THE ROLE OF ANTIBODY DURING OVINE LENTIVIRUS DISEASE IN EXPERIMENTALLY INFECTED SHEEP

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ABSTRACT

Infection of sheep by Ovine Lentivirus (OvLV) is a significant disease throughout the world, with the exception of Australia and New Zealand. OvLV infection is manifested by an initial acute phase of viral swarming, followed by the emergence of quasi-species, which are able to evade neutralizing antibody responses. Frequently, neutralizing antibodies are slow to develop, of low affinity and titre, and unable to effectively clear virus. In addition to escape from host antibodies, latency of proviral DNA in host monocytes, and restricted viral gene expression in macrophages, are some of the currently proposed mechanisms of *in vivo* life long persistency. Of interest is the so far unexplained observation, that parental virus can be isolated months to years post infection, despite the presence of type-specific neutralizing antibodies. This prompted us to speculate regarding an additional, previously unrecognized mechanism of persistence.

In this study, the sera of four experimentally infected sheep were analysed over a two-year time period, with respect to neutralizing antibody (NT-Ab) titres and non-neutralizing ELISA antibodies against both the whole virus and the immuno-dominant recombinant proteins; capsid (CA), and trans-membrane peptide (TM). It was hypothesised that a further mechanism of persistence of OvLV may be mediated by interference of non-neutralizing antibodies with neutralizing antibody via conformational change of viral surface envelope proteins, which contain Nt-Ab epitopes. The postulated phenomenon of virus-obstructing antibody activity was investigated by analysis of correlation between neutralizing and non-neutralizing antibody responses (*i.e.* vs capsid/transmembrane), as well as by a neutralizing assay where high and low neutralizing antibody titre sera were analysed in a 1:1 mixture. A lack of correlation between neutralizing Ab and non-neutralizing (ELISA) Ab in 3 of 4 animals is indirectly supportive of the postulate. The result of the serum neutralization assay on mixtures of sera revealed that in at least one combination the neutralizing activity of one serum was suppressed by another serum, and this more directly supports the hypothesis.

The report of an IgG_1 restricted antibody response in OvLV infection was investigated by using monoclonal antibodies, specific to the predominant ovine IgG_1 and IgG_2 subclasses. In 2 of 2 animals tested, the IgG_2 subclass response was suppressed. The phenomenon was thus confirmed, and it too may also be a contributing factor to OvLV persistence.

Also, an un-characterised OvLV inhibitory serum factor was found in heat-inactivated pre-immune sera at 1:4-1:8 titre. Considering the relative potency of the factor, it is surmised that this serum factor plays a role in OvLV pathogenesis.

In summary, OvLV is possibly able to persist through a number of mechanisms, thus presenting considerable challenges in control and/or eradication.

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ABBREVIATIONS

Abbreviations	Terms
Ab	antibody(ies)
ABTS	2'2-azino-bis 3-ethylbenz thiazoline-6-sulfonic acid
AGID	agarose gel immuno-diffusion
CAEV	caprine arthritis-encephalitis virus
CA	capsid
cm	centimeter
CPE	cytopathic effect
DEPC	diethyl pyrocarbonate
D-MEM	Dulbecco's modified Eagle's medium
(c)DNA	(complimentary)deoxy ribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme linked immuno-sorbent assay
FBS	fetal bovine sera
FLchor	fetal lamb choroid plexus
g	gravities
GST	glutathione-s-transferase

LIP	lymphoid interstitial pnuemoniae
MVV	Maedi-Visna virus
µg	microgram
µl	microliter
µM	micromolar
ml	milliliter
mM	millimolar
mAb	monoclonal antibody
NaCl	sodium chloride
ng	nanogram
nm	nanometer
NT	neutralizing
OD	optical density
OPPV	ovine progressive pneumonia virus
ORF	open reading frame
OvLV	ovine lentivirus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	post infection
RNA	ribonucleic acid
RT	reverse transcriptase

SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
ss	single stranded
SU	surface envelope protein
$TCID_{50}$	median tissue culture infectious dose
TM	transmembrane
Tris	tris (hydroxymethyl) aminomethane
VN	virus neutralization

1. General Introduction

Maedi-Visna virus (MVV), is a non-oncogenic, exogenous ovine lentivirus (OvLV), of the *Retroviridae* (sub-family, *Lentivirinae*). OvLV is a significant pathogen of many sheep-producing countries throughout the world (1, 2, 3), though exact numbers of infected animals and total losses are not known. Originally isolated in Iceland by Bjorn Sigurdsson, Maedi-Visna is genetically and phenotypically related to Ovine Progressive Pneumonia virus (OPPV), found in the American Mid-west, Caprine arthritis encephalitis virus (CAEV), and to OvLV which is endemic in North America, Europe and other areas, excluding Australia and New Zealand (4).

MVV was the first lentivirus to be extensively characterized, resulting in a fundamental understanding of other important Lentiviruses, including human immunodeficiency virus (HIV). In terms of nucleotide sequence homology and morphology, OvLV most closely resembles caprine arthritis-encephalitis virus (CAEV), equine infectious anemia virus (EIAV), and HIV (5, 6, 7). MVV is the causative agent of a persistent, multi-systemic disease of sheep, most often characterized by a protracted period between initial infection and the development of lymphoproliferative interstitial pneumonia (LIP), indurative mastitis, non-suppurative arthritis, and de-myelinating leukoencephalmyelitis (8, 9, 10, 11). Many of the original concepts of MVV pathobiology and disease were elucidated in Iceland, through studies involving both naturally and experimentally infected animals. A summary of the important concepts include: 1)

identification and characterization of the etiological agent

as a retrovirus; 2) observations that productive MVV replication was restricted to terminally differentiated, non-dividing macrophages; 3) findings that infection becomes systemic after long incubation periods; 4) observations on cytopathic effect by cell fusion and lysis; and; 5) that antigenic variation occurred throughout chronic infection (10, 11,12, 13, 14, 15, 16). It is important to make the distinction that the Icelandic strain of MVV causes a lytic infection of ovine and caprine fibro/endothelial cells *in vitro*, as well as severe encephalitis and Lymphoid interstitial pneumonia in susceptible Icelandic breeds. In contrast, the more predominant OvLV strains are non-lytic in vitro, and pathology is usually characterized by anemia, wasting, lymphoid interstitial pneumonia and generalized immunopathology from inflammatory responses, while a demylinating encephalitis is rare (17).

Lentiviruses in general have adapted several evolutionary strategies allowing them to become significant pathogens of higher vertebrate hosts. Firstly, they include a tropism for specific leukocyte subsets such as monocyte/macrophages and dendritic cells (18). Secondly, lentivirus employs the mechanism of latency by utilization of the viral gene integrase, which allows insertion of the proviral DNA into the host genome (19). OvLV gene expression was originally thought to be limited to terminally differentiated macrophages. However, antigen processing dendritic cells have recently been also implicated in OvLV pathogenesis (20).

OvLV infection triggers a vigorous cellular and humoral immune response, however,

the virus is able to evade clearance by the host through mechanisms which have been previously discussed (e.g. proviral DNA) and remains latent for the life of the host (21). In addition to latency, OvLV infection is characterized by persistency, which is defined as long term presence of infectious virus. The ability of OvLV to establish a life-long persistence, despite vigorous host immune responses, has posed serious challenges in the control and/or eradication of the virus (22).

Several mechanisms of persistency have been proposed. Viral swarming, or the emergence of quasi-species is one feature of infection by OvLV that occurs during the acute phase, due to the inherently high mutation rate of ssRNA viruses, especially retroviruses (*i.e.* lack of proof reading mechanism in RNA dependant DNA polymerase), resulting in high levels of heterogenous virus (23). Despite the presence of such large numbers of heterogenous virus, antigenic variants do not seem to be essential for long term survival of virus (24). In early work, the role of antigenic variation was studied by Narayan and colleagues (25), using plaque-purified virus, followed by the addition of two distinct antisera to ovine cell cultures, the first was "early " (approximately 6 months PI), and the second antiserum was "late " (approximately 3 years PI). The study concluded that 1) parental virus was neutralized by both early and late sera, 2) early serum variants were not neutralized by early serum, but were neutralized by late serum, and 3) late serum variants were not neutralized by early sera but were weakly neutralized by late sera. Lastly, and also of special interest was the observation that all variants were neutralized by sera obtained from sheep immunized with a detergent-disrupted virus. They reported that late sera

contained a wider spectrum of neutralizing antibodies which were able to neutralize early variants. Of importance, was also the observation that epitopes which were masked due to the complex glycosylated oligomeric structure of the envelope, and were presumably exposed by detergent disruption, may have broadly neutralizing capabilities (26).

High mutation rates of the retroviral RNA genome are estimated to be 10^{-3} to 10^{-5} per nucleotide, which translates to a substitution rate of 0.0017, per site per year (27). Though most of these mutations likely result in lethal consequences for the virus, mutations which allow infectious virus to escape host immune responses may confer an evolutionary advantage. Specific mutations in the outer glycosylated envelope proteins have been documented to alter neutralizing epitopes, allowing for viral escape from host humoral responses (28, 29). This latter mechanism is thought to be a significant factor during initial viral infection, when antigenic variants are able to escape from host neutralizing antibodies. Clements and colleagues (30) used RNA fingerprint analysis to further elucidate earlier work on the role of antigenic variation in OvLV infection. The authors concluded that the mechanisms of antigenic variation were single base substitutions and deletions, which accumulated in the viral RNA as the genotype became more resistant to neutralization. Of particular interest is that parental virus has been isolated months after original infection, despite the presence of neutralizing antibodies titres (31). This observation has yet to be satisfactorily explained as it suggests that antigenic drift is not the only reason for establishment of persistency. In summary, the ability of OvLV to; 1) infect leukocytes, such as macrophages and dendritic cells; 2) escape host immune mechanisms

through antigenic variation of neutralizing epitopes during early acute viremia, and 3) by integrating as provirus with viral gene expression activated upon maturation of monocytes into macrophages, are the currently recognized mechanisms of OvLV persistence (32, 33). However, the presence of parental virus months after infection, despite the presence of neutralizing antibodies, suggests that an unrecognized mechanism of persistence must occur. This led us to postulate another mechanism of persistency (outlined under 1.6), one that would be compatible with co-existence of virus neutralizing antibody and of infectious virus.

1.2 Structural, Biochemical and Genetic Organization of OvLV

OvLV is an exogenous, replication competent, enveloped virus which buds from the host cell membrane. OvLV is approximately 80 - 100 nanometers (nm) in size and possesses a dense isometric nucleocapsid core structure (34). The genome is a single stranded, positive sense ribonucleic acid genome (+ ss RNA), of which there are two copies, coding for several major structural, regulatory and accessory proteins in small open reading frames (ORF). The three main genes encode major structural and essential accessory proteins in the order of; 5' --*gag*--*pol*--*env*-- 3' (Figure 1). The major structural proteins include the generalized group antigen (*gag*) gene, which encodes the capsid (CA), matrix (MA) and nucleocapsid (NC) proteins, with molecular weights, of 25, 16 and 14 kDa respectively. The capsid protein is the most abundant viral protein and forms the

hydrophobic core of the virus. Within the CA is the nucleocapsid protein which coats the RNA. Though necessary for packaging of viral RNA, it is dispensable for production of virus progeny. The matrix protein is myristilated and considered essential for virus assembly and generation of progeny virus. The *gag* proteins are processed from a 53-55 kDa precursor polyprotein by a virion-encoded protease (35, 36, 37).

The proviral DNA of OvLV is flanked on both ends of the genome by long terminal repeats (LTR) which contain enhancer and promoter elements important for tropism as well as initiation of transcription. There are four non-structural genes in total: *vif, vpu, tat* and *rev*. *Tat* regulates viral transcription, RNA transport and translation; *vif* and *vpu* control infectious viral particle production and unknown infectivity factors, while *rev* encodes reverse transcriptase, the RNA-dependent DNA polymerase (38, 39). Amino acid and nucleotide sequence homology of regulatory and *env* genes between lentiviruses are relatively un-conserved, despite conserved functionality. In contrast, the *gag* and *pol* genes, have reported homology of 75 and 78% respectively (40, 41).

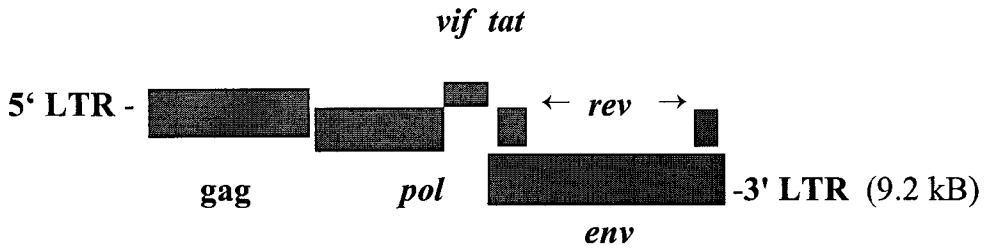

Figure 1. Genetic organization of OvLV, showing major ORF(*gag/pol/env*) and accessory genes (*vif/tat/rev*) .

OvLV has a large envelope (*env*) gene which encodes two major glycoproteins. The highly glycosylated surface (SU) protein gp135 - containing receptor sites, neutralizing epitopes and antibody-mediated/complement-dependant cytotoxic response epitopes - is non-covalently linked to a hydrophobic trans-membrane (TM) protein (gp 41), the second glycoprotein (42). The precursor *env* protein, a high mannose protein synthesized on the endoplasmic reticulum, is first transported to the Golgi body, where some high mannose residues are converted to a more complex type, followed by cleavage into the two predominant subunits, the surface glycoprotein and the hydrophobic transmembrane protein (43). The final stage is the transport of the mature glycoprotein complex to the plasma membrane where it is incorporated into the budding virus.

The third major gene, *pol*, encodes for RNA and Mg^{2+}-dependent DNA polymerase (reverse transcriptase; RT) and integrase/endonuclease proteins, which mediates integration

of viral double stranded DNA into the host genome. As well, this gene also encodes for dUTPase activity (44, 45).

The OvLV infectious cycle begins with fusion of the virus with the cell membrane, a MHC-II molecule is believed to function as a receptor, by receptor-mediated endocytosis (46). The predominant target cells are monocytes/macrophages, while other cells implicated in infection are CD4+ lymphocytes, dendritic cells and fibro/epithelial cells derived from the natural host, though the latter has been observed *in vitro* only (47, 48, 49). Following entry into the host cell, ssRNA released from the virus is transcribed to DNA, via Mg^{2+}-dependent reverse transcriptase. Double stranded DNA is then transported to the nucleus where it is integrated into the host genome, to form proviral DNA (50). Proviral DNA can be transcribed to make full-length viral RNA copies, or spliced to make messenger RNA (mRNA) for translation to viral proteins. Progeny virus is assembled within cytoplasmic vacuoles and bud from the cell surface as enveloped virus, completing the life cycle in 20-24 hours (51).

1.3 Epidemiology and Transmission

The sero-prevalence of ovine lentivirus infection varies significantly on a world-wide basis. With the exception of Australia and New Zealand, OvLV infection causes significant economic losses in sheep producing nations such as Belgium, Canada, Denmark, Spain, Italy, Germany, Greece, Holland, Russia, South Africa and the USA (52). Although

sero-prevalence is widespread in certain cases (*e.g.* North America), epizootics, though rare, may occur following the introduction of new animals into existing herds. The classic example is that of the original introduction of MVV, through the importation of twenty infected rams from Germany into Icelandic herds during the 1930's. Attrition due to disease is low, with adult sheep showing symptoms after an incubation period of 2 to 3 years.

In many parts of the world, OvLV infection continues to cause significant economic losses to domestic sheep (53, 54). Since there are no commercially available antiviral treatments for OvLV infection, specific and sensitive diagnosis of infected animals followed by culling and/or isolation, is the most practical means of eradication (55). Factors which may prevent accurate diagnosis of OvLV infection include subclinical infections and insensitive diagnostic procedures (56). Husbandry practices also play a role in the transmission of OvLV (57). In one U.S.A. study of OPPV, sero-prevalence ranged from 1-90%. Of 16,827 sheep tested, 26% were seropositive, with the highest prevalence in the American Midwest (49%) and lowest in the north eastern Atlantic region (9%). The distribution of seropositive sheep was age-dependent, ranging between 4% seropositive at less than 1 year, to 34% sero-positive at 4 years of age (58, 59). In Canada, one serological study surveyed 14,047 sheep over the age of 1 year and found an average sero-prevalence of 19% and a mean prevalence within a flock of 12%, while 63% of the flocks tested had one or more seropositive sheep. An increase in sero-prevalence with increasing age was also observed (60). Another study, examined 103 randomly selected flocks in Ontario and

found an average sero-prevalence of 21 % (61). Johnson and colleagues (62) used *in situ* hybridization for detection of viral RNA in two flocks of purebred sheep, one with a history of OvLV disease, and one without. ELISA and immunoblotting showed sero-prevalence of 25% and 33% respectively. However, when *in situ* hybridization was employed, viral RNA was detected in 72% and 67% of sheep respectively. When a selected sub-group of 20 yearling sheep were tested by ELISA, using tissue culture derived antigen and by co-cultivation of macrophages with target cells, 10% were sero-positive, as compared with 70% which were positive by *in situ* hybridization, PCR and co-cultivation, suggesting that OvLV infection may not be detected using conventional serological tests (*e.g.* AGID).

Transmission of OvLV occurs from ewe to lamb through ingestion of infected colostrum and/or milk, and it may be enhanced by the practice of placing orphan lambs with infected sheep (63). Transmission also occurs via infected respiratory exudates within the herd, which is primarily dependent upon rearing practices, hence the wide range in sero-prevalence. Sheep raised in low density situations, such as large open grazing tracts, exhibit lower rates of OvLV infection than those in more crowded sheep rearing areas (64). One study suggests that venereal transmission, via OvLV-infected semen could also in some cases be relevant in the transmission of OvLV (65)

1.4 Pathology of OvLV Infection

The unique pathogenesis of OvLV infection is due in part to mechanisms which

control viral gene expression, as well as the complex viral genome, and the ability of OvLV to exist in a proviral state. The clinical signs of OvLV disease are characterized by the presence of a chronic progressive respiratory distress, caused by Lymphoid interstitial pneumonia, mastitis and cachexia (66, 67), while the demyelinating leukoencephalomyelitis and/or neuro-degenerative paralytic disease are rare with North American OvLV isolates (68). Lymphadenopathy and follicular hyperplasia in lymph nodes and in the spleen are predominant lesions in natural OvLV infection, characterized by the presence of OvLV-infected macrophages (69). Lesions are often caused by cytokines produced by infected lymphocytes and/or macrophages (70, 71). The subclinical phase usually lasts months to years, but the infection ultimately leads to death of the host. Infected sheep usually develop Lymphoid interstitial pneumonia, with thickened inter-alveolar septa and dense interstitial perivascular and peribronchial infiltrates of lymphocytes and macrophages (72). In addition, a significant proportion of animals develop indurative mastitis, with periductal and interstitial lymphocytic infiltrates. Arthritis is also seen, and it is characterized by inflamed carpal joints. The affected joints usually have hyperplastic synovial membranes, characterized by large numbers of lymphocytes and/or macrophages in the subsynovial tissue, though this is less pronounced than in the closely related caprine arthritis encephalitis virus infection (73, 74). OvLV pathology may be viewed as a chronic systemic disease of which the central feature is infection of cells of the monocyte/macrophage lineage, combined with organ-specific immuno-pathological changes. The host response to OvLV infection leads to humoral immune response and to

lymphocytic infiltration of targeted tissues. Lesion formation and chronic inflammatory response may be further exacerbated by viral antigen-antibody complexes and T cell dysfunction (75). Infected macrophages and/or dendritic antigen presenting cells present OvLV peptides resulting in a cascade of cellular immune responses, including proliferation and accumulation of CD8+ lymphocytes as well as production of interferons, resulting in immunopathology of various tissues (76). OvLV isolates differ in their *in vitro* cytopathic effect, compared with Icelandic MVV strains. MVV strains induce syncyticia and cause lysis in cell lines of fibro/endothelial cells, as well as *in vivo* in susceptible Icelandic sheep. In contrast, most OvLV strains including OPPV from North America, induce Lymphoid interstitial pneumonia and syncyticia, but are generally non-lytic in nature (77).

Cheevers and colleagues (78) have investigated the phenotypic differences between OvLV strains by using sets of isogenic twin lambs, infected with two distinct phenotypic strains of OvLV. Analysis of antibody response and the degree of lymphoid interstitial pneumonia suggested unknown host genetic factors may play an important role in determining the level of inflammatory response to Lymphoid interstitial pneumonia.

1.5 Diagnosis and Detection

Currently, detection of serum anti-OvLV antibodies, viral nucleic acid and virus isolation in susceptible cell lines, are all methods employed to detect OvLV infection. The sensitivity of these different methods can be affected by viral strain differences and the

time between infection and testing (79). Some tests may be only suitable for particular strains of the virus. Agar gel immuno-diffusion (AGID) has been the standard test for screening sheep for infection with OvLV (80), though complement fixation, virus neutralization, virus isolation and immunofluorescent antibody staining have all been employed (81, 82, 83). AGID has disadvantages compared to ELISA, including lower sensitivity, long incubation period, and reproducibility (84). Occasional ambiguity in the interpretation of results is also a disadvantage (85). ELISA and immuno-blotting are known to be more sensitive and specific, and in the case of ELISA, there is the advantage of high throughput automation (86).

Indicative of recent OvLV infection is the presence of antibodies directed against the capsid (p24) protein, though these antibodies are not virus neutralizing (87). Immune response to OvLV infection generates neutralizing and fusion-blocking activities, which are discussed in detail in section 1.6. The Ab, as detected by ELISA, are directed to the hydrophobic TM (gp 41) protein, as well as other structural proteins, such as the capsid (p24); nucleocapsid (p16) and matrix (p14) (88, 89, 90, 91).

Initially, ELISA and Western blot analysis employed whole virus and/or virus proteins derived from tissue culture, using concentrated viral protein from diluted tissue culture supernatant as antigen (92). However, using tissue culture as a source of virus antigen suffers from several disadvantages, including cost and time needed to produce large scale viral antigen preparations (93), the need for downstream purification, and variability in lot to lot reproducibility (94). With the advent of recombinant DNA technology, the

cloning of major OvLV genes (*e.g.* CA and TM) into various plasmids, and subsequent expression of these recombinant constructs in prokaryotic (*E. coli*) or eukaryotic (baculovirus, yeast) expression systems has resulted in production of more standardized antigenic viral proteins for use in serological diagnostics (95, 96, 97, 98, 99). ELISA employing recombinant viral proteins (*e.g.* CA and TM) or synthetic peptides has become the diagnostic test of choice in detection of antibodies to OvLV infection (100, 101, 102, 103).

Nucleic acid based testing, such as polymerase chain reaction (PCR) and *in situ* hybridization, utilizing primers and probes from the conserved *gag* and *pol* genes regions respectively, may also be used (104, 105, 106). Although this type of testing (*e.g.* RT-PCR) is inherently more sensitive, it is not currently used for routine diagnosis for several reasons, including cost and time.

There are several factors which ultimately determine the outcome of accurate diagnosis. They include host genetic factors, particularly those which govern immune response, viral strain effects, and sampling time subsequent to infection. Antibody response usually results in appearance of anti-capsid within 2- 4 weeks post-infection, while those directed against transmembrane and surface envelope proteins may not appear until several weeks later (107, 108, 109). Diagnostic tests, such as ELISA and Western blotting, employing recombinant viral antigens may use capsid and transmembrane proteins for increased sensitivity (110).

1.6 Humoral Immune Response to OvLV Infection

Antibody response to OvLV infection usually appears within 1-2 weeks post infection, while neutralizing antibodies rarely appear before 4 weeks post infection, and typically appear at 6-12 months PI (111, 112, 113). Apart from virus neutralization, various other functions of specific anti-OvLV antibodies were described; 1) anti-fusion antibodies which are measured by their ability to prevent syncytia *in vitro*, but are not virus neutralizing; 2) enhancing antibodies, which bind to virus and enhance infection of macrophages via the Fc receptor on the target cells, and 3) complement-dependent cytotoxic antibodies (114, 115, 116). Antibodies directed against capsid proteins, which are non-neutralizing in nature, are usually detected early in infection, are high in titre and remain for the life of the host. In contrast, antibodies against surface and transmembrane epitopes, some of which are neutralizing, usually do not appear until 6-12 months PI (117). The specific mechanisms of neutralizing antibody on OvLV have been examined in several studies (118). In general, neutralizing antibodies have poor binding efficiency when tested in vitro, using macrophage and fibroblast cell cultures (119) . It has been reported that neutralizing antibodies were able to block replication of virus internalized into cells, as well as disrupt virus/receptor binding (120). Here, the neutralization was presumed to take place through inhibition of viral uncoating. In one study, ultra structural examination revealed that neutralizing antibodies caused capping of budding OvLV particles, with subsequent clumping of the virus into large aggregates (121). However in macrophage

cultures, antibodies also enhanced entry of virus into cell by Fc receptor-mediated endocytosis (122).

Antibodies to surface envelope protein may neutralize parental virus, but are often present in low titres with binding affinity and usually appear after extended periods (123). However, type-specific antibodies, while able to neutralize laboratory strains *in vitro*, are often not effective against strains which emerge by antigenic drift (124, 125). Antigenic drift is documented to occur and give rise to small changes (*i.e.* mis-sense mutations) within peptides of the surface envelope protein (gp135), allowing mutated strains to escape neutralizing antibody directed against parental strain epitopes. While this is generally considered to be an important mechanism for lentivirus evasion of clearance by antibody, there is still speculation as to the extent this phenomenon contributes to persistence of OvLV infection (126, 127, 128). Most lentivirus neutralizing antibodies are directed towards epitopes on the surface envelope glycoprotein (gp135), and possibly to epitopes on the hydrophobic transmembrane (gp 41) protein, which anchors the SU within the viral envelope (129). The mechanism of neutralization in OvLV is not known. Neutralizing antibodies may interfere with virus/cell surface receptor binding, or with the fusion of virus envelope and cell plasma membrane, as is the case in HIV antibody-mediated neutralization (130).

Studies on two OvLV linear neutralizing epitopes in the surface variable region (V4), have been mapped to a specific region of the *env* gene. The pepscan analysis revealed that a disulphide bridge between the two cysteine residues results in formation of a

conformational epitopes by bringing the linear epitopes into proximity with each other. Skraban and colleagues (131) found that antigenic variants had one of the cysteines mutated, resulting in an antibody escape phenotype.

Stanley and colleagues (132) used monoclonal antibodies and polyclonal antisera to study topographical rearrangement of *env* epitopes in visna virus, after undergoing antigenic drift. While polyclonal serum against the parental strain was unable to neutralize 5 of 6 of the virus progeny mutants isolated from the same sheep, mAb were able to distinguish antigenic differences on five partially overlapping epitopes of the mutated progeny virus. The authors remarked that topographical rearrangement of the tertiary structure of *env* glycoproteins may magnify the primary amino acid sequence changes, brought about by a small number of point mutations in the *env* gene.

Narayan and colleagues (133) reported that sheep which had been immunized with a non-ionic detergent disrupted parental virus isolate developed broadly neutralizing antibodies against both parental and mutant isolates. This suggested that the epitope(s) contained on the mutant strain are also present on the parental strain, but are conformationally blocked or masked, and may be exposed by detergent disruption. Since virus proteins undergo conformational change *in vivo* due to Ab binding, the resulting conformational change might be unfavourable to virus neutralization by the idiotypic Ab repertoire mounted during infections.

Another study utilized two characterized OvLV isolates on sets of monozygotic twin lambs to study neutralizing antibody responses as they correlated with development of

Lymphoid interstitial pneumonia. The two neutralization serotypes, classified as "rapid/high" and "slow/persistent", were distinct with regard to their replication rates, *in vitro* cell lysis and pathogenesis (134). The authors reported that unknown host genetic factors played a role in the progression of pathogenesis in one lamb. The authors also highlighted three important observations. Firstly, they observed that macrophage-associated OvLV neutralization escape variants arose in the absence or presence of neutralizing antibodies, which led to conclusion that cell-to-cell transfer of virus between macrophages may be one mechanism by which OvLV may avoid neutralizing antibodies (135). OvLV variants persisted in macrophages despite neutralizing antibody response. Secondly, OvLV variants arose without correlation to development of Lymphoid interstitial pneumonia. Drawing upon similar work with other closely related lentiviruses (*e.g.*, CAEV, EIAV and HIV), and including the observation that variants may arise in the absence of detectable neutralizing antibody titres, the authors suggested that the formation of antigenic variants can occur without the selection pressure of neutralizing antibodies (136, 137, 138).

There are several features of OvLV neutralizing antibody response which differ from that of conventional virus neutralization mechanisms. First, neutralizing antibodies may not appear until months to years after initial infection, and are often low in titre. Second, the time required for *in vitro* neutralization is often 24 - 48 hours at decreased temperatures (*e.g.* 4 - 25°C), whereas more conventional neutralizing antibodies require 30-60 minutes at 37°C. The explanation might be the previously reported low affinity of epitopes binding (139, 140). Although the development of neutralizing antibody escape variants has been

thought to be the most likely mechanism responsible for evasion of virus antibody, this mechanism does not account for the delay in appearance of neutralizing antibodies. In addition, this mechanism cannot explain continued long term presence of parental (i.e. non-mutated) virus, despite type-specific neutralizing antibodies.

We hypothesized that the host's inability to produce neutralizing antibody idiotypes of high affinity was responsible for the delay in the appearance of neutralizing antibody titres, thereby rendering the host incapable of effectively neutralizing parental virus. We hypothesized that the antibody idiotype in the early phase of infection might induce conformational changes in the virus envelope glycoprotein (*i.e.* gp-135). Further, the result of an induced conformational change of envelope glycoproteins may lead to the masking of the relatively few neutralizing epitopes. In other words, we hypothesized that in spite of the presence of the appropriate neutralizing Ab idiotypes, neutralizing effects were abrogated by non-neutralizing antibodies. For further discussion, the hypothesis of 'induced fit' should be considered (141). Conformational adaptation during antigen and antibody binding arises from an induced fit, the net result of which is a conformational change in both the antibody and antigen. This concept is well documented and accepted (142 - 146). The presence of conformational epitopes has been documented and reviewed in related lentiviruses, such as HIV (147), as well as in other non-related viruses (*e.g.* canine calcivirus) (148) and the tick-borne encephalitis virus (149). A recent study using HIV, found that non-neutralizing mAb to epitopes of the *env* glycoproteins which are conformational or discontinuous in structure, interfered with neutralizing mAb (150). In

another study, Park and colleagues (151) concluded that a conformationally-dependent epitopes in the V3 region was an important target for resistant strains of HIV-1. Watkins and colleagues (152) found that escape by HIV neutralizing-resistant variants was a direct result of single amino acid changes at several epitopes, which altered envelope conformation, an observation which would seem to corroborate similar observations with OvLV (153). Burioni *et. al.* (154) found that some non-neutralizing antibodies (*e.g.* human recombinant Fab) to a major antigenic glycoprotein (E2) of Hepatitis C virus (HCV) exerted conformational changes on the HCV E2 glycoprotein, resulting in diminished ability of other antibodies to neutralize by blocking of virus attachment. Cepica and colleagues (155) used pairs of monoclonal antibodies (mAb) to induce and assess conformational changes in the potato virus X (PVX) protein, using a blocking ELISA with native and glutaraldehyde treated virus. A double antibody sandwich ELISA (DAS- ELISA) was employed to detect possible inter-molecular spread of conformational-induced change in PVX. Using the blocking ELISA, they demonstrated one-way blocking, with a pair of mAb, specific for non-overlapping epitopes. The conformational change was confirmed when the one-way blocking was prevented using conformationally-restrained virus. Further, inter-molecular spread of the conformational change of PVX protein was confirmed using the DAS-ELISA, when capture mAb inhibited binding of detecting Ab, in the absence of any stearic hindrance.

The focus of the present study was the previously discussed postulation that an additional mechanism for OvLV persistence may be due to conformational change exerted

by non-neutralizing antibodies, on or in proximity to conventional neutralizing epitopes, resulting in their modulation, thus abrogating the host's ability to neutralize virus. Two approaches were taken. The first approach was indirect, and it involved correlation analysis of the neutralizing and ELISA anti-CA/TM antibody levels, which was performed with samples of sera of four experimentally infected sheep, collected sequentially over the period of over two years post infection. This approach had the following rationale. If certain serum samples contained specific antiviral Ab with the ability to obstruct virus neutralization by other antibody idiotypes, then the levels of neutralizing and ELISA Ab titres in these samples should show a lack of correlation, as it would be expected that a sustained antibody response to OvLV infection would result in increased levels of Ab to both CA and/or TM as well as to neutralizing epitopes on the gp-135 envelope .

The second approach used for demonstrating that virus neutralization obstructing Ab activity might exist was a direct one, with the following rationale. If serum samples with low virus neutralizing titres and high ELISA titres were identified, these would be presumed to contain virus obstructing antibody. The presence of such Ab would then be tested by mixing this serum with an equal amount of a serum of opposite activities, *i.e.* high neutralizing titre/low ELISA titre, and the mixture would be subjected to what is called a "modified virus neutralization assay". If the serum presumed to contain the neutralization obstructing Ab activity would suppress the virus neutralization activity of the other serum, this would be taken as the direct evidence of virus neutralization-obstructing Ab.

In addition to previously discussed mechanisms of viral persistence, it was reported that OvLV may impair immune system function. It has been reported that a IgG_1 restricted neutralizing antibody response occurs in MVV infected sheep (156, 158). IgG_2 is a large component of virus neutralizing antibody isotype and plays a key role in antibody dependent cell mediated cytotoxicity (ADCC) mechanisms during viral clearance, and in addition, the ovine IgG_2 isotype activates complement (157). It has been reported that isotypic restriction of IgG_2 response to OvLV infection may also contribute to persistence (156, 158). In the study of Pétursson et al. (158), the two predominant ovine IgG isotype subclasses (i.e. IgG_1 and IgG_2) were analysed in three groups of sheep, inoculated with the following: 1) cell-free MVV; 2) parapox ORF virus; or 3) immunized with MVV CA antigen. Using western blotting techniques and monoclonal antibodies specific to both ovine IgG_1 and IgG_2, ratios of both isotypes were established over the course of infection and/or immunization. Although total Ab responses in sheep immunized with adjuvant/CA, as well as sheep inoculated with parapox ORF resulted in vigorous IgG_1 and IgG_2 responses, the IgG_2 response remained undetectable in sheep inoculated with infectious MVV. Bird and colleagues confirmed the absence of an IgG_2 response in MVV experimentally infected animals, but not in those immunized with the recombinant CA antigen. They speculated that the lack of IgG_2 may limit ADCC response against infected macrophages and may play a role in OvLV persistence. Because to our knowledge there are only two reports on the IgG_1-restricted Ab response in OvLV infection (156, 158), we decided to re-investigate this phenomenon with mAb specific to both ovine IgG_1 and IgG_2, in the panel of sera of

experimentally-infected sheep available to us.

In summary, we propose to test the hypothesis of the existence of neutralizing and neutralizing-obstructing antibodies by using convention serum neutralization assays as well as measurement of Ab to recombinant CA/TM antigen, using indirect ELISA.

A sera panel, spanning approximately 2 years PI, collected from 4 Italian Massese/Sarda mixed breed sheep (ewes of approximately 6 months of age) were utilized. All four sheep had been pre-screened for OvLV infection by AGID and ELISA, and were found to be negative, prior to inoculation with 100 $TCID_{50}$ of OvLV.

In addition, correlation analysis of Ab titres obtained by these methodologies may add indirect evidence to the existence of Ab-obstructing idiotypes. To add further evidence, conventional and modified serum neutralization assays were used, the latter employing serum exhibiting opposite Ab repertoires (*i.e.* serum with high anti-CA/TM and low neutralizing Ab or early PI, combined with serum with low anti-CA/TM and higher neutralizing Ab or late PI). In addition, the phenomenon of an IgG1-restricted Ab response will be investigated using mAb specific to the two predominant ovine Ig subclasses. The rationale being that OvLV infection may employ a number of novel mechanisms for persistency within the host, in addition to those which have been well studied by previous researchers (reviewed earlier).

2. Methods & Materials

2.1 Growth and expression of recombinant *E. coli* (JM-109) clones

Recombinant proteins were produced for the detection of anti-capsid and anti-transmembrane antibodies. Recombinant fusion pGEX 2T clones: (MVV TM/GST); (MVV CA/GST); and fusion pGEX 2T (GST only) clones were expressed as follows. Clones, containing the ligated gene of interest, either CA/GST (58 kDa); TM/GST (38 kDa) or GST only (26 kDa) were inoculated into sterile 10 ml Luria broth (LB: 10 g/l peptone; 5 g/l yeast extract; 10 g/l NaCl, pH 7.2; 100 μg/ml ampicillin) cultures and grown overnight at 37° C, in a shaker-water bath. The 10 ml culture were then used to innoculate a 500 ml volume of LB in a baffled shaker flask, containing 100 μg/ml ampicillin.. *E. coli* (JM 109 strain) clones, containing the above-described pGEX 2T plasmids were grown to an optical density of approximately 0.8 - 1.0 @ 600 nm, over a 4-6 hour time period, then inoculated with 1 ml of 100 mM isopropyl-β-D-thiogalatopyroanoside (IPTG), giving a final concentration of 0.5mM IPTG, for induction of the *lac* repressor gene, resulting in initiation of transcription for specific recombinant fusion proteins. OD was monitored for 4 hrs and cells harvested afterwards by centrifugation at 5000 rpm in a refrigerated GSA rotor (Beckman-Coulter Industries) for 10 minutes. Pellets were gently washed in ice cold sterile phosphate buffered saline, pH 7.4 (PBS: 136 mM NaCl; 2.68 mM KCl;10 mM Na_2HPO_4; 1.76 mM KH_2PO_4, pH 7.4) and centrifuged again at 5000 rpm for 10 minutes to remove LB, then repeated once more. Pellets were stored immediately at -80° C.

2.2 Semi-purification of expressed CA/GST and TM/GST recombinant proteins

Recombinant GST fusion proteins accumulated as insoluble, misfolded protein aggregates, called inclusion bodies, and purification protocols were employed to address this. The use of purified recombinant proteins would alleviate potential background (*i.e.* non-specific binding of Ab to *E. coli* proteins) when used in ELISA.

After centrifugation and washing, bacterial pellets were resuspended in 25 ml of 100 mM NaCl; 20 mM Tris-HCL, pH 8.0; 5mM EDTA (STE) buffer, containing hen egg white derived lysozyme (Sigma Chemical Co.) at 200 μg/ml final concentration. Resuspended pellets were allowed to incubate on ice for 30 minutes to facilitate digestion of outer cell membrane. The suspension was then sonicated, using a micro-tip probe sonicator (Branson Industries) for 30 second bursts, then repeated eight times at a 50% duty cycle setting. The micro-tip was pre-chilled and the bacterial suspension was kept on ice between sonication rounds. After sonication, the spheroplasts, containing recombinant inclusion bodies, were centrifuged in a Sorvall RC-5A refrigerated centrifuge, using a GSA rotor, at 10,000 gravities (g) for 30 minutes. Spheroplasts were resuspended in ice cold wash buffer containing; 300 mM NaCl; 20 mM Tris-HCL, pH 8.0; 1 M urea; 1% triton X-100 for 10 minutes to solubilize lipid and cell wall debris from previous sonication. Washed spheroplasts (*i.e. E. coli* cells deficient in cell wall and having a spherical shape), were centrifuged for 30 minutes at 10,000 g. Washing and centrifugation was repeated twice and the resulting pellet was resuspended in 20 ml of 300 mM NaCl; 20 mM Tris-HCL, pH 8.0;

1M urea buffer, without triton X-100. The semi-purified pellet, containing recombinant GST fusion proteins as inclusion bodies, was dialysed overnight at 4° C and stored at -80° C in PBS, pH 7.4, for further affinity chromatography purification.

2.3 Affinity purification of CA/GST and TM/GST recombinant proteins

Dialysed and solubilized recombinant GST fusion proteins were purified using a batch purification process. An aliquot of 1 ml of glutathione resin (Amersham-Pharmacia Biotech) was washed with three volumes of 5 ml, de-ionized water, then equilibrated with three volumes of 5 ml of PBS, pH 7.4 and allowed to gravity flow in a disposable column (Bio-Rad Industries). A 10 ml volume of dialysed, GST fusion proteins, in sterile PBS, pH 8.0 was then added to the plugged column and the mixture was gently resuspended and allowed to incubated for overnight at 4 °C, on a platform rocker apparatus. The GST fusion protein and glutathione resin mixture was allowed to settle and subsequently washed with five column volumes (10 ml each) of PBS (0.3 M NaCl, pH 7.4). Wash fractions were collected for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), to ensure recombinant protein was not eluted. Elution of bound GST fusion proteins was performed by incubation and gentle resuspension of resin with 5 ml of PBS, pH 7.4 containing 20 mM reduced Glutathione for a 10 minute incubation period.
The resulting mixture of elution buffer and resin was allowed to settle and elution fractions of 1 ml each were collected for analysis by SDS-PAGE and spectrophotometry.

2.4 Propagation of Fetal lamb choroid plexus cell line in tissue culture

Fetal lamb choroid plexus cells (FLchor), an adherent primary fibroblast cell line were used for propagation of OvLV 130-91. Cells were originally obtained from the American Type Culture Collection (ATCC) and were grown in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with the following: 10% fetal bovine serum (FBS) for growth, and 2% FBS for maintenance; 5 μM non-essential amino acids; 4 μg/ml folic acid; 500 units/ ml penicillin; 500 μg/ml streptomycin. Cells were grown and/or maintained in an incubator at 37° C, with 5% CO_2 and 90% relative humidity (Rh) in membrane-capped 25, 75 and/or 225 cm^2 tissue culture flasks (Falcon Industries Inc).

2.5 Fusion of OvLV 130-91 in FLchor cells using polybrene

Ovine lentivirus strain 130-91 is a characterized isolate obtained from Professor Francesco Tolari, Department of Veterinary Pathology, University of Pisa, Italy. Polybrene (hexadimethrine bromide; Sigma Chem Co.) was utilized to facilitate cationic fusion of OvLV 130-91 to FLchor cells and was used at a final concentration of 8 μg/ml. FLchor cells grown to confluence after being freshly passaged two days prior, were washed twice with pre-warmed Dulbecco's PBS (D-PBS). Initial propagation of the Italian stock OvLV 130-91 took place as follows. Virus aliquots, supplied in 200 μl volumes were resuspended to a final 1000 μl volume of D-MEM, containing 8 μg/l polybrene was added

to a T-25 flask, containing freshly confluent FLchor cells, at an approximate volume of 3-4 ml. Flasks were then incubated at 37° C for 2 hours, with gentle agitation every 15-20 minutes. After incubation for two hours, media were carefully removed and replaced with D-MEM, containing 2% FBS. Flasks were checked daily over a two week period to monitor development and/or progression of CPE, as defined by syncytia (the formation of giant/multi-nucleated cells). After development of 50-75 % CPE, flasks were trypsinized using 2.0 ml of 0. 25 mM trypsin/EDTA (Gibco/BRL), for 5 minutes. Infected cells were centrifuged at 1000 g in a Sorvall refrigerated centrifuge for 10 minutes. The media was removed and the stock cell pellets were frozen to -80° C, at a rate of one degree per minute in 1 ml aliquots of D-MEM, containing 10% dimethly sulphoxide (DMSO) and 20% FBS

2.6 Serial end-point titration of OvLV 130-91

End-point serial titration of stock OvLV 130-91was performed as follows. D-MEM aliquots of 1.8 ml were added to 15 ml Falcon conical tubes. Stock OvLV 130-91 virus was thawed at 37° C and gently vortexed. A 200 µl aliquot was aseptically removed and added to the first tube, designated "10^{-1}" and gently pipetted up and down to ensure complete mixing. From this tube (i.e. 10^{-1} tube), a 200 µl aliquot was removed and transferred to a second tube, using a fresh pipette tip to a tube labelled "10^{-2}" and the contents pipetted up and down to ensure mixing. The procedure was repeated until a serial log dilution of 10^{-10} was achieved. Next, five 200 µl aliquots of the end-point titration were removed and added

individually to a 24 well tissue culture plate (CoStar; Corning Industries Ltd.), containing freshly confluent FLchor cells . This step was sequentially repeated from 10^{-10} to 10^{-1}, giving five replicates of each dilution. Plates were monitored daily to check for development and/or progression of CPE. Wells exhibiting CPE were sampled and frozen at -80° C for calculation of 100 $TCID_{50}$, following the method of Reed and Muench (1938), or for further analysis by RT-PCR or RT-ELISA assay.

2.7 Precipitation and concentration of OvLV 130-91 from FLchor cells

Large scale production of whole virus utilized T-75 (CoStar; Corning Industries Ltd.) flasks. Briefly, freshly passaged FLchor cells, grown in D-MEM, with 10 % FBS, were washed twice with D-PBS. A volume of D-MEM containing 100 $TCID_{50}$ and 8 μg/ml polybrene was added and incubated for two hours, with gentle agitation every 15-20 minutes. Media was then removed and replaced with D-MEM, with 2 % FBS and flasks were monitored daily until development of CPE.

Harvesting of OvLV 130-91 from infected cells and cell-free virus in D-MEM, occurred when CPE was observed in approximately 90 % of the monolayer. Briefly, infected FLchor cells were trypsinized and the resulting cells and medium were spun at 500 g's for 10 minutes at 4° C. The supernatant was removed and transferred to a sterile 100 ml tissue culture flask. A volume of sterile polyethylene glycol 8000 (PEG 8000); 1.2 M NaCl, was slowly added, to a final ratio of 1:2 (v/v) to the tissue culture supernatant and the

mixture was gently stirred overnight at 4° C. D-MEM containing PEG 8000/1.2 M NaCl was centrifuged at 10,000 g for 30 minutes, using a JA-14 (Beckman-Coulter Industries) rotor and Beckman Avanti model J-25 refrigerated centrifuge. The supernatant was carefully removed and the tubes containing precipitated OvLV, washed gently in cold PBS. After removal of all traces of PEG 8000, pellets were resuspended in sterile PBS, pH 7.4 and frozen at -80° C for further analysis by SDS-PAGE.

2.8 Purification of OvLV using sucrose density gradient ultracentrifugation

Purification of OvLV 130-91 was performed in order to use whole virus for the determination of Ab specific to outer surface envelope proteins. Density-gradient ultracentrifugation of OvLV 130-91 utilized a discontinuous 20% sucrose/50% sucrose; PBS system. First, 5 ml of ice cold, sterile 50% sucrose; PBS, pH 7.4 was added to a 14.0 ml ultracentrifuge tube (Beckman Industries Inc). Next, 5 ml of 20 % sucrose; PBS, pH 7.4 was carefully added to create a distinct overlay with interface. 1-2 ml of the concentrated OvLV 130-91 tissue culture material (section 2.7) was then added to each tube. The balanced tubes were then centrifuged at 35,000 rpm for 90 minutes, using a Beckman SW 40 Ti ultracentrifuge rotor and Beckman ultracentrifuge. The tubes were removed and examined under shadow-box light source, for the presence of bands.

The two bands in the 50% sucrose phase, were extracted separately with a sterile 2 cc syringe, added to 10 ml of sterile 20 % sucrose; PBS, pH 7.4 and spun at 35,000 rpm

for 2 hours to pellet purified virus, using the above described rotor and centrifuge. After careful removal of sucrose/PBS solution, pellets were carefully resuspended in 100 µl of sterile PBS, pH 7.4 and frozen at -80 °C until further analysis and/or use in ELISA plates.

2.9 Neutralization Assay using OvLV 130-91 and FLchor cells

To determine neutralizing antibody titres, 100 µl of sera from the four experimentally infected sheep (no. 3, 5, 6, 11) ranging from day 0 PI to day 796 PI., as well as reference OvLV negative, OvLV positive, FBS and Bovine (certified pathogen free reference sera; IDEXX Industries Ltd) - were mixed with 100 µl of D-MEM, containing 100 $TCID_{50}$ of OvLV 130-91 virus, using a 96 well tissue culture plate (CoStar, Corning Industries Ltd.). Controls consisted of mock-infected FLchor (negative control); 100 µl of D-MEM and 100 $TCID_{50}$ OvLV 130-91; as well as a 2 log back titration of 100 $TCID_{50}$ OvLV 130-91 (*i.e.* 10 and 1 $TCID_{50}$ OvLV 130-91). Starting from the original 1:1 mixture of serum and D-MEM and virus, two-fold serial dilutions were performed using a multi-channel pipette. Serial dilutions from 1:2 to 1: 5096 were performed in duplicate, from left side of the plate to the right. The 96 well plates were then incubated at 25° C for 24 hours in a 5% CO_2, humidified incubator to facilitate neutralization reactions. After incubation, freshly grown FLchor cells were washed twice in D-PBS, trypsinized briefly then resuspended in D-MEM containing 2% FBS. Cells were carefully added at 100 µl per well with a seeding density of approximately 10,000 cells per ml. Plates were monitored daily

for CPE and aliquots of all wells were collected at termination of neutralization for determination of RT activity. Neutralization titre was determined as the reciprocal dilution where 50% of the FLchor cells in the wells exhibited CPE.

2.10. Neutralization Assay using combinations of high and low neutralizing serum

The neutralization assay used for determination of interaction between OvLV infected sera was used in order to determine the effect of non-neutralizing Ab on neutralizing Ab. The assay, using high and low neutralizing Ab titres was performed as per the previous section, with one exception. Sera with low and high neutralizing titres were added at a 1:1 ratio, in duplicate and added in serial dilution exactly as other test sera. In addition to standard positive and negative controls, the paired sera were tested separately.

2.11 ELISA-based reverse transcriptase assay

The use of a commercially available ELISA-based RT assay was used to determine RT levels *in vitro*. Measurement of reverse transcriptase (RT) activity was performed by using the commercially developed assay by Roche Molecular Biochemicals (Catalogue 1468120), which utilizes a colorimetric enzyme immunoassay (EIA) for quantitative determination of RT activity. Aliquots containing virus-infected cells/supernatant, mock-infected control and D-MEM only were centrifuged at 250 g for 10 minutes at 4° C. The

supernatant was collected and added at a ratio of 2:1 to 100 µl of sterile-filtered 30% PEG 8000; 1.2 M NaCl and allowed to precipitate overnight at 4° C. The precipitated solution was then centrifuged at 8000 g in a 4° C Eppendorf micro-centrifuge for 10 minutes. The supernatant was carefully aspirated by vacuum to remove all traces of PEG, and the sample pellets were kept for analysis by SDS-PAGE. The pellets were resuspended in 40 µl of lysis buffer (50 mM Tris, pH 8.0; 5mM EDTA; 1% NP-40) and kept on ice for 30 minutes to solubilize viral particles.

For preparation of template and nucleotide mixture, nucleotides - supplied by the manufacturer - were diluted in sterile de-ionized water to a concentration of 46 mM Tris-HCl, 266 mM potassium chloride; 27.5 mM magnesium chloride; 9.2 mM DDT; 10 µM digoxigenin (DIG) and biotin labelled dUTP/dTTP template/primer hybrid, 750 mA 260nm per ml. A final template/nucleotide mixture was achieved by diluting the stock template/primer hybrid mixture to a final concentration of 9 mA 260nm, containing; dATP; dGTP and dCTP, 30 µM each. A 20 µl aliquot of template/nucleotide mixture was then added to all samples, containing 40 µl of either virus serial titrations, mock-infected titrations or D-MEM only. The 60 µl reaction mixtures were then transferred to 96 well microtiter plates, pre-coated with streptavidin. The reaction was allowed to proceed overnight (16 hrs.) at 37° C. A positive control was utilized as follows. Serial dilutions of recombinant-derived HIV-1 RT (rHIV-1 RT) were established as follows. 10 µl of lysis buffer containing 2.0 ng of rHIV-1 RT was added to 390 µl of lysis buffer, then serially diluted 1:2 in lysis buffer (2.0/1.0/0.5/0.25/0.125/0.0625 ng/ml) to a final concentration

of 0.0625 ng of rHIV-1 RT. A total of seven tubes, one containing no rHIV-1 RT and six containing the serially diluted rHIV-1 RT were added to the microtiter plate and incubated overnight at 37° C. After overnight RT incubation, the samples, controls and standards were transferred to labelled microtiter plates, covered with a plate sealer and allowed to incubate for 60 minutes at 37° C. The solutions in each well were removed and the plate washed 5 times in PBS/0.05% Tween 20 at 250 µl per well, with a 30 second soak cycle between each wash. 200 µl of anti- DIG-POD was then added to each well and the reaction was then allowed to incubate for 60 minutes. The plate was washed with PBS/Tween-20 as described above. For colorimetric development, 200 µl of 2,2'- azino-bis 3-ethylbenzthizoline-6-sulfonic acid (ABTS; anhydrous; F.W. 548.7), in citric acid, H_2O_2 was added to each well and colorimetric development was allowed to proceed for 20 minutes. Reactions were measured at 405nm, with a 490 nm reference wavelength. Mean absorbencies were calculated by subtraction of pre-immune and/or negative control serum OD from individual sample OD.

2.12 Reverse transcriptase assay in neutralization procedure

The ELISA-based RT assay, as described in section 2.11 was incorporated into the neutralization assay, inorder to lend confidence to visual CPE readings. After titres were read by appearance of CPE, duplicate wells containing FLchor cells were pooled. The cells were centrifuged at 2000 g for 10 minutes and the resulting cell pellet was utilized for

determination of RT activity as described above.

2.13 Reverse transcriptase ELISA assay in virus titration procedure

The ELISA-based RT assay, as described in section 2.11 was incorporated into the end-point titration assay to compare the sensitivity of visual CPE , as compared with that of the RT assay. After end-point titres were read by appearance of CPE, all five wells containing FLchor cells were pooled. The cells were centrifuged at 2000 g for 10 minutes and the resulting cell pellet was utilized for determination of RT activity as described above. Controls included mock infected FLchor cells, as well as a no template control.

2.14 Sodium dodecyl sulphate polyacrylamide electrophoresis of recombinant viral proteins and PEG 8000 precipitated OvLV 130-91 preparations

SDS-PAGE was utilized to analyse specific recombinant as well as tissue culture derived OvLV structural and/or non-structural proteins. Recombinant OvLV CA and TM proteins, concentrated OvLV 130-91 via PEG 8000 precipitation, then purified by previously described ultracentrifugation, were analysed by SDS-PAGE as follows. A 4 % stacking gel (4% acrylamide; 0.12% bis-acrylamide in 0.125 M Tris-PO_4, pH 6.8) and a 12% resolving gel (12% acrylamide; 0.16% bis-acrylamide in 0.375 M Tris-HCl, pH 8.8) was assembled according to manufacturer's directions, using a Bio-Rad Mini protean II electrophoresis system (Bio-Rad Industries). Samples were mixed with Laemmli four

times (4 X) reducing sample buffer (62.5 mM Tris-HCl, pH 6.8; 20% glycerol; 2% SDS; 5% β-mercaptoethanol; 37.5% H_20) and heated for 5 minutes at 95° C. The electrophoresis buffer consisted of 0.025 M Tris; 0.192 M Glycine; 0.1% SDS. The gels were run at 180 volts (constant), at 4°C. Pre-stained molecular weight markers (Bio-Rad Industries) were used for molecular weight markers.

2.15 Western Blotting of recombinant and whole viral proteins with monoclonal, hyper-immune and pre- immune serum.

Western blotting using either polyclonal or monoclonal sera, was used to confirm the presence of either recombinant or tissue culture derived OvLV proteins. Recombinant and purified OvLV proteins which were resolved using SDS-PAGE, were electrophoretically transferred from gels to 0.22 μm nitrocellulose membrane (Scheicher & Schuele Inc) using a Bio-Rad Mini Transfer unit (Bio-Rad Industries) as follows. Pre-chilled immunoblotting Towbin transfer buffer consisted of 0.025 M Tris, pH 8.3; 0.192 M Glycine: 20% methanol. Gels were placed onto a 9 cm by 9 cm 1 mm thick blotting paper (Whatman). A pre-cut 7 cm by 9 cm piece of pre-wetted membrane (transfer buffer) was carefully overlaid on the gel, followed by a second piece of blotting paper. The assembled gel/nitrocellulose was then rolled gently on a flat surface, with a clean Pasteur pipette to remove any air bubbles which may have been trapped between the gel and membrane. The assembled gel and membrane were placed between the comb apparatus and immersed into the transfer tank, with the orientation of the gel towards the

anode. Electrophoretic transfer was 100 volts constant for 1 hour at 4° C.

After transfer, membranes were rinsed in deionized water for 5 minutes, then transferred to 30 ml of Tris-buffered saline (TBS; 20mM Tris, pH 7.4; 0.135 M NaCl) and equilibrated for 5 minutes. Following equilibration, membranes were blocked in 5% non-fat skim milk powder (Carnation Brand) dissolved in TBS and incubated for 1 hour on a rotary shaker. After blocking, membranes were rinsed in TBS, containing 0.05% Tween 20 (Sigma Chemical Company) for 5 minutes, then drained to remove buffer. Membranes were then probed for 16 hours at 1:100 using ovine immune sera in TBS, containing 1% non-fat milk powder on a rotary shaker (approximately 90 RPM). All overnight incubations were performed at 4°C. Control membranes were probed under identical conditions using ovine pre-immune sera (day 0 PI) from the same animal. Following primary incubation, membranes were washed three times, 10 minutes per wash with TBS, containing 0.05% Tween 20. Membranes were drained and then probed with a commercially-available donkey anti-ovine IgG (whole molecule) alkaline phosphatase conjugate (Sigma Chemical Company, catalogue A-5187). Membranes were probed for 1 hour on a rotary shaker at a dilution of 1: 20,000. Following probing with the secondary conjugate, membranes were washed three times, at ten minutes per wash, with TBS, containing 0.3% Tween 20. Membranes were allowed to drain thoroughly, then equilibrated for 1 minute in 5 ml of alkaline phosphatase buffer (APase: 100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM $MgCL_2$). Membranes were developed using a chromagenic substrate kit (Gibco BRL, Catalogue 18280-016). A 44 μl aliquot of nitroblue tetrazolium chloride (NBT) stock solution was

added to 10 ml of APase buffer, followed by 33 µl of 5-Bromo, 4-clair-3-indolyphosphate p-toluidine salt (BCIP) and the contents mixed by gentle vortexing. A 5 ml aliquot was added to each membrane and colour development proceeded for 5-15 minutes in a light proof chamber. Colour development was terminated when purple bands were observed, by the addition of 30-40 ml of 50 mM Tris, pH 7.5, containing 5 mM EDTA.

2.16 ELISA assay for detection of serum antibodies to recombinant viral proteins and whole OvLV 130-91

ELISA was used to determine the presence of Ab, specific to either recombinant proteins or to tissue culture derived OvLV proteins. Analysis of recombinant GST fusion proteins utilized ELISA procedure as follows. Recombinant viral proteins: 1) TM/GST and, 2) CA/GST and, 3) GST only were coated at a dilution of 1:700 in 50mM carbonate/bicarbonate buffer, pH 9.5 (2.93 g $NaHCO_3$; 1.59 g Na_2CO_3 / litre in de-ionized H_2O) using 96 well Immulon-2 polystyrene ELISA plates (Immulon Industries), at 100 µl per well. Plates were incubated at 37° C for 1 hour, then stored at 4° C for use within 1-2 days, or stored at -80° C for long term. Plates coated with ultracentrifuge purified OvLV 130-91 virus were coated at a working dilution of 10 µg/ml whole virus in PBS, pH 7.4 using the above described conditions. Plates were allowed to incubate at 37° C for 1 hour, then either stored at - 80° C for future use or stored, sealed at 4° C for use within 1-2 days.

Serum was diluted 1:100 in PBS, pH 7.4 containing 1% skim milk powder/0.05%

Tween -20 and allowed to incubate at 37 C for one hour. Plates were then washed for 5 cycles of 300 µl volume per well, with a 30 second soak cycle per wash, using a Dynatech Plate washer. Plates were then gently hand washed with sterile de-ionized water to remove traces of wash buffer. Next, 100 µl of horse radish peroxidase (HRPO)-conjugated secondary antibody, specific to ovine IgG whole molecule (Sigma Chemical Co., Catalogue 5187), diluted to a working dilution of 1:1300, was added to each well. Plates were allowed to incubate for 60 minutes at 37° C and then washed five times, as described above. Development of ELISA plates utilized the following chromagenic substrate. Each well received 100 ul of 2' 2-azino-bis (3-ethylbenz thiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Co; Catalogue A-3219) substrate and was incubated with rapid agitation, using a high speed shaker platform, in the dark for 20 minutes. Plates were read at 405 nm wavelength, using a Molecular Devices V-Max kinetic plate reader and Soft Max Pro software (version 2.6.1).

2.17 RT-PCR for detection of OvLV cDNA in tissue culture

RT-PCR was used to lend confidence to *in vitro* observations of end-point titration of OvLV 130-91stock. Following end-point titration, individual wells were trypsinized, aspirated and the tissue culture material was centrifuged at 2000 g for 10 minutes. Supernatant was removed and the cell pellets were processed for DNA extraction using a Qiagen DNA kit, as per manufacturer's directions. The resulting DNA, presumably

containing proviral OvLV 130-91 DNA was stored at -20° C until PCR analysis. A negative control consisted of FLchor cells extracted, as described above. Visna *Pol* primers were synthesized, using a Beckman 1000 M oligonucleotidesynthesizer (Beckman-Coulter Industries), using standard phosphoamadite chemistry, as follows. Vis *Pol* 2 (antisense): 5' ATC ATC CAT AGT ATA TCG TCC AAA TTG 3' and Vis *Pol* 3 (sense): 5' GAT TTA ACA GAG GCA CA 3'. Anticipated product size for amplification is 303 base pairs (bp). For a 35 cycle PCR reaction, the following 10 times (500 µl total volume) mixture was made: 1) 390 µl of sterile de-ionized H20; 2) 50 µl PCR 10 X stock buffer (Gibco-BRL); 3) 15 ul 50mM $MgCl_2$ (Gibco-BRL); 4) 10 µl 10 mM dNTP; 5) 10 µl of 100 mM Vis *Pol* 2 primer; 6) 10 µl of 100 mM Vis *Pol* 3 primer; 7) 2 µl Taq DNA polymerase (5 U/µl; Gibco-BRL Catalogue 10342-020). After thoroughly mixing the stock reaction mixture, 49 µl was dispensed per tube (10 tubes total). Each tube received 1 µl of DNA template, extracted from a specific end-point titration. Controls included 1 tube with DNA from mock-infected FLchor cells and 1 tube with no template added. The file method for a PTC-200 (Peltier thermal Cycler, MJ DNA Engine) is as follows. First a 96° C cycle for 3.0 minutes; 2) 95° C for 10 seconds; 3) 53° C for 30 seconds; 4) 72° C for 60 seconds; 5) followed by 34 cycles of steps 2 - 4. Completion of the file resulted in a 4° C hold cycle until the PCR unit was shut-off. A 2% agarose gel was cast using 100 mls of 40 mM Tris-Acetate, pH 8.5; 2mM EDTA buffer (TAE); 8 µl of ethidium bromide stock solution (1% ethidium bromide; Fisher Biotech Catalogue BP1302-10) and 4 g of agarose (Bio-Rad Catalogue 162-0126). Samples, consisting of 15 µl of each PCR tube were pre-mixed with

3 µl of 6 times gel loading buffer and the 18 µl volume was loaded into individual wells. A 2 µl volume of a 100 bp ladder (Gibco-BRL Catalogue 15628-050) was added to the outside lane. The gel was run at 70 volts constant voltage for approximately 75 minutes, using a Bio- Rad Wide mini-Sub GT system. The gel was photographed using a Bio-Rad Gel Doc 2000 system.

2.18 ELISA assay for detection of ovine IgG_1 and IgG_2 antibodies using mAb

ELISA was performed, using mAb specific to the two IgG subclasses in order to determine if either IgG response to recombinant proteins was dampened. Serum was diluted 1:100 in PBS, pH 7.4 containing 1% skim milk powder/0.05% Tween -20 and allowed to incubate at 37° C for one hour. Plates were washed for 5 cycles of 300 µl volume per well, with a 30 second soak cycle per wash, using a Dynatech plate washer. Plates were then gently hand washed with sterile de-ionized water to remove traces of wash buffer. Next, 100 µl of mAb, specific to ovine IgG_1 and 100 µl of mAb diluted 1:4 in PBS/blocking diluent (described above), specific to ovine IgG_2 were added in duplicate to each well and allowed to incubate at 37° C for one hour. Washing was repeated, as above. Next, 100 µl of horse radish peroxidase (HRPO)-conjugated secondary antibody, rabbit anti- mouse IgG whole molecule (Sigma Chemical Co., Catalogue A 9044), diluted to a working dilution of 1:10,000 in PBS/blocking diluent, was added to each well. Plates were allowed to incubate for 60 minutes at 37° C and then washed five times, as described

above. Development of ELISA plates utilized the following chromagenic substrate. Each well received 100 ul of 2' 2-azino-bis (3-ethylbenz thiazoline-6-sulfonic acid (ABTS; Sigma Chemical Co; Catalogue A-3219) substrate and was incubated with rapid agitation, using a high speed shaker platform, in the dark for 20 minutes. Plates were read at 405 nm wavelength, using a Molecular Devices V-Max kinetic plate reader and Soft Max Pro software (version 2.6.1).

3. Results

3.1 Growth and expression of recombinant *E. coli* (JM-109) clones

Expression of MVV TM/GST resulted in greater yields of recombinant protein, as compared with MVV CA/GST cultures. Expression of all recombinant OvLV protein clones, including GST control protein, resulted in aggregates of mis-folded, insoluble recombinant proteins, called inclusion bodies. These insoluble recombinant proteins were subsequently solubilized in PBS, pH 8.0, containing 1% SDS, which allowed for affinity chromatography purification.

3.2 Affinity purification of CA/GST and TM/GST OvLV recombinant proteins

Purification of solubilized CA/GST and TM/GST recombinant proteins was most effective using a batch-style affinity chromatography, with 15 ml disposable columns, containing 1 ml of glutathione resin (Amersham-Pharmacia Inc). Binding was most effective with an overnight incubation at 4°C, as compared with room temperature incubation for 30 minutes, using solubilized recombinant proteins diluted 1:10 in sterile PBS, pH 8.0. A rocker platform was used to facilitate contact of recombinant proteins with the glutathione resin. No protease degradation was observed using this procedure. A minimum of 5 column volumes of PBS, containing 270 mM NaCl was required to wash out weakly bound non-specific contaminating proteins from host *E. coli* (data not

shown). Eluted proteins were found in the first 1-3 fractions (1ml per fraction in 20mM reduced glutathione PBS, pH 8.0). Spectrophotometric analysis of recombinant GST fusion proteins, using a Beckman DU-640 (Beckman-Coulter Ind.), allowed for analysis of affinity purified recombinant protein at 280 - 300 nm (Figure 2).

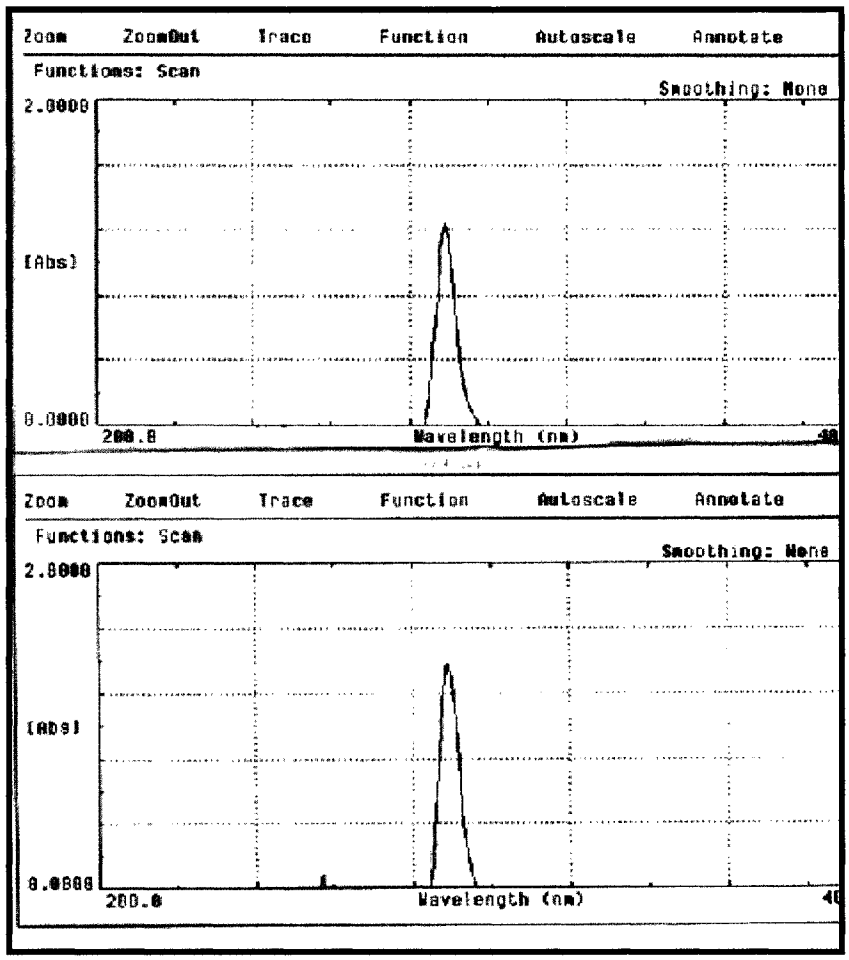

Figure 2. Spectrophotometric analysis (200 - 400 nm) of affinity purified CA/GST (top) and TM/GST (bottom) recombinant proteins.

3.3 Propagation of FLchor cells and purification of OvLV 130-91 isolate

FLchor cells, split at a 4:1 ratio from seed stock T-75 flasks, grew to 100 % confluence within 2 - 4 days, with D-MEM plus 10% FBS. When D-MEM containing 2 % FBS was added to confluent FLchor cells, they were able to maintain for 1-2 weeks.

3.4 Infection of FLchor with OvLV 130-91 isolate using polybrene

CPE occurred between 5 and 12 days post infection, with the peak CPE between days 7 and 10. The formation of syncytia (giant, multi-nucleated cells exhibiting long dendritic like processes), was the most common manifestation though cell lysis was seen in advanced cases of CPE.

3.5 Calculation of end-point titration for OvLV 130-91

In order to perform neutralization assays, a working stock of 100 $TCID_{50}$ of OvLv 130-91 was required. Serial log dilutions, using 200 µl of tissue culture derived OvLV and 1800 µl of D-MEM, were performed in replicates, using T-25 membrane capped flasks. After day 12, the number of infected flasks and uninfected flasks were counted, based upon appearance of CPE. Table I summarizes the cumulative data for serial dilutions ranging from 10^{-1} to 10^{-8}. A FLchor control remained negative throughout.

Table I: Results of ten-fold serial end-point titration of OvLV 130-91 strain

log virus dilution	Infected test units	infected (A)/CPE	non-infected (B)	Ratio A/B	% infected
10^{-5}	5/5	8 +++	0	8/8	100 %
10^{-6}	2/5	3 ++	3	3/6	40 %
10^{-7}	1/5	1 +	4	1/5	20 %
10^{-8}	0/5	0 -	9	0/9	0 %

CPE is scaled as follows: 1) ++++ 75-100 % of monolayer with CPE; 2) +++ 50-75 % CPE; 3) ++ 25-50 % CPE;4) + < 25 % CPE; 5) - no visible CPE

3.6 Serial end-point dilution of OvLV 130-91:Calculation of $TCID_{50}$:

Following the method of Reed and Muench (1938), the $TCID_{50}$ was calculated:

$$\frac{(\%\text{ positive above } 50\%) - 50\ \%}{(\%\text{ positive above } 50\ \%) - (\%\text{ positive below } 50\%)}$$

$$= \frac{100\ \% - 50\ \%}{100\ \% - 40\ \%} \times \log 10 \text{ dilution}$$

$$= 0.838 \times 1 \text{ or, } 0.838 \text{ to add to optimal dilution (i.e. } 10^{-5})$$

Therefore, since the log of the dilution, above 50 % is 10^{-5}, the proportionate distance is 0.838. The log of the dilution factor is 10^{-1}, and the 50 % endpoint is calculated as follows: $(-5) + 0.838 \times (-1) = -5.838$. Therefore the $TCID_{50} = 10^{-5.838}$, or the end point dilution which will infect 50 % of test units inoculated, equals 1 $TCID_{50}$ therefore the log dilution for 100 $TCID_{50}$ is $10^{-3.838}$.

3.7 Precipitation and enrichment of OvLV 130-91 from FLchor cells

Propagation and subsequent precipitation of OvLV was performed several times. Results of western blotting of the enrichment technique using PEG 8000/1.2 M NaCl demonstrated that this protocol was effective at concentrating OvLV from the large volumes tissue culture supernantant. The predominant band was approximate 24-26 kDa in size, corresponding to the actual size of OvLV CA protein. Other bands were observed

with molecular weights as follows: 32, 55-58, 90-95 and 130-150 kDa (Figure 7, page 71).

3.8 Purification of OvLV 130-91 using sucrose density gradient centrifugation

Ultracentrifugation of PEG 8000 precipitated OvLv 130-91, via 20%/50% discontinuous sucrose density resulted in two bands. The first was approximately 1.6 cm into the upper 20% sucrose phase, the second band in the 50% phase, was approximately 3.8 cm from the bottom of the centrifuge tube . The extracted lower band, upper band and the inter-phase material were collected separately and centrifuged at 100,000 g for 60 minutes to pellet the virus, presumed to be in the lower band. A small, visible pellet was collected from each centrifuged sample and gently re-suspended in 200 µl of sterile PBS, pH 7.4. Protein concentration was determined by spectrophotometric measurement at 260 and 280 nm, using the following formula: $(1.55 \times Abs_{280}) - (0.76 \times Abs_{260})$. Table II summarises the protein concentrations of the two bands and interface material collected.

Table II. Spectrophotometric analysis of ultracentrifuged OvLV 130-91 bands.

band	absorbance 260 nm	absorbance 280 nm	protein concentration[1]
upper	.045	.053	0.048 mg/ml
lower	.046	.037	0.023 mg/ml
inter-phase	.007	.005	0.0025 mg/ml

[1]protein concentration = (1.55 X absorbency 280 nm) - (0.76 X absorbency 260 nm)

3.9 Neutralization assay using OvLV 130-91 and FLchor cells

Conventional neutralization assays were performed using 100 $TCID_{50}$ of OvLV 130-91 with sera of all four sheep. The results showed a wide range of neutralizing titres, from 1:4 to 1:256 (Table III), over the course of approximately two years. Sheep 3 exhibited the earliest sero-conversion (the presence of antibodies in blood serum, as a result of infection or immunization) day 34 PI when neutralizing titres reached 1:16. In contrast sheep 5 and sheep 6 showed sero-conversion between day 50 and 79 PI (sheep 5) and approximately day 255 PI (sheep 6). Neutralizing titres were in the range of 1:2 to 1:32, with average titres at 1:8 (Table IV).

Sheep 11 showed relatively higher neutralizing titres, with 1:256 observed on day 261 PI. Tables III - VI , summarize neutralizing titres for all four sheep, including pre-immune, FBS, negative and positive reference ovine sera. Pre-immune sera from all four sheep (day 0) gave neutralizing titres between 1:2 and 1:8. FBS and negative control bovine sera from a commercially available ELISA test kit for BLV were also tested and gave neutralizing titres of 1:8 and 1:64 respectively.

Table III. Summary of neutralization titres, ELISA OD for recombinant CA/GST and TM/GST for OvLV 130-91 infected sheep 3 and 5.

sheep no.	NT Ab titre	ELISA virus	CA/TM rAg	ELISA no virus	GST only	days PI
sheep 3	1:4	.333	.110	.093		pre-immune
sheep 3	1:4	.318	.145	.088		20
sheep 3	1:16	.317	.146	.088		34
sheep 3	1:8	.326	.152	.085		53
sheep 3	1:16	.324	.158	. 087		53
sheep 3	1:2	.644	.913	.083		185
sheep 3	1:128/1:256	.695	.523	.092		255
sheep 3	1:128	.747	.460	.113		255
sheep 3	1:256	.663	.951	.115		462
sheep 3	1:256	.592	.931	.109		462
sheep 3	1:64	.652	.642	.104		735
sheep 5	1:2	.291	.173	.104		pre-immune
sheep 5	1:4	.277	.095	.083		5
sheep 5	1:2	.300	.134	.071		20
sheep 5	1:4	.366	1.393	.095		50
sheep 5	1:16	.523	1.395	.118		79

Table IV. Summary of neutralization titres, ELISA OD for recombinant CA/GST and TM/GST for OvLV 130-91 infected sheep 5, 6 and 11.

sheep no.	NT Ab titre	ELISA virus	CA/TM-GST rAg	ELISA no virus	GST only	days PI
sheep 5	1:2	1.135	1.597	.291	.052	82
sheep 5	1:16	1.598	3.26	--	.058	352
sheep 5	1:2	1.349	3.46	--	.072	721
sheep 5	1:2	1.706	3.309	--	.084	721
sheep 5	1:32	2.287	3.71	--	.068	796
sheep 6	1:2	0.224	0.132	.224	.056	pre-immune
sheep 6	1:2	0.236	0.126	–	.061	5
sheep 6	1:8	0.656	0.69	--	.051	20
sheep 6	1:4	0.461	1.695	--	.064	50
sheep 6	1:8	0.423	1.684	--	.045	82
sheep 6	1:32	0.415	0.821	--	.043	255
sheep 6	1:32	0.462	1.298	--.	.071	462
sheep 6	1:8	0.390	0.677	--	.063	721
sheep 6	1:64	0.312	0.431	--	.073	781
sheep 11	1:8	0.124	0.105	--	.095	pre-immune
sheep 11	1:32	0.160	0.076	–	.063	20

Table V. Summary of NT-Ab titres (CPE-read & RT-ELISA), ELISA OD values for whole virus, CA/TM/GST for infected sheep 11, control & reference sera.

sheep no.	NT Ab titre CPE	ELISA virus	CA/TM rAg	no virus	GST only	NT Ab titre RT-ELISA OD	days PI
sheep 11	1:16	.392	.207		.095	1:16 = .88	50
sheep 11	1:64	.381	.310		.087	1: 64 = 1.47	82
sheep 11	1:64	.383	.464		.084	1:64 = 1.17	261
sheep 11	1:256	.390	.481		.087	1:256 = 2.1	261
sheep 11	1:64	.185	.202		.073	ND	470
sheep 11	1:128	.234	.336		.092	ND	523
sheep 11	1:32	.240	.383		.115	ND	756
sheep 11	1:4	.405	.784		.142	ND	713
s367 +++	1:16	1.31	3.435		.073	see Table VI	see below
s359 neg	1:8	.532	.128		.117	see Table VI	see below
bovine	1:64	ND		ND		ND	see below
FBS	1:8	ND		ND		see Table VI	see below
ovine 21	1:128	ND	4.09	ND	.148	ND	ELISA +
ovine 30	1:8	ND	3.74	ND	.087	ND	ELISA +
ovine neg	1:8	ND	.178	ND	.130	ND	ELISA -
ovine neg	1:8	ND	.194	ND	.141	ND	ELISA -

RT OD_{405} ++ control = 0.43; RT OD_{405} neg control = .046 + 2 SD; 1) S367 reference serum = "strong positive, source: CFIA/ CAPH; 2) S359 reference serum = "negative"; source (as above); 3) FBS: heat inactivated (Cansera, Toronto, Canada); 4) Bovine negative serum, (IDEXX Laboratories Ltd: BLV-ELISA negative reference serum; 5) Ovine +++: ovine serum, from diagnostic submission: ELISA OD greater than 1.0; Sour; 6) ovine serum, from diagnostic submission: ELISA OD less than 0.05; Source: CFIA
ND = no data.

Table VI. Neutralizing antibody titres of pre-immune and reference ovine sera and fetal bovine serum in conventional neutralization assays

Serum	NT Ab titre	RT-ELISA OD
ovine # 3 (pre-immune)	1:4	ND
ovine # 5 (pre-immune)	1:2	ND
ovine # 6 (pre-immune)	1:2	ND
ovine # 11 (pre-immune)	1:8	ND
ovine negative reference serum s359	1:8	1:8 = 0.110
ovine strong positive ref. serum s367	1:16	1:16 = 0.062
bovine negative serum (BLV test kit; IDEXX Laboratories Ltd.)	1:64	ND
FBS (heat inactivated @ 56° C for 30 minutes)	1:4/1:8	1:4 = 0.043 1:8 = 1.138

Although all four sheep exhibited distinct patterns of neutralization titres, two trends emerged for the four infected sheep. Sheep 3 and 11 displayed higher neutralizing titres as compared with sheep 5 and 6 (Figure 3). Sheep 3 displayed the highest titres, which peaked at 1:256 (462 days PI), though the RT values suggests a titre of 1:128. Sheep 11 showed the earliest induction of neutralizing antibodies, after 34 days PI. The titres for sheep 5 and 6 were low and ranged between 1:2 and 1:32, although a titre of 1:64 was observed for sheep 6 at day 781 PI, but the trend for both sheep was an increase at the end of the sampling period.. Neutralizing antibody titres for sheep 5 and 6 appeared later in the course of infection and rose to titres ranging from 1:8 to 1:32. By the end of the sampling period, both of these animals exhibited a slight increase in neutralizing titres. In contrast, the neutralizing antibody titres in sheep 3 and 11 dropped towards the end of the sampling period (approximately 735 days PI).

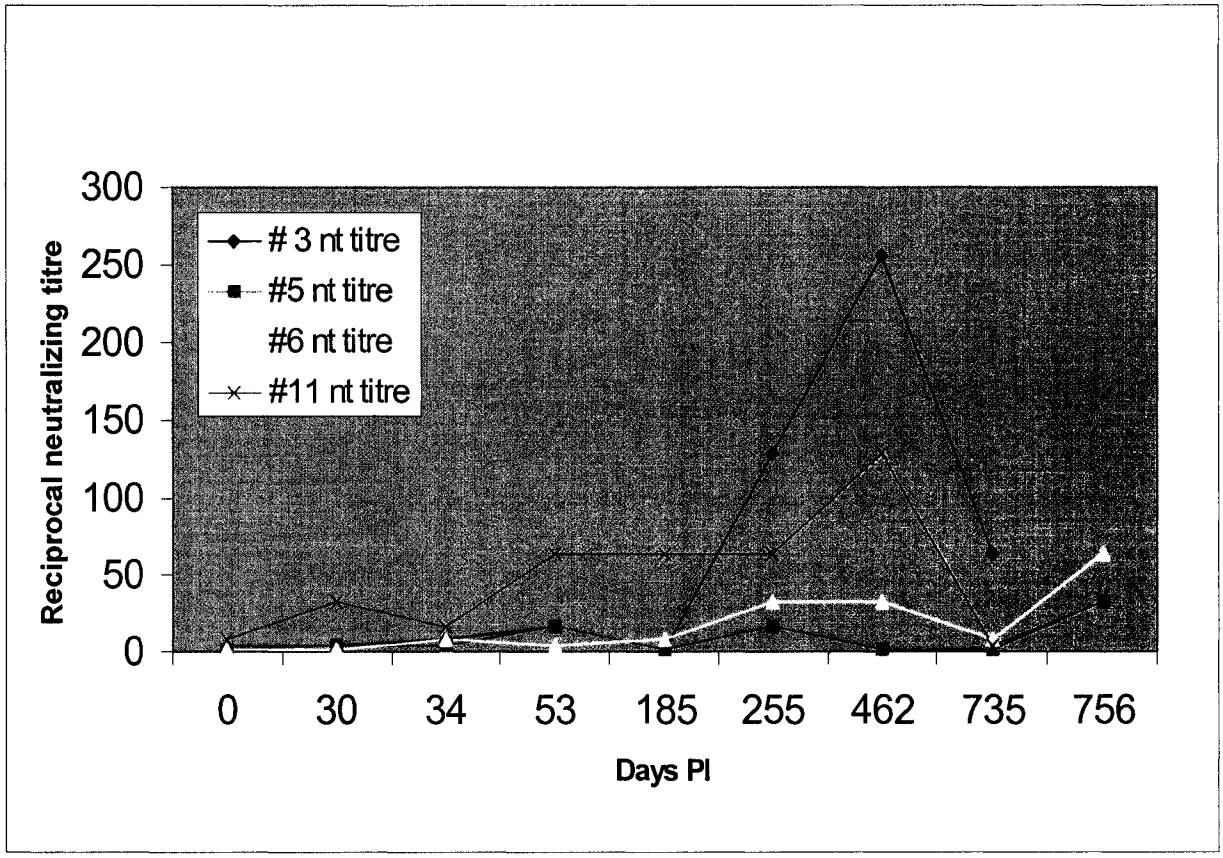

Figure 3. Neutralizing antibody titres for OvLV infected sheep 3, 5, 6 and 11.

3.10 Modified neutralization assay using mixtures of high and low neutralizing serum for determination of neutralization-obstructing activity

Serum from sheep 3, day 20 PI gave a neutralization titre of 1:4 (CA/TM-GST ELISA OD =.145), while serum from day 185 PI gave a 1:2 titre (CA/TM-GST ELISA OD =.913). Late serum (day 735 days PI) had a titre of 1:128 (CA/TM-GST ELISA OD = .642). When the sera 185 PI and 735 days PI were combined at a 1:1 ratio, the resulting titre dropped 3 serial dilutions, to 1:16 (Table VII).

Table VII. Modified neutralization assay for sheep 3, using 1) intermediate (185 days PI); 2) late sera (735 days PI); and 3) 1:1 of 185 days /735 days PI.

	1:2[2]	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
185[1]	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos
185	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos
735	neg	neg	neg	neg	neg	neg	+/-	pos	pos	pos
735	neg	neg	neg	neg	neg	neg	neg	pos	pos	pos
185/735	neg	neg	neg	neg	pos	pos	pos	pos	pos	pos
185/735	neg	neg	neg	neg	pos	pos	pos	pos	pos	pos

Legend: [1] Days post infection; [2] serum dilutions; pos = positive for CPE; neg = no CPE; +/- = equivocal. Controls :1) FLchor cells only (neg control ; 2) FLchor cells plus working dilution (100 $TCID_{50}$ OvLV 130-91); 3) back titration of working dilution of OvLV 130-91.

3.11 Correlation analysis of neutralizing antibody titres with CA/GST and TM/GST ELISA antibody titres

Individual sheep were analysed for correlation between neutralizing antibody titres and non-neutralizing ELISA (*i.e.* CA/TM-GST) antibody titres over the course of sampling. Sheep 3 showed a strong correlation ($r = 0.894$; $p = 0.003$) between neutralizing antibody titres and non-neutralizing titres, while sheep 5, 6 and 11 showed no correlation between neutralizing and non-neutralizing antibodies titres. Table VIII. summarizes the correlation coefficients (r) and probability (p) values for the data.

Table VIII. Summary of correlation analysis: NT Ab titres vs CA/TM-GST titres.

Sheep	Correlation (r): NT titre vs. CA/TM-GST titre	p value
3	0.894	0.003
5	0.505	0.202
6	- 0.070	0.858
11	-0.161	0.697

3.12 Reverse transcriptase ELISA assay

The RT-ELISA assay was used to determine the correlation of visual CPE and levels of reverse transcriptase enzyme in neutralization assays, using sera from sheep 3 and 11. Correlation was shown to occur between CPE observed in tissue culture and RT values using the ELISA-based assay ($p < 0.01$). However, in one instance, sheep 3 gave a neutralizing titre of 1:128 at 465 days PI, though the RT-ELISA value of 0.716 indicated a positive value for RT (mean neg = $0.0418 \pm .018$), thus reducing the titre to 1:64. At 1:64, the CPE was negative and the RT-ELISA value was also negative (0.048). At a dilution of 1:256, the RT-ELISA value increased to 2.39. The pre-immune serum from the same sheep, gave a neutralizing titre of 1:4 (RT = 0.366) and at 1:16 the value increased approximately five-fold to 1.938. Table IX summarizes the RT values and neutralizing titres for sheep 3 and 11, including negative (mock-infected FLchor cells) and positive (FLchor infected with 100 $TCID_{50}$ OvLV) controls.

On occasion, CPE was determined to be negative, though RT-ELISA values gave moderately positive values. Three sera from sheep 11 were analysed using the RT-ELISA. At day 50 PI the conventional neutralizing titre was determined to be 1:16, though the RT value was still 0.88 at this dilution. At day 82 PI, the RT value was 0.82 at 1:16 dilution of the serum, though conventional CPE was observed only at 1:32 and above. At day 225 PI, the RT value (0.41) was again moderately positive at a titre of 1:16, and at the conventional neutralizing titre of 1:64, the RT value was approximately three-

fold greater (1.17). At a maximum dilution of 1:2048, the RT value was 3.25, approximately three-fold greater than at the determined neutralization titre. Average values for RT from mock-infected FLchor cells were 0.046 - 0.048 .

In addition to observations involving sera from experimentally infected animals, negative control sera (*e.g.* FBS, ovine pre-immune and bovine ELISA negative reference sera) used in the RT-ELISA assay confirmed observations from the neutralization test read by CPE, that "virus neutralization" occurred in sera that did not contain OvLV specific antibodies. Using heat-inactivated FBS, neutralizing titres were seen at 1:4, as read by both CPE and RT-ELISA value = 0.043. In contrast, at the 1:8 dilution, the RT-ELISA value was 1.138. With the negative reference serum (s359), a neutralizing titre was observed at the 1:8 dilution (RT ELISA = 0.110), while the dilution 1:16 gave an RT-ELISA value of 1.598. The RT-read, and CPE-read neutralization titres, were also observed in pre-immune serum from sheep 3. At a neutralizing titre of 1:8, the RT value was 0.298 and at 1:16, the RT-ELISA value increased approximately eight-fold to 1.938. Using the strong positive serum (s367), a neutralizing titre was observed at a dilution of 1:16 (RT ELISA = 0.062), which was only a single serial dilution above the negative serum. At a dilution of 1:32, the RT value was 0.786. RT activity was also used to assess the effect of log dilution of OvLV 130-91 on the virus yield from FLchor cells, in order to determine the inoculum for production of the stock virus. RT activity was greatest at serial log dilutions of 10^{-2} to 10^{-4}, (see page 64, Figure 4.). Average values of RT from mock-infected FLchor cells were 0.046.

Table IX: RT-ELISA vs CPE-read neutralizing antibody titres for sheep 3 sera: pre-immune and day 465 PI.

Days PI	Serum Dilution	CPE	RT-ELISA raw OD
sheep 3/465 days PI	1:2	neg	0.057
	1:4	neg	0.051
	1:8	neg	0.052
	1:16	neg	0.051
	1:32	neg	0.059
	1:64	neg	0.048
	1:128	pos	0.716
	1:256	pos	2.39
sheep 3/ pre-immune	1:2	neg	0.067
	1:4	pos/neg	0.266
	1:8	pos	0.298
	1:16	pos	1.938
	1:32	pos	1.669
	1:64	pos	2.483
	1:128	pos	2.515
	1:256	pos	2.561
FLchor cells only	N/A	N/A	0.048
OvLV 100 $TCID_{50}$	N/A	pos	1.915
OvLV 10 $TCID_{50}$	N/A	pos	0.76
OvLV 1 $TCID_{50}$	N/A	neg	0.32
OvLV 10^{-1} $TCID_{50}$	N/A	neg	0.059
OvLV 10^{-2} $TCID_{50}$	N/A	neg	0.047

Table X: RT-ELISA vs CPE-read neutralizing antibody titres for sheep 11: 50, 82 and 225 days PI.

Days PI	Serum Dilution	CPE	RT-ELISA raw OD
sheep 11/50 days PI	1:2	neg	0.035
	1:8	neg	0.099
	1:16	neg	0.88
	1:32	pos	1.2
	1:64	pos	1.15
	1:128	pos	1.95
	1:256	pos	1.56
sheep 11/82 days PI	1:2	neg	0.135
	1:16	neg	0.82
	1:32	pos	2.3
	1:64	pos	1.47
	1:128	pos	1.4
	1:256	pos	2.2
sheep 11/225 days PI	1:2	neg	0.04
	1:16	neg	0.41
	1:32	pos	1.07
	1:64	pos	1.17
	1:128	pos	1.16
	1:256	pos	1.32
	1:512	pos	1.28
	1:1024	pos	1.68
	1:2048	pos	3.21

Table XI. RT-ELISA vs CPE-read neutralizing Ab titres for FBS and reference sera.

Serum	Serum dilution	CPE	RT-ELISA raw OD[1]
Fetal Bovine Serum[2]	1:2	neg	0.04
	1:4	neg	0.043
	1:8	pos	1.138
	1:16	pos	2.786
	1:32	pos	2.638
s367 strong + serum	1:2	neg	0.132
	1:4	neg	0.067
	1:8	neg	0.125
	1:16	neg	0.062
	1:32	pos	0.786
	1:64	pos	2.495
	1:128	pos	2.474
	1:256	pos	2.723
s359 neg serum	1:2	neg	0.036
	1:4	neg	0.047
	1:8	neg	0.11
	1:16	pos	1.598
	1:32	pos	1.839
	1:64	pos	1.932
	1:128	pos	2.276
	1:256	pos	N/D

[1]Neg control: PBS only = 0.047
[2]FBS: heat inactivated @ 56 °C for 30 minutes

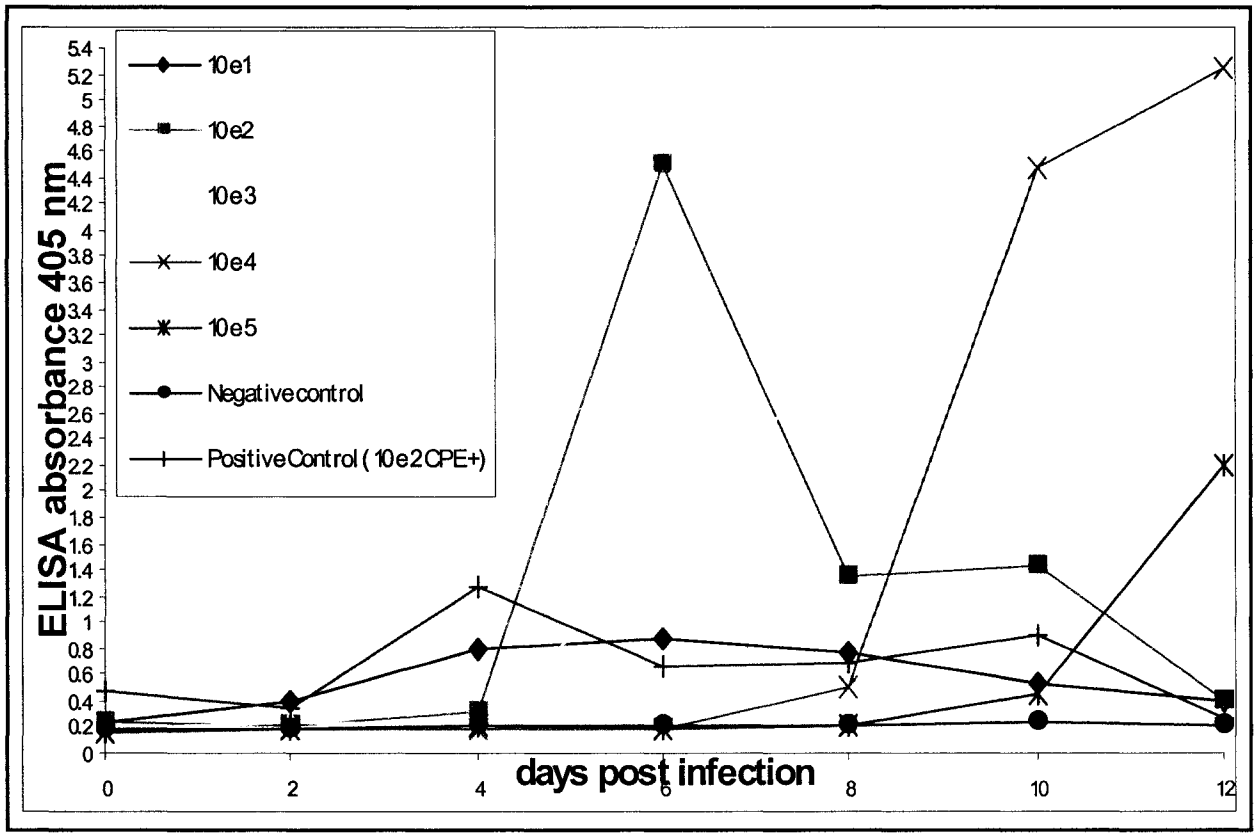

Figure 4. Development of reverse transcriptase activity in FLchor cells infected with ten-fold serial log dilutions of OvLV 130-91, from day 0 PI to day 15 PI.

3.13 Correlation of virus neutralization titres read by RT-ELISA with virus neutralization titres read by CPE.

The correlation of virus neutralization titres as read by RT-ELISA assay with virus neutralizing assay and by cytopathic effect were studied. Selected sera from sheep 11 and sheep 3 were used together with the negative serum (FBS) (Table XIII). The correlation of RT-ELISA vs. *in vitro* CPE, using the virus neutralization assay was also performed on sera exhibiting non-specific inhibition of OvLV 130-91 replication (*i.e.* FBS, s359 negative and s367 positive ovine sera) and two sera were found to have a significant correlation, sheep11 (day 225 PI) and sheep 3 (day 465 PI) . Table XII. summarizes the correlation (r) and p values using Minitab® statistical soft ware.

Table XII. Correlation between virus neutralization read by RT levels and CPE.

Sheep - days PI	r value	p value	CPE NT Ab titre	RT NT Ab titre
11 - 50 PI	0.711	0.072	1:16	1:8
11 - 82 PI	0.589	0.219	1:64	1:2
11- 225 PI	0.914	0.001	1:64	<1:16
3 - pre-immune	0.690	0.058	1:4	<1:4
3 - 465 PI	0.960	0.001	1:256	1:64
FBS	0.855	0.065	1:8	1:4
S367 +++	0.834	0.010	1:16	1:16
S359 ---	0.636	0.090	1:8	1:8

3.14 SDS-PAGE of OvLV 130-91, recombinant CA/GST and TM/GST proteins

Analysis of recombinant CA/GST, TM/GST and GST, by 12.5% resolving SDS-PAGE confirmed expression of recombinant GST fusion proteins, with approximate molecular weights of 58 (CA/GST), 38 (TM/GST) and 26 (GST only) kDa . IPTG-induced LB cultures and resulting expression levels of recombinant CA/GST and TM/GST resulted in overall different levels of recombinant protein, although starting OD of *E. coli* JM-109 clones at expression (i.e. 0.5 L @ OD_{600} ~ 0.8) were almost identical in value. Expression of TM/GST was approximately five-fold over that of CA/GST, as is apparent in the coomasie-stained SDS-PAGE (Figure 5).

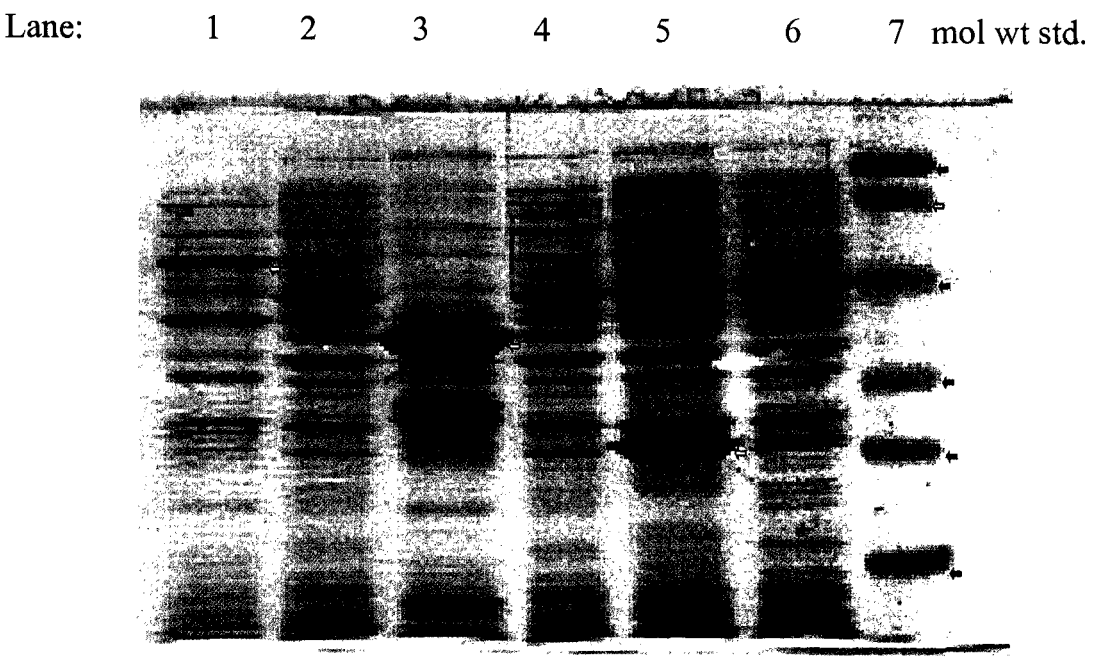

Figure 5. Coomassie-stained SDS-PAGE of pGEX-2T CA/GST and TM/GST clones

Lane 1: induced pGEX 2T/CA-GST (~58 kDa); **Lane 2**: un-induced pGEX 2T/CA-GST; **Lane 3**: induced pGEX 2T/ TM-GST (~ 40 kDa); **Lane 4**: un-induced pGEX 2T/ TM-GST; **Lane 5**: induced pGEX 2T/GST(~26 kDa); **Lane 6** = un-induced pGEX 2T (26 kDa/GST only); **Lane 7**: mol. wt. markers: **107;76; 52; 36.8; 27.2** kDa

3.15 Western blotting of PEG 8000/NaCl precipitated OvLV 130-91, recombinant CA/GST and TM/GST proteins

Immunological detection of recombinant OvLV GST fusion proteins and purified whole OvLV 130-91 utilized positive and negative reference ovine sera and a monoclonal antibody specific to a continous/linearized OvLV/CAEV capsid epitope (VMRD, Pullman WA; clone 10AE1). Utilization of strong positive reference serum (s367) as well as experimentally infected ovine sera from the test panel showed immunological detection of recombinant proteins and major structural proteins from purified OvLV 130-91. Expressed but non-purified recombinant *E. coli* cultures, probed with polyclonal anti-sera, often resulted in non-specific binding of ovine antibodies to bacterial proteins, though binding to CA/GST and TM/GST resulted in stronger staining. Analysis of a Western blot employing mAb 10AE1, specific to CAEV/OvLV capsid epitope demonstrates the specificity of the mAb to recombinant CA (Figure 6).

Tissue culture derived whole OvLV130-91, which had been PEG precipitated and subsequently analysed via Western blot with OvLV positive antisera, revealed the presence of several structural OvLV proteins, with the CA (p24 gag = 24 kDa) being most predominant. Other proteins which appear with less abundance are higher molecular weight proteins of approximately 90-100 kDa (= gp90). Figure 7 shows several PEG precipitated OvLV 130-91 fractions and a mock-infected/PEG precipitated fraction, probed with s367 anti-OvLV sera.

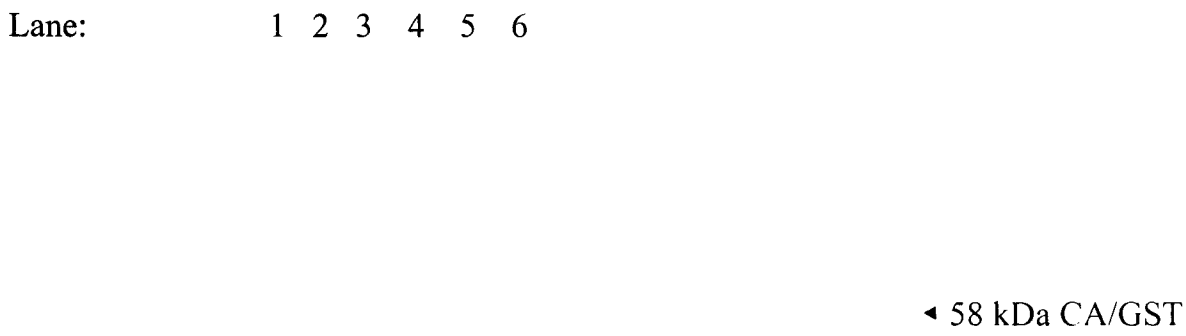

Figure 6. Western Blot Analysis: Affinity purified and semi-purified recombinant OvLV CA/GST preparations probed with anti-capsid mAb 10AE1

Lane 1: Uninduced *E.coli* clone (CA/GST); **Lane 2**: 1st wash fraction - batch column with glutathione resin **Lane 3**: 2nd Wash fraction - batch column with glutathione resin; **Lane 4**: Eluted fraction, CA/GST in 20 mM reduced glutathione ; **Lane 5**: Positive control: recombinant CA/GST ELISA antigen; **Lane 6**: Solubilized CA/GST from inclusion bodies.

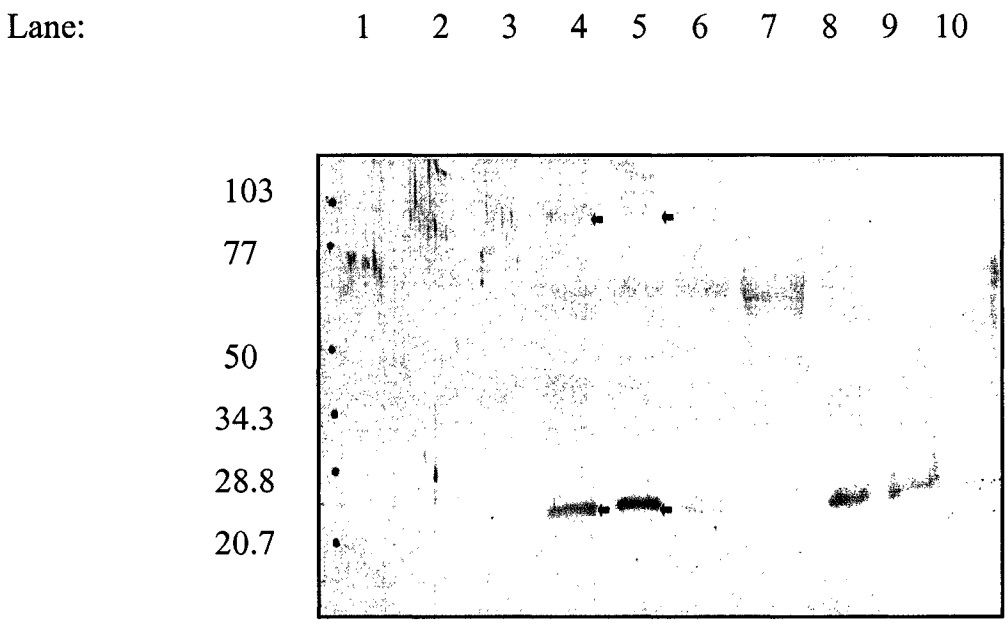

Figure 7. Western Blot of PEG/NaCl precipitated OvLV 130-91 preps.

Lane 1: mol. wt. markers: 103; 77; 50; 34.3; 28.8; 20.7 kDa; **Lane 2**: mock-infected PEG/NaCl ppt.; **Lane 3**: supernatant from PEG ppt.; **Lane 4**: solubilized pellet from PEG/NaCl ppt. OvLV 130-91 infected cells; **Lane 5**: solubilized pellet from PEG/NaCl ppt. OvLV 130-91 infected cells; **Lane 6**: solubilized pellet from PEG/NaCl ppt. OvLV 130-91 infected cells; **Lane 7**: supernatant from PEG/NaCl OvLV 130-91 ppte; **Lane 8**: solubilized pellet from PEG/NaCl ppt. OvLV 130-91 infected cells; **Lane 9**: solubilized pellet from PEG/NaCl ppt. OvLV 130-91 infected cells; **Lane 10**: semi-pure GST.

N.B. arrows in Lane 4 and 5 indicate OvLV 130-91 gp90/gp135 (SU~90/135kDa) and p24 (CA ~24 kda)

3.16 ELISA analysis of antibody response to OvLV 130-91

ELISA employing recombinant TM/GST, CA/GST and purified whole OvLV 130-91 was performed on the experimental sera panel in order to assess total IgG response to OvLV 130-91. Purified whole virus, coated at 4.8, 9.7 and 19.4 μg/ml in PBS, pH 7.4, were utilized in a direct ELISA protocol, in order to determine optimal coating concentration of whole virus, PEG/NaCl precipitated from infected FLchor cells, using PEG 8000/NaCL (Table XIII). Results indicated high background levels at 19.4 μg/ml. Therefore, the next lower dilution was chosen for the analysis. In addition, two mAb specific to the two predominant ovine isotypes (*i.e.* IgG_1 and IgG_2) were also utilized in a indirect ELISA to measure the specific isotype IgG (*i.e.* IgG_1 & IgG_2) response to OvLV 130-91, in sera from sheep 11 and 5.

Table XIII. Determination of whole OvLV 130-91 coating concentration for ELISA

Sheep/ days PI	OvLV@ 4.8 μg/ml	OvLV @9.7 μg/ml	OvLV@ 19.4 μg/ml
sheep 6 pre-immune	0.332	0.434	0.587
sheep 6 day 82 PI	0.561	0.892	1.1014
sheep 6 day 255 PI	0.493	0.848	0.998
sheep 6 day 723 PI	0.551	0.772	0.975
ELISA ++ serum 21[1]	0.658	0.964	1.163
ELISA ++ serum 15[1]	0.437	0.604	0.555
S367 ++ reference	0.49	0.618	0.796
S359 neg reference	0.345	0.464	0.609
PBS only	0.017 - 0.054	0.033 - 0.083	0.017 - 0.037

N.B[1]. OvLV serum 21 and 15 were diagnostic submissions and pre-determined to have a strong positive ELISA OD value for anti-OvLV antibodies.

3.17 Immune response to whole OvLV 130-91 using ELISA

ELISA was performed, using ultracentrifuge purified whole OvLV 130-91, coated at 9.7μg/ml in PBS, pH 7.4 in order to determine Ab response to whole virus. Over the course of approximately 780 days PI, the four sheep displayed varied Ab responses to whole OvLV 130-91. Sheep 6 displayed a strong and early response, which was evidenced between day 20 and 50, followed by a gradual decline until day 782 PI.. In contrast, sheep 5 displayed a strong Ab response to whole OvLV over the course of the sampling period, with a positive ELISA reading initiating between day 50 and 79. The response continued until termination of the sampling (day 796 PI). Sheep 3 displayed a lower Ab response than either sheep 5 or sheep 6. Between days 53 and 185, a positive ELISA reading was observed, and the response remained moderate for the duration of the infection. In contrast, sheep 11 displayed an Ab response to OvLV infection which could be described as weak in nature, as compared with that of the other three sheep (Figure 9). Positive and negative reference sera to a heterogenous strain of OvLV gave an ELISA OD of 1.31 and 0.532 respectively.

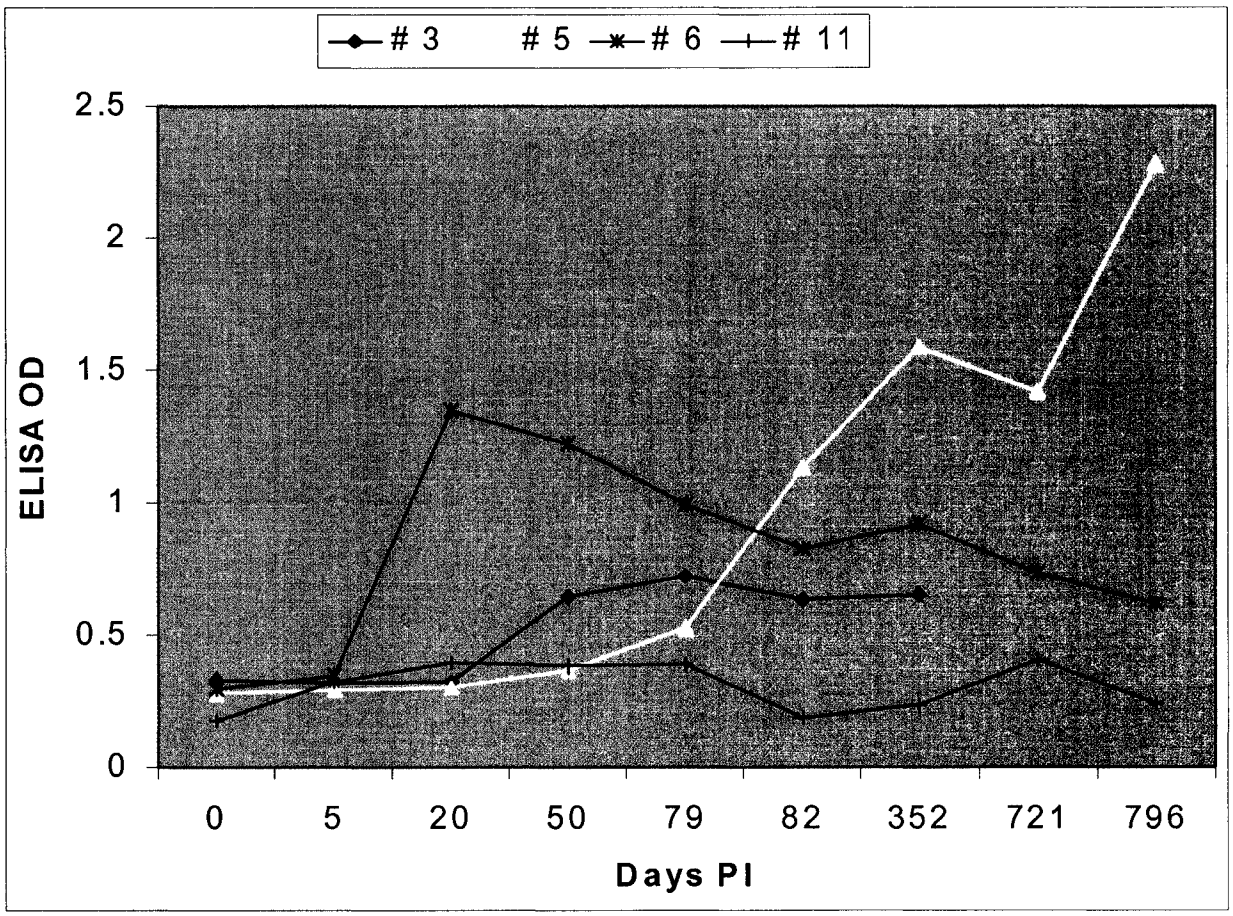

Figure 8. IgG response to whole OvLV-coated ELISA: sheep 3, 5, 6 and 11.

3.18 ELISA analysis of antibody response to CA/GST, TM/GST and GST.

Recombinant CA/GST and TM/GST were used to assess the IgG response in all four sheep, from day 0 to approximately 756 days PI. The sheep displayed a varied IgG response to CA and TM GST fusion proteins, with three of four animals mounting anti-TM/CA responses between day 20 and 50, while the fourth animal (sheep 3) did not show an anti-TM/CA until after 82 days PI (Figure 9). Sheep 5 displayed the strongest IgG response to TM/CA-GST recombinant proteins and maintained high levels of anti-TM/CA antibodies until termination of sampling. Sheep 6 displayed a moderate response, while the two remaining sheep (3 and 11) displayed lower levels of IgG response throughout the sampling period. In this study, antibody responses to CA and TM epitopes, though varied, were sustained for the duration of the sampling period.

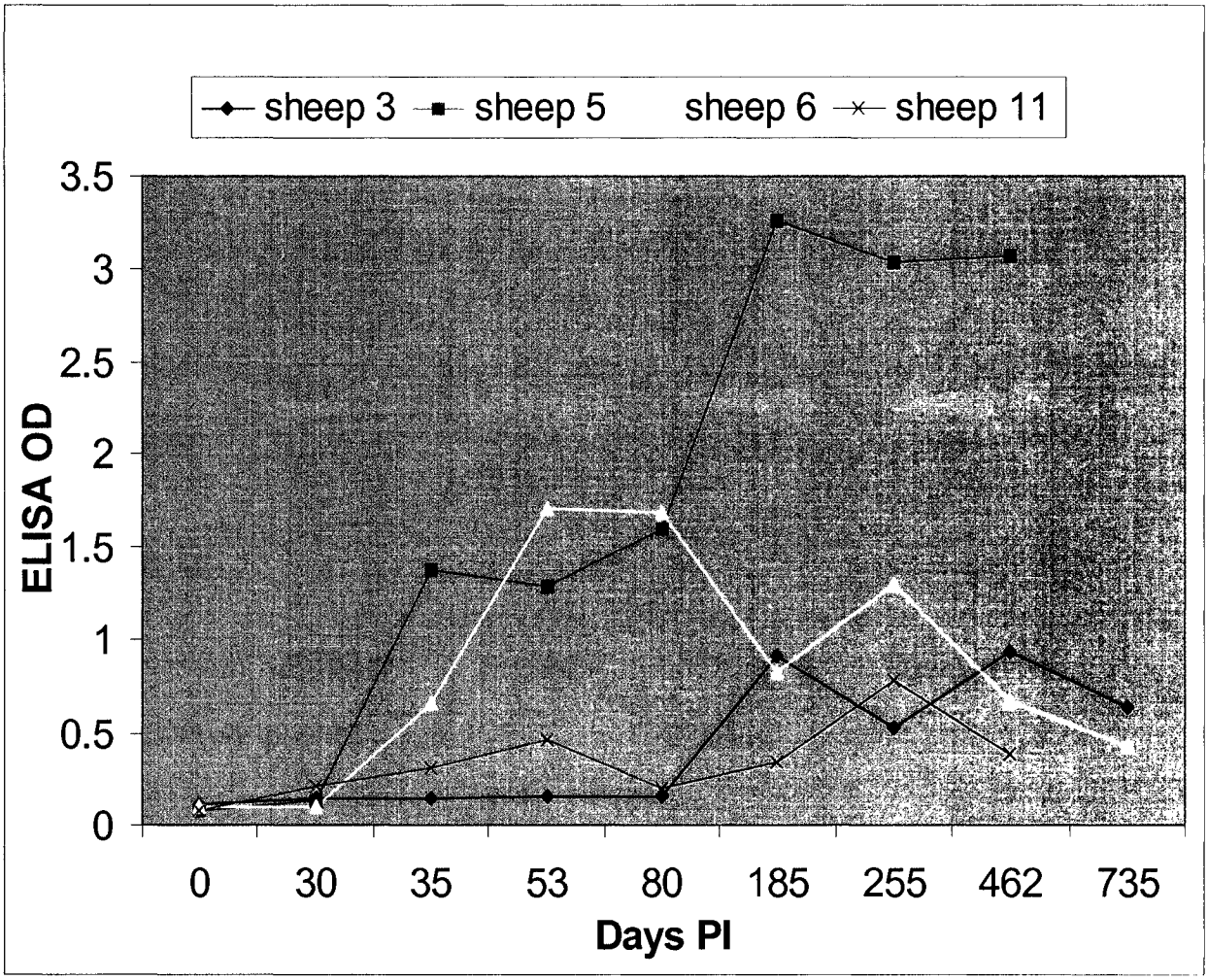

Figure 9. IgG response to CA/GST and TM/GST-ELISA: sheep 3, 5, 6 and 11.

3.19 ELISA analysis of Ovine IgG_1 and IgG_2 isotypes

In order to test the hypothesis that an IgG_1 restricted antibody response occurred in the course of OvLV infection, mAb specific to the two IgG isotypes (IgG_1 & IgG_2) were employed in an indirect ELISA. Sera from sheep 11 and 5 were tested over the course of 454 days for the presence of IgG_1 and IgG_2 isotypes, specific to OvLV. In sheep 11, equivocal values of IgG_1 response to CA/GST appeared between day 20 and 50 PI , and between day 80 and 292 for TM/GST. In sheep 5, the appearance of IgG_1 antibodies also occurred between day 20 and 50 PI for CA/GST and between day 50 and 80 PI for TM/GST (Table XIV).

Using the second mAb, specific to ovine IgG_2, the appearance of either equivocal or positive ELISA values for IgG_2 response to either CA/GST or TM/GST was not detected over the entire range of sampling (454 days PI), except for an equivocal value of 0.061 at day 454, for CA/GST, in sheep 5 (Table XV). Positive and negative reference control sera were used, as well as GST only. In addition, a blind panel of OvLV positive and negative sera was tested using the two mAb as an additional control (data not shown).

Table XIV. ELISA OD for IgG_1 and IgG_2 responses in Sheep 11 to CA & TM/GST

Sheep 11	CA-IgG_1	CA-IgG_2	TM-IgG_1	TM-IgG_2
day 0	.042	.039	.040	.040
20 days PI	.040	.040	.039	.041
50 days PI	*.066*	.041	*.055*	.040
80 days PI	*.075*	.040	*.065*	.039
292 days PI	**.238**	.047	**.221**	.040
454 days PI	**.351**	.041	**.354**	.043
+/- cont sera	**.316**/.039	.041/.040	**.160**/.038	.039/.036
GST control: .035 to .041 range for all of above IgG_1 values and .034 to .046 range for IgG_2 values. Mean = 0.0418 $\pm$ .0056; positive OD = Bold; negative OD = regular; italic = equivocal				

Table XV. ELISA OD for IgG_1 and IgG_2 responses in Sheep 5 to CA & TM/GST

Sheep 5	CA-IgG_1	CA-IgG_2	TM-IgG_1	TM-IgG_2
day 0	.042	.046	.043	.042
20 days PI	.043	.042	.041	.041
50 days PI	**.106**	.041	**.078**	.040
80 days PI	**.126**	.040	**.097**	.041
292 days PI	**.478**	.045	**.451**	.044
454 days PI	**.687**	*.061*	**.683**	*.052*
+/- cont sera	**.623**/.041	.047/.039	**.306**/.039	.039/.036
GST control: .035 to .045 range for all of above IgG_1 values and .035 to .049 range for IgG_2 values positive OD = bold; negative OD = regular; italic = equivocal				

3.20 RT-PCR for detection of OvLV 130-91 cDNA

RT-PCR was employed for confirmation of the presence of OvLV 130-91 cDNA in the FLchor end-point titration using primer pairs from the conserved region of the *pol* gene. The expected amplification product of VisPol 1/VisPol3 was 475 bp and for VisPol2/VisPol3 is 302 bp. PCR amplification using VisPol2/VisPol3 yielded the 302 bp band in the positive control and in serial titrations showing CPE in tissue culture (Figure 10). PCR analysis of serial ten-fold titrations revealed detection of OvLv 130-91 at 2 log dilutions past visible CPE (*i.e.* 10^{-8}). At 10^{-6} log dilution, CPE was evident by day 9, but remained negative at 10^{-8} dilutions, until termination at day 15. However PCR analysis of 10^{-8} log dilution showed a faint positive band of the expected size of 302 bp, after 40 cycles. All samples, including controls were tested using cDNA generated from RNA extracted tissue culture samples, utilizing the SuperScript ® (Gibco-BRL) system for synthesis of cDNA. The negative control, consisting of RNA and subsequent cDNA synthesis, from mock-infected FLchor, did not give a 302 bp product. PCR amplification using VisPol 1 and VisPol 3 (sense/antisense) primers did not yield a 475 bp amplification product (data not shown).

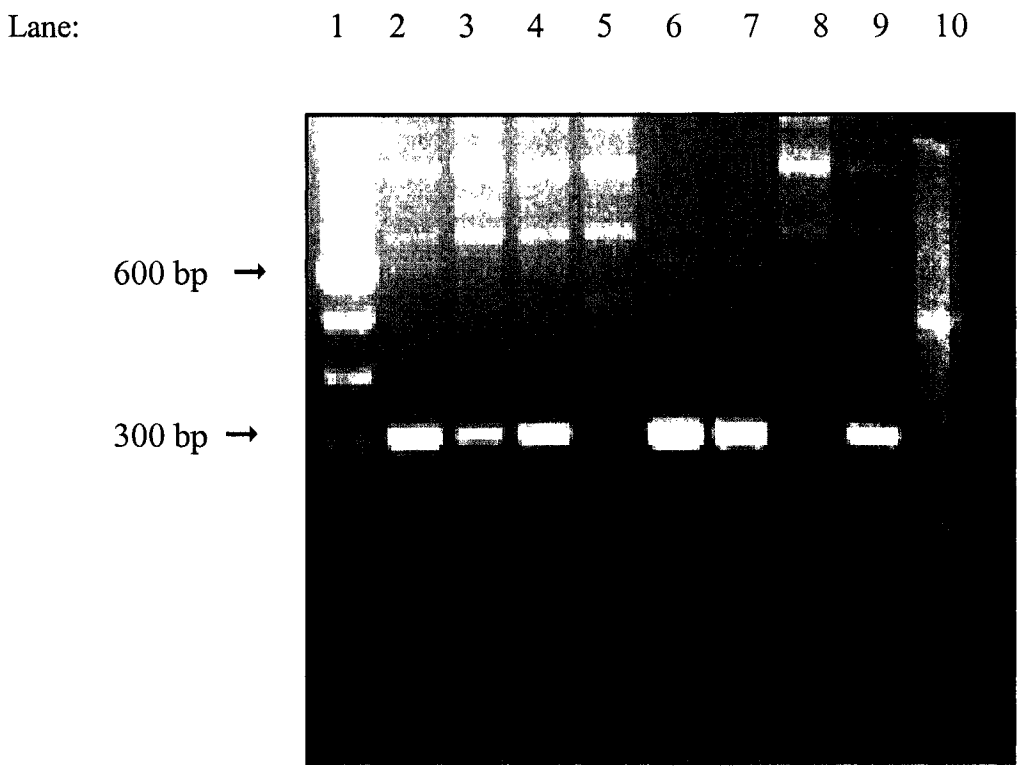

Figure 10. RT-PCR analysis of end-point titration in OvLV-infected FLchor cells.

Lane 1: 100 bp ladder; **Lane 2**: 100 $TCID_{50}$ + control (stock); **Lane 3**: 100 $TCID_{50}$ OvLV 130-91; **Lane 4**: 10^{-1} titration OvLV 130-91; **Lane 5**: Mock Infected FL chor; **Lane 6**: 10^{-3} titration; **Lane 7**: 10^{-4} titration; **Lane 8**: End-point titration (10^{-8}, no CPE); **Lane 9**: PEG precipitated OvLV 130-91; **Lane 10**: 20 bp ladder

4. Discussion

4.1 Recombinant ELISA for detection of OvLV 130-91 antibodies

Recombinant protein expression, utilizing prokaryotic-based hosts (*e.g. E coli*), frequently results in the over expression of recombinant proteins. Mis-folded or aggregated proteins of this nature form inclusion bodies (159, 160). Although inclusion bodies are insoluble, they may be semi-purified using density gradient centrifugation, and then solubilized using detergents (*e.g.* SDS). Alternatively, chaotropic agents (*e.g.* 4-8 M urea or 6 M guanidine hydrochloride) may also be used from the onset to solubilize recombinant proteins. In the present study, recombinant proteins, derived from conserved regions of the capsid and transmembrane regions of the Icelandic 1514 isolate, as previously described (161). These recombinant proteins, expressed from CA/GST and TM/GST clones formed inclusion bodies which were solubilized, as previously described. Solubilized recombinant proteins were re-suspended in PBS buffer, and were subsequently affinity-purified, using the GST fusion 'tag' as a binding site to glutathione resin, and were used in an indirect ELISA..

Current serological diagnosis of OvLV infection frequently utilizes recombinant proteins and/or peptides, derived from important regions of viral proteins. In particular, antibodies directed against the capsid region, which appear 3-6 weeks PI, are considered an important indicator of infection (162). Other major antigenic regions of OvLV also elicit strong antibody responses, including the transmembrane protein (gp41) and to a lesser

extent, the matrix (p16) and nucleocapsid (p14) proteins (163). Accurate diagnosis of OvLV infection can be affected by the selected sampling time post-infection, and by the genetic and antigenic variability of OvLV strains, both of which may result in a particular assay being ineffective in detecting the infection (164). Recently, two distinct serotypes of Italian OvLV have been identified according to differences in three major epitopes in the variable region of the gag gene, encoding the capsid protein (165). While serological diagnosis has been widely utilized, some reports suggest that at least in young animals, serology may not always be indicative of active OvLV infection. For example, one study employed ELISA and *in situ* hybridization aimed at proviral DNA, to diagnose OvLV infection in 20 lambs, 1 year of age or less. ELISA showed only 2 positive animals out of 20, while using *in situ* hybridization showed 14 of them to be positive (166).

In the present study, for the purpose of correlating virus neutralizing activities and total anti-virus antibody response, we used whole virus ELISA, and two recombinant OvLV GST fusion proteins CA and TM to assess IgG response to an Italian strain of OvLV. The CA and TM fusion proteins were derived from a heterologous Icelandic isolate 1514. While originally, it was our intention to use the whole virus ELISA for determination of Ab titres, and the recombinant protein ELISA was intended to be used as controls, the results showed that the recombinant protein ELISA not only correlated well with the whole virus ELISA, but gave results that were easier to interpret. In spite of the use of a purified virus in the whole virus ELISA, this format showed higher non-specific backgrounds than the recombinant protein based ELISA. For this reason, the recombinant protein ELISA was

used for the assessment of antibodies specific to CA and TM regions, and correlation with neutralizing Ab titres. The results showed a varied magnitude of immune response to CA and TM antigen in the four sheep tested. This was in spite of the fact that they were of the same breed, similar age, and that they were all serologically negative prior to experimental infection with the identical dose of OvLV 130-91(167). GST was used as a control in all recombinant protein ELISA assays, and the negative and positive reference sera were also used as controls in order to ensure the analytic specificity of ELISA readings. In order to minimize the chance of false positives, the background levels generated by antibodies binding non-specifically to GST protein were evaluated for all sera tested. A mean ELISA OD = 0.0531 for GST only (SD =0.0014) was subtracted from test sera .

Boshoff and colleagues (168) used a CA/TM-GST fusion construct with favorable results, and DeMartini *et. al.* (169) utilized a similar recombinant protein ELISA in a longitudinal study of both experimentally and naturally infected sheep (n =128). In the hands of these authors, the ELISA assay detected OvLV serum antibodies 5-10 weeks post infection, while AGID did so in 3-5 weeks PI. However, another study reported 23-60% higher seropositive rates for ELISA as compared with AGID (171). In a more recent study, Celer and Celer (172) utilized CA and TM-histidine tagged recombinant proteins for detection of MVV antibodies in 260 infected sheep. By using both antigens, they found higher sensitivity, as compared with whole virus ELISA.

In this work we show that the recombinant viral proteins were capable of assessing the Ab response to an infection with a heterologous OvLV strain, and this is in agreement

with the work of others (172). In sheep 6, antibodies specific to CA and TM appeared between 20 and 50 days PI, in sheep 3 and 5, they appeared after day 50, while in sheep 3 they did not appear until after day 82 PI (see Figure 9, page 76). Saman and colleagues (173), utilizing sera from the same infected sheep as was used in this study, were able to detect antibodies to CA and TM within 2-6 weeks PI.

4.2 Whole virus ELISA for detection of OvLV 130-91 antibodies

The results obtained in whole virus ELISA were compared with ELISA utilizing CA/TM. While the whole virus ELISA resulted in higher background levels -despite the fact that OvLV was purified using standardized density gradient ultra-centrifuge techniques - making the values in some cases equivocal. The results correlated with CA/TM ELISA ($p < .05$). Subsequently, ELISA values acquired from CA/TM were used in the study of correlation with VN Ab activities. The reason for the high backgrounds acquired in whole virus ELISA is not known, and values acquired from non-immune sera were subtracted from all values acquired from immune sera, to correct for high background levels. The whole virus ELISA based on density gradient ultracentrifuged virus may not only measure Ab to intact viral particles, as the virus may undergo significant degradation of complex glycosylated envelope oligomeric structures, resulting in exposure of epitopes not exposed on an intact virus (174, 175). Statistical analysis of correlation of virus neutralizing and antibodies (i.e. CA/TM based ELISA) demonstrated a significant correlation in sheep 3 (r =

0.894, p = 0.003). In the other three sheep (5, 6, 11), there was no correlation (see Table VIII, page 59).

One currently recognized phenomenon that may have contributed to the observed lack of correlation in three of four sheep is the production of quasi-species during viremia (176,177). While the neutralizing antibody levels against the parental virus might not be necessarily affected by the continued antigenic stimulation of quasi-species, the continued exposure to the highly immunogenic conserved capsid epitopes may lead to sustained high levels of Ab measured by ELISA. Another possible explanation for the discordance between the neutralizing and anti-CA/TM Ab levels is the postulated co-existence of neutralizing and neutralization-obstructing Ab idiotypes. The ELISA readings would be expected to correlate with VN activities, if the following unspoken conventional dogmas were true. While it is generally accepted that some viral proteins within a virus may be more immunogenic than others, it is believed that the quantities of the individual Ab idiotypes produced in response to individual virus epitopes, neutralizing and non-neutralizing, are proportionate. As a result, it is generally expected that assessment of virus neutralizing activity in any given serum sample should be in correlation with ELISA Ab readings. In other words, it is implied, that virus neutralization antibodies are merely understood as a subset of total idiotypic Ab repertoir, and as such are expected to be reflective of the overall Ab response. If, on the other hand, as we hypothesized, both VN and VN-obstructing Ab idiotypes are present in immune sera, but in varied proportions, then the VN levels and ELISA Ab levels would not necessarily be expected to correlate. The lack of correlation

between ELISA and VN tests was observed in this study in three out of four animals. While this is in agreement with the hypothesis, it constitutes only an indirect evidence of VN-obstructing antibody. The fact that correlation between ELISA and VN tests was present in one out of four animals could relate to the genetic differences between the animals. Correlation between VN and ELISA antibody titres was previously reported for a number of other viral infections, e.g., bovine viral diarrhea and transmissible enteritis virus(178, 179). However, these viruses belong to Flaviviridae and Coronaviridae, respectively.

4.3 CPE and RT-read virus neutralization and modified neutralization assay.

While the neutralizing antibody response in many viral infections has clearly been demonstrated to play a major role in viral clearance, as well as in protective immunity (180), neutralizing Ab against OvLV are detected in low levels, take long periods of time to develop, and their presence is not accompanied by virus clearance (181). The Icelandic MVV isolates were reported to be better inducers of neutralizing antibodies than the North American isolates of OvLV, in both natural and experimental infections, though the NT-Ab may still not appear for years (182). The North American OvLV is genetically more closely related to CAEV than to MVV (183). There is evidence that antibodies binding to envelope glycoproteins of CAEV cause enhancement of infection in macrophages via attachment of the Ab-virus complex to the Fc receptors (184, 185, 186). In view of the chronic progressive nature of OvLV infection and failure to control viral replication, it is clear that the effectiveness of neutralizing antibodies, as well as other immune mechanisms is insufficient. The failure to accomplish virus clearance may be in part due to the low affinity of binding between the virus and the neutralizing Ab (187, 188). Another and perhaps more important mechanism of evasion of immune clearance is the high rate of point mutations in the genomic regions (e.g. *env*), which encode for neutralizing epitopes (189). These neutralizing variants are, however, reportedly capable of persistence in the face of homologous neutralizing antibodies. Interestingly, it has been reported that even the

parental virus, to which a type-specific Ab response is present, establishes long term persistence (190). This prompted us to speculate that further mechanisms of persistence might exist. Our hypothesis was based on a previous report of Ab-induced conformational change in a virus, and its intra-molecular and inter-molecular spread (191), and on reports of mediation of virus neutralization via conformational change in a viral protein (192, 193). The hypothesis assumes that Ab idiotypes binding to a virus exert conformation modifying forces on the protein. These forces induce conformation of viral proteins leading to neutralization, but they might induce viral conformation compatible with infectivity. Thus, Ab idiotypes favouring overall conformation compatible with infectivity as opposed to neutralization, could be seen as virus neutralization obstructing Ab. According to this hypothesis, it is the final conformation assumed according to the sum of Ab-exerted forces that would determine whether neutralization or infectivity occur. Since the final viral conformation in the presence of the total virus-specific Ab would depend on the balance of the conformational forces, rather than on the total antiviral Ab, lack of correlation between virus neutralizing activity and the total anti-virus Ab (ELISA) would be expected.

Several studies have been undertaken to further define the role of neutralizing antibodies in OvLV infection (194). Type-specific neutralizing antibodies of low titre generally appear within 1-6 months PI, while more broadly neutralizing antibodies may take several years to develop (195). This is sometimes referred to in the context of lentiviruses, as maturation of virus neutralizing antibody response. In the early study with the lytic MVV 1514 strain, neutralizing antibodies took 2 - 4 years to appear, with titres averaging 1:4 to

1:16, but in some instances they never appeared at measurable levels (196). Another study (197) reported that the neutralization conditions required longer incubation periods (24 - 48 hours), and lower temperatures (4° C and/or 25° C). This differs dramatically from conventional conditions of virus neutralization used for the majority of viruses, which use 30-120 min. incubation times at 37° C or at room temperature (198). The unusually slow and inefficient virus neutralization with MVV and OvLV suggest that NT-antibodies may be of low affinity, an observation supported by Kennedy-Stoskopf *et al.* (199), who demonstrated that the rate of binding of the virus to the cellular receptor was greater than that of antibody to virus, thus allowing for virus to spread before it can be neutralized.

Non-lytic or slow/progression OvLV strains typically produce lower levels of neutralizing antibodies, as compared with lytic and more rapidly progressing OvLV strains (200). Non-lytic OvLV strains have been observed to persist in cells of monocyte/macrophage lineage, despite the presence of neutralizing antibodies. Cheevers and colleagues (201) inoculated two sets of monozygotic twin lambs with two distinct OvLV neutralization serotypes. The aim of the study was to evaluate the role of neutralizing antibodies in the selection and persistence of antigenic variants and to relate the severity of lymphoid interstitial pneumonia (LIP) to the development of neutralizing variants. They concluded that macrophage-associated OvLV neutralization variants may arise both in the presence and absence of neutralizing antibodies directed against the parental strain, and that escape variants persist in the presence of homologous neutralizing antibodies. This finding would argue that appearance of neutralization escape mutations are not the predominant

mechanism OvLV persistence. The authors suggested that cell to cell transfer among macrophages may circumvent neutralization by antibodies. The *in vitro* mechanism of OvLV neutralization in macrophages is different to that which occurs in fibroblastoid cells. Antibodies enhance entry of virus into the macrophage by Fc-mediated endocytosis, and neutralization occurs after viral uncoating (201). On the other hand, in fibroblastoid cell cultures used in the present study, antibodies were reported to either block virus receptor binding or to prevent replication after viral internalisation (202, 203).

In the present study neutralizing antibody titres in four experimentally infected sheep were analysed over the course of approximately two years. Conventional neutralization assays with incubation of virus and serum for 36 hours at 25° C and modified neutralization assays utilizing the same conditions but a mixture of two sera, were employed using 100 $TCID_{50}$ of the virus. Only one cell culture passage was made for production of the virus used in the neutralization assays from the virus stock used in animal inoculations. This should have assured minimal amount of antigenic change due to mutations.

Similar to the recombinant CA/TM ELISA, variable responses among sheep were also found in terms of the levels of neutralizing antibody titres and the time of their appearance. Sheep 3 exhibited the highest neutralizing antibody titres, reaching 1:128 ~ 1:256 at 462 days PI, with seroconversion occurring between days 80 and 185 PI. Of interest is the observation that this animal displayed a moderate ELISA response to both whole virus and recombinant protein. The rise in the neutralizing Ab occurred at the time

when IgG response to CA and TM antigen rose from an ELISA OD of 0.158 to 0.913. Sheep 11, which also showed a moderate neutralizing antibody response, had the lowest ELISA values to both whole virus and recombinant antigen. In contrast, sheep 5 and 6, which exhibited lower neutralizing antibody titres, displayed high levels of ELISA IgG response to both whole virus and recombinant antigen. The neutralizing antibody titre of sheep 5 at 352 days PI was only 1:2. In view of the general presence of a non-specific viral inhibitor, with a titre of 1:4 - 1:8 (discussed later), this serum may have in fact been negative. The CA/TM ELISA OD for the same sample was relatively high at 3.257, exceeding even the OD of the pooled strong positive reference sera. Sheep 6, which developed a neutralizing titre of 1:4, like sheep 5, showed early and vigorous IgG response to CA/TM antigen (ELISA OD = 1.708). In summary, the four sheep showed two different trends with respect to neutralizing antibody titres. In the case of sheep 3 and 11, seroconversion occurred within six months PI, and neutralizing titres were variable with maximum titres of 1:128. In contrast, sheep 5 and 6 showed lower levels of neutralizing titres (average of 1:8 - 1:16, maximum = 1:32), and seroconversion did not appear until approximately six months (sheep 6) and two years (sheep 5). The lack of correlation between ELISA and the VN-assay in 3 out of 4 sheep, and in particular, low or non-existent VN Ab activities in the samples containing high levels of ELISA Ab, is in agreement with our hypothesis. Another factor that may have contributed to the observed lack of correlation between anti-CA/TM and neutralizing antibody responses could be found in the highly immunogenic nature of the lentiviral capsid core protein, which comprises approximately 30 % of the total mass of the virion. Anti-CA/TM IgG

responses are often quite high in lentivirus infections, and therefore because of that, are often used for diagnostic purposes.

4.4 Assessment of the use of RT determination for reading of VN assay.

The RT-ELISA assay was used as an aid to make the reading of the VN test less equivocal, in view of the difficulty with reading OvLV minimal CPE. RT-read virus neutralization assay was performed with various sera of OvLV experimentally infected sheep, control reference positive and negative sera, as well as with FBS. Sheep 3 which expressed relatively high VN titres was chosen for this purpose. The RT assay was applied in reading of VN test of the pre-immune serum and day 465 PI serum of sheep 3 (see Table XII, page 67). Further analysis of correlation between RT levels with CPE-read NT-Ab titres was performed on sera from sheep 11 at 50, 82 and 225 days PI (see Table XII, page 67). RT assay was performed for titres ranging from 1:2 to 1:256. The results of the comparison between CPE and RT reading demonstrated overall correlation between the two. However, in some instances the levels of neutralizing antibody titres as determined by CPE were higher than those indicated by RT levels. This indicates that RT determination is slightly more sensitive in detection of the OvLV in cell culture than CPE. Because of the good correlation between CPE and RT reading of the VN test, and because the CPE reading gave slightly higher neutralizing titres, only CPE reading was used for the determination of the VN levels. The correlation of visual CPE readings with RT reading was also performed

in order to increase the confidence in the accuracy of CPE-read neutralizing titres, as the low levels of CPE were sometimes difficult to determine unequivocally.

RT-PCR was employed on end-point titration serial log dilutions, to corroborate CPE with *in vitro* OvLV infection (204). Primers from the *pol* gene were used, and the assay showed a two log-fold increased sensitivity over visual CPE. Specifically, CPE was observed to 10^{-6} serial dilution end-point, but PCR analysis extended this to 10^{-8} serial end-point titration, although no CPE was seen at this dilution. One explanation of the increased sensitivity of the RT-PCR may be due to high titre of non-infectious particles, rather than the presence of free genomes.

4.5 Abrogation of cell culture OvLV infection by non-immune sera.

The presence of an uncharacterised compound, which is able to abrogate viral replication or infection is of note, as evidenced by "neutralizing[1]" titres of 1:4 - 1:8 in FBS, negative reference ovine sera, 1:16 - 1:32 in bovine serum, and of 1:2 - 1:4 in pre-immune serum. The observation of identical titres achieved by both strong ELISA positive and ELISA negative ovine reference sera was unexpected. Although serum specific to a North American OvLV isolate may not neutralize a heterologous Italian OvLV isolate, and this might be an explanation for the low titre of the strong ELISA positive reference serum, the low titre (1:4-1:8) in the negative reference serum is incompatible with the view that these titres were antibody mediated. An un-characterized serum inhibitory compound was reported in an early study, but the authors did not elaborate as to what the specific compound or mechanism of anti-viral activity might be (205). Innate, antiviral compounds found in serum have been reported previously and they include a high density lipoprotein compound and the compound, UT1β (206). They were found effective against a number of DNA viruses, including enveloped and un-enveloped RNA viruses (207), members of the Picornavirus, Paramyxovirus, Alphavirus, Flavivirus, and Rhabdovirus families. Welsh and colleagues (208) reported complement-activated lysis of cells infected by oncoronavirus. However the serum used in this study was heat inactivated, and therefore complement

[1]Virus neutralization is by definition mediated by specific antiviral antibody, therefore, in this case, in the absence of OvLV Ab, we can not speak of true virus neutralization.

activation could not have been involved. Substances displaying anti-viral activity against lentiviruses have been described, including a serum leukocyte protease inhibitor (209), UT1β, a 60 -70 kDa glycoprotein which possesses 40 plaque inhibitory units per ml (210), and a high density lipoprotein compound, apolipoprotein A-1. This compound has been reported to inhibit HIV infection *in vitro,* though the effect was of low activity - approximately 4 plaque inhibitory units per ml (211).

4.6 Interpretation of OvLV neutralization

By general definition, virus neutralization is described as antibody binding to virus, resulting in a loss of infectivity (212). Though this definition is widely accepted, it requires substantial elaboration in the context of this study. In general, virus particles may be neutralized by several mechanisms, including the interference of antibody with the ability of the virus particle to bind to cell receptors (213). Antibodies may also neutralize by preventing secondary or tertiary un-coating events, after virus has bound to a cellular receptor and has undergone endocytosis (214). Various parameters of virus neutralization, such as the nature of the neutralizing epitope, the binding affinity of antibody, as quantified by an association constant, Ka (M^{-1}); the isotype of NT-antibody; the ratio of antibodies to virus, and whether the Fab binding is mono or bivalent, have been studied in various virus groups (215).

The mechanisms of neutralization have been well studied in a number of virus groups. It is agreed that virus neutralization may involve the following: 1) obstruction and/or inhibition of receptor/ligand binding; 2) aggregation of virus by multi-binding antibodies; 3) inhibition at primary or secondary uncoating stages of virus via bivalent binding of antibody or cross-linking of pentamers (216, 217, 218). It has been shown that not all antibodies that bind to virus particles neutralize (219), and this has even been suggested for the closely related HIV. Non-neutralizing antibody attachment to non-neutralizing epitopes in proximity to neutralizing epitopes reportedly inhibited binding of neutralizing antibodies, thus allowing for potential escape of virus from humoral immune response (220). While this report did not elucidate the mechanism by which non-neutralizing Ab inhibited virus neutralization, the observation supports our hypothesis that non-neutralizing Ab may interfere with Nt-Ab activities.

Previous research has shown that the role of neutralizing antibodies in their ability to abrogate in vivo OvLV infection is limited (221).The presence of neutralizing antibodies does not confer protection on the host. Gudnadottir's work with Icelandic Maedi-Visna (222), utilizing experimentally infected lambs, showed that although complement-fixing antibodies developed within 4-6 weeks to average titres of 1:512, the corresponding development of neutralizing antibodies took on average, 2.5 to 4 years, with average titres of only1:8-1:16. In addition, these authors were the first to report that the conventional neutralization used for other viruses did not work, and instead, reproducible assays required 24 - 48 hours of incubation of the virus-serum mixture at 4° C (or 25°C), as opposed to 1-

2 hours at 37° C used for many other virus groups (discussed earlier). The phenomena of low avidity antiviral antibody and slow development of neutralizing antibodies remain poorly understood, as is another proposed mechanism of OvLV persistency, namely the poorly immunogenic nature and complex glycosylated structure of the gp135 envelope (223).

One mechanism, however, which is relatively well studied, is the development of neutralization escape mutants. Antigenic drift, and its role in lentivirus persistency, was originally elucidated in maedi-visna infection several decades ago, and it was recently confirmed (224). During the early phase of acute infection, viral swarming or the emergence of quasi-species ensures that some OvLV isolates may escape neutralizing antibody responses. Stanley and colleagues (225) utilized polyclonal sera and mAb specific to five gp135 epitopes to characterize the role of antigenic drift, caused by point mutations in six characterized escape mutants. They concluded that antigenic variants arise more frequently than may be detected by serological tests, as evidenced by disappearance of parental epitopes on escape mutants, as well as by evidence of poor exposure of epitopes on the parental virus, which were exposed on plaque purified escape variants. The authors also concluded that the extent of phenotypic changes caused by point mutations may also be greater than previously thought and that topographical re-arrangement of the envelope tertiary structure may be magnified by point mutations in the primary amino acid structure.

The main objective of this project was to investigate the possible existence of the virus neutralization obstructing Ab idiotypes. This hypothesis arose from the recognition of

the phenomenon of antibody-induced conformational change in a virus (previously discussed). Although a number of virus evasion mechanisms have been identified for lentiviral infection (discussed above), no explanation has been put forward to explain the delay in the onset of neutralizing antibodies, even while antibodies have developed (*e.g.* anti-CA). The isolation of parental virus months after initial infection, and despite the presence of type-specific antibodies, has never been satisfactorily elucidated (226). One proposed contributing factor may be the rate at which virus is able to bind to macrophage (~ 2 min), as compared with the average rate of antibody to bind with virus NT epitope (~15 - 30 min) (227). We postulated that the antibody idiotype repertoire early in infection, as well as in some later stages of infection, may induce conformational changes in neutralizing epitopes, or prevent induction of certain conformation necessary for virus neutralization, thus effectively obstructing virus neutralization. The relatively few neutralizing epitopes are located within 1 or 2 variable domains (*e.g.* V-4), and are conformational or discontinuous in nature (228). Apart from already discussed indirect evidence of the postulate through constituted by the lack of correlation VN and ELISA antibody levels in 3 sheep, the existence of neutralization obstructing Ab idiotypes was investigated directly by a modified neutralization assay. In this assay, a serum with high titre of non-neutralizing Ab (ELISA) and low titre of neutralizing Ab activity were mixed with an equal volume of a serum exhibiting high neutralizing titres and low non-neutralizing titres. The objective was to see if the sera with low neutralizing antibody but high total antiviral Ab (ELISA) would inhibit the level of neutralization displayed by the other serum. Several pairs

were used; however one drawback was the limited availability of sera with high neutralizing antibody titres. Considering that low neutralizing antibody activity is an inherent feature of small ruminant lentiviral infection, this is not unexpected. Sera from sheep 3, at 185 days PI and 735 days PI were used at 1:1 ratios. The neutralization titre of 1:2 at day 185 PI indicates no neutralization capability, since this activity may have been in fact the activity of the Ab-independent inhibitor, detection of which is discussed elsewhere, while the serum collected 735 PI had the VN titre of 1:128. When combined at 1:1 ratios, the resulting virus neutralizing titre was 1:16, which is four-fold less than expected (1:64), should the 185 days PI serum have no VN obstructing effect. This is considered significant, because in serology, wherever two fold dilutions of sera are used, a fourfold change in titre is considered significant while two fold change can be due to a technical error. Unfortunately, due to low volumes of sera, no other pairs of serum samples fulfilling the selection criteria were available. Although this data must be considered preliminary, the significant four-fold decrease in the neutralization titre of the serum sample collected 735 PI, suggests that VN obstructing activity was present in the serum sample collected at 185 PI.

Several speculations have been put forward to explain why a lack of high neutralizing antibody titres occurs during HIV infection. This is especially relevant in cases where immunization has involved envelope immunogens. Firstly, many anti-envelope Ab are generated to either precursors of gp160 or to shed monomeric gp120, instead of the structural oligomeric envelope moiety, which is composed of a trimer of gp120 (229,

230). The nature of the exposed HIV envelope spike, renders it weakly immunogenic and the complex structure of the envelope acts as a shield, hindering the ability of many potential neutralizing antibodies to bind to conformational epitopes within the variable domain (231). Wyatt and colleagues (232) studied both neutralizing and non-neutralizing antibody responses against HIV gp120 and concluded that the latter are often directed against epitopes on the occluded portion of the envelope trimer (i.e. spike), which are exposed when the trimer is shed. Neutralizing antibodies must access epitopes, which are near the receptor-binding regions and are difficult to access due to heavily glycosylated core and loop structures. The analogous structure and function of the OvLV envelope gp135 to the gp120 of HIV is worth noting. As in the gp120 of HIV, the gp135 envelope in OvLV may shield NT-Ab from accessing epitopes, and combined with the conformational nature of shielded neutralizing epitopes, thus may contribute to the ability of OvLV to evade significant NT-Ab actions (233). This is in addition to other previously described mechanisms of persistency such as NT-Ab escape mutants, proviral latency; tropism for monocytes, macrophage and dendritic cells, and the low binding affinity of NT-epitopes. The question remains, as to whether there are other mechanisms which may contribute to the observed low neutralizing antibody titres in experimental and natural infection. Although it is generally accepted that neutralizing Ab bind to virus envelope, it is less clear which antibodies out of those that bind to the envelope actually neutralize the virus. The question also remains, are there antibodies which bind to envelope, but do not neutralize (234)? The question we tried to answer was whether non-neutralizing antibodies were able

to inhibit neutralization. We observed this phenomenon in at least one case. We speculate that this may have occurred through the binding of the VN-obstructing Ab to envelope epitopes and the ensuing conformational change of a virus protein. This could affect the conformational integrity of the true neutralizing epitopes, and thus prevent the neutralizing idiotypes from binding, or the resulting conformational change may have prevented assumption of the virus conformation necessary for virus neutralization. Although the data presented here is of a preliminary nature, it is suggestive of the role non-NT Ab may play in disrupting the conformational integrity of conformational NT epitopes, and thus contributing to OvLV persistence.

4.7 Restricted IgG_1 immune response in OvLV infection

The mAb, specific to ovine IgG_1 and IgG_2 which were used in the original report (235), were also used in the present study. We performed an indirect ELISA to evaluate if an IgG_1-restricted response occurred. Due to limitations in both the amount of mAb available as well as serum, only a small portion of the OvLV sera panel could be tested (*i.e.* 2 of 4 sheep). A blind panel of control sera, consisting of OvLV infected and non-infected serum was also tested to add further confidence. Sera from sheep 5 and 11 had no detectable levels of IgG_2 to OvLV 130-91 over the course of 454 days PI, and it was concluded that the IgG_2 isotype did not appear in the sera tested. The control panel showed that the lack of IgG_2 to OvLv was not due to a methodological failure. Normally, IgG_2

comprises a significant portion of the ovine neutralizing antibody response in virus neutralization (236). In the study, using whole virus, recombinant CA (p25) and parapox virus ORF, Bird and colleagues corroborated a previous report (237) which suggested a IgG_2-restricted antibody response may be at least partially responsible for OvLV persistence. The data from the present study, using the mAb from the original report, supports the previously published work on a putative mechanism of OvLV persistence (238), through dampening of the IgG_2 isotype. The alteration of the IgG_2 isotype response suggests that persistency in OvLV infection involves a number of mechanisms.

5.0 Summary and Conclusions

We tested the hypothesis that non-neutralizing antibody may exist which may interfere with virus neutralization. Two approaches were taken, the indirect one of correlating VN and ELISA Ab levels, and the direct one of assessing the effect of one serum on the VN activity of another. The sequential sera of four experimentally infected sheep were analysed for correlation between the VN Ab levels and the levels of total antiviral Ab using a recombinant antigen-based ELISA, over approximately 2 years post-infection. We reasoned that if the VN activities in some samples were influenced by the simultaneous presence of VN-obstructing activities, a lack of correlation between the two systems of Ab assessment would be observed. ELISA was performed using the recombinant capsid and transmembrane OvLV proteins. The serum neutralization tests were read by cytopathic effect (CPE), after the suitability of this reading was verified in preliminary experiments by correlation with ELISA-based reverse transcriptase (RT) data. The majority of the experimental animals (3 of 4) showed no correlation between VN Ab activities and the corresponding ELISA Ab titres throughout the observation period. This is consistent with the postulated existence of VN obstructing Ab activity. The hypothesis was further directly investigated by a modified VN assay, where the effect of one serum on the VN activity of another was assessed. Both sera had similar ELISA Ab levels, but they differed in terms of the VN activity. One mixture of such sera exhibited significantly lower VN activity than would be expected due to the dilution effect, thus providing indirect evidence

for the existence of VN-obstructing Ab activity.

According to previous reports, another contributing factor in OvLV latency may be the fact that the Ab response is IgG_1 restricted. Since there were only two reports on this subject, we analysed the two predominant ovine IgG isotypes of anti-OvLV antibody in two animals over the course of 454 days post-infection. The results indicate that the OvLV Ab response, in the two animals tested, was indeed IgG_1 restricted.

Finally, we report the existence of an unidentified inhibitor of OvLV in ELISA negative ovine pre-immune sera, the reference OvLV Ab-negative ovine serum, and fetal bovine serum, at titres up to 1:8. The virus inhibitory activity was seen by both the conventional CPE-read VN test, as well as by the RT-read VN test. The possible existence of an inhibitor had been previously mentioned, but the methodological description was completely lacking. Therefore, it is unclear whether the two inhibitory substances are identical. We believe that further characterization of this inhibitory compound may aid in understanding the pathogenesis OvLV infection.

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