

Short Communication

The vascular lesions of a cow and bison with sheep-associated malignant catarrhal fever contain ovine herpesvirus 2-infected CD8⁺ T lymphocytes

Susan Simon,^{1†} Hong Li,² Donal O'Toole,³ Timothy B. Crawford¹
and J. Lindsay Oaks¹

Correspondence

J. Lindsay Oaks
loaks@vetmed.wsu.edu

^{1,2}Department of Veterinary Microbiology¹ and Pathology and Animal Diseases Research Unit,² USDA-ARS, Washington State University, Box 647040, Pullman, Washington 99164-7040, USA

³Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, Wyoming 82070, USA

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Malignant catarrhal fever (MCF) is a herpesvirus disease syndrome of ruminants. The microscopic pathology of MCF is characterized by lymphoid proliferation and infiltration, necrotizing vasculitis and epithelial necrosis. Because previous attempts to detect viral antigen or nucleic acids in lesions have been unsuccessful, the pathogenesis of the lesions in acute MCF has been speculated to involve mechanisms of autoimmunity and lymphocyte dysregulation. In this study, the vascular lesions in the brains of a cow and a bison with acute MCF were evaluated by *in situ* PCR and immunohistochemistry. The results demonstrated that the predominant infiltrating cell type in these lesions was CD8⁺ T lymphocytes and that large numbers of these cells were infected with ovine herpesvirus 2. The lesions also contained macrophages, but no detectable CD4⁺ or B lymphocytes.

Malignant catarrhal fever (MCF) is a lymphoproliferative disease syndrome of certain ruminant species (Plowright, 1990). The causative agents of MCF include several closely related gammaherpesviruses that exist in nature as sub-clinical infections in carrier ruminants (Crawford *et al.*, 1999a). MCF in susceptible ruminant species is most commonly associated with exposure to wildebeest (wildebeest-associated, WA-MCF) or sheep (sheep-associated, SA-MCF), caused by alcelaphine herpesvirus 1 (AIHV-1) and ovine herpesvirus 2 (OvHV-2), respectively (Plowright, 1965; Reid & Buxton, 1984).

The primary lesions in ruminants with acute MCF are lymphoid proliferation and infiltration, necrotizing vasculitis with perivascular lymphoid accumulation and necrosis of mucosal epithelium (Kalunda *et al.*, 1981; Piercy, 1952a, b; Pierson *et al.*, 1979; Plowright, 1968). Although these lesions are highly characteristic, their pathogenesis is poorly understood (Burrells & Reid, 1991; Plowright, 1990; Rossiter, 1985; Swa *et al.*, 2001). Several studies have shown that, in cattle, CD8⁺ T lymphocytes are the predominant cells associated with the vascular lesions (Ellis *et al.*, 1992; Lagourette *et al.*, 1997; Nakajima *et al.*, 1992, 1994). Limited data from *in vitro* studies of cell lines derived from cattle and deer indicate that T lymphocytes may also be targets of infection in these two species (Burrells & Reid,

1991; Cook & Splitter, 1988; Schock *et al.*, 1998). However, in affected animals, although viral DNA can be detected in circulating mononuclear cells and most tissues by PCR, the cellular site(s) of virus replication *in vivo* is unknown (Crawford *et al.*, 1999b; Plowright, 1990). Previous studies using immunofluorescence and *in situ* hybridization have been unable to detect either viral antigens or nucleic acids in lesions (Edington *et al.*, 1979; Rossiter, 1980, 1985). Consequently, it has been proposed that the lymphoproliferative and vascular lesions are due to lymphocyte dysregulation and autoreactive immune responses rather than virus cytopathology or antiviral immune responses (Plowright, 1990; Rossiter, 1985; Swa *et al.*, 2001).

This study examined tissues from a cow and a bison with SA-MCF using *in situ* PCR and immunohistochemistry to determine whether the infiltrating cells in the lesions were infected with OvHV-2. Both animals had a history of exposure to sheep and clinical signs typical of acute MCF, including fever, nasal-ocular discharge, oral erosions and bilateral corneal opacity. Both cases were confirmed as MCF by the presence of typical histopathological lesions (Innes & Saunders, 1962; Summers *et al.*, 1995) and MCF-specific antibody (Li *et al.*, 2001) and by PCR for OvHV-2 DNA (Li *et al.*, 1995). In both animals, prominent MCF lesions were dispersed throughout the neuraxis of the brain, consisting of mild, widely scattered perivascular aggregates of large mononuclear cells and small lymphocytes. Affected vessels were located primarily in the leptomeninges, Virchow–Robin spaces around arterioles and venules and choroid

[†]Present address: Institute of Pathology and Experimental Cancer Research, Semmelweis University, H1085 Budapest, Ulloi Street 26, Hungary.



plexus. Swollen endothelium, exuded perivascular fibrin, extravasated erythrocytes and pyknotic inflammatory cells were associated with larger cellular cuffs. Small, ill-defined foci of gliosis were also present throughout the brain. In the bison brain, the perivascular infiltrates also contained neutrophils and eosinophils. Fibrinoid vasculitis was absent in brain and leptomeninges of both animals.

In situ PCR was performed on formalin-fixed and frozen sections of brain to detect OvHV-2-infected cells. Sections (5 μm) of formalin-fixed, paraffin-embedded brain were mounted on silane-coated glass slides (Sigma), deparaffinized in xylene, rehydrated through aqueous ethanol solutions and permeabilized with 0.1% Triton-X for 5 min, 0.2 M HCl for 5 min and 2 mg pepsin ml^{-1} dissolved in 0.02 M HCl for 20–30 min at room temperature. For frozen brain, 4 μm sections were mounted on silane-coated glass slides, fixed in a solution of 75% absolute methanol, 25% (v/v) 37% formaldehyde and 300 μl acetic acid for 5 min, washed with PBS, air-dried and stored at -80°C . The frozen sections were then post-fixed with 4% paraformaldehyde for 20 min and permeabilized by digestion with 2 mg pepsin ml^{-1} for 20 min at room temperature. *In situ* PCR with direct incorporation of labelled nucleotide was performed by applying 30 μl of a mixture containing the following to each tissue section: 0.6 μg MCF-specific primers μl^{-1} (primers 556, 5'-AGTCTGGGGTATATGAATCCAGATGGCTCTC-3', and 775, 5'-AAGATAAGCACAGTTATGCATCTGATAAA-3'; Li *et al.*, 1995), 1.4 mM deoxynucleoside triphosphates, 0.02 mM digoxigenin-11-2'-deoxyuridine-5'-triphosphate, 2.2 mM MgCl_2 , 15 μl Self-Seal Reagent (MJ Research) and 9 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in Perkin Elmer PCR Buffer II. Coverslips were then applied and hot-start activation of the *Taq* DNA polymerase was performed by heating the slides to 95°C for 10 min. Amplification was performed with a GeneAmp *In situ* PCR System 1000 (Perkin Elmer) using 1 cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. Post-amplification, the slides were washed in 0.15 mM NaCl and 0.015 mM sodium citrate solution at 60°C for 10 min. Labelled amplicons were detected by incubating the slides with anti-digoxigenin F(ab) fragments conjugated with alkaline phosphatase and NBT/BCIP (Roche) according to the manufacturer's directions. *In situ* PCR detected numerous OvHV-2-infected cells, both in the perivascular infiltrates and in cells diffusely scattered throughout the surrounding neuropil (Fig. 1A, B). OvHV-2 DNA was localized to the nuclei of infected cells with the morphology of lymphocytes. Infected cells were best demonstrated

in formalin-fixed, paraffin-embedded tissue due to the improved preservation of cellular and tissue architecture. The *in situ* PCR procedure on frozen brain sections from the bison resulted in significant destruction of the tissue architecture, but positive cells were nevertheless identified adjacent to blood vessels. Control sections, including tissues from an uninfected calf, MCF-infected tissues using irrelevant PCR primers (Fig. 1C–F) and MCF-infected tissues with no *Taq* DNA polymerase (not shown), contained no positive cells.

Immunohistochemical staining was used to determine the cell type of the inflammatory cells. Initial immunohistochemical staining was performed for CD3 antigen, a pan-T lymphocyte marker (MacHugh *et al.*, 1997), on formalin-fixed, paraffin-embedded tissues. Sections of brain were deparaffinized and rehydrated into PBS containing 1% BSA, 0.05% Tween and 1% normal goat serum. Blocking steps included 3% hydrogen peroxide for endogenous peroxidase, avidin-biotin blocking solution (Vector Laboratories) for endogenous biotin and 1% BSA, 0.05% Tween and 5% normal goat serum for non-specific protein binding. Antigen retrieval was performed with 0.2 mg pepsin ml^{-1} for 20 min at room temperature and the sections were then incubated overnight at 4°C with 5 μg of an anti-human CD3 antiserum ml^{-1} (A0425; Dako). Bound antibody was detected with a streptavidin-horseradish peroxidase system (UltraStreptavidin Detection System; Signet) followed by development with AEC (Dako). To verify that the anti-human CD3 antisera would react with ruminant CD3 antigens, immunohistochemistry was also performed on lymph node sections from a normal calf and the bison. This demonstrated specific staining in the cortical and paracortical areas expected to contain T lymphocytes. In the vascular lesions in the brain, CD3⁺ T lymphocytes were the primary infiltrating cell type and were also diffusely scattered within the adjacent white matter, but were absent in the grey matter.

Further characterization of the phenotype of the T lymphocytes was performed by immunohistochemical staining of frozen brain sections with a panel of cell-specific markers. The sections were rehydrated and blocked as described above and then incubated for 30 min at room temperature with 7.5 μg of one of the following primary monoclonal antibodies ml^{-1} , provided by W. C. Davis, Washington State University: anti-CD4 (ILA11.A), anti-CD8 (CACT80C), anti-monocyte/macrophage (DH59B and CAM36A) and anti-B cell (BAQ44A). Isotype-matched monoclonal antibodies were used as negative controls. Bound antibodies

Fig. 1. *In situ* PCR on brain sections demonstrating OvHV-2 infected mononuclear cells using direct incorporation of digoxigenin-labelled dUTP and visualized with NBT/BCIP. Positive cells are indicated by purple staining of nuclei. (A, B) Formalin-fixed, paraffin-embedded (A) and frozen (B) sections of brain white matter from the MCF-infected calf. Bars: 25 μm (A); 100 μm (B). (C, D) Formalin-fixed, paraffin-embedded (C) and frozen (D) sections of brain white matter from the uninfected calf as negative controls. Bars: 25 μm (C); 100 μm (D). (E, F) Formalin-fixed, paraffin-embedded (E) and frozen (F) sections of brain white matter from the infected calf with irrelevant PCR primers in the *in situ* PCR reaction as negative controls. Bars: 25 μm (E); 100 μm (F).

were detected as described above. The lymphocytes in the brain lesions were predominantly CD8⁺. In addition, low numbers of monocytes and macrophages were also present, while CD4⁺ T and B lymphocytes were not detected.

To confirm that the CD8⁺ T lymphocytes were infected, *in situ* PCR for OvHV-2 was performed on brain sections previously labelled for cell-specific markers by immunohistochemistry. Immunohistochemistry was performed as described above on both frozen and formalin-fixed tissues. The sections were then prepared for *in situ* PCR by washing in PBS and post-fixing for 60 min in 4% paraformaldehyde. The frozen sections were permeabilized with 0.002 mg pepsin ml⁻¹ for 20 min at room temperature. The formalin-fixed tissues were permeabilized with 2 mg pepsin ml⁻¹ dissolved in 0.02 M HCl for 20 min at room temperature. *In situ* PCR was then performed as described above. In the formalin-fixed tissues, OvHV-2 DNA and CD3 antigen could be co-localized in the same cells (Fig. 2A). Because there were no CD4⁺ cells or B lymphocytes detected in these lesions, it was assumed that the infected cells were CD8⁺ T lymphocytes. Attempts to demonstrate co-localization of OvHV-2 DNA and CD8 antigen required the use of frozen tissue sections, since the CD8 epitope detected by the CACT80C monoclonal antibody was sensitive to formalin fixation. Although the tissue morphology was poor, a few CD8⁺ cells infected with OvHV-2 could be identified (Fig. 2B). The specificity of these assays was demonstrated by negative *in situ* PCR results on the following controls: uninfected brain, uninfected brain stained by immunohistochemistry for CD3 or CD8, infected brain using irrelevant primers and infected brain omitting *Taq* DNA polymerase from the reaction.

These results demonstrate that the vascular and lymphoproliferative lesions in the brains of a cow and bison with acute MCF are infiltrated primarily with CD8⁺ T lymphocytes and that many of these cells are also infected with OvHV-2. The finding that CD8⁺ lymphocytes are the predominant infiltrating cell type, with lower numbers of monocytes and macrophages, is consistent with the findings of other studies (Ellis *et al.*, 1992; Lagourette *et al.*, 1997; Nakajima *et al.*, 1992, 1994). Dual staining for macrophages and OvHV-2 DNA was not attempted, but the morphology of the *in situ* PCR-positive cells did not resemble macrophages. Previous studies searching for viral antigens using immunofluorescence and for viral DNA using *in situ* hybridization were not able to find evidence of virus in MCF lesions (Bridgen *et al.*, 1992; Edington *et al.*, 1979; Rossiter, 1980, 1985). The success in demonstrating viral DNA in this study is most likely due to the greater sensitivity of *in situ* PCR relative to the sensitivity of *in situ* hybridization. The inability to detect viral antigens by immunofluorescence in the studies cited, as well as others, suggests that the infected cells in these lesions are either latently infected or express very limited amounts of viral protein. Although other authors have suggested that the lesions of MCF do not contain virus (Buxton *et al.*, 1984; Schock & Reid, 1996), the current study demonstrates that OvHV-2-infected cells are in fact present. The current study is also consistent with the previous finding that OvHV-2 genomic DNA can be detected in extracts of multiple tissues of affected cattle by solution PCR (Crawford *et al.*, 1999b).

The pathogenesis of the lesions of acute MCF has been the subject of considerable debate. Most proposed scenarios have been constructed to accommodate the apparent lack of

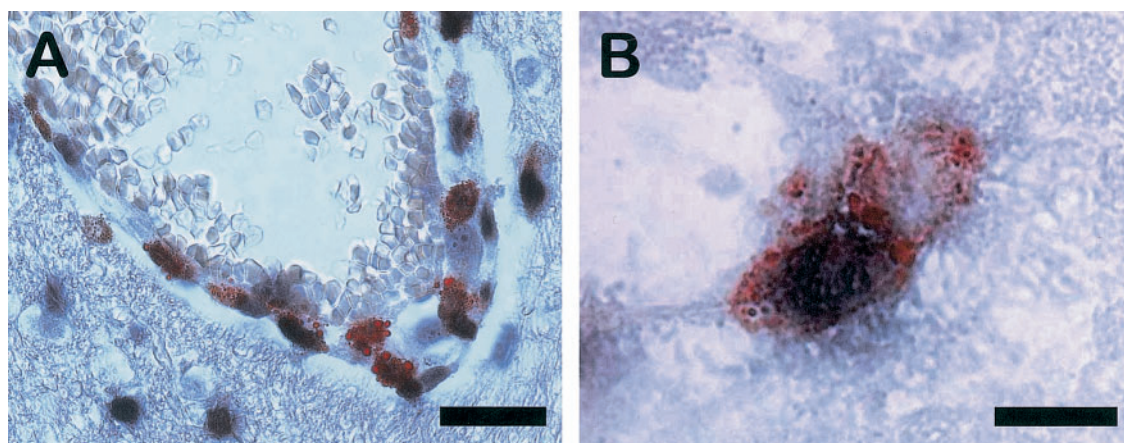


Fig. 2. Dual staining by *in situ* PCR for OvHV-2-infected cells and immunohistochemistry for cell-specific markers. (A) Dual staining for OvHV-2 and CD3, showing numerous OvHV-2-infected cells that are also positive for CD3 in a formalin-fixed, paraffin-embedded section of brain white matter from the MCF-infected calf. OvHV-2-infected cells are indicated by the purple staining of nuclei and CD3⁺ cells are indicated by red staining of the cytoplasm. Bar, 35 µm. (B) A dual-stained cell positive for both OvHV-2 and CD8 in a frozen section of brain white matter from the MCF-infected bison. An OvHV-2-infected cell is indicated by the purple-stained nucleus and the CD8 antigen is indicated by the red staining of the cytoplasm. Bar, 10 µm.

virus or viral protein expression in the vascular or lymphoproliferative lesions and thus hypothesize indirect mechanisms such as autoimmunity or lymphocyte dysregulation (Plowright, 1990; Rossiter, 1985; Schock & Reid, 1996). Although the mechanisms of CD8⁺ lymphocyte recruitment and tissue damage are still unknown, the findings of this study demonstrate that the predominant infiltrating cells in the lesions of acute MCF are infected with OvHV-2. These results make more plausible the possibility that the pathogenesis of this disease is primarily related to direct virus–cell interactions or perhaps immune-mediated responses directed against infected cells.

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