Journal of NeuroVirology (1998) 4, 38–48
© 1998 Journal of NeuroVirology, Inc.
http://www.jneurovirol.com

Neuroinvasion by ovine lentivirus in infected sheep mediated by inflammatory cells associated with experimental allergic encephalomyelitis

Yahia Chebloune[2], Bradley M Karr[1], Ravi Raghavan[1], Dinesh K Singh[3], Kevin Leung[1], Darlene Sheffer[1], David Pinson[1], Larry Foresman[1] and Opendra Narayan[1]

[1]*Marion Merrell Dow Laboratory of Viral Pathogenesis, Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160-7424, USA*

Maedi Visna Virus (MVV) is a prototypic lentivirus that causes infection only in cells of macrophage lineage, unlike the primate lentiviruses which infect both CD4+ T lymphocytes and macrophages. In primates, the earliest viral invasion is associated with the ability of the virus to infect and activate T cells which convey virus to the brain. Infected monocytes in blood rarely cause CNS infection in absence of activation of CD4+ T cells. In the face of lack of infection or activation of T cells by MVV in sheep, the question arises, how does MVV gain access to the brain to cause the classical lesions of visna? In previous studies on experimental induction of visna, sheep were inoculated with virus directly in the brain. In this study, we asked whether neuroinvasion by MVV would occur if sheep were inoculated with virus in a non-neural site. Nine sheep were inoculated intratracheally and all developed systemic infection when examined 3 weeks later. At this time, five were injected intramuscularly with brain white matter homogenized in Freund's complete adjuvant to induce EAE. None of the four animals inoculated with virus alone developed CNS infection despite typical lentiviral infection in lungs, lymphoid tissues and blood-borne mononuclear cells. In contrast, all five of the sheep injected with brain homogenate developed infection in the brain. Virus was produced by macrophages associated with the EAE lesions. This study illustrated that both activated T cells specific for antigen in the CNS and infected macrophages are essential for lentivirus neuropathogenesis.

Keywords: Visna; lentiviral encephalitis; Neuro AIDS; macrophages; EAE;

Introduction

Visna is a wasting paralytic disease that occurred as a complication of progressive dyspnea which broke out among sheep in Iceland during the 1950s (Sigurdsson, 1954; Sigurdsson *et al*, 1957). Histologically, the disease complex was characterized by chronic-active inflammation with mononuclear cells and activated macrophages which, in the CNS, was associated with destruction of myelinated tracts and in the lung, with obliteration of alveoli (Sigurdsson, 1954; Sigurdsson *et al*, 1957; Gudnadottir, 1974). The viral etiology of the disease complex was established by Sigurdsson (1954) and the lentiviral etiology by Lin and Thormar (1972). Virus could always be obtained from cell-free homogenates of affected lung or brain tissues where it was produced almost exclusively by macrophages (Narayan *et al*, 1982; Gorrell *et al*, 1992). Experimentally, maedi was reproduced by intratracheal inoculation of virus but such animals rarely developed neuropathological changes in the brain (Lairmore *et al*, 1987). Visna lesions were reproduced only after intracerebral inoculation of the virus (Sigurdsson *et al*, 1957; Petursson *et al*, 1976; Narayan *et al*, 1984; Kennedy *et al*, 1988; Georgsson *et al*, 1989). The pathogenesis of this neurological disease has never been studied following exposure of sheep to the virus via a natural route.

Neurological disease is common in lentiviral infections of humans and macaques and more than 60% of HIV-1 infected individuals develop neuro-

Correspondence: O Narayan
[2]Visiting scientist from Institut National de la Recherche Agronomique (INRA), Ecole National Veterinaire de LYON. B.P. 83 69280 Marcy l'Etoile, France
[3]Visiting scientist from High Security Animal Disease Laboratory, IVRI, Bhopal, 462021, M.P. India
Received 22 October 1997; accepted 21 November 1997

logical syndromes of varying severity (Price, 1994). The discovery that maedi-visna virus (MVV) is prototypic of the lentiviruses and that both MVV and HIV cause neurological disease in which virus in brain is produced mainly by microglia and perivascular macrophages, reopened the question of the mechanism of neuropathogenesis of visna as a model of HIV disease. Early studies had shown that animals with visna never developed cell-free viremia and that the virus caused infection only in cells of macrophage lineage (Gendelman *et al*, 1985; Gorrell *et al*, 1992). Therefore, infected monocytes or dendritic cells in blood were the only source of virus in the brain. On the other hand, early pathogenesis on HIV infection showed that the virus caused viremia and had tropism for both $CD4^+$ T cells and macrophage-lineage cells (Rosenberg and Fauci, 1989). Therefore, any or all of these three could be the source of virus for the brain. However, infected monocytes have been favored by many as the primary source because of productive replication of the virus in brain macrophages (Gendelman *et al*, 1989; Kure *et al*, 1990). Speculation on the monocytic source of brain virus was even more attractive because the ovine-caprine lentivirus model had shown that latently infected monocytes in blood matured into virus-producing macrophages in culture and probably in tissues (Narayan *et al*, 1982; Gendelman *et al*, 1985).

The SIV-macaque model of HIV-disease proved to be a more accurate analog model of HIV disease than MVV infection in sheep, since plasma viremia and dual tropism of the virus for T cells and macrophages are all part of SIV pathogenesis as it is in HIV pathogenesis (Sharer *et al*, 1988; Chakrabarti *et al*, 1991; Lackner *et al*, 1991; Sharma *et al*, 1992). However, use of molecular clones of SIV that were predominantly lymphocyte-tropic or macrophage-tropic, respectively, produced paradoxical results. SIV_{mac}239 is a lymphocyte-tropic virus that replicates poorly in macrophages (Sharma *et al*, 1992; Anderson *et al*, 1993; Mori *et al*, 1993; Stephens *et al*, 1995). This virus caused activation of infected T cells *in vivo* and these cells disseminated to various tissues including the brain where the virus caused wide spread infection as indicated by PCR on brain DNA. However, it was incapable of replicating in brain because the brain has resident macrophages but not T cells. On the other hand, SIV_{mac}239/17E or SIV_{mac}LG1 which caused a lytic infection in macaque macrophages, similar to MVV infection in sheep macrophages, failed to cause activation of T cells and was poorly neuroinvasive even though the virus was genetically capable of replicating in brain macrophages (Joag *et al*, 1995a; Stephens *et al*, 1997). These experimental data suggested that activated infected T cells may cause the initial seeding of virus in the brain and simultaneously produce chemotactic factors for bringing in infected monocytes later.

Extending our experience with the SIV_{mac} infection in macaques, in the present study we reexamined the question of neuropathogenesis of visna virus from the perspective that the neurological disease is a complication of systemic infection. The results showed that intratracheally-inoculated MVV caused widespread systemic infection characterized at the cellular level by infection in macrophage-lineage cells in the lungs and lymphoid tissues. Animals developed interstitial pneumonia but there was no virus invasion in the brain. However, injection of infected sheep with a homogenate of uninfected brain emulsified in Freund's complete adjuvant, resulted in induction of experimental allergic encephalomyelitis (EAE) with neuroinvasion by activated T cells presumably sensitized to myelin antigen as described by Panitch *et al* (1976). This was accompanied by appearance of macrophages infected with visna virus in the inflammatory perivascular cuffs in the brain.

Results

Nine sheep were inoculated intra-tracheally with MVV strain OvLV 85/34. Four were inoculated with virus alone and the other five were injected intramuscularly with a homogenate of sheep brain white matter emulsified in Freund's complete adjuvant three weeks later. Macrophage cultures from each animal were derived from peripheral blood mononuclear cells (PBMC) at 3 weeks after inoculation and cocultured with GSM cells. Cultures from all nine animals developed syncytial cytopathic effects (CPE) indicating that all nine animals had developed systemic infection with the virus. All of the animals were observed for 3 months following inoculation with virus and examination for development of clinical signs of lentiviral-induced pneumonia and/or neurological disease. Among the five animals inoculated with brain homogenate, only one (sheep 235) developed clinical neurological disease. Approximately 3 weeks after injection with brain material the animal developed ataxia of the hind legs and its condition deteriorated within 24 h to complete paralysis at which point it was euthanized. The other four animals inoculated with brain did not develop any signs of neurological impairment during the 3 month observation period. At necropsy, only the spinal cord from sheep 235 had macroscopically visible lesions. The other animals had no gross signs of neuropathological change.

Productive virus replication in lung of all nine infected sheep and in the CNS of brain-inoculated animals

Following necropsy of each animal in this study, explant macrophage cultures were derived from samples of lung, lymph node, thymus, spleen and

multiple regions of the CNS. GSM cells were added to all of the macrophage cultures. Examination of these cultures 7 days later showed that macrophage cultures derived from lung, spleen, lymph nodes and blood from all nine animals caused CPE. However, there was a sharp disparity in virus yield from CNS macrophages. None of the brain macrophages from the virus (only) animals caused CPE, whereas cultures containing macrophages from brain of all five animals that were injected with brain developed CPE (Table 1). These findings correlated with histological assessment of CNS since none of the animals receiving virus alone had histological abnormalities in the brain and spinal cord. Examination of lung tissue from eight of the nine animals showed multiple focal interstitial and peribronchial accumulations of inflammatory cells comprising monocyte-macrophages and lymphocytes (Figure 3d) and a follicular hyperplasia of tracheo-bronchial lymph nodes. Sheep 235 had severe maedi lesions in the form of areas of consolidation. Thus, these animals had typical signs of interstitial pneumonia. Interestingly, sheep 235 which had the most severe EAE response also had the most severe lesions in lung.

Histologically, the brains and spinal cords from the five infected animals inoculated with brain had multiple lesions of variable severity in the CNS (Table 2). These lesions were consistent with those of EAE and consisted of compact perivascular cuffs of mononuclear inflammatory cells (Panitch *et al*, 1976) (Figure 1a–d, Figure 2a and b) made up of lymphocytes and monocyte-macrophages. In the cerebral hemispheres, white matter involvement was more pronounced than the grey matter, with evidence of focal perivascular myelin pallor (Figure 2c) and axonal damage (Figure 2d) in two of the five animals inoculated with brain homogenate. In sheep 235, which had the most severe EAE reaction among the five animals, the intense inflammatory

Table 1 Recovery of infectious cytopathic virus from explanted macrophage cultures.

	Lungs	*L Nodes*	*Thymus*	*Spleen*	*CNS**
EAE					
Sheep 230	+	+	+	+	+
231	+	+	+	+	+
232	+	+	+	+	+
235	+	+	–	+	+
236	+	+	+	+	+
Non-EAE					
Sheep 227	+	+	+	+	–
228	+	+	+	+	–
233	+	+	+	+	–
234	+	+	+	+	–

Note: Explant cultures were derived from the indicated tissues of the EAE (230, 231, 232, 235, 236) and non-EAE (227, 228, 233, 234) sheep were derived immediately following necropsy. Cultures considered positive for replicating virus showed typical CPE with 10 days after the addition of GSM cells. *Explant cultures were derived from 15 regions of brain and spinal cord (Table 2). Animals were considered positive for CNS virus if any of these regions produced cytopathic virus in culture.

Table 2 Distribution of CNS lesions (EAE) and virological data on sheep inoculated with OvLV-34 and brain homogenate/Freund's adjuvant.

Regions	*Sheep 230*	*Sheep 231*	*Sheep 232*	*Sheep 235*	*Sheep 236*
Pre-frontal	+	+	+	++	+
Mid-frontal	+	++	–	++	+
Parietal	+	++	++	++	+
Temp/Pyriform	+	–	–	++	+
Occipital	–	–	–	++	+++
Deep white	–	–	++	+++	+++
Basal ganglia	–	+	++	+++	+++
Thalamus	–	+	++	+++	+++
Midbrain	–	+++	+++	++	+
Pons	–	–	+++	++	–
Medulla	–	+++	+++	++	+
Cerebellum	–	–	–	++	+
Cerv cord	–	+	++	++	+
Thor cord	–	+	–	++	+
Lumb cord	–	–	++	++	+
Explant Virus	6 of 14	4 of 15	3 of 15	6 of 12	7 of 14
%regions positive	(43%)	(27%)	(21%)	(50%)	(50%)
Severity of EAE*	Mild	Mild	Moderate	Severe	Moderate

–: no perivascular mononuclear cell infiltrates; +: >50% of lesions show Grade 1 intensity i.e. 1–3 layers of perivascular mononuclear cell infiltrates. ++: >50% of lesions show Grade 2 intensity i.e. 4–6 layers of perivascular mononuclear cell infiltrates. +++: >50% of lesions show Grade 3 intensity i.e. >6 layers of perivascular mononuclear cell infiltrates. See methods section for full criteria for the assessment of EAE severity based on topography of disemination and morphological grading. Note: Regions considered positive for replicating virus showed CPE within 10 days after cocultivation with GSM cells.

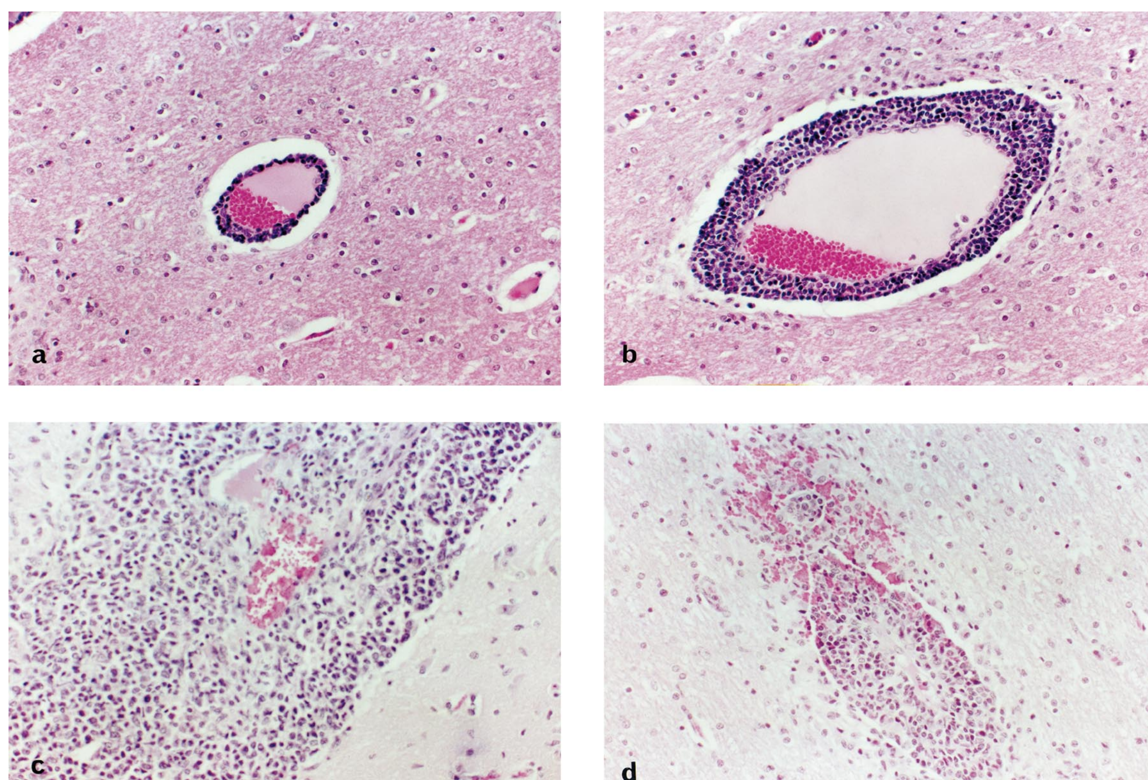

Figure 1 (**a**) Grade 1 EAE lesion showing 1–2 perivascular layers of mononuclear cells in the cerebral white matter of a sheep which was inoculated with brain homogenate (H&E stain. ×266). (**b**) Grade 2 EAE lesion showing a 3–6 perivascular layers of mononuclear cells (H&E stain. ×266). (**c**) Grade 3 EAE lesion showing a dense perivascular infiltrate (>6 layers) of mononuclear cells (H&E stain. ×266). (**d**) Grade 2 EAE lesion in white matter of sheep 235 with focal perivascular extravasation of red cells (H&E stain. ×266).

response was associated with focal perivascular hemorrhage (Figure 1d). Sections from the midbrain, pons and medulla all had pathological changes with varying degrees of severity, except for the cerebellum which showed severe changes only in sheep 235. The lesions in the spinal cord were similar to those of the brain, with the bulk of the pathologic changes being restricted to white matter tracts (not shown). This was clearly observed in sheep 235, in which the intensity and distribution of the spinal cord lesions correlated with the severity of clinical signs terminally.

Localization of virus expression

To determine the cellular localization of virus in lung and CNS tissues, we performed *in situ* hybridization using a cloned PCR product of the OvLV 85/34 *env* gene as a probe and immunocytochemical stains. Viral RNA was identified in a portion of the large cells that had lysozyme (muramidase) within the perivascular cuffs (Figure 3b and c) indicating infection in macrophages. The more severe the inflammatory response, the more were the cells that expressed viral RNA. Most of the inflammatory cells were T lymphocytes (Figure 3a) that lacked (as expected) viral RNA. We did not find evidence of virus expression in any other cell type within the CNS, suggesting that at least at this stage of the experiment the perivascular macrophages were the only cells that had signs of productive infection with the virus.

Correlation between the severity of disease and virus dissemination

To confirm the correlation between the severity of the EAE response and dissemination of the virus in different regions of brain as well as lungs, we performed quantitative PCR on DNA from lung, midbrain and deep white matter tissues of sheep 230, 236 and 235. These animals had different degrees of EAE-induced inflammation (Table 2 and Figure 1a, b and c). Interestingly, sheep 235, which had the most severe EAE response had the highest viral DNA load in the three tissues examined compared to animals 230 and 236, which had mild and moderately severe EAE responses respectively (Figure 4). These results suggested that the severity of the cellular EAE responses correlated with the extent of viral dissemination in both the CNS and lung tissues. Viral DNA was not detected in the brains of the four animals that were inoculated with virus alone.

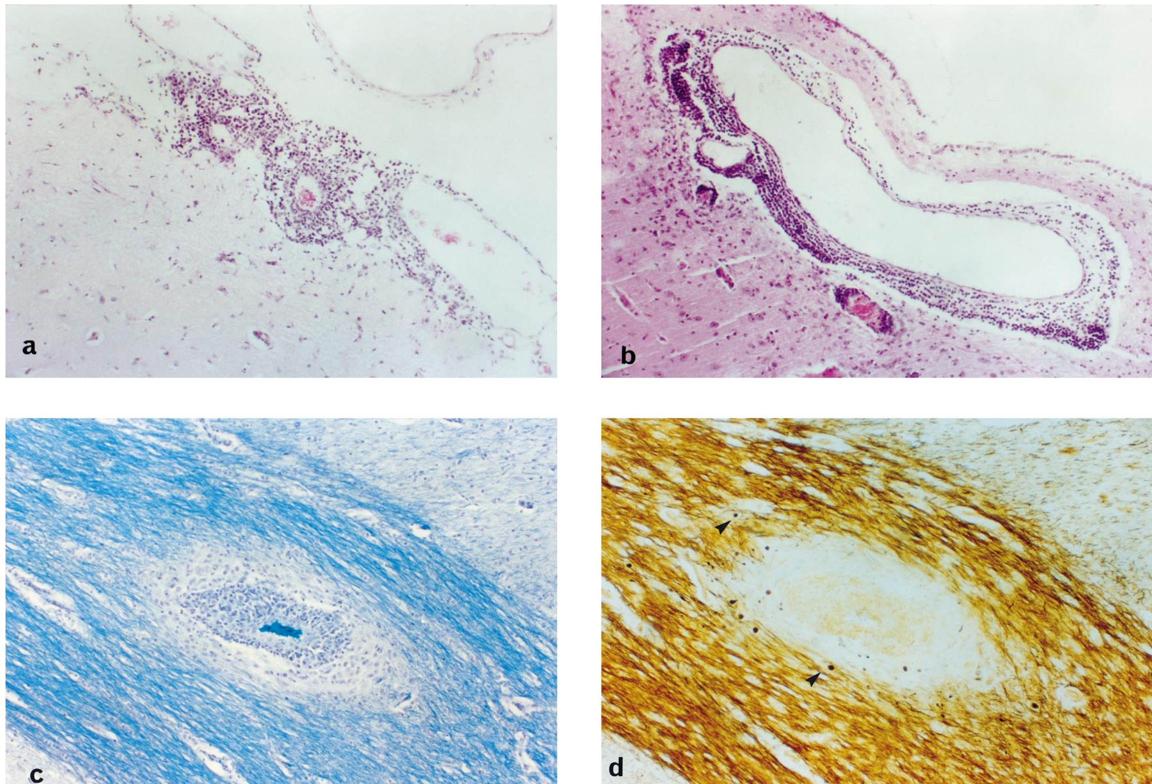

Figure 2 (**a**) Severe disseminated EAE lesions in sheep 235 including inflammatory cell infiltrates in the leptomeninges (H&E. stain × 133). (**b**) EAE lesion in the periventricular region of the brain in the same sheep shown in **a** (H&E. stain × 133). (**c**) Focal myelin pallor in relation to EAE lesion in the deep white matter of sheep 235 (LFB stain. × 133). (**d**) Serial section of the same lesion shown in **c** demonstrating poor staining in peri-lesional regions and few silver-stained axonal spheroids (arrow-heads) indicating focal axonal damage (Sevier-Munger stain. × 133).

Discussion

In this report, we show that intra-tracheal inoculation of sheep with maedi-visna virus resulted in establishment of a typical low-grade infection which, at the cellular level, consisted of restricted expression of the virus in cells of macrophage lineage in lymphoid tissues and blood as reported previously (Gendelman *et al*, 1985; Narayan *et al*, 1983). However, latently infected cells in blood did not cross into the brain, thus sparing the organ from infection. This however was overcome by induction of EAE in the animals. Whereas no neuroinvasion occurred in any of the four infected virus control animals, neuroinvasion occurred in all five of the infected animals injected with brain homogenate. Presumably, specifically sensitized T lymphocytes infiltrating the neuropil provided chemotactic signals that promoted migration of latently infected monocytes and/or dendritic cells into the brain where further signals promoted differentiation and activation of the cells into macrophages in which the virus life cycle now went to completion. These data contrast with SIV and HIV infected macaques and humans, respectively, in whom the lentiviruses themselves cause activation and infection in T cells and thus provide the mechanism for neuroinvasion (Joag *et al*, 1995b). The use of macrophage-tropic ovine lentivirus which does not infect or activate T cells illustrated the obligatory need for brain-bound activated T lymphocytes to provide the signals for promoting the migration of infected monocytes into the brain and the subsequent differentiation of these cells into virus-producing macrophages. Both cell types therefore seem equally important for neuropathogenesis of lentiviruses. These results agreed with our previous findings in the SIV-macaque system in which molecularly-cloned SIV that is macrophage-tropic, similar to MVV, failed to cause activation of T cells and was poorly neuroinvasive (Joag *et al*, 1995a). All previous studies on attempts to induce visna in sheep were performed by intracerebral inoculation of the animals with either infectious brain homogenate or infectious virus. Earlier studies had shown that the encephalitic lesions developing after IC inoculation of MVV were immune-mediated and were similar in histological characteristics to those of EAE (Nathanson *et al*, 1976; Panitch *et al*, 1976). Further, the lesions

were prevented by treatment of the animals with cyclophosphomide, a drug commonly used to prevent clonal expansion of specifically sensitized T lymphocytes. Subsequent studies on pathogenesis of visna showed that neuro-adaptation of visna virus by rapid passage of the virus intracerebrally resulted in a more rapid onset of neurological disease (Petursson *et al*, 1989) but such viruses

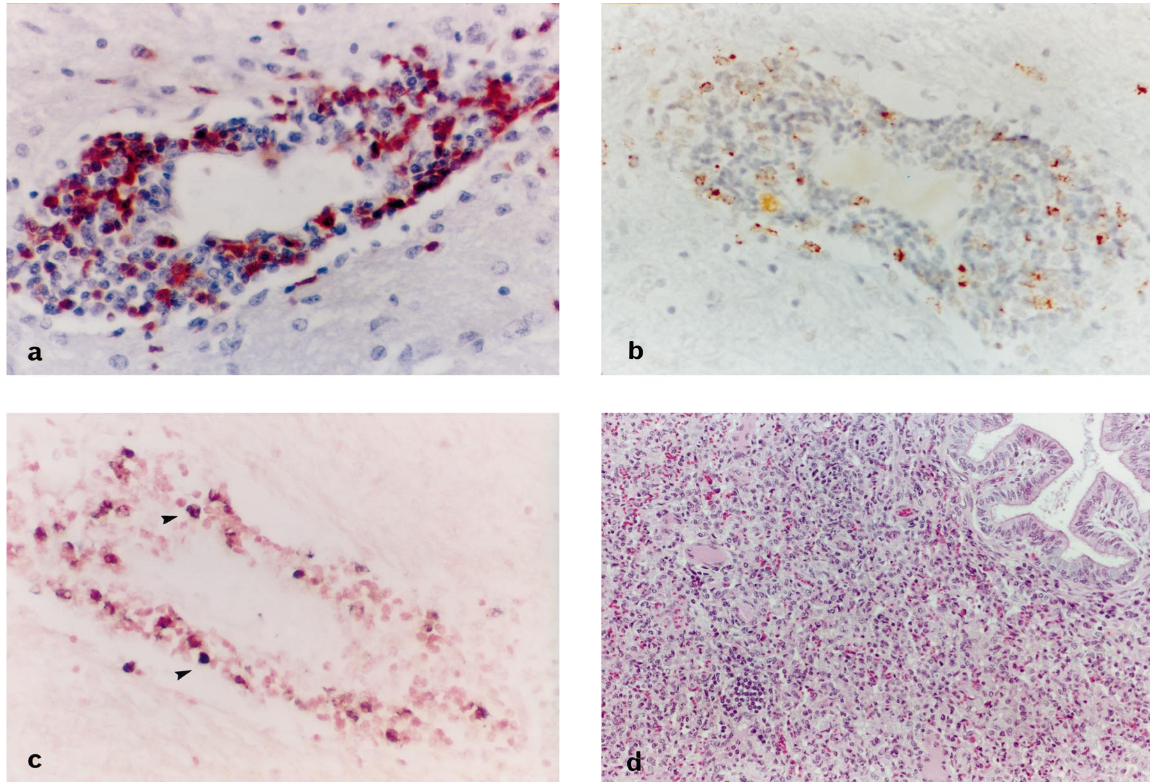

Figure 3 (**a**) Dense infiltrate of $CD3^+$ T lymphocytes in EAE lesion of sheep 236 (immunohistochemical staining with anti-CD3 antibody; color of reaction product: red; nuclei: light purple. ×532). (**b**) Fewer cells of monocyte-macrophage lineage within serial sections of the same infiltrate shown in **a** (immunohistochemical staining with anti-muramidase [lysozyme] antibody; color of reaction product: red; nuclei: light purple. ×532). (**c**) Serial section of the same lesion shown in **a** and **b** demonstrating positive hybridization signals (arrow-heads) for OvLV *env* mRNA. Note that the number of positive cells are roughly proportional to the number of monocyte-macrophages present in the lesion depicted in **b** (*in situ* hybridization using an OvLV 85/34 *env* probe; color of reaction product: blue-black; nuclei: red. ×532). (**d**) Dense interstitial pneumonia in sheep 235 (H&E stain. ×266).

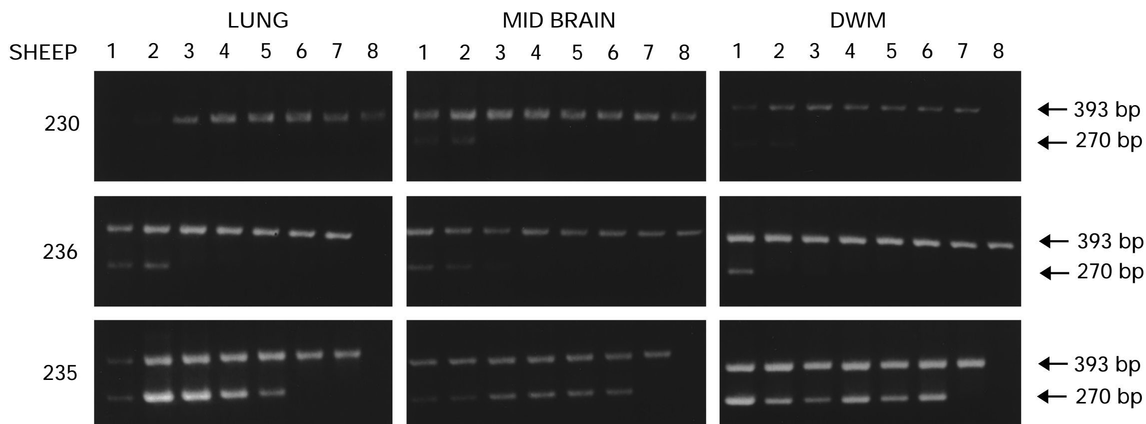

Figure 4 Quantitation of viral DNA by PCR. Total cellular DNA was extracted from lung, mid brain and deep white matter (DWM) tissues of EAE sheep 230, 236, and 235. Serial tenfold dilutions of the total DNA, ranging from 10 μg (lane 1) to 1 pg (lane 8), was prepared and subjected to two rounds of PCR using primers specific to the cellular β-actin gene and the *env* region of OvLV 85/34. The expected sizes of the β-actin and *env* PCR products are 393 bp and 270 bp respectively (Indicated by arrows on the right).

lacked neuroinvasive properties when inoculated peripherally (Zink *et al*, 1997, submitted). Since neuroinvasion is an obligatory step of viral neuropathogenesis, failure of virus to invade the brain from non-neural sites would abrogate all claims for neurovirulence of the agent provided that neurological disease is considered as one of the complications of the systemic infection.

In the intratracheally-inoculated sheep, the failure of the infected cells to cause infection in the CNS of the sheep agrees with the world-wide epizootiology of maedi-visna which is characterized predominantly by pneumonia and mastitis and only rarely by encephalopathy (Narayan and Cork, 1985). In retrospect, the classic CNS disease observed in natural outbreaks of maedi-visna in Iceland could have been initiated by T cells that were induced by other neuropathogens. In our studies reported here, EAE provided the surrogate inducer of the activated T cells.

It is well established that cell mediated immunity plays a significant role in the induction of EAE. In normal healthy mammals, T cells are rarely found in the CNS and expression of MHC class II by microglia in the brain is expressed at very low levels (Barker and Billingham, 1977; Hickey and Kimura, 1987). In contrast, it is also well known that during immune-mediated illnesses such as multiple sclerosis, post-infectious incephalomyelitis, viral encephalitis and EAE, lymphocytes and MHC II-expressing macrophages are found commonly in the CNS (Paterson *et al*, 1981; Hickey *et al*, 1985; Lassmann, 1991). Whereas resting T cells rarely enter the brain, activated T cells are neuroinvasive and recent studies have shown that such activated cells enter brain regardless of their specificity for antigen, their phenotype, or their recognition of antigen being expressed by activated macrophages in the brain (Hickey *et al*, 1991). Activated lymphocytes which enter the CNS from blood exit within 1 to 2 days, but when they locate their antigen, they persist at the site and produce lymphokines and chemokines that attract and activate other mononuclear cells in the blood vessels to extravasate into the neuropil. Monocytes are one such cell type that, responding to such signals, migrate and mature on site into macrophages. Thus, both activated T cells and activated macrophages are important for development of encephalitis. An intriguing observation from this study was that the severity of EAE-induced immune responses in the brain correlated not only with increase in virus load in the brain, but also that severe EAE/visna correlated with severe interstitial pneumonia and a corresponding increase in virus burden in the lungs. A possible mechanism for this phenomenon involves the non-specific principle of dissemination of activated T cells throughout the body. In the lungs containing infected alveolar macrophages, this presence of such activated T cells with their chemokine-producing capacity could have provided the signals to enhance the inflammatory process. Such autocrine and paracrine signals could have caused amplification and acceleration of onset of lesions with concurrent increase in virus production by increasing numbers of invading susceptible macrophages, the outcome of which was more severe pneumonia in the virus/EAE animals.

Materials and methods

Virus

The Maedi Visna virus (MVV) strain OvLV 85/34 used in this study was originally isolated from a sheep that had lymphoid interstitial pneumonia (LIP) (Lairmore *et al*, 1987) and was kindly provided by Dr Jim DeMartini, Colorado State University. This virus was plaque purified and expanded in goat synovial membrane (GSM)-derived cell cultures. Virus stock had an infectivity titre of 10^6 $TCID_{50}$/ml.

Animals

One year old Dorset lambs were purchased from local flocks in the Kansas City area. Four criteria were used to ensure that these sheep were free of lentiviral infection. First, blood monocyte-derived macrophages matured *in vitro* were co-cultivated with indicator GSM cells and observed for the development of typical lentivirus-induced syncytial cytopathic effects (CPE) (Narayan *et al*, 1980; Chebloune *et al*, 1996b). Second, culture medium and cell lysates from parallel macrophage cultures were analyzed for the presence of viral proteins by a radio-immunoprecipitation assay (RIA) using a goat hyper-immune serum against MVV and caprine arthritis-encephalitis virus (CAEV) (Chebloune *et al*, 1996a). Third, serum from each animal was examined for the presence of antibodies to MVV and/or CAEV using a similar RIA. Fourth, DNA extracted from the cultured macrophages was subjected to PCR using oligonucleotide primers specific to sequences in the CAEV *gag* gene (Chebloune *et al*, 1996a). All of the animals used in this study were negative by the above criteria and were thus considered free of endogenous lentiviral infection.

Inoculation of animals with OvLV-85/34 and induction of EAE

One ml of stock virus containing 1×10^6 $TCID_{50}$ was diluted in 5 ml RPMI and inoculated intra-tracheally. Three weeks later, five of the sheep were each injected intramuscularly with a 2 ml suspension containing 1 ml of a 10% homogenate (wt/vol) of normal sheep brain white matter emulsified in 1 ml of complete Freund's adjuvant (CFA) (Difco Laboratories).

Derivation of macrophages from peripheral blood mononuclear cells and tissue explants
Blood collected in EDTA from each animal was sedimented to remove plasma (saved for antibody detection) and the buffy coat cells suspended in Hanks balanced salt solution (HBSS) were centrifuged on Ficoll-Hypaque gradients to obtain mononuclear cells (PBMC) as previously described (Narayan *et al*, 1983). Approximately 10^8 PBMC were resuspended in 30 ml of macrophage differentiation medium (MDM) consisting of RPMI supplemented with 10 mM Hepes buffer (pH 7.3), 50 μg/ml gentamicin, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine and 20% heated lamb serum, and cultivated in Teflon bottles at 37°C for 2 weeks with a complete medium change at the end of the first week. This method yielded suspensions of macrophages which when seeded into tissue culture dishes, became adherent and differentiated into typical esterase-positive cells, fully capable of Fc receptor-mediated endocytosis of antibody-coated RBC at 37°C within 4 h. Mature macrophages were transferred from Teflon bottles to tissue culture dishes and maintained in MDM for another 2 weeks. Samples of lung, lymph node, thymus, spleen, and CNS regions were aseptically removed from animals and placed in ice-cold HBSS. Portions of the tissues were subsequently minced, suspended in MDM and explanted in 2 ml of MDM in 25 cm^2 tissue culture flasks. After 5–7 days at 37°C, adherent macrophages were washed and incubated an additional 10-days in fresh MDM. The macrophage cultures derived by explantation were then analyzed for presence of virus as described below.

Two dishes of PBMC-derived macrophages or two flasks of tissue explant-derived macrophages were maintained and examined for degenerative changes (cytolysis) and/or syncytial CPE after cocultivation with indicator GSM cells as previously described (Chebloune *et al*, 1996a, b). Some of the macrophage cultures were also used for detection of viral proteins by immunoprecipitation and for detection of viral DNA by PCR as described below. Selected tissue samples were homogenized and examined for viral DNA or infectious virus.

Indicator cell line
Derivation of goat synovial membrane (GSM) cell culture from explanted carpal synovial membrane of colostrum-deprived, newborn goats has been described previously (Narayan *et al*, 1980). These cells were expanded by cultivation in Minimum Essential Medium+10% fetal bovine serum, passaged at 1 : 3 split ratios and used for 7–10 passages.

Necropsy
Sheep were anesthetized by administration of Rompum at a concentration of 0.1 mg/kg followed by laparotomy and exsanguination via the abdominal aorta. Portions of the apical, middle and diaphragmatic lobes of the lungs, spleen, mesenteric and dorsal tracheal lymph nodes, and brain and spinal cord were removed aseptically for virological and histopathological analyses.

Brain dissection and neuropathological methods
The brain was bisected and the right half was transferred into 10% buffered formalin. The left half of brain and spinal cord were sliced and dissected into 15 anatomically distinct regions as indicated in Table 2. Samples intended for virus or viral DNA assays were free of contamination with meninges or choroid plexus. Portions of all samples were snap-frozen over dry ice and saved at −70°C for PCR analysis of viral DNA. Other portions were explanted into culture to obtain macrophages and others homogenized to yield 10% cell-free extracts (wt/vol) in MEM+1% serum. The remaining portions of the left half of the brain and spinal cord were fixed in 10% buffered formalin for 3–4 days. Blocks taken from areas adjacent to the previously sampled parts of the fixed left half of the brain, and from identical parts of the right half were embedded in molten paraffin wax and processed according to established procedures. A preliminary screening for morphological abnormalities was performed on paraffin sections stained with hematoxylin and eosin (H&E). Serial sections were stained with Luxol Fast Blue (LFB) and Sevier Munger stains respectively, for the assessment of suspected myelin and axonal abnormalities in relevant parts of the central nervous system. The remaining sections were set aside for immunostains and *in situ* hybridization.

All visceral tissues examined were fixed in 10% formalin and blocks from selected areas were embedded in paraffin. These were processed for routine histological examination and sections stained with H&E.

Evaluation of histopathological changes in the CNS
We graded the EAE lesions semi-quantitatively on a 4-point scale (0–3) based on the number of layers of mononuclear inflammatory cells in the perivascular compartment of each lesion (Traugott, 1989). Absence of a cellular infiltrate was graded as 0; lesions with one to three layers were scored as grade 1; four to six layers were scored as grade 2; and the presence of more than four layers of perivascular mononuclear cells was scored as grade 3. Hematoxylin and eosin stained sections from each CNS region were examined under a 10× objective lens, and the lesions were scored within 40 contiguous microscopic fields in the case of larger CNS sections (i.e. cerebral hemispheres), and within 20 microscopic fields in the case of smaller sections (i.e. brain stem and spinal cord). Presence of grade 1 lesions in over 50% of the area examined in each section would be given a composite score of (+) for that particular CNS

region; presence of grade 2 lesions in over 50% of the area an overall score of (++); and the presence of grade 3 lesions in over 50% of the area, a score of (+++). This data from each CNS region was analyzed in conjunction with the extent of dissemination of lesions within the fifteen CNS regions examined, and the overall severity of the EAE reaction in each animal was assessed as: Mild- $<$50% of the CNS regions were affected and fewer than 50% of the regions involved had a composite score of (+); Moderate-$>$50% of the CNS regions were affected and the majority of regions involved had composite scores of ++ or +++; Severe-all CNS regions were affected and all received composite scores of ++ or +++.

Immunohistochemistry
Paraffin sections were reacted overnight with primary antibodies including, rabbit-anti-human-CD3 (Dako, 1:50 dilution) used to stain T-cells, rabbit-anti-muramidase (Dako, 1:750 dilution) to stain macrophages, and rabbit anti-GFAP (Dako, 1:50 dilution) for staining astrocytes. After repeated washing, the sections were incubated with biotinylated goat anti-rabbit antibody [Vector, 1:200 dilution]. The reaction product was detected using avidin-biotin-peroxidase enzyme complex [Vector] followed by the substrate AEC [Biomeda].

In situ *hybridization*
Paraffin sections were digested with pepsin [Boehringer], and hybridized overnight with a digoxigenin labelled OvLV-*env* DNA probe. The probe was prepared from a 1.6 kb PCR fragment isolated from the *env* gene of OvLV 85/34 (Joag *et al*, 1995b). After post-hybridization washes, the hybridization product was detected by treating the sections with alkaline phosphatase conjugated anti-digoxigenin fragments [Boehringer], followed by incubation with NBT/BCIP alkaline phosphatase substrate [Pierce] to obtain a blue-black product.

DNA extraction and quantitative PCR
Minced frozen tissues were homogenized into a lysis buffer containing 50 mM Tris, 5 mM EDTA and 7 μM SDS, and proteins were digested following incubation with 100 μg/ml of proteinase K. The lysates were then phenol/chloroform extracted and DNA ethanol precipitated using standard methods. Purified DNA was resuspended in sterile distilled water and the concentration was determined by spectrophotometry. Two sets of oligonucleotide primers specific for the *env* gene of OvLV 85/34 were used detection of viral DNA. Primers 5′34B 914-CAATTGTACTAGGTCAGGCAATCACTT-940 and 3′34A 1511-ATTAGCAACACCGAGTCCAGCT-CCT-1487 were used for first round PCR, and primers 5′D34 1158-CTATGGACATACTCGGCCC-GAAGAA-1182 and 3′34C 1419-GCTGTAGCTCGG-TGGATCTCTTCTTC-1394 were used for nested PCR as previously described (Chebloune *et al*, 1996a; Karr *et al*, 1996).

To quantitate the amount of viral DNA relative to the total cellular DNA, tenfold serial dilutions of each sample ranging from 10 μg to 1 pg DNA were subjected to PCR using the viral *env* primers and primers directed to the cellular β-actin gene (Stephens *et al*, 1995; Joag *et al*, 1995a). PCR reactions were performed as previously described (Chebloune *et al*, 1996a; Karr *et al*, 1996). Following the second round of amplification, 10 μl aliquots of each reaction were electrophoresed on 1.5% agarose gels. Specific PCR products were visualized by staining with ethidium bromide.

Acknowledgements

This work was supported by Public Health Service Grant NS-12127 from the National Institutes of Health. We are grateful to Dr James C DeMartini of Colorado State University for generously providing ovine lentivirus strain 85/34. We thank the Phillippe Foundation and Institut National de la Recherche Agronomique (INRA) for providing partial financial support of Dr Yahia Chebloune. We also thank the Indian Veterinary Research Institute (IVRI) for partial support of Dr DK Singh.

References

Anderson MG, Hauer D, Sharma DP, Joag SV, Narayan O, Zink MC, Clements JE (1993). Analysis of envelope changes acquired by SIV_{mac}239 during neuroadaptation in rhesus macaques. *Virology* **195:** 616–626.
Barker CF, Billingham RE (1977). Immunologically privileged sites. *Adv Immunol* **25:** 1–54.
Chakrabarti L, Hurtrel M, Maire MA, Vazeux R, Dormont D, Montagnier L, Hurtrel B (1991). Early viral replication in the brain of SIV-infected rhesus monkeys. *Am J Pathol* **139:** 1273–1280.
Chebloune Y, Karr B, Sheffer D, Leung K, Narayan O (1996a). Variations in lentiviral gene expression in monocyte-derived macrophages from infected farm sheep. *J Gen Virol* **77:** 2037–2051.
Chebloune Y, Sheffer D, Karr B, Stephens EB, Narayan O (1996b). Restrictive type of replication of ovine/caprine lentiviruses in ovine fibroblast cell cultures. *Virology* **222:** 21–30.

Gendelman HE, Narayan O, Molineaux S, Clements JE, Ghotbi Z (1985). Slow, persistent replication of lentiviruses: role of tissue macrophages and macrophage precursors in bone marrow. *Proc Natl Acad Sci USA* **82:** 7086–7090.

Gendelman HE, Orenstein JM, Baca LM, Weiser B, Burger H, Kalter DC, Meltzer MS (1989). The macrophage in the persistence and pathogenesis of HIV infection. *AIDS* **3:** 475–495.

Georgsson G, Houwers DJ, Palsson P, Petursson G (1989). Expression of viral antigens in the central nervous system of visna-infected sheep: an immunohistochemical study on experimental visna induced by virus strains of increased neurovirulence. *Acta Neuropathol* **77:** 299–306.

Gorrell MD, Brandon MR, Sheffer D, Adams RJ, Narayan O (1992). Ovine lentivirus is macrophagetropic and does not replicate productively in T lymphocytes. *J Virol* **66:** 2679–2688.

Gudnadottir M (1974). Visna-Maedi in Sheep. *Prog Med Virol* **18:** 336–349.

Hickey WF, Osborn JP, Kirby WM (1985). Expression of Ia molecules by astrocytes during acute experimental allergic encephalomyelitis in the Lewis rat. *Cell Immunol* **91:** 528–535.

Hickey WF, Hsu BL, Kimura H (1991). T-Lymphocyte entry into the Central Nervous System. *J Neurosci Res* **28:** 254–260.

Hickey WF, Kimura H (1987). Graft-vs.-host disease elicits expression of class I and class II histocompatibility antigens and the presence of scattered T lymphocytes in rat central nervous system. *Proc Natl Acad Sci USA* **84:** 2082–2086.

Joag SV, Stephens EB, Galbreath D, Zhu GW, Li Z, Foresman L, Zhao LJ, Pinson DM, Narayan O (1995a). Simian immunodeficiency virus SIVmac chimeric virus whose env gene was derived from SIV-encephalitic brain is macrophage-tropic but not neurovirulent. *J Virol* **69:** 1367–1369.

Joag SV, Stephens EB, Narayan O (1995b). *Virology* (Fields BN, Knipe DM and Howley PM (eds). 3rd edition. Lippincott-Raven Publishers, Philadelphia. pp 1977–1996.

Karr B, Chebloune Y, Leung K (1996). Genetic characterization of two phenotypically distinct North American ovine lentiviruses and their possible origin from caprine arthritis-encephalitis virus. *Virology* **225:** 1–10.

Kennedy PGE, Narayan O, Zink MC, Hess J, Clements JE, Adams RJ (1988). *Clinical and Molecular aspects of Viral illness of the Nervous System* (Gilden DH and Lipton H (eds)). Martinus Nijhoff, Boston. pp 394–421.

Kure K, Lyman WD, Weidenheim KM, Dickson DW (1990). Cellular localization of an HIV-1 antigen in subacute AIDS encephalitis using an improved double-labeling immunohistochemical method. *Am J Pathol* **136:** 1085–1092.

Lackner AA, Smith MO, Munn RJ, Martfeld DJ, Gardner MB, Marx PA, Dandekar S (1991). Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *Am J Pathol* **139:** 609–621.

Lairmore MD, Akita GY, Russell HI, DeMartini JC (1987). Replication and cytopathic effects of ovine lentivirus strains in alveolar macrophages correlate with *in vivo* pathogenicity. *J Virol* **61:** 4038–4042.

Lassmann H (1991). Inflammation and the blood-brain barrier. *Brain Pathol* **1:** 88.

Lin FH, Thormar H (1972). Properties of maedi nucleic acid and the presence of ribonucleic acid- and deoxyribonucleic acid-dependent deoxyribonucleic acid polymerase in the virions. *J Virol* **10:** 228–233.

Mori K, Ringler DJ, Desrosiers RC (1993). Restricted replication of simian immunodeficiency virus strain 239 in macrophages is determined by env but is not due to restricted entry. *J Virol* **67:** 2807–2814.

Narayan O, Clements JE, Strandberg JD, Cork LC, Griffin DE (1980). Biological characterization of the virus causing leukoencephalitis and arthritis in goats. *J Gen Virol* **50:** 69–79.

Narayan O, Wolinsky JS, Clements JE, Strandberg JD, Griffin DE, Cork LC (1982). Slow virus replication: the role of macrophages in the persistence and expression of visna viruses of sheep and goats. *J Gen Virol* **59:** 345–356.

Narayan O, Kennedy-Stoskopf S, Sheffer D, Griffin DE, Clements JE (1983). Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. *Infect Immun* **41:** 67–73.

Narayan O, Strandberg JD, Griffin DE, Clements JE, Adams RJ (1984). *Symposium on viruses and demyelinating diseases* (Mimms CA, Cuzner ML and Kelly RE (eds). Academic Press, New York. pp 125–140.

Narayan O, Cork LC (1985). Lentiviral diseases of sheep and goats: Chronic pneumonia leukoencephalomyelitis and arthritis. *Rev Infect Dis* **7:** 89–98.

Nathanson N, Panitch H, Palsson PA, Peturrson G, Georgsson G (1976). Pathogenesis of visna II. Effect of immunosuppression upon the central nervous system lesions. *Lab Invest* **35:** 444–451.

Panitch H, Petursson G, Georgsson G, Palsson PA, Nathanson N (1976). Pathogenesis of Visna. III. Immune responses to central nervous system antigens in experimental allergic encephalomyelitis and visna. *Lab Invest* **35:** 452–460.

Paterson PY, Day ED, Whitacre CC (1981). Neuroimmunologic diseases: effector cell responses and immunoregulatory mechanisms. *Immunol Rev* **55:** 89–120.

Petursson G, Nathanson N, Geysson G, Parritch H, Palsson PA (1976). Pathogenesis of Visna 1. Sequential Virologic, serologic and pathologic studies. *Lab Invest* **35:** 402–412.

Petursson G, Palsson PA, Georgsson G (1989). Maedi-visna in sheep: host-virus interactions and utilization as a model. *Intervirology* **30 Suppl 1:** 36–44.

Price RW (1994). *HIV, AIDS, and the Brain* (Price RW and Perry SW, eds). Raven Press, New York. pp 1–45.

Rosenberg ZF, Fauci AS (1989). The immunopathogenesis of HIV infection. *Adv Immunol* **47:** 377–432.

Sharer LR, Baskin GB, Cho E-S, Murphey-Corb M, Blumberg BM, Epstein LG (1988). Comparison of simian immunodeficiency and human immunodeficiency virus encephalitides in the immature host. *Ann Neurol* **23**(Suppl): S108–S112.

Sharma DP, Zink MC, Anderson M, Adams R, Clements JE, Joag SV, Narayan O (1992). Derivation of neurotropic simian immunodeficiency virus from exclusively lymphocytetropic parental virus: pathogenesis of infection in macaques. *J Virol* **66:** 3550–3556.

Sigurdsson B (1954). Observations on three slow infections of sheep: Maedi, paratuberculosis, rida, a slow encephalitis of sheep with general remarks on infections which develop slowly, and some of their special characteristics. *Br Vet J* **110:** 255–270.

Sigurdsson B, Palsson PA, Grissom H (1957). Visna, a demyelinating transmissable disease of sheep. *J Neuropathol Exp Neurol* **16:** 389–403.

Stephens EB, McClure HM, Narayan O (1995). The proteins of lymphocyte- and macrophage-tropic strains of simian immunodeficiency virus are processed differently in macrophages. *Virology* **206:** 535–544.

Stephens EB, Galbreath D, Liu ZQ, Sahni M, Lamb-Wharton RJ, Foresman L, Joag SV, Narayan O (1997). Significance of macrophage tropism of SIV in the macaque model of HIV disease. *J Leukoc Biol* **62:** 12–19.

Traugott U (1989). Detailed analysis of early immunopathologic events during lesion formation in acute experimental autoimmune encephalomyelitis. *Cell Immunol* **119:** 114–129.