Ty-p25 fusion protein (wild type VLP, on SDS-PAGE have an apparent M_r of 50kd.). p25 expression was confirmed by immunoblot analysis using a p25 specific monoclonal antibody, mAb 396 (Houwers & Schaake, 1987), which recognised a single strong band of

Figure 5.4 Expression and purification of Ty-p25 protein

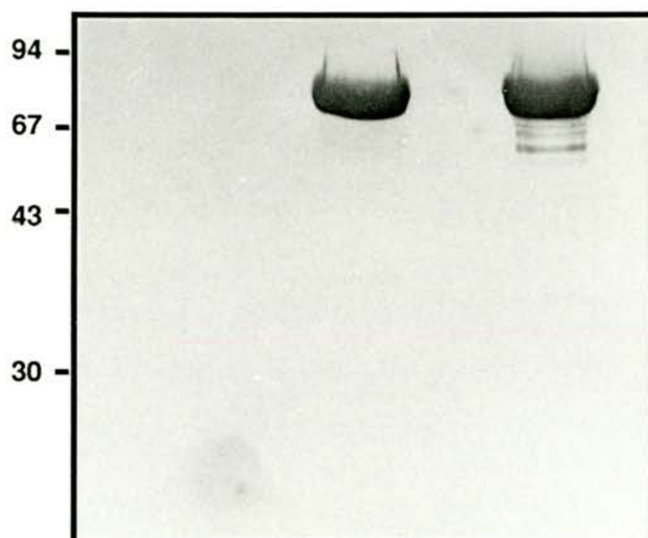
Crude extracts (see section 2.6.4) of untransformed yeast, induced TEV1P25.U and gradient purified Ty-p25 (from left to right tracks 1 to 3 respectively) were electrophoresed on 10% SDS-PAGE and the gels either stained with Coomassie blue (A) or electroblotted and developed with a p25 specific monoclonal antibody (B). Positions of molecular weight standards are indicated.

Figure 5.4

A



B



approximately 75kd. in extracts of TEV1P25.U, but not untransformed BJ 2168 (Figure 5.4B) or yeast transformed with pOGS 40 alone (data not shown). The same band was recognised when these samples were probed with sera from visna infected sheep.

Hybrid Ty-p25 virus-like particles (VLP's) could be purified, by centrifugation through sucrose gradients, to greater than 95% purity (Figure 5.4A, track 3), as judged by densitometric examination of Coomassie stained gels. Batch cultures of induced TEV1P25.U yielded 50-60mg of fusion protein per litre of culture fluid (estimated using a BioRad dye-binding assay) and immunoblotting confirmed that the sucrose-gradient purified particles carried p25 antigen (Figure 5.4B, track 3).

Recombinant p25 could be further purified by cleavage from the Ty-p25 particle using Factor Xa protease (Nagai & Thorgerson, 1984). Under optimum conditions (10mM $CaCl_2$, 100mM Tris pH 7.4, 0.05% CHAPS) established by preliminary time course and titration experiments, complete cleavage of p25 from the Ty-p25 VLP's could be achieved and the soluble p25 purified from the VLP's by ultracentrifugation (Figure 5.5), yielding 15-20mg of p25 per litre of culture fluid. Non-specific degradation of VLP's was not observed.

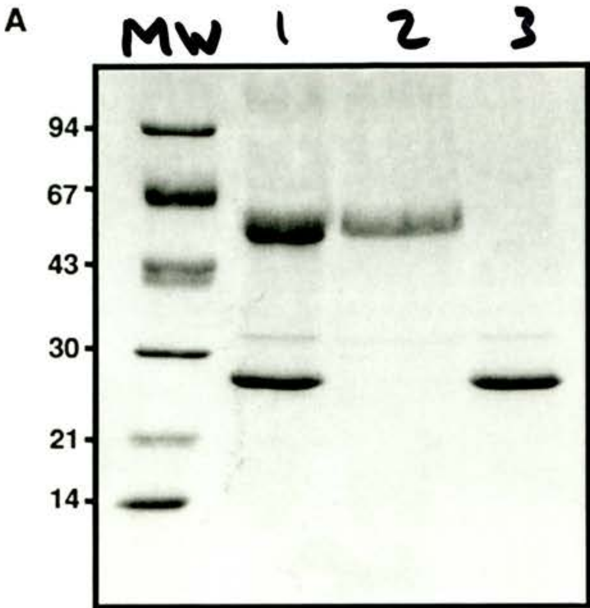
5.2.2 Antigenic authenticity of recombinant p25 protein

The cleaved, purified recombinant p25 migrated as native p25 protein on SDS-PAGE and was reactive with an anti-p25 monoclonal antibody (Figure 5.5). To further examine the antigenicity of the recombinant p25 rabbits were immunised with purified p25 protein. When tested in immunoblot analysis against a lysate of virus infected cells these antisera showed reactivity not only against p25, but also recognised the ***gag*** precursor protein, p55, and processing intermediates between p55 and p25 (Figure 5.6). A similar pattern of reactivity was seen when these antisera were used in immunoprecipitation analysis of visna infected skin cells (data not shown). In immunofluorescence experiments these antisera specifically stained EV1 infected monolayer cultures and not uninfected controls. This staining was

Figure 5.5 Purification of p25 protein

Ty-p25 VLP's were treated with *Factor Xa* protease and then centrifuged to separate solubilised p25 from the VLP's. Samples of the digest (track 1), centrifuge pellet (track 2) and p25 containing supernatant (track 3) were electrophoresed on 5%-20% linear gradient SDS-PAGE and the gels either stained with Coomassie blue (A), or developed with an anti-p25 monoclonal antibody (B), (Houwers & Schaake, 1987). The weakly staining band seen seen in tracks 1 & 3 of gel A is Factor Xa (M_r-34kD). Positions of molecular weight standards are indicated.

Figure 5.5



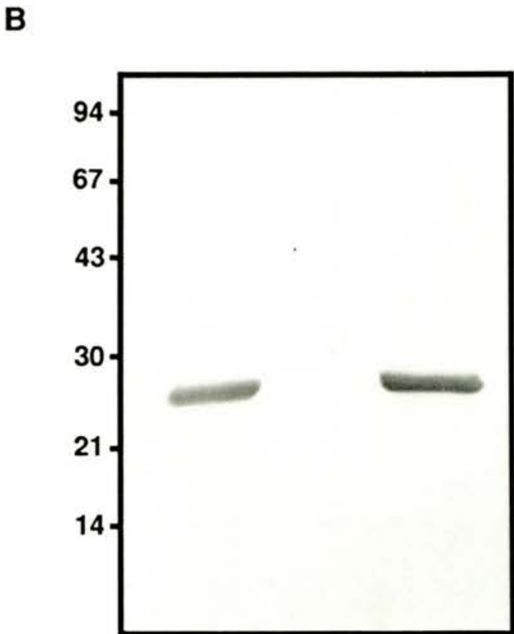
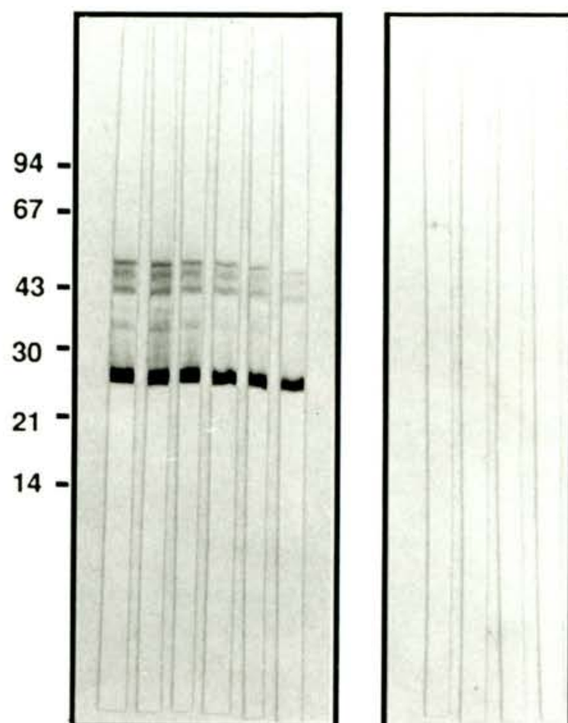


Figure 5.6 Rabbits immunised with recombinant p25 protein produce antibodies reactive with native viral protein

A lysate of EV1 virus infected cells (see section 2.8.1) was separated on 5%-20% linear gradient SDS-PAGE and electroblotted onto a nitrocellulose membrane. These blots were then probed with either immune (Rabbit 187) or non-immune rabbit antisera. The left-hand panel shows a titration, in doubling dilutions from 1/100, of rabbit anti-p25 antiserum. A titration, again from 1/100, of non-immune rabbit antiserum is shown in the panel on the right.

Figure 5.6



mainly confined to the cytoplasm of the cell although a ring of patchy staining at the plasma membrane was also seen (see Figure 3.9).

5.2.3 Monoclonal antibody production

Balb/c mice were immunised with 10µg of purified recombinant p25 emulsified in Freund's complete adjuvant. Secondary immunisations were given by intraperitoneal injection of 10µg p25 in PBS. Three days before fusion mice were given an intravenous injection of 5µg of p25 antigen. The procedure of cell fusion was performed as described in Chapter 2.

A number of hybridomas were positive in ELISA assay against purified p25 antigen. The majority of these were of IgM isotype, but one hybridoma was found to secrete IgG_1 anti-p25 antibody. This hybridoma, designated 1D10, was cloned and the monoclonal antibody further characterised (Figure 5.7). This antibody is reactive with p25 from EV1 infected cells in both immunoblotting (5.7A) and immunofluorescence assays (5.7B & C).

Interestingly, while testing sera from mice immunised with the recombinant proteins prior to hybridoma production it was noted that mice immunised with Ty-p25 VLP's had made lower titre antibody responses than those immunised with similar amounts of purified, soluble p25 (Figure 5.8). It can be seen that there is an approximate four-fold difference in anti-p25 antibody titre between the two groups. It has been suggested that antigen presented in a polyvalent, particulate form is particularly immunogenic (Adams *et al*, 1987*a*), yet here that is clearly not the case. Antigenic competition between p25 and the p1 protein may be one explanation for this finding. Another possible explanation is that mice, if previously exposed to yeast, may have already made an immune response to p1 protein on the 'wild-type' VLP's present at low levels in yeast cells and the secondary anti-p1 response predominates in the response to Ty-p25 . Lastly, this result may be related to physical distortion of p25 in the hybrid Ty-p25 particle, leading to loss or masking of discontinuous epitopes.

In this expression system the virus-like particle is formed by self assembly of multiple copies of a truncated form of the yeast TYA gene product, designated p1, which is analogous

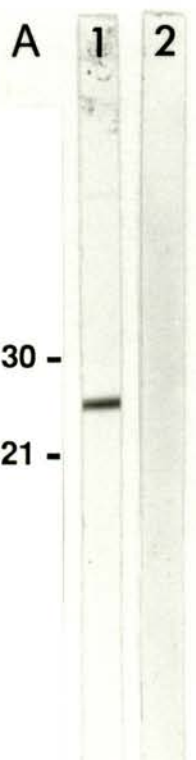
Figure 5.7 Characterisation of monoclonal antibody 1D10

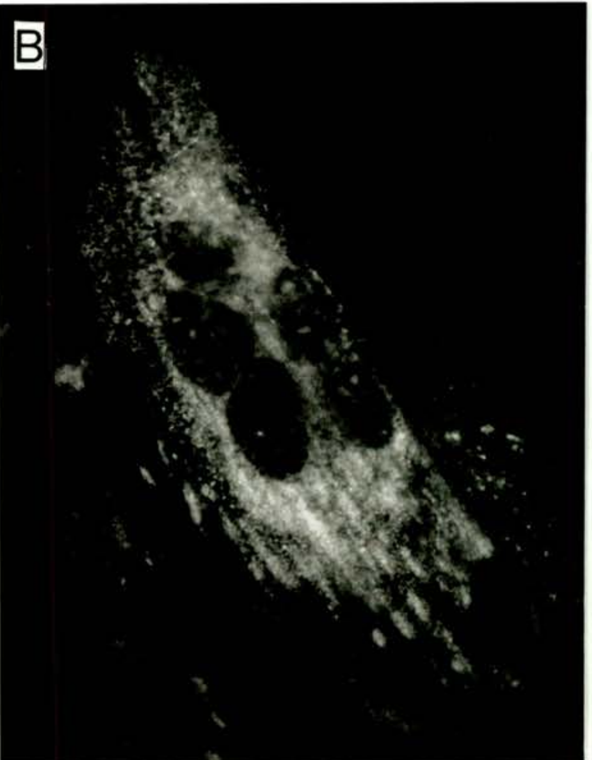
A. Immunoblot analysis

Visna antigen, prepared as described in section 2.8.1, was separated on 5-20% linear gradient SDS-PAGE run under reducing conditions and electroblotted onto a nitrocellulose membrane. Strips, cut from these blots, were incubated with either neat culture supernatant of 1D10 (track 1), or a control, isotype-matched irrelevant antibody (track 2). After washing, bound antibody was detected with rabbit anti-mouse Ig conjugated to alkaline phosphatase. Positions of molecular weight standards (kDa) are indicated.

B. & C. Indirect immunofluorescence analysis

Monolayer cultures of either EV1 infected (B) or mock-infected (C) skin cells were grown on glass coverslips, fixed in 80% acetone/0.15M NaCl and then incubated in neat 1D10 culture supernatant. After washing, cells were incubated with biotinylated anti-mouse Ig. Bound antibody was visualised using avidin-FITC. Visna infected cultures stained with a control, isotype matched mouse antibody showed similar fluorescence to C. Magnification for both panels is **x**1,714.


A
1
2
30
21


B

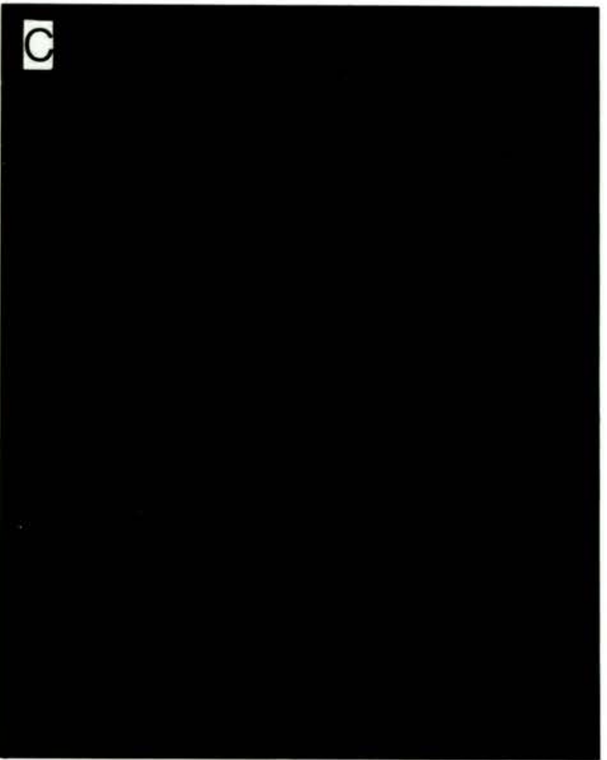
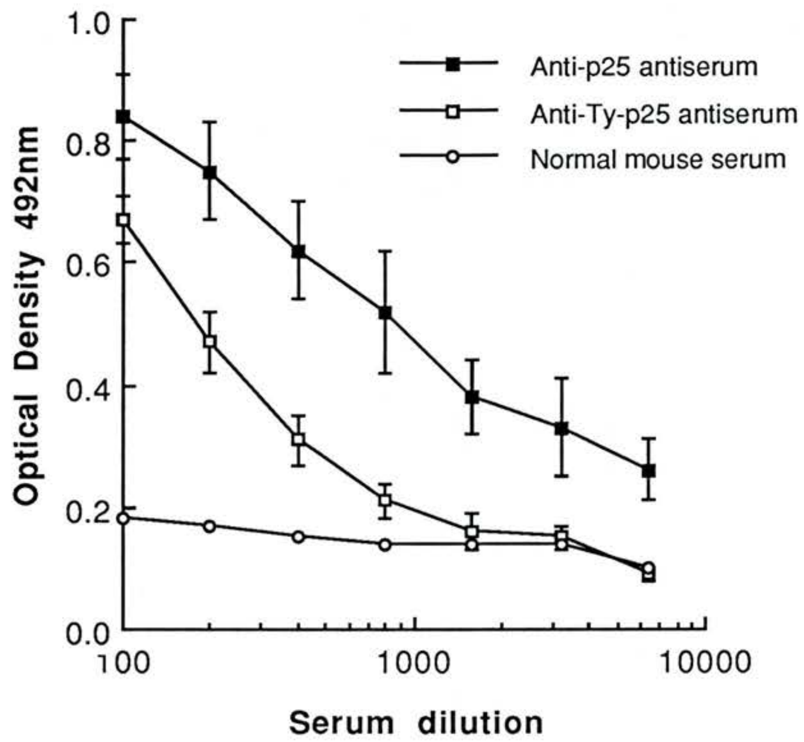

C

Figure 5.8 Relative immunogenicity of soluble p25 and hybrid Ty-p25 VLP's

Serial dilutions of sera from mice immunised with either soluble p25 or particulate Ty-p25 were tested in ELISA assay against purified maedi-visna virus. Mice were immunised with approximately 30µg of Ty-p25 or 10µg p25, ie.equivalent amounts of p25 antigen. This assay was performed as described in the legend to Figure 3.1, except that a rabbit anti-mouse Ig HRP conjugate (diluted 1/4000) was used to detect bound antibody.

Values are geometric means, ± 1 standard deviation, of groups of three mice.


1.0
0.8
0.6
0.4
0.2
0.0
Optical Density 492nm
100
1000
10000
Serum dilution
Anti-p25 antiserum
Anti-Ty-p25 antiserum
Normal mouse serum

to retroviral ***gag*** proteins (Adams *et al*, 1987*b*). p25, in visna, forms the core of the viral particle (Narayan & Clements, 1990) and it is possible that interactions between p25 and p1, each attempting to assemble into a preferred conformation, results in the observed distorted morphology of hybrid Ty-p25 VLP's (Figures 5.9 & 5.10).

Negatively stained Ty-p25 VLP's, when examined by transmission electron microscopy, show considerable heterogeneity in size and shape (Figure 5.9). In contrast to Ty-VLP's composed only of p1 protein, which have a characteristic regular morphology (Adams *et al*, 1987*b* and Figure 5.10A), many of the Ty-p25 particles had a distorted morphology. They appeared irregular in shape with a distinct outer shell, and in general were larger than non-recombinant VLP's. Many of them appeared to have internal core structures (Figure 5.10B). These distortions in p25 and p1 may have resulted in loss and/or masking of antibody epitopes on p25.

5.2.4 An assay for detection of p25 antigen

A specific and sensitive assay commonly used for the assessment of lentivirus replication in HIV and SIV systems is the measurement of p25 antigen levels. In order to develop such an assay for maedi-visna virus, the polyclonal and monoclonal anti-p25 antisera described previously were tested, in various combinations, in antigen-capture assays.

The most sensitive assay was found to be one based on the use of affinity-purified polyclonal anti-p25 for both antigen capture and antigen detection. Titration experiments using soluble, recombinant p25 antigen, diluted in sheep serum, show that this assay is sensitive down to 150-300pg of p25/ml. There is a linear relationship between the concentration of p25 protein and the OD_{492} observed in this assay (Figure 5.11A). A sandwich ELISA using ID10 antibody for antigen captire was sensitive down to around 10ng/ml only (data not shown).

Figure 5.9 Electron microscopy of negatively stained VLP's

Purified hybrid Ty-p25 VLP's, diluted to 1mg/ml in PBS, were placed onto Formvar coated grids and negatively stained with uranyl acetate. The bar indicates 100nm.

Note the extensive heterogeneity in size and shape of the Ty-p25 particles.

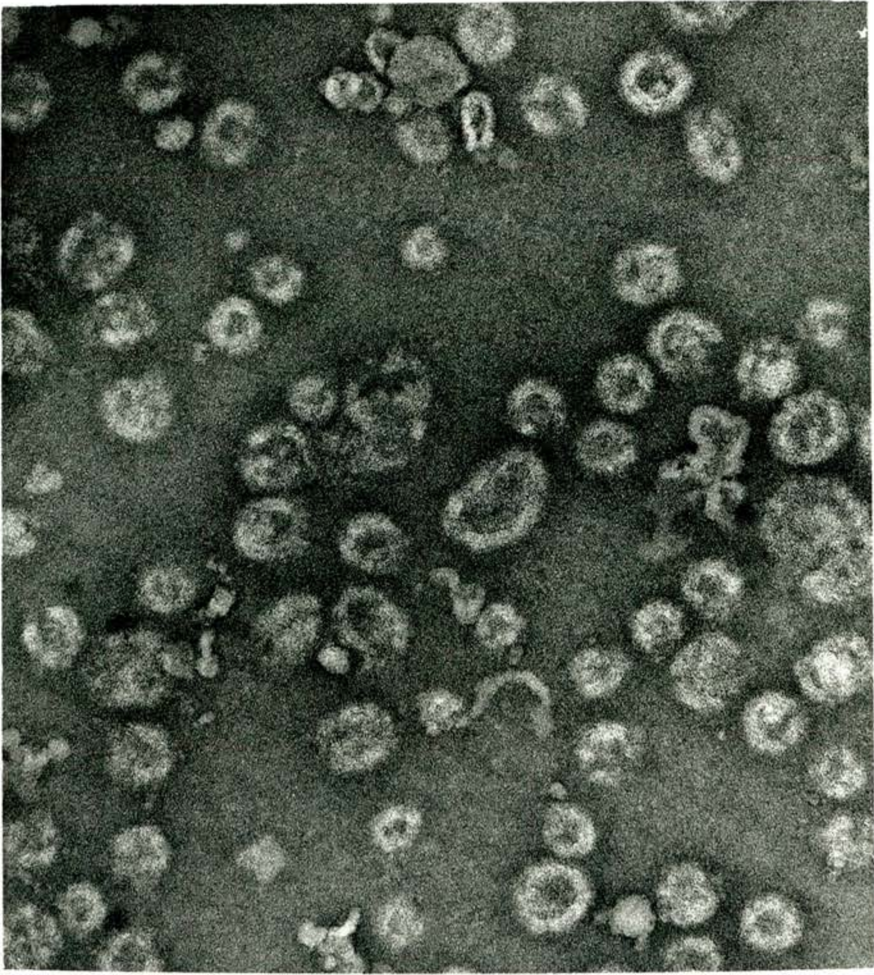
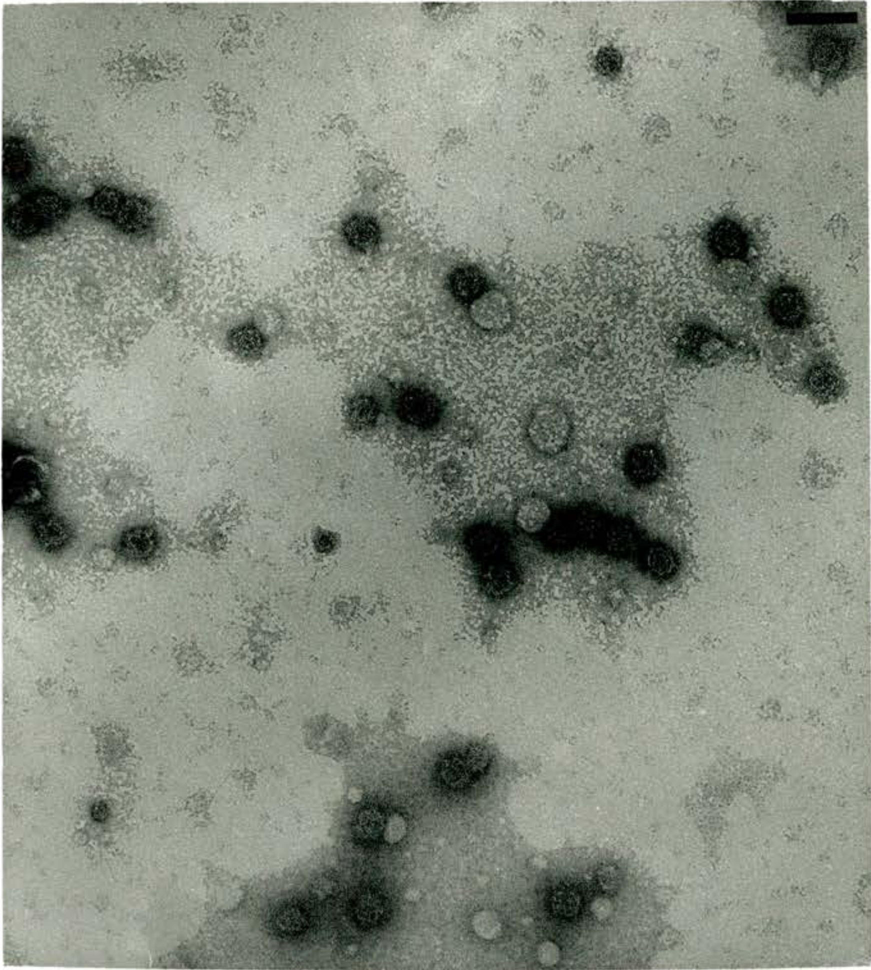


Figure 5.10 Electron microscopy of negatively stained VLP's

Purified preparations of either p1 particles (A) or hybrid Ty-p25 VLP's (B) were diluted to 1mg/ml and then placed onto Formvar coated grids and negatively stained as described previously (section 2.10.2).

The bars indicate 100nm. Arrows indicate particles with obvious core structures.



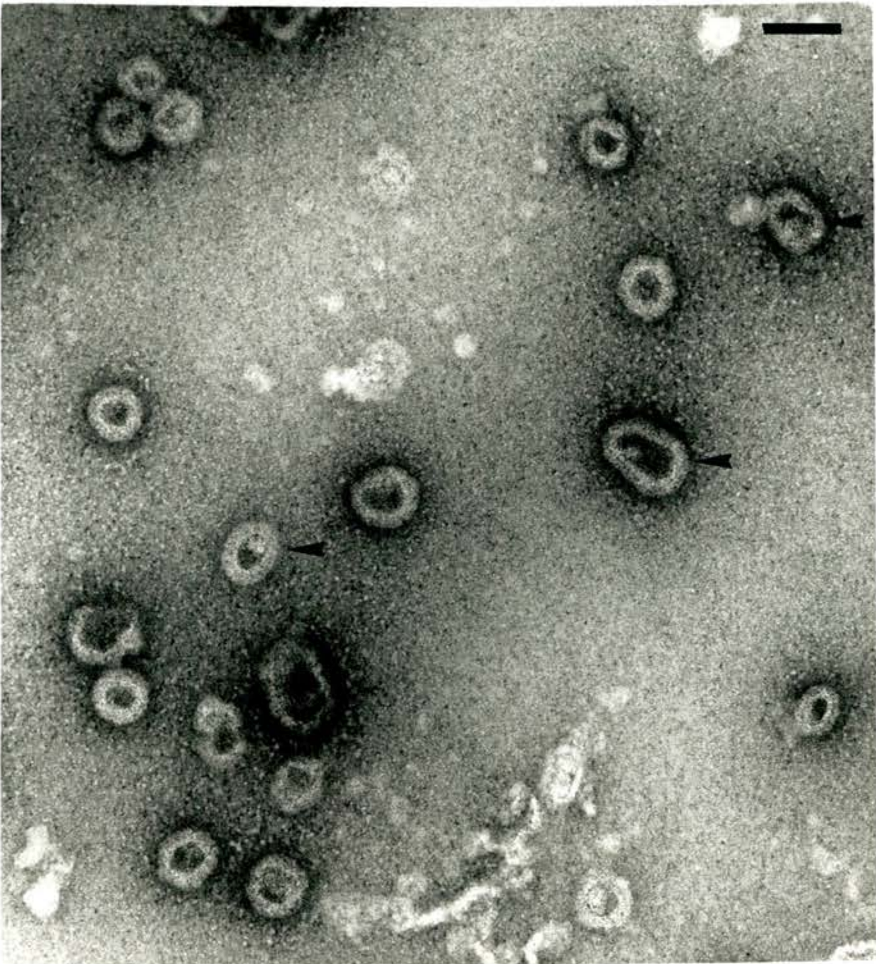


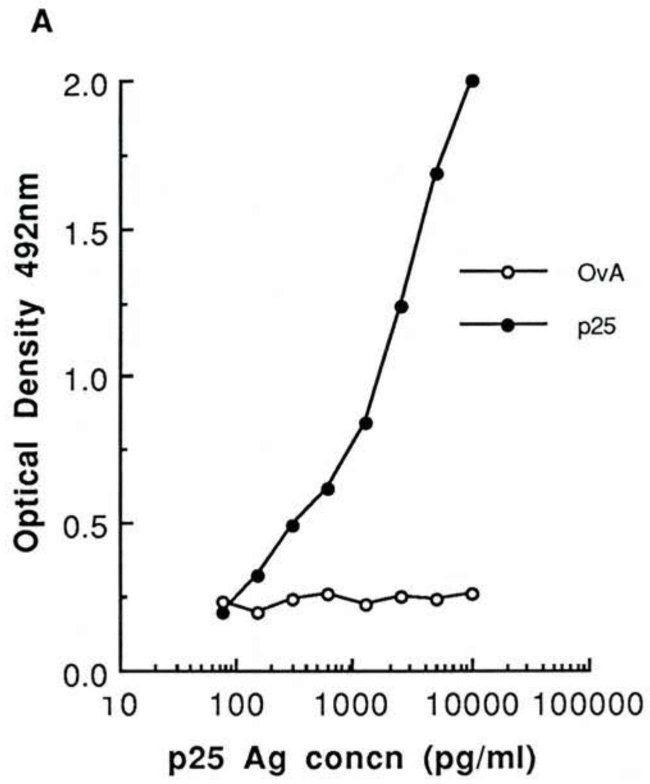
Figure 5.11 Maedi-visna virus p25 antigen detection assay

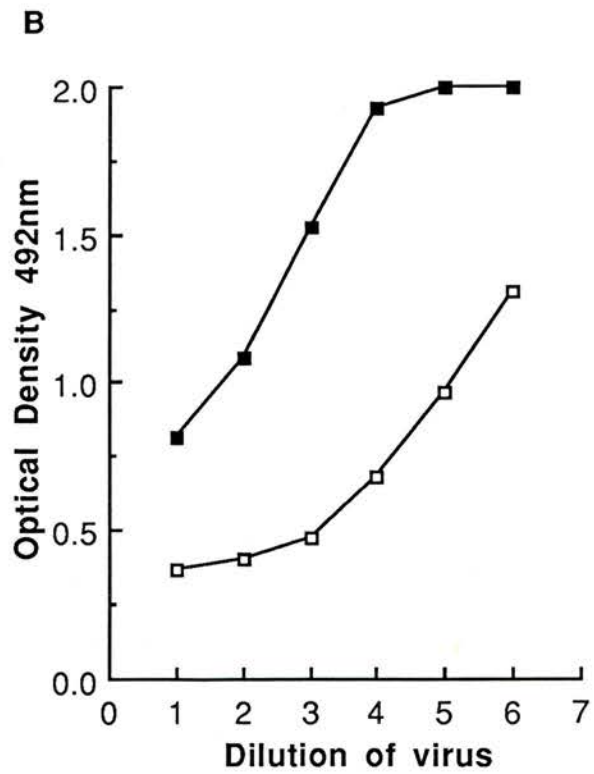
A. Sensitivity and specificity of the p25 antigen detection assay

Rabbit anti-p25 antibody (10µg/ml in BBS), which had been affinity-purified on a p25-sepharose column, was coated onto ELISA plates by overnight incubation at 4°C. Plates were then blocked using PBS/1% BSA/1% rabbit serum (PBR) and incubated for 1hour with samples of sheep serum containing either p25 antigen or a control antigen, ovalbumin. After washing six times with PBS/0.05% Tween 20, the plates were incubated for 1hour with biotinylated rabbit anti-p25 (20µg/ml in PBR), washed again and then incubated with avidin-HRP (1/4000 in PBS/1% BSA). After a final wash plates were developed with OPD and absorbance at 492nm read using a Titertek ELISA reader.

B. Titration of virus-containing culture supernatants in p25 antigen detection assay

Titration of high titre (10^6 $TCID_{50}$/ml) and low titre (10^3 $TCID_{50}$/ml) stocks, in DMEM 2% FCS, of EV1 diluted in DMEM. The p25 antigen detection assay was carried out as described in A.


A
2.0
1.5
1.0
0.5
0.0
Optical Density 492nm
OvA
p25
10
100
1000
10000
100000
p25 Ag concn (pg/ml)


B
2.0
1.5
1.0
0.5
0.0
Optical Density 492nm
0
1
2
3
4
5
6
7
Dilution of virus

Titration of virus-containing supernatants of high and low infectivity titres (5.11B) showed that over the dilution ranges tested the slopes of these titration curves are broadly parallel to each other. These experiments suggest that no factors present in either tissue culture conditioned medium or sheep serum interfere with this assay.

Interpolation of the OD_{492} values shown in Figure 5.11B onto a standard curve such as that shown in Figure 5.11A allows calculation of the absolute concentration of p25 antigen in these virus containing supernatants; the p25 antigen concentration of the high titre virus stock (10^6 $TCID_{50}$/ml) was 71ng/ml, while the low titre virus stock contained 6.8ng of p25/ml. Thus although there was a thousand-fold difference in infectivity titre between these virus stocks, there was only a 10-fold difference in p25 antigen concentration.

5.3 Summary of Chapter 5 results

1. The gene encoding the major core protein of maedi-visna, p25, has been cloned and expressed using the yeast Ty-VLP system.
2. Recombinant p25 protein has been used to generate both polyclonal and monoclonal anti-p25 antisera.
3. A specific and sensitive p25 antigen detection assay has been developed.

5.4 Discussion

The low level of sequence variability between the p25 gene isolated here and that previously reported for the EV1 p25 gene is about the expected level of 'within-isolate' sequence variation seen in lentiviral populations (Balfe *et al*, 1990), and confirms that ***gag*** p25 is among the most highly conserved of maedi-visna virus proteins (Sargan *et al*, 1991).

A notable feature of the cloning strategy described here is the design of PCR primers to facilitate subsequent steps in cloning and protein purification. This approach allows a great

deal of flexibility to be introduced into cloning and expression strategies and obviates the need for extensive DNA manipulations during cloning. Moreover the cloning and expression strategy employed here should be well suited for the production of overlapping fragments of viral and other proteins for use in epitope mapping studies.

A number of modifications to the expression and purification methodologies previously described for this system (Burns *et al*, 1991) were employed in this study. These modifications helped to achieve the high levels of Ty-p25 expression (50-60mg/l) observed in cultures of TEV1P25.U. The major modification resulting in increased levels of fusion protein expression was probably the increased time of culture in galactose containing medium, but the choice of Ty-VLP vector utilised in this system also affected the yields of fusion protein obtained. The combination of the galactose inducible pOGS 40 vector and the pUG 41S 'helper' plasmid generated the highest yield of p25 fusion protein. This was approximately three times the yield achieved when Ty-p25 was expressed using the constitutive Ty-VLP vector pMA 5620 (Adams *et al*, 1987*a*), and approximately six times greater than that obtained when p25 was expressed from the pOGS 40 vector in the absence of pUG 41S (data not shown).

The purification of these hybrid Ty-p25 VLP's was relatively simple and straightforward, consisting, after breaking open the yeast cells and clarifying this lysate, of a single step of differential centrifugation through a sucrose gradient. Ty-p25 VLP's were recovered from the sucrose gradients at 90-95% purity and further purification steps were judged unnecessary. p25 protein could be released from the VLP's by Factor Xa protease digestion with a final yield of soluble p25 protein of 15-20mg/l of culture fluid. An advantage to using Factor Xa enzyme is that it cleaves immediately carboxy-terminal to its recognition sequence thus the released protein does not contain additional amino acids, however the amino-terminal proline residue of p25 had to be omitted from the recombinant protein as this would otherwise have inhibited Factor Xa cleavage (Gilmour *et al*, 1989., S.E. Adams, *personal communication*).

The reactivity of this purified protein with monoclonal antibody 396 (Houwers & Schaake, 1987) indicates that the protein contains at least one antigenic determinant recognised by an

antibody raised against native viral protein. The ability of the recombinant p25 protein to elicit mouse and rabbit antibodies which recognise native viral protein is further evidence of the antigenic authenticity of this protein.

There are no previous reports of expression of a visna virus structural protein, but a related protein, HIV p24, has been expressed in a variety of systems including *E. coli* (Dowbenko *et al*, 1985., Shoeman *et al*, 1987), insect cells (Madisen *et al*, 1987) and mammalian cells infected with vaccinia virus recombinants (Flexner *et al*, 1988). The yield of p25 obtained in the yeast Ty-VLP system described here compares favourably with these reports and the purification of Ty-p25 protein in this system is considerably simpler than for these other systems. In some cases the expressed protein has been purified by immunoaffinity chromatography or preparative SDS-PAGE (Dowbenko *et al*, 1985), but in most cases the protein has been used in biochemical and immunological assays as crude lysates (Madisen *et al*, 1987., Flexner *et al*, 1988) or as partially purified material following solubilisation of *E. coli* derived inclusion bodies (Shoeman *et al*, 1987). The use of crude material in immunoassays may result in non-specific cross reactions, particularly if the recombinant protein is produced in bacterial cells. In addition, it is possible that harsh purification techniques and subsequent renaturation may affect some important antigenic determinants. Use of the Ty-VLP system for expression has the major advantage that purification of the recombinant protein is relatively straightforward.

The p25 protein generated in this system has been used in immunological assays of T & B cell function in visna infected sheep (see Chapters 3 & 4). Given a supply of pure, soluble p25 the epitopes being recognised in these assays could be further characterised by assessing the reactivity of T cells and anti-p25 antibodies with fragments of p25 generated by chemical or proteolytic cleavage of the recombinant protein. That said, given the current state of knowledge regarding the ovine MHC, experiments mapping T cell epitopes within p25 would be difficult to interpret.

Given the highly conserved nature of p25 between visna isolates (Sargan *et al*, 1991) an ELISA assay based on recombinant ***gag*** protein should prove useful as a sensitive and

specific diagnostic tool for the detection of lentiviral infection of sheep and goats. Such an assay would, however, require considerable work-up and validation with respect to the incidence of false-negative and false-positive results. This would require the testing of large numbers of samples from known visna seropositive and seronegative sheep. Recombinant p25 protein may also be of use in immunocytochemical detection of visna p25 specific B cells within lymphoid tissue (Laman *et al*, 1991) and in estimating the functional affinity of antibodies to p25 in visna infected sheep.

The recombinant p25 protein has been useful in generating p25 specific antisera (Figures 5.6 & 5.7). The polyclonal antiserum obtained recognises both p25 and the ***gag*** precursor p55 and other intermediate processed forms. This reagent will be useful for studying the processing of p55 during viral replication. In contrast, monoclonal antibody 1D10 appears to recognise mature p25 protein strongly, but precursor forms of the ***gag*** polyprotein only weakly by immunoblot analysis (faint band on Figure 5.7A), implying that this antibody may recognise an epitope which is more accessible after processing of p55. However, both the rabbit anti-p25 antiserum and 1D10 will be of use in immunocytochemical localisation of p25 antigen within tissue sections. In this connexion it is interesting to note the pattern of p25 staining seen in fibroblasts, in tissue culture, infected with visna virus (Figure 3.9A). Virus specific signals appear as linear arrays within the cells, a similar striated pattern is observed when infected fibroblasts are probed for viral RNA by *in situ* hybridisation (Dr. D.J. Roy, *personal communication*). These striated structures are reminiscent of cytoskeletal components such as microtubules and actin filaments, and it is interesting to note that *in vitro* infection of fibroblasts with visna virus has been reported to result in a redistribution of actin-containing filament bundles within fibroblasts (Pautrat *et al*, 1980). It is tempting to speculate on a role for intermediate filaments in intracellular viral protein trafficking and/or virion assembly, however, two colour immunofluorescence analysis using antibodies to visna antigens and intermediate filaments plus electron microscopic analysis would be necessary to test the worth of this hypothesis.

The rabbit anti-p25 antibody has also proved useful in setting up a sensitive and specific assay for maedi-visna p25 antigen (Figure 5.11). That an assay based on monoclonal antibody 1D10 is less sensitive than one based on affinity-purified rabbit antiserum is probably a reflection of differences in the avidity of interaction between the antisera. The higher avidity, multivalent interaction between p25 and the polyclonal antiserum resisting the mechanical stresses of repeated washing of ELISA plates better than the single interaction between 1D10 and p25 protein.

The p25 antigen detection assay described here is capable of detecting visna p25 antigen at levels down to 150-300pg/ml and has proved to be of use in quantitating viral replication both *in vitro* (Figure 5.11) and *in vivo* (see Chapter 4, Figures 4.5A & 4.6). The availability of the recombinant p25 protein and monospecific antisera described in this chapter will enhance studies of maedi-visna virus replication *in vitro* and *in vivo* and will also be of use in further defining the immune response to visna virus infection.

Chapter 6

Discussion

6.1 Introduction

The central feature of the pathogenesis of lentiviral disease is viral persistence *in vivo* despite the induction of both virus-specific antibody and T cell responses (Petursson *et al*, 1976., reviewed in Fauci, 1988., McCune, 1991). It is thus important to precisely define the immune mechanisms activated in response to lentiviruses and to attempt to understand the strategies employed by lentiviruses to evade and subvert the immune system. The aims of this thesis were therefore two-fold: to analyse the acute phases of the immune response to lentiviral infection, and secondly, to re-examine and investigate more closely the immune response to maedi-visna in persistently infected sheep.

6.2 Maedi-visna as a model system for lentiviral pathogenesis

Studies of maedi-visna virus infection of sheep, because of the opportunity to directly access both lymphoid tissue and the lymphatic output of lymph nodes of sheep via lymphatic cannulation, provide a unique *in vivo* model system for investigation of acute immune responses to (lenti-)viral infection. Moreover, since the ruminant lentivirus maedi-visna is restricted in its tropism to cells of the monocyte-macrophage lineage (Gendelman *et al*, 1985. 1986) there exists the potential for study of the significance of accessory cell infection in the pathogenesis of lentiviral disease in the absence of the concomitant infection of other cell types which complicates study of the pathogenesis of disease caused by the immunodeficiency-viruses, such as HIV, FIV and SIV (Rosenberg & Fauci, 1990., Narayan & Clements, 1990). This, however, assumes that infection, of for example a macrophage, by visna will have the same effect(s) on the infected cell as infection of a macrophage by HIV. It is not yet clear whether this is a justifiable assumption. Further, *in vivo* it is possible that the dysfunction and dysregulation of $CD4^+$ lymphocytes which is the hallmark of HIV infection may feedback, through the release of factors such as IFN-γ, IL-2, IL-4, M-CSF and GM-CSF, and have profound effects on accessory cell phenotype and function. Thus although studies on the pathogenesis of maedi-visna disease may provide insights into the role of accessory

cell infection in the biology of lentiviral disease generally; these should be interpreted only cautiously when discussing the specifics of any particular lentiviral disease.

6.3 A model of the acute immune response to lentiviral infection

Naturally occurring stimulation of the mammalian immune system usually results from the entry of antigens, such as viruses, bacteria and allergens, through intact or damaged skin and mucosa. Having gained access to the tissue spaces, a proportion, at least, of the antigen enters the lymphatic capillaries draining the site of entry, and is transferrred, via afferent lymphatic vessels to the regional lymph node where an immune response is initiated and subsequently disseminated by the lymphatic and circulatory systems. An important component of this immune response is believed to be the movement of Langerhans cells (or other antigen-presenting cells) from the site of infection into the node which they enter as interdigitating cells (Silberberg-Sinakin & Thorbecke, 1980., Hoefsmit *et al*, 1982., Kraal *et al*, 1986). The function of these cells is believed to be to carry antigen, from the site of entry of the antigen/pathogen, to the paracortical areas of the node for presentation to T cells (Macatonia *et al*, 1987., Kripke *et al*, 1990) and to play a role in the movement of antigen and immune complexes from the marginal sinus to the follicles (B cell zones) of the lymph node (reviewed in Szakal *et al*, 1989). Antigen-activated $CD4^+$ T cells then move to the interface between the paracortex and the primary follicle (Rouse *et al*, 1982) and a rapid oligoclonal B cell response occurs. This results in the formation of a germinal centre containing $CD4^+$ T cells, a few antigen-bearing germinal centre and follicular dendritic cells and large numbers of dividing B cells which give rise to antibody producing plasma cells and memory B cells (Szakal *et al*, 1989).

The above summarises the pattern of events which, based on studies of immune responses to inert protein antigens, is thought to occur normally in response to antigenic challenge. In most instances, the T and B cell immune responses initiated in the lymph node by this process are sufficient to clear infection by a pathogen.

Analysis of the acute immune responses of lymphoid tissue to infection by a lentivirus, studied via lymphatic cannulation, has revealed that lentiviral infection activates many of the immune mechanisms seen in a 'normal' immune response to antigen, but that in this case these fail to prevent the establishment of a persistent viral infection.

The early events in lentiviral infection, once the virus has gained access to the body across a mucosal and/or epithelial surface, are likely to include localised infection of permissive cells with subsequent migration, via afferent lymphatic vessels, of infected cells, antigen-presenting cells carrying viral antigen and free virus into draining lymph nodes. In visna this will usually mean the transfer of infected monocytes/macrophages to regional lymphoid tissue after aspiration/ingestion of either virus-infected cells in colostrum or aerosolised virus exhaled by an infected sheep (Narayan & Cork, 1985).

After the entry of lentivirus infected cells into the paracortex antigen presentation to T cells, in the context of MHC class I, would be expected to occur and result in the activation and proliferation of $CD8^+$ T cells. Indeed a marked increase in the number of $CD8^+$ blast cells, both within the lymph node and exiting it, is a striking feature of the early immune response to lentivirus infection (Reimann *et al*, 1991., Appendix A, Figure A.2). However, the extent of this $CD8^+$ lymphocytosis does not appear to correlate with the development of cytotoxic T cell activity (Reimann *et al*, 1991., Dr. B. A. Blacklaws, *personal communication*). This is in contrast to the situation observed in mice acutely challenged with LCMV or HSV, where 5-6 days after infection high levels of MHC class I restricted CTL activity can be generated from lymph nodes draining the site of infection (Pfizenmaier *et al*, 1977., Lynch *et al*, 1989). There are two possible explanations for this observation; firstly the $CD8^+$ cells may be specific for viral antigen, but not able to mediate cytolysis *in vitro*. It is, for example, possible that these $CD8^+$ T cells are producing cytokines such as IFN-g and TNF that may affect viral replication without causing lysis of infected cells *in vitro*. Secondly, it is possible that only a proportion of these T cells are specific for viral antigens. Given the available data, it is not possible to distinguish between these alternative, although not mutually exclusive, explanations.

The basis for any suggested non-specific activation of CD8^{+} T cells is not clear, but the appearance of non-antigen-specific blast cells prior to the detection of antigen-specific efferent lymphocytes has been described previously (Poskitt *et al*, 1977., Issekutz, 1985). This phenomenon has been suggested to result from cytokine release within the lymphoid micro-environment leading to proliferation of both antigen-specific and non-specific lymphocytes. It is possible that such non-antigen specific blast cells, if IL-2R^{+}, may act to modulate the cellular response by acting as passive sinks for cytokines such as IL-2. This, however, does not explain why the majority of the activated T cells in lymphoid tissue after lentiviral infection are CD8^{+} rather than a mixture of CD4^{+} and CD8^{+} phenotypes. Interestingly however, other viral infections eg. Epstein-Barr virus and cytomegalovirus infections in man can also result in a CD8^{+} lymphocytosis (Carney *et al*, 1981., Tomkinson *et al*, 1987), which may suppress various immunologic functions (Reinherz *et al*, 1980).

Association between virus antigen and antigen-presenting cells in the paracortex of the lymph node would also be predicted to result in the activation of CD4^{+}, MHC class II restricted 'T-helper' cells specific for viral antigen. *In vivo* antigen-activated CD4^{+} lymphocytes are believed to be the main source of cytokines such as IL-2, IL-4, IL-5, IFN-γ, and, despite increasing evidence that CTL activity can develop independently of CD4^{+} T cell function (Bennink & Doherty, 1979., Leist *et al*, 1987., Ahmed *et al*, 1988) such 'T-helper cells' are thought to play an important role in the generation and maintenance of both specific antibody-producing B cells and optimal cytotoxic T cell (CTL) responses (reviewed in Vitetta *et al*, 1991). For example, it has recently been shown, using an adoptive transfer system, that protection from a murine retrovirus disease (Rauscher leukaemia-sarcoma virus complex) requires both immune CD4^{+} and CD8^{+} T cells (Hom *et al*, 1991).

Analysis of the occurrence of virus antigen-specific CD4^{+} T cells in HIV and SIV infection is complicated by the fact that these viruses infect, and subvert the function of, CD4^{+} lymphocytes and this may explain the difficulties reported in detecting HIV antigen-specific CD4^{+} T cells by *in vitro* analysis (Wahren *et al*, 1987). However, *in vitro* lymphocyte proliferation to maedi-visna antigen, presumably mediated by CD4^{+} T cells (see section

3.4.1) was clearly demonstrated during the acute immune response to visna in 2/2 sheep tested (section 4.3).

Acute maedi-visna infection also induced a relatively normal humoral immune response, including the production of virus-specific neutralising antibodies (section 4.2). The appearance of these antibodies coincides with the disappearance of infectious virus in efferent lymph, as judged by co-cultivation experiments (Figure 4.7 and Appendix A, Figure A.1), but the basis of this correlation is unclear. All the infectious virus exiting the node was cell-associated (Bird *et al, in preparation*). Therefore if neutralising antibody is directly causing the decrease in infectious virus production this implies that *in vivo* cell:cell transmission of virus may not be an important mechanism in the spread of viral infection. Alternatively this correlation may be related to the fact that neutralising antibody is mainly directed against the viral ***env*** protein (Scott *et al*, 1979) and it may be that anti-***env*** antibody is acting against virus infected cells, perhaps via complement activation and cell lysis (see section 3.2.3).

In summary, acute infection with the ruminant lentivirus maedi-visna activates both cellular and humoral arms of the immune system, but the local immune response, while able to modulate the initial burst of viral replication and dissemination to an extremely low level (section 4.2 & Appendix A, Figure A.1), is not sufficient, as judged by the continued low level production of p25 antigen (section 4.2), to completely eliminate lentiviral infection. This is probably due to the establishment of a persistent lentiviral infection associated with the 'restricted replication' phenotype characteristic of lentiviral pathogenesis *in vivo* (Haase, 1986).

6.4 Host-virus interactions in persistently infected animals

"The best current explanation for the persistence of lentiviruses is the immunologically silent nature of the infection." (Haase, 1986)

The seminal studies of Haase and coworkers have demonstrated the existence of a striking dichotomy between the behaviour of lentiviruses *in vitro* and *in vivo*. In tissue culture

visna virus reproduces rapidly to high titre in a lytic cycle of infection: infected cells contain thousands of copies of genomic and messenger RNA's, these are translated into millions of copies of virion proteins and more than 50-100 progeny virions are produced by each infected cell (Brahic *et al*, 1977., Haase *et al*, 1982). In contrast replication of the virus in animals is characterised by a markedly restricted replication cycle. Only a few cells are infected and these contain relatively low numbers of copies of viral RNA, only 50-150 per cell. Interestingly, less than 0.02% of infected cells contain detectable quantities of viral antigens (Haase *et al*, 1977., Brahic *et al*, 1981., Peluso *et al*, 1985). On the basis of these observations Haase and his coworkers have proposed that "most infected cells harbour the virus in a latent state in which viral antigens are not produced in sufficient quantities for detection and destruction by immune surveillance mechanisms."

Taken at face value this hypothesis would seem to indicate little, if any, role for the immune system in controlling viral replication in persistently infected animals. However there are a number of provisos to this hypothesis. Firstly, the conclusion that *in vivo* only a small percentage of virus infected cells are expressing viral antigens was based on immunofluorescence analyses using antisera specific for viral structural proteins such as ***gag*** (Haase *et al*, 1977., Brahic *et al*, 1981). However the levels of expression of the regulatory proteins, encoded by the short ORF's within the lentivirus genome, was not investigated.

Visna, at least *in vitro*, does not appear to integrate (Harris *et al*, 1984) and *in vivo* virtually every cell containing viral DNA also contains viral RNA (Brahic *et al*, 1981), suggesting that true latency does not occur in visna infection *in vivo*. The nature of the RNA transcripts expressed *in vivo* is not known, however *in vitro*, cell lines chronically infected with HIV, but not producing viral particles have been shown to contain mainly doubly spliced transcripts (Michael *et al*, 1991), consistent with the hypothesis that expression of viral regulatory proteins may be a feature of the restricted replication state observed *in vivo*. The validity of this hypothesis is not known, but, using techniques such as immunocytochemistry and *in situ*-PCR (Haase *et al*, 1991), it is is fully testable.

If expression of regulatory proteins such as ***tat*** and ***rev*** is a feature of restricted lentiviral replication *in vivo* then it would be interesting to investigate the occurrence of T cells specific for these proteins in visna infected sheep. In HIV infection, one of the proteins encoded by short ORF's, ***nef***, has been shown to be a target for CTL activity (Chenciner *et al*, 1989), but I am not aware of any published work on the occurrence of CTL to other regulatory proteins.

The second proviso to the hypothesis that lentiviral infection is largely immunologically silent is that leukocytes have a finite life span *in vivo* and therefore the maintenance of viral burden in the infected host can be predicted to require continual recruitment of newly infected cells. A logical corollary of this prediction is that in some infected cells viral replication with expression of viral structural proteins and production of progeny virions must be occurring. Such cells, and any free virus produced by them, would be expected to be fully susceptible to immune attack by CTL, natural killer cells and anti-viral antibody. It is possible that such cells may represent the isolated visna infected cells, surrounded by clusters of inflammatory cells, described by Stowring *et al* (1985).

The occurrence of occasional cells in which a complete viral replication cycle takes place could also serve to provide a chronic trickle of antigenic stimulation. Such a phenomenon could contribute to the infiltration and proliferation of mononuclear cells in the active-chronic inflammatory process typical of the histopathological lesions associated with visna infection (Narayan & Cork, 1985). Since, in visna, the majority of virus infected cells are monocyte-macrophages it is also conceivable that disordered monokine secretion may play a role in the genesis of these inflammatory lesions.

Lentiviruses may employ a number of strategies to evade such immune surveillance. The immune dysfunction subsequent to infection of $CD4^+$ T cells by lentiviruses such as HIV and SIV is one obvious mechanism for escaping immune responses. However lentiviral infection may also be associated with other more subtle immune defects eg. the unusual pattern of isotypic restriction of anti-visna antibodies to the IgG_1 subclass (section 3.2.2), which may prevent activation of anti-viral ADCC mechanisms (see section 3.2.3).

Another potential mechanism by which lentiviruses may escape immunological control is antigenic variation. The occurrence of lentiviral variants, including visna virus, which evade the antiviral antibody response has been described (reviewed in Narayan *et al*, 1987., Clements *et al*, 1988), but the significance of these variants is unclear. In visna, antigenic variants do not replace the infecting serotype and in most long-term infections the inoculum virus strain persists and spreads (Lutley *et al*, 1983., Thormar *et al*, 1983). Mutation in CTL epitopes has been described in HIV infection (Phillips *et al*, 1991). This was associated with loss of recognition by the patient's CTL of that epitope; however this resulted not in the generation of viral escape mutants, but rather recognition of a different epitope in the viral antigen. Therefore the significance of this mechanism in pathogenesis of lentiviral disease remains unclear.

6.5 Future prospects

Several major points emerge from the preceding discussion with regard to the role of the immune system in lentiviral infection. Firstly, there is a clear correlation between the appearance of antibodies to the viral envelope proteins/virus neutralising antibodies and a marked reduction in the dissemination of infectious virus (section 4.2., Petursson *et al*, 1976). Secondly, local immune responses are able to down-modulate viral replication and dissemination, but once virus has entered a cell and adopted a 'restricted replication' state these immune mechanisms are not able to clear the pathogen (sections 4.2 & 4.3).

Lastly, although T cell and antibody responses specific for visna virus structural proteins such as the ***gag*** antigens can be demonstrated in persistently infected animals (sections 3.2 & 3.4), these are unlikely to be of major significance in clearing the pathogen. In persistently infected animals the predominant viral replication state is not associated with expression of these antigens in infected cells (Haase *et al*, 1977., Brahic *et al*, 1981).

Bearing the above in mind, it is clear that the major candidate viral protein for inclusion in any vaccine is the envelope protein of the virus. ***Env*** is the major target antigen eliciting virus

neutralising antibodies (Scott *et al*, 1979., Rosenberg & Fauci, 1989), moreover it has been reported that in SIV infected macaques that ***env*** is also a major target for CTL responses (Yamamoto *et al*, 1990). This may be related to relatively inefficient transport of ***env*** protein to the cell surface from the ER and Golgi (Willey *et al*, 1988) resulting in the production, after protein degradation, of a pool of ***env*** peptides in the cytosol which may re-enter the ER and associate with nascent MHC class I molecules (Townsend *et al*, 1989).

Given the above, it is a not unreasonable hypothesis that a pre-existing high titre anti-***env*** antibody response, together with high levels of ***env*** specific T cells, may be able to prevent the establishment of a persistent infection.

That said, genetic diversity in the lentiviral genome is largely associated with the ***env*** genes and most anti-***env*** antibody responses are type specific. This is a major potential obstacle to the development of a successful vaccine based on ***env***. Interestingly, however, sequential immunisations with recombinant gp120's from different HIV strains has been reported to result in the development of a broadly reactive ***env***-specific antibody response (Klinman *et al*, 1991). This observation may represent an approach to dealing with the problem of antigenic diversity in ***env***.

Once a persistent infection has been established, the potential for immune intervention to alter the course of disease appears limited. If viral regulatory proteins are the major viral antigens expressed by infected cells *in vivo* then it would be logical to try and promote the development of a CTL response to these antigens. It is however, perhaps more likley that pharmacological intervention, throught the use of compounds such as AZT (Hirsch, 1988., Volberding *et al*, 1990) may prove a more profitable approach to the treatment of established lentiviral disease.

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Appendix

Figure A.1 Detection of infectious visna virus, by cocultivation, in efferent lymph

Washed efferent lymph cells and lymph plasma were titrated, in triplicate, onto a preformed monolayer culture of sheep skin cells in 24 well plates. Co-cultures were maintained for 14 days, after which time the skin cell monolayers were fixed in acetone/salt and stained for visna ***gag*** antigen by immunofluorescence (see section 2.9.3). Virus titres were calculated according to Reed & Muench (1938).

Cells and plasma were assayed separately and only cell associated virus was detected. Data is expressed as 50% tissue culture infectious doses ($TCID_{50}$) exiting the node daily.

Figure A.1

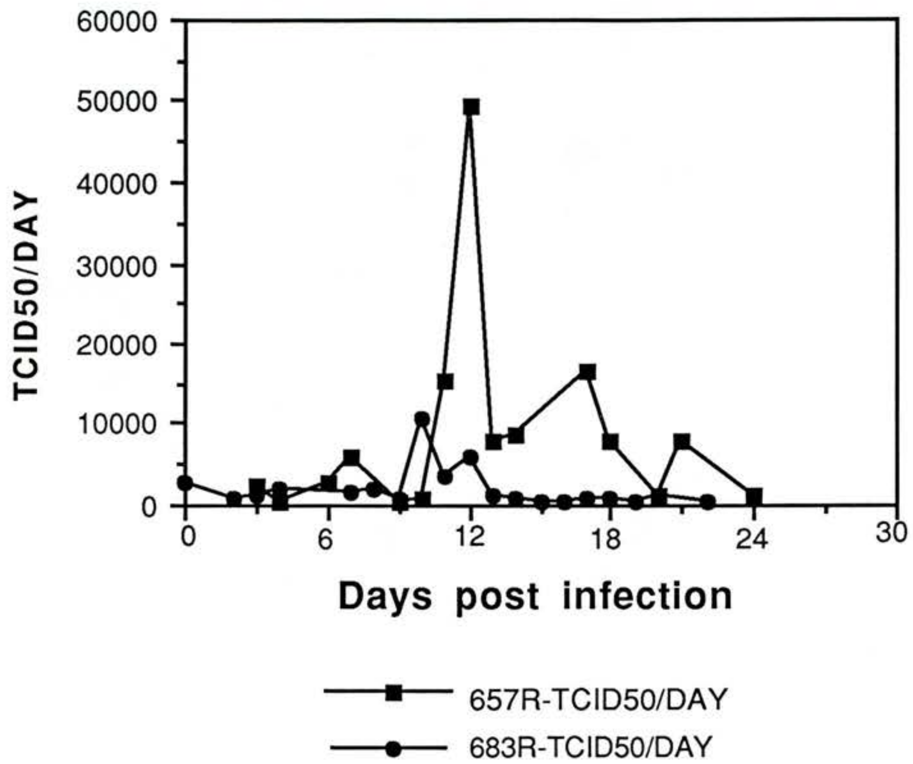


Figure A.2 Kinetics of efferent lymph cells exiting prefemoral lymph nodes challenged with either maedi-visna virus (A,C,E,G) or 'mock-infected' with control antigen (B,D,F,H).

Total cell output (A,B), lymphoblast output (C,D), % lymphoblasts CD8+ (E,F) and % small lymphocytes CD8+ (G,H) are shown against time for visna infected sheep 657R & 683R and for mock infected sheep 1060P & 663R. Arrows indicate time of challenge.

Lymphoblasts were defined by their forward scatter/side scatter FACS profiles (see below). FACS staining (see section 2.13.3) for CD8 was done using mAb SBU-T8 (Maddox *et al*, 1985).

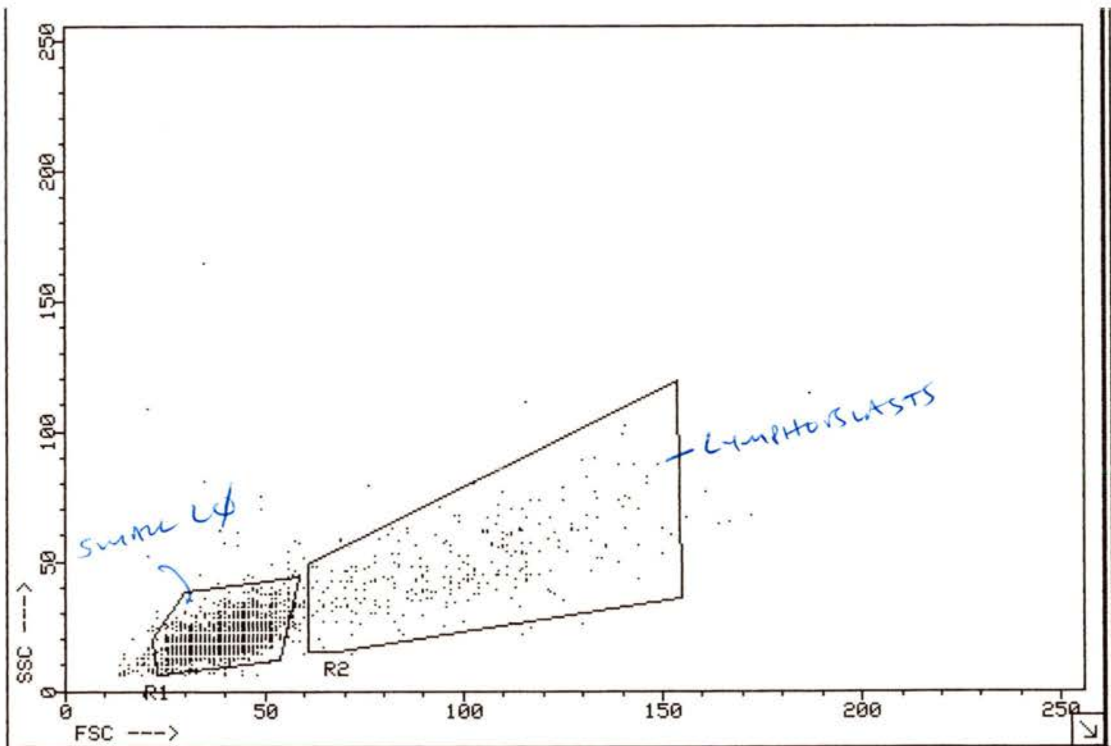


Figure A.2

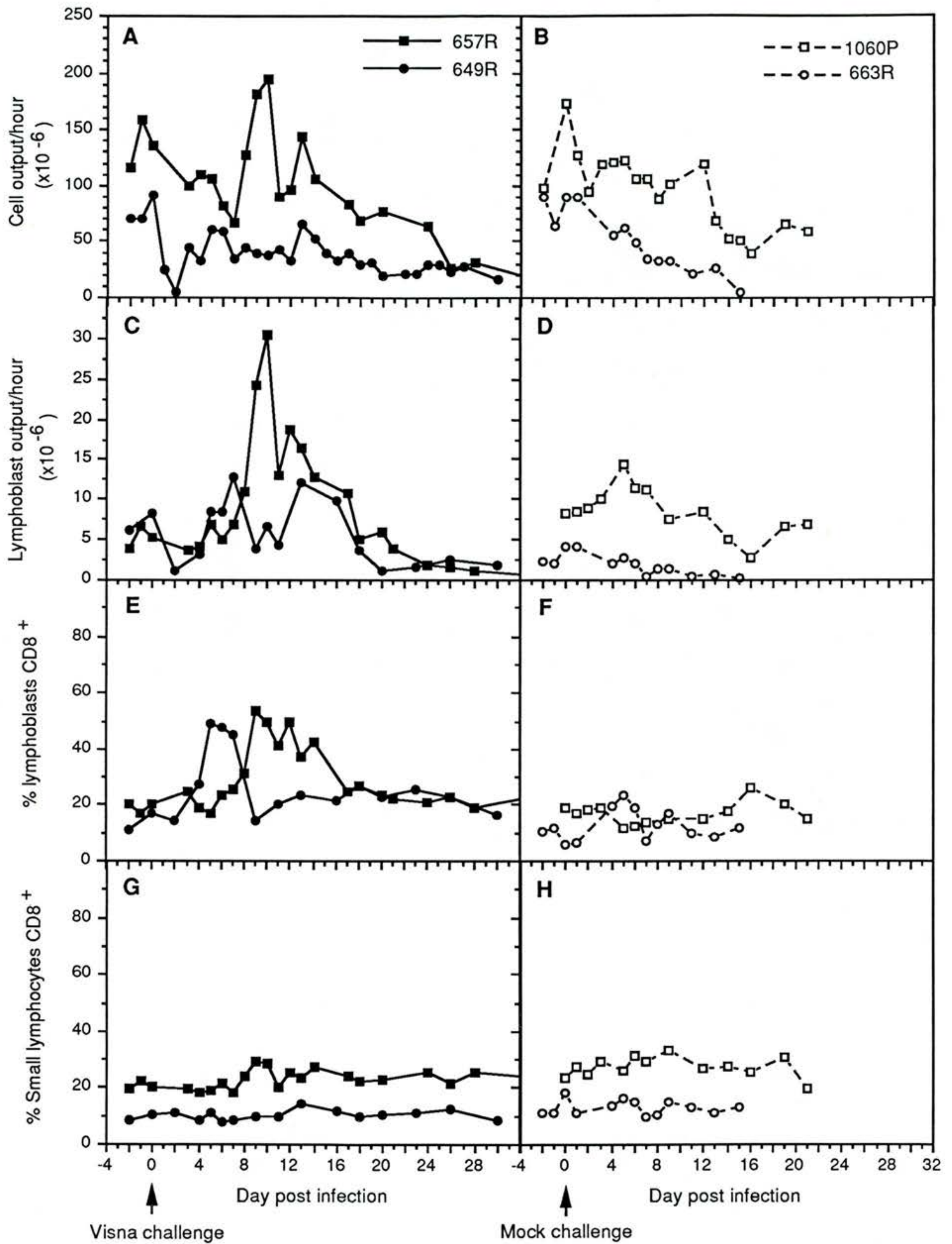
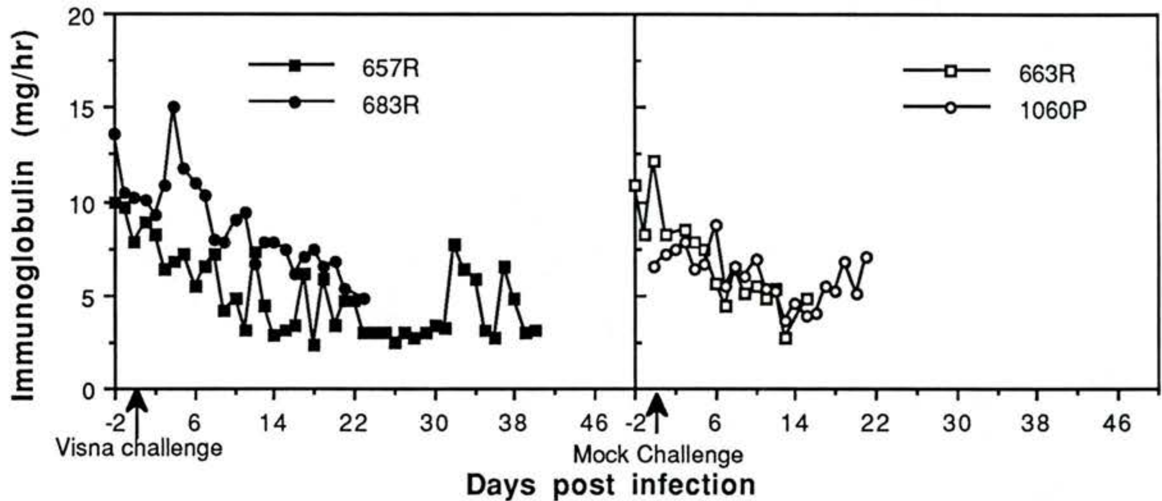


Figure A.3 Immunoglobulin levels in efferent lymph (A) and synthesised spontaneously by efferent lymph B cells *in vitro* (B).

A. Total immunoglobulin was measured by radial immunodiffusion with purified preparations of ovine Ig as standards.

B. 10^6 washed efferent lymphocytes were cultured in vitro for seven days. Culture supernatant was then assayed for immunoglobulin in a capture ELISA using VPM8, a monoclonal antibody specific for light chain of ovine Ig (Bird *et al*, *in preparation*).

A. Immunoglobulin in efferent lymph



B. Ig synthesised by efferent lymphocytes in vitro