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Phenotypic characterisation and infection of ovine microglial cells with Maedi-Visna Virus

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Maedi-Visna Virus (MVV) infection of the central nervous system (CNS) results in pathological changes, the mechanisms of which are poorly understood. MVV preferentially infects cell of the monocyte/macrophage lineage *in vivo*. The neuroparenchymal microglial cells are the resident tissue macrophages in the CNS and therefore likely targets for MVV infection. However, no information is currently available on the susceptibility of these cells to MVV infection or their contribution to neuropathological changes as a result of MVV infection. Highly enriched primary ovine microglial cell cultures were set up from brain tissues of lambs. These cells were amoeboid or bipolar with spikes, a morphology consistent with microglial cells of other species, and stained positive for CD1, CD11a, CD11c, CD14, MHC-class I, MHC-class II, and β-N-acetyl galactose, but not with markers of astrocytes or oligodendrocytes. These sheep microglial cells were permissive for MVV infection. Productive MVV infection resulted in selective transcriptional up-regulation of the pro-inflammatory cytokines TNFα and IL-6. In contrast, there was no change in levels of transcripts for TGFβ1, IL-1β, GM-CSF, IL-10, or IL-12. These data provide the first evidence that ovine microglial cells can support productive infection with MVV, and that this leads to a selective up-regulation of proinflammatory cytokines. These may contribute to visna neuropathology. *Journal of NeuroVirology* (2000) **6,** 320–328.

Keywords: CNS; cytokine; cell culture; lentivirus

Introduction

The *lentivirus* subfamily of Retroviridae includes the human, simian, feline, and bovine immunodeficiency viruses (HIV, SIV, FIV, BIV respectively), equine infectious anaemia virus (EIAV), caprine arthritis-encephalitis-virus (CAEV) and maedi-visna virus (MVV). One outcome of lentiviral infection can be central nervous system (CNS) disease (reviewed by Georgsson, 1994).

The CNS disease in sheep naturally infected with MVV presents a sub-acute encephalomyelitis with an insidious outset which develops into rapidly deteriorating hind leg paralysis (reviewed by Palsson *et al*, 1974). A common feature of the CNS pathology which can be reproduced by intracerebral inoculation of MVV is extensive perivascular cuffing and lymphocytic infiltration of the neuroparenchyma. Although no correlation has been established between the extent of the inflammatory response in the CNS and the degree of clinical disease, early CNS disease is believed to be immune mediated (Nathanson *et al*, 1976; Torsteinsdottir *et al*, 1992; 1994). MVV infection of the CNS results in lesions of demyelination similar to those observed in multiple sclerosis in humans (Palsson, 1974). In both MVV and HIV-1, encephalitis may be an early feature. However, the chronic stages of HIV-1 infection include multinucleated giant cells and white matter pallor (Budka, 1989), whereas multinucleated giant cells are a rare occurrence in MVV and there is no report of white matter pallor associated with visna (reviewed by Georgsson, 1994).

Intracerebral infection experiments with MVV have established an association between extent of pathological changes and frequency of virus isolation from affected tissues (Petursson *et al*, 1976). However, an anomaly exists between the number of virus antigen-positive cells in the brain parenchyma and the extent of the neuropathology (Georgsson *et al*, 1989). This disparity is not confined to ruminant

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lentiviruses; similar observations have also been made in HIV-1 and SIV encephalopathies (Gabuzda *et al*, 1986; Wiley *et al*, 1986; Kure *et al*, 1991; Brinkman *et al*, 1993; Nuovo and Alfieri, 1996).

Increased intrathecal expression of pro-inflammatory cytokines has been associated with neuropathological changes in primate lentivirus infections (Genis *et al*, 1992; Koka *et al*, 1995; Lane *et al*, 1996). Similarly, MVV infection has been shown to induce expression of TNFα within brain tissues (Craig *et al*, 1997). Furthermore, lentiviral-encoded components, such as the MVV and HIV-1 transactivator of transcription (tat) can induce the expression of TNFα, IL-6, and IL-1β in rodent brains (Philippon *et al*, 1994). The apparent discordance between the number of virus antigen-positive cells and the severity of the neuropathology may, at least in part, be explained by cytokines and be independent of direct virus infection (reviewed by Gendelman *et al*, 1994).

Microglial infections by HIV-1, SIV, FIV, and CAEV have been demonstrated (Gabuzda *et al*, 1986; Wiley *et al*, 1986; Lackner *et al*, 1991; Dow *et al*, 1990, Baszler *et al*, 1994). However, MVV infection of ovine microglial cells has not been conclusively established, and consequently studies on the effects of MVV infection of microglial cells are lacking. The aims of this work were to establish and characterise cultures of ovine microglial cells, to determine whether these could be infected by MVV and to investigate any subsequent changes in the expression of cytokine transcripts.

We report here the characterisation of highly enriched ovine microglial cell cultures, and their infection by MVV. MVV infection of these cells resulted in a selectively increased transcription of microglial-derived TNFα and IL-6. The implications of these findings for visna neuropathology are discussed.

Results

Morphological features of cultured cells

Ovine microglial cell cultures have not previously been established and characterised. To establish cultures of cells with a morphology consistent with that observed in cultured microglia from other species, a number of tissue culture procedures were tried. The technique eventually used is detailed in the Materials and methods section.

High density primary glial cultures were prepared from lamb brains. Floating non-adherent cells were removed and brief plating of these cells resulted in selection and enrichment of putative ovine microglial cells.

Twenty four hours after enrichment, and on the basis of morphology, the vast majority ($>98\%$) of cells could broadly be divided into either amoeboid or process bearing (Figure 1). The amoeboid cells had membrane spikes evenly distributed around the cell body. The process bearing cells were often bipolar and had asymmetric membrane spikes which were focally distributed on the tip of the long processes and on the main cell body (Figure 1). These morphological features are entirely consistent with those described for both rodent and human cultured microglial cells (Giulian *et al*, 1995).

These putative microglial cultures were characterised by immunostaining with a panel of cell markers. The greater majority of cells stained positive for CD1, CD14, MHC-class I, CD11a, CD11c, and β-NacGal (Figures 2 and 3 and the

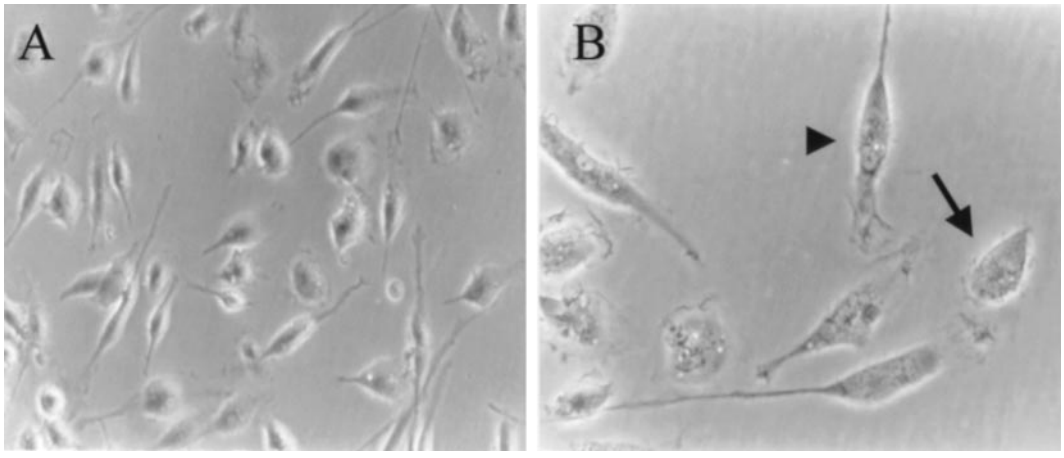

Figure 1 Morphological characterisation of ovine microglial cultures. Glial cells were obtained from brains of new born lambs and enriched for microglial cells by adherence. Twenty-four hours post-enrichment, these glial cells showed both flat and bipolar morphologies. Both (**A**) and (**B**) show phase contrast photographs of live cells. (**A**) shows microglial cells with typical bipolar morphology. (**B**) Shows a higher magnification of cells in (**A**), demonstrating both bipolar (flat arrow head) and apolar cells (arrow). Magnifications (**A**) $\times 125$; (**B**) $\times 250$.

intensity of staining is given in Table 1), 4–7 days after enrichment. The surface immunostaining for MHC class II was comparatively weaker and was only detected on a minority of microglial cells (less than 50%). Further, the cultured microglial cells did not stain with antibodies to GFAP, O4, galactocerebroside, or fibronectin, markers for astrocytes, oligodendrocytes and fibroblasts, respectively (Table 1). Interestingly, surface antigen staining on adherent cells was similar in all cases and was most intense on the cell body and at the distal end of the elongated cell processes (Figures 2 and 3).

Effect of L-methyl ester treatment on cultured cells

L-methyl ester (LME) is a potent toxin for cells of the monocyte/macrophage lineage (Thiele *et al*, 1985). Treatment of the cell cultures with LME resulted in cytotoxicity. The initial toxic effects of LME were apparent within 30 min of treatment in the form of cytoplasmic ballooning (Figure 4). Prolonged LME treatment resulted in loss of these cells. In contrast, LME treatment did not have any detrimental effects on astrocytes and other cells in mixed glial cultures.

Taken together the morphology, the cell surface phenotype and the sensitivity to LME confirm that the cultures established were highly enriched for ovine microglial cells.

Infection of microglial cells with MVV

To assess the tropism of MVV for these ovine microglial cells, cultures were infected with the K1514 strain of MVV on day five post-enrichment. Uninfected cultures were used as controls for immunostaining and cytokine RT–PCR assays. The earliest cellular changes were first apparent by light microscopy at 2–3 days post-infection, as an increase in cytoplasmic vacuoles/vesicles. By day five post-infection, the cell membrane appeared discontinuous and irregular, and by days 8–9 the

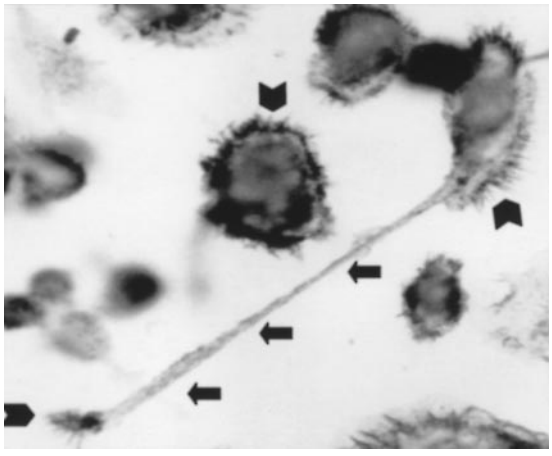

Figure 2 Cell surface staining for CD14 on cultured ovine microglial cells. Highly enriched microglial cells were immunostained for cell surface expression of antigens common to cells of the monocyte/macrophage lineage. Cells were stained 4–7 days after enrichment. Staining was localised to the cell membrane of the cell body and the tip of elongated processes (arrowhead). Note lack of staining of long processes (arrows). Magnification × 300.

Table 1 Immunocytochemical reactivity of cultured ovine microglial cells

Antibody	*Specificity*	*Microglia*
SBUT-6	CD1	+++
VPM19	MHC-I	+++
VPM36	MHC-II DR	+
VPM54	MHC-II DQ	+
LFA-1	CD11a	+++
OM1	CD11c	+++
VPM65	CD14	+++
[a]GSI-B4	[b]β-NacGal	+++
[c]Anti-O4	Oligodendroctyes	–
[c]Anti-Galc	Oligodendrocytes	–
[c]Anti-GFAP	Astrocytes	–

Enriched microglial cells were immunostained at 7–10 days post-culture. (+++) indicates strong, (+) indicates weak and (–) indicates no staining above background levels, respectively. [a]β-N-acetyl galactose; [b]lectin; [c]cross reacts with ovine tissues.

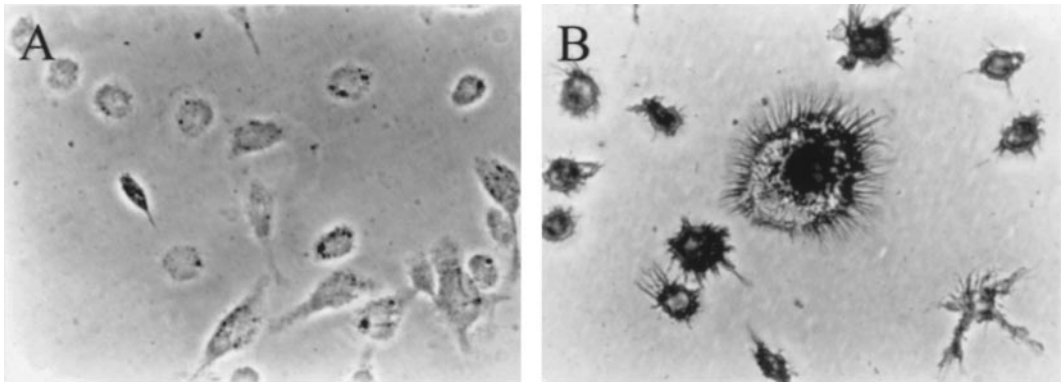

Figure 3 Cultured ovine microglial cells express surface CD1. Highly enriched microglial cultures were stained for the expression of CD1 on 4–7 days after enrichment. (**A**) The negative control where cultures were stained with an isotype control antibody. Cells in (**B**) were stained with anti-CD1 antibody. Note the surface staining of CD1 and localisation of CD antigens on membrane spike. Magnifications: (**A**) × 125; (**B**) × 250.

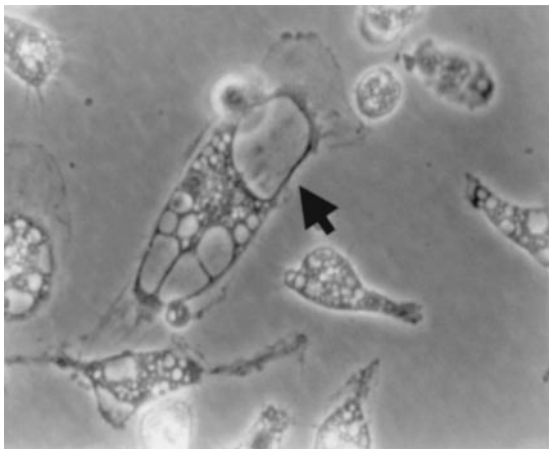

Figure 4 L-methyl ester is toxic to cultured microglial cells. Microglial cultures were treated with this lysosomo-tropic agent (7.5 mM in serum-free DME) and morphological changes were followed by light microscopy. The initial sign of toxicity was intracellular ballooning was apparent within 30 min of LME treatment (arrowhead). LME treatment resulted in eventual loss of cells from culture. Magnification × 250.

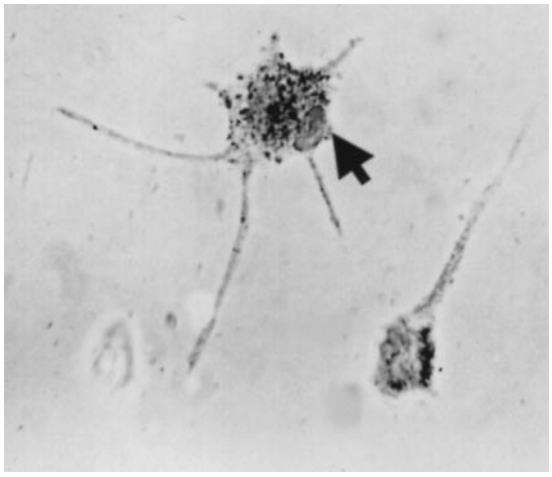

Figure 5 Immunostaining for viral p25 in MVV-infected microglial cells. Microglial cultures infected with MVV and were immunostained on days 4–7 post-infection with a monoclonal antibody against the major viral core protein (p25). Note punctate cytoplasmic staining of p25 in infected cells. The arrow indicates the location of the nucleus. Magnification × 300.

cell membrane had shrunk extensively with cells becoming detached from the monolayer. By day 4 post-infection, the majority of cells in the culture stained positive with a monoclonal antibody against the major viral core protein p25. In infected cultures, p25 staining was punctate and always localized to the cytoplasm (Figure 5). Multinucleated giant cells were observed only rarely in infected cultures. Infectious virus was detectable in the supernatants of infected cultures within 48 h and increased with time (Figure 6).

Effect of MVV infection on microglial-derived cytokines

It has been suggested that microglial released cytokines are major contributors to the neuropathology in retroviral neuropathies (Baszler *et al*, 1994; Nuovo and Alfieri, 1996; Sopper *et al*, 1996; Craig *et al*, 1997). To investigate this, changes in cytokine gene expression in infected ovine microglial cells were investigated. Semi-quantitative RT–PCR analyses of cytokine transcripts were carried out by comparison of PCR cycle numbers, determined empirically, against an internal control gene, ATPase. Infection of cultured microglial cells with MVV resulted in increased expression of TNFα and IL-6 (Figure 7). TGFβ-1 transcripts were present at relatively high levels in both control and MVV-infected cultures, and there was no statistical difference in transcript levels (Figure 7). No transcripts were detected for IL-1β, GM-CSF, IL-10, or IL-12 in control or infected cells. To test whether these PCR assays could potentially amplify their intended brain-derived transcripts, we tested these assays on a brain material derived from a sheep with a non-specified mononuclear cell inflammatory response. Most cytokines were detected from the brain of this

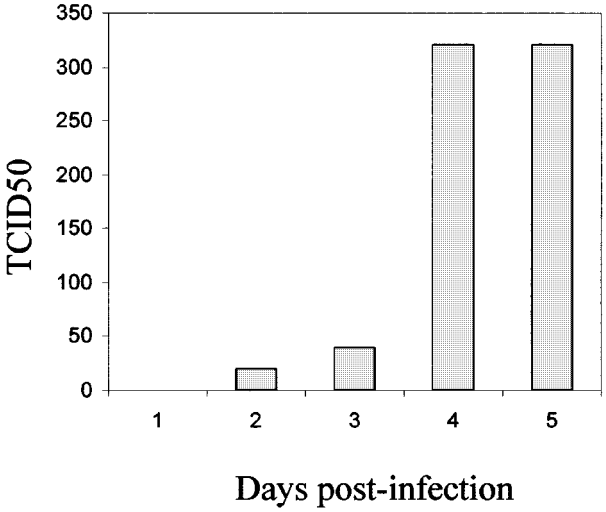

Figure 6 Productive infection of microglial cultures with MVV. Enriched microglial cells (100 000 per 35 mm plate) were infected with 100 $TCID_{50}$ of MVV. Supernatants from infected cultures were harvested at different time points post-infection and titred on sheep fibroblasts for infectious virus.

sheep (Figure 8). Therefore, the PCR assays could potentially amplify their intended targets.

Discussion

Techniques for the preparation of highly enriched cultures of ovine microglia were established. These cells were characterised as microglial cells by their morphology, cell surface phenotype and sensitivity to LME treatment. Similar immunostaining patterns and morphological features have been observed with microglial cells from other species (Akiyama and

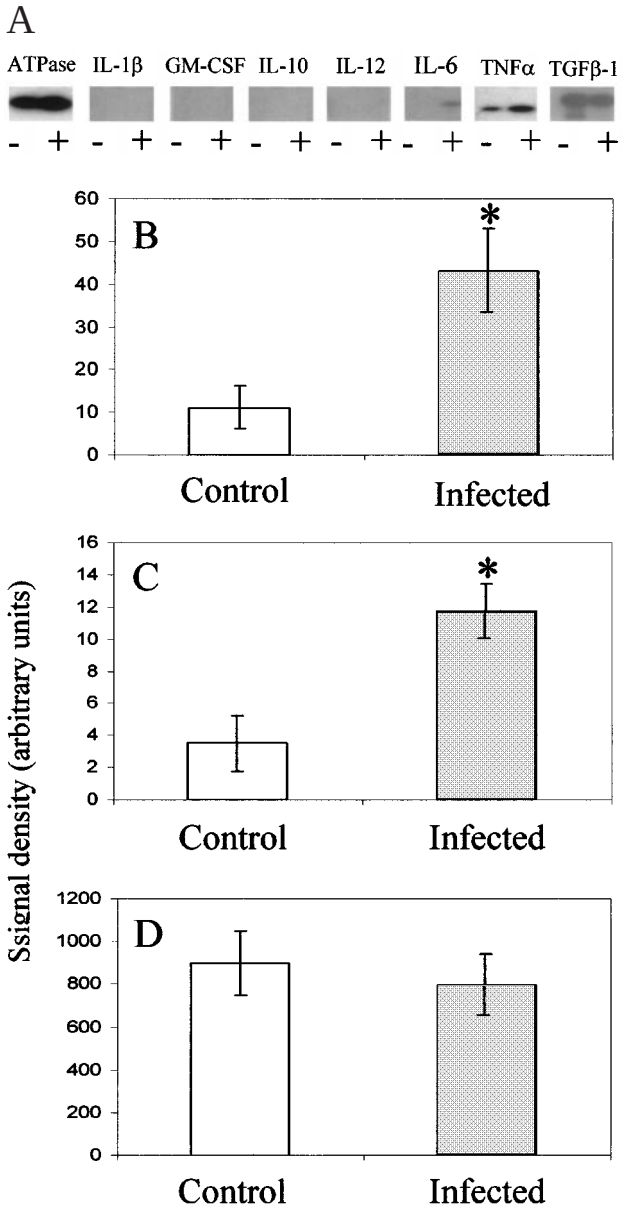

Figure 7 Effect of MVV infection on mRNA expression of microglial-derived cytokines. Enriched microglial cell cultures were either mock-infected (control) or MVV-infected. Total RNA was harvested from cultures on day 4 post-infection, DNase-I-treated and then reverse-transcribed and used for amplification of cytokine transcripts and the housekeeping gene ATPase. PCR products were resolved by gel electrophoresis and blotted. The Southern blots were probed with gene-specific internal primers and resultant signals scanned, and normalised against the signal for the ATPase transcript. (**A**) is a representative Southern blot, where (−) and (+) correspond to mock-infected and MVV-infected microglial cultures, respectively. (**B**), (**C**) and (**D**) are the means ± one standard deviation compiled from four separate experiments, and refer to TNFα, IL-6, and TGFβ-1, respectively. A significant increase ($P<0.05$, *) was observed in the expression of TNFα and IL-6 in MVV-infected cultures compared to control cultures. In contrast, no significant change was observed in the expression of TGFβ-1 between control and infected cultures.

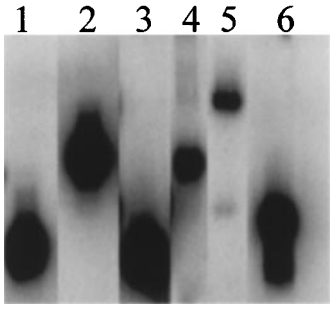

Figure 8 Detection of brain-derived ovine cytokines using gene-specific PCRs. Total cellular RNA isolated from the brain of a sheep known to contain inflammatory infiltrates by histology was used to test the specificity and sensitivity of the PCR assays. PCR products were resolved by gel electrophoresis and blotted. The Southern blots were probed with gene-specific internal primers. The figure is the autoradiograph of the probed blots. 1: ATPase (168 bp); 2: IL-1β (466 bp); 3: TNFα (144 bp); 4: IL-10 (452 bp) 5: IL-6 (622 bp); and 6: TGFβ-1 (336 bp).

McGeer, 1990; Colton *et al*, 1992; Baszler *et al*, 1994; Ford *et al*, 1995; Becher and Antel, 1996; Havenith *et al*, 1998). The enriched ovine microglial cultures were used to study infection with MVV.

To our knowledge, this if the first study to demonstrate the presence of CD1 on microglial cells from any species. The CD1 family of cell surface glycoproteins are expressed on most antigen presenting cells and have been shown to present non-protein antigens to the immune system (reviewed by Porcelli, 1995). At present, it is not clear what role, if any, microglial CD1 may play in the CNS environment in general or in MVV infection specifically. Expression of MHC was detected on cultured ovine microglial cells, with MHC-class I staining more prominent than MHC-class II staining. This is consistent with previous *in vivo* studies in which ovine parenchymal microglial cells were shown to express both MHC-class I and class II molecules (Torsteinsdottir *et al*, 1994; Bergsteinsdottir *et al*, 1998).

The punctate pattern of viral p25 immunostaining in infected microglial cultures demonstrates that MVV infects and replicates in cultured ovine microglial cells. This pattern of punctate p25 immunostaining is consistently observed in cells of the monocyte/macrophage lineage infected with MVV. Both alveolar macrophages and blood monocyte-derived macrophages, but not fibroblasts, show similar immunostaining patterns (Lee *et al*, 1996). The infection of microglial cells was productive since culture supernatants contained infectious virions. Together, these findings demonstrate not only that microglial cells can become infected with the virus, but also that the packaging and egress pathways for the progeny virions are intact in these cells.

Cytokine production has been closely linked with lentiviral infections of cells of the monocyte/macrophage lineage and has been suggested to contribute to the CNS pathology (Baszler *et al*, 1994; Nuovo and Alfieri, 1996; Sopper *et al*, 1996; Craig *et al*, 1997). The profile of cytokines produced in MVV-infected primary ovine microglial cells

under our experimental conditions demonstrated a restricted pattern of gene expression. This restriction at the transcriptional level is more stringent than that observed with other tissue macrophages. For example, infection of freshly isolated ovine blood monocyte-derived macrophages with MVV resulted in up-regulation of mRNA for IL-1β, TNFα, IL-6, and IL-10 (Z Zhang and G Harkiss, personal communication). This restricted cytokine output from microglial cells could be appropriate to the CNS environment, which is an immunologically specialised site. In support of this notion, cultured ovine microglial cells constitutively expressed high levels of TGFβ-1 mRNA, a cytokine with potent immunoinhibitory effects (reviewed by Bogden and Nathan, 1993). Infected microglial cells produced mRNA for the pro-inflammatory cytokines TNFα and IL-6. This is consistent with a previous study in which TNFα immunoreactivity in MVV-infected brain tissues was demonstrated to associate with brain macrophages (Craig *et al*, 1997). Intrathecal expression of TNFα has also been demonstrated in primate lentivirus infections (Nuovo and Alfieri, 1996; Sopper *et al*, 1996). Increased transcription of IL-6 mRNA has also been reported in caprine microglial cultures infected with CAEV (Baszler *et al*, 1994) and in the brains of SIV-infected macaques (Sopper *et al*, 1996). Over-expression of IL-6 in the murine CNS has been shown to result in reactive astrocytosis and an increase in ramified microglial cells (Fattori *et al*, 1995). Interestingly, inoculation of HIV-1-infected monocytes into the brains of severe combined immunodeficient mice resulted in microgliosis. These activated microglial cells produced IL-6 (Persisky *et al*, 1996). These *in vitro* studies demonstrate that MVV can productively infect ovine microglial cells resulting in transcription of proinflammatory cytokine genes. These results are consistent with the hypothesis that in sheep brain direct infection of microglial cells by MVV results in production of pro-inflammatory cytokines which may be important mediators of neuropathology.

Materials and methods

Primary ovine glial cultures

Mixed breeds of British lambs (male and female and less than 1 week old) were used to set up cell cultures. The brain stem region was the source of glial cells. This region included diencephalon, metencephalon (excluding cerebellum), and myelencephalon (excluding cervical portion of spinal cord caudal to obex). After euthanasia with Euthatol the brain stem was removed and placed in Leibovitz L-15 medium (GibcoBRL), supplemented with benzylpenicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (2.5 mg/ml). The meninges and major blood vessels were removed. The tissue was cut into approximately 1–2 mm fragments and triturated using a 10 ml glass pipette. The suspension was spun at 500 r.p.m. ($50 \times g$) in a bench-top centrifuge for 1 min and the supernatant containing mixed glial cells was removed and kept. The cell pellet was re-suspended in 4 ml of fresh L-15 medium and the procedure repeated another three times. The supernatants from each cycle were pooled and centrifuged at 1000 r.p.m. ($150 \times g$) for 3 min. The pellet containing glial cells was resuspended in 5 ml of Dulbecco's minimum essential medium (DME) containing HEPES (10 mM), $NaHCO_3$ (10 mM), 10% heat-inactivated foetal calf serum (FCS) and antibiotics (see above). The resuspended cells derived from one lamb brain were plated at high density in a T25 tissue culture flask (Nunc, Denmark), pre-treated with poly-D-lysine (0.2 mg/ml for 30 min, rinsed with sterile water and air-dried). The cells were cultured at 37°C, at 5% CO_2 in a humidified (95%) atmosphere, and left undisturbed for 4 days. Due to the high density of cells, a large number of cells remained floating.

After 4 days of culture, the floating cells were removed and plated on 35 mm poly-D-lysine coated dishes for 15 min at 37°C. The medium was then removed and the adhered cells rinsed once with serum-free DME and then cultured in DME plus 10% FCS. This short incubation step allowed the adherence of putative microglial cells without significant adherence of other glial cells or fibroblasts. After overnight culture, the FCS was reduced to 2% to reduce growth of any fibroblasts.

Antibodies

Monoclonal antibodies SBUT-6 (Hopkins and Dutia, 1991), VPM 65 (Gupta *et al*, 1996), LFA-1 (Gupta *et al*, 1995), IL-A15 and OM1 (Gupta *et al*, 1993) were derived from hybridoma supernatants and were used undiluted (Table 2). VPM65, LFA-1, IL-A15, OM-1, VPM19, and VPM54 all are ovine-specific antibodies. SBUT-6 was raised against bovine CD1 and cross-reacts with ovine tissues. The monoclonal antibody O4 clone 81 (Boehringer Mannheim, Germany) and polyclonal anti-galactocerebroside (Sigma Chemical, UK) markers for immature and mature oligodendrocytes (Raff *et al*, 1978; Sommer and Schachner, 1981) were used at final dilutions of 1 : 20 and 1 : 100, respectively. The monoclonal antibody A2B5 (Boehringer Mannheim), a marker for cells of neuroglial origin (Eisenbarth *et al*, 1979) was used at a final dilution of 1 : 10. The biotin conjugated isolectin *Griffonia simplicifolia* GSI-B_4 (Sigma Chemicals), a marker for brain microglial cells (Colton *et al*, 1992) was used at a final dilution of 1 : 100. Polyclonal rabbit anti-bovine glial fibrillary acidic protein (GFAP; Dako, UK), a marker for astroglial cells (Bignami *et al*, 1972) was used at a final dilution of 1 : 100. The same dilution was used with rabbit anti-human

fibronectin (Sigma Chemicals), for detection of fibroblasts. The antibodies against astrocytes, oligodendrocytes and fibroblasts were tested on ovine brain-derived cultures. These antibodies cross-reacted with ovine tissues (data not shown).

Indirect immunostaining
Cells grown on 35 mm tissue culture dishes were used for immunostaining 7–10 days post-plating. For cell surface markers, live cells were first incubated with primary antibody, rinsed with phosphate-buffered saline (PBS), fixed with 4% (w/v) para-formaldehyde in PBS (PFA/PBS), followed by a blocking step with 3% (v/v) normal goat serum (NGS) in PBS. Cells were then incubated with fluorescein or alkaline-phosphate-conjugated secondary antibody, diluted in 1% NGS/PBS.

For immunostaining of intracellular antigens, cells were first fixed and then permeabilised with 0.3% (v/v) Triton X-100 in PBS before incubation with primary antibody. Immunostaining of microglial cells with anti-CD markers was performed using goat-anti-mouse alkaline phosphatase conjugate as secondary antibody, with nitroblue tetrazolium and X-phosphate as detection system (Boehringer Mannheim). The negative control was normal mouse serum as primary antibody in place of immune serum. Presence of virus in microglial cells was detected using a monoclonal antibody against the viral p25 major core protein (Reyburn *et al*, 1992).

Virus infection
The neural adapted Icelandic K1514 strain of MVV was used (Staskus *et al*, 1991). One hundred thousand cells were infected with 100 TCID50 by incubation in serum-free DME for 2 h at 37°C, followed by three rinses with serum-free DME and finally incubated in DME plus 2% FCS.

Virus titer determination
Virus titers from infected cultures were determined using sheep fibroblasts as indicator cells. Fibroblasts were seeded in 96-well flat-bottomed plates at a density of 1×10^4 cells/ml in DME plus 2% FCS. The medium was removed and serial dilutions of test samples were added to each well. Each dilution was tested in triplicate. Supernatants from mock-infected cultures were used as negative controls. After incubation for 7 days, cells were fixed and stained with Giemsa's stain. The cytopathic effect was scored by the presence of syncytium and/or lysis. The tissue culture infectious dose 50 ($TCID_{50}$) was determined by the method of Reed and Muench (1938).

Amplification of cytokine gene transcripts
Total cellular RNA was isolated from mock-infected and virus-infected microglial cultures on day four post-infection. Briefly, RNA lysis buffer (Qiagen) was added to cells on the day of harvest and total RNA extracted using RNeasy columns (Qiagen) according to manufacturer's instructions. To eliminate possible contaminating genomic DNA, the isolated RNA samples were pre-treated with DNAse-I (RNAse-free, Pharmacia Biotech), before reverse transcription. The DNAse-treated samples were then reverse transcribed with SuperScript II (GibcoBRL).

Amplification of microglial-derived cytokine genes and the housekeeping gene ATPase was carried out using ovine gene-specific intron-spanning RT–PCR assays (Table 2) (Dutia *et al*, 1994; Ebrahimi *et al*, 1995; Woodall *et al*, 1997). All PCR reactions were performed using a denaturing step at 95°C for 1 min, an annealing temperature at 55°C for 1 min, and an extension step at 72°C for 2 min, with an extension step at 72°C for 5 min was introduced to allow for the completion of DNA strand synthesis. PCR reactions were performed in 2 mM $MgCl_2$ for amplification of all cytokine transcripts and in 3.5 mM $MgCl_2$ for amplification of ATPase transcripts.

The ubiquitous ovine housekeeping gene alpha sub-unit of the ATPase (Na+/K+) pump was used as the internal control for cytokine PCR reactions (Woodall *et al*, 1994).

PCR products were resolved by running 20 μl aliquots from PCR reactions on 1.6% (w/v) agarose gels. Southern blots were performed on resolved PCR products with ^{32}P-3′-end-labelled probes with high specific activities. The gene-specific signal intensities were determined using the ImageQuaNT™ phosphorimage analyzer (Molecular Dynamics, UK). The definition of pixel intensity, as given in the ImageQuaNT™ user's guide is 'the intensity, which reflects signal strength. The pixel

Table 2 PCR Primers and internal probe sequences for ovine cytokines and the housekeeping gene ATPase

Gene	*Forward primer*	*Reverse primer*	*Internal probe*	*Product (bp)*
IL-1β	TACGAACATGTCTTCCG	CCAGTTAGGGTACAGGA	CGATGAGCTTCTGTGTGATGCAGC	466
TNFα	ATGAGCACCAAAAGCATGATCC	GAAGAGCGTGGTGGCTCC	AGGAGGTGCTCTCCAACAAAGCA	144
TGFβ-1	GCCCTGGACACCAACTACTG	TCAGCTGCACTTGCAGGAG	CCTTCCGGAAGTCAATGTAGAGCTG	336
IL-6	ATGAACTCCCTCTTCACAAGCG	ACTTCATCCGAATAGCTCTC	AAATGACACCACCCCAAGC	622
GM-CSF	AGTCCTCAACAGGATGTGGC	CGTCTGTGAGGTAAGCTT	AACGACAGCACTGACACTGCTGCTGTG	495
IL-10	ATGCCACAGGTGAGAAC	TTCACAGAGAAGCTCAG	ACCTGCTCCACCGCCTTG	452
IL-12	AGATGCTGGGCAGTACACCT	CAATGGGCAGGCTCTCCTC	GCTCGTGGCTGACAGCAATCATGACT	380
ATPase	GCTGACTTGGTCATCTGC	CAGGTAGGTTTGAGGGGATAC	CATCCCCTGCTGGAAGACGGAATT	168

intensity units depend on which instrument was used to collect the image and in the case of a phosphorImager, the unit is counts.'

The normalisation of cytokine PCR signals were carried out according to previously published protocols (Woodall *et al*, 1997). Briefly, PCR cycle numbers that produced subsaturation levels of amplified products (31 cycles for ATPase and all cytokines except TGFβ-1, and 29 cycles for TGFβ-1) were determined empirically by performing PCR for each assay on mixed cDNA samples. The results from each cytokine assay were then normalised against the reading for the housekeeping gene ATPase.

Ovine cytokine genes cloned in plasmid vectors were used to test for specificity of PCR assays (data not shown). Furthermore, to test the sensitivity of the PCR assays on RNA isolated from brain tissues, expected to be at comparatively minute quantities, total RNA was isolated from an ovine brain known to contain inflammatory infiltrates by histology. This material was then used in the PCR assays (Figure 8). The results from these PCRs showed that these assays could amplify their intended targets and that the methodology worked.

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