The Effects of Pharmacological and Lentivirus-Induced Immune Suppression on Orbivirus Pathogenesis: Assessment of Virus Burden in Blood Monocytes and Tissues by Reverse Transcription-In Situ PCR

SCOTT J. BRODIE,^{1,2*} WILLIAM C. WILSON,² PATRICIA M. O'HEARN,¹ DAVID MUTHUI,¹
KURT DIEM,¹ AND LEONARD D. PEARSON³

Virology Division/Retrovirology Laboratory, University of Washington School of Medicine, Seattle, Washington 98144¹; Arthropod-Borne Animal Disease Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Laramie, Wyoming 82071²; and Department of Microbiology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523³

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We investigated the effects of pharmacological and lentivirus-induced immunosuppression on bluetongue virus (BTV) pathogenesis as a mechanism for virus persistence and induction of clinical disease. Immunologically normal and immunosuppressed sheep were infected subcutaneously with BTV serotype 3 (BTV-3), a foreign isolate with unknown pathogenicity in North American livestock, and with North American serotype 11 (BTV-11). Erythrocyte-associated BTV RNA was detected earlier and at greater concentrations in sheep treated with immunosuppressive drugs. Similarly, viral RNA and infectious virus were detected in blood monocytes earlier and at higher frequency in immunosuppressed animals: as many as 1 in 970 monocytes revealed BTV RNA at peak viremia, compared to <1 in 10⁵ monocytes from immunocompetent sheep. Animals infected with BTV-3 had a higher virus burden in monocytes and lesions of greater severity than those infected with BTV-11. BTV RNA was detected by in situ hybridization in vascular endothelial cells and cells of monocyte lineage, but only in tissues from immunocompromised animals, and was most abundant in animals infected with BTV-3. In contrast, reverse transcription-in situ PCR showed BTV RNA from both viral serotypes in high numbers of tissue leukocytes and vascular endothelial cells from both immunosuppressed and, to a lesser extent, immunocompetent animals. Collectively, these findings show that BTV infection is widely distributed during acute infection but replication is highly restricted in animals with normal immunity. These findings also suggest that in addition to virulence factors that define viral serotypes, immunosuppression could play a role in the natural history of orbivirus infection, allowing for higher virus burden, increased virus persistence, and greater potential for acquisition of virus by the arthropod vector.

Bluetongue virus (BTV) is the causative agent of bluetongue, a noncontagious, often sporadic disease of domestic and wild ruminants (3). BTV represents 1 of 13 serogroups in the genus *Orbivirus*, family *Reoviridae*. All members of this genus replicate in the cytoplasm of infected cells (12) and have a double-layered protein capsid consisting of seven polypeptides, each of which is encoded by one of 10 double-stranded RNA viral segments (12). In temperate climates, outbreaks of BTV occur seasonally in association with the arthropod vector of the *Culicoides* species (51). The outcome of infection varies between animals, ranging from subclinical or mild disease to acute and fatal disease. Acute disease, as seen in sheep and some wild ruminants, is characterized by inflammation, hemorrhage, and/or necrosis of mucosal surfaces in the oronasal and alimentary systems (6). Animals that survive acute infection may develop chronic dermatitis and vesicular and erosive lesions at interdigital and mucosal surfaces.

Prolonged viremia occurs in many BTV-infected animals and persists despite a rapid and specific humoral (35, 48) and cellular (30) immune response. Although BTV is ultimately eliminated, mechanisms allowing for prolonged infection are

poorly understood. Unlike most single-stranded RNA viruses, orbiviruses are genetically and antigenically stable throughout infection (14, 18, 19, 29). Genetic recombination can occur via BTV gene segment reassortment; however, point mutations (viral escape mutants) do not arise in vivo, at least at the high frequency noted with many nonsegmented single-stranded RNA viruses. Still, there is strong evidence for pathogenetic differences in BTV isolates (45); however, it is not clear if these differences result from variations in the virus or if host factors that determine susceptibility to infection ultimately determine the outcome of infection.

BTV binds the surface of erythrocytes, and cell-associated viral RNA can be detected for several months following infection, usually long after infectious virus has been eliminated (36). Virus and viral antigens are internalized within erythrocyte vesicles and concealed from the immune response. This likely contributes to virus persistence but does not explain the wide variation in the host response to infection. Recently, massive covert infection with epizootic hemorrhagic disease virus, an orbivirus and close relative of BTV, was shown to precede virus-specific immunity and to facilitate rapid disease progression (11). Animals with a high viral burden that did not succumb to infection had weakened immune responses and ultimately developed severe diseases. Deficiencies in BTV immunity could explain the differential pathogenesis of BTV, including why prolonged viremia is observed in some animals and not others.

* Corresponding author. Mailing address: University of Washington School of Medicine, Department of Laboratory Medicine, Vaccine/Virology Division, Room T293X, Seattle, WA 98195. Phone: (206) 685-6894. Fax: (206) 685-3639. E-mail: sjbrodie@u.washington.edu.

TABLE 1. Experimental groups

Group	Animal no.	Virus inoculum ^a	Antibody titer ^b on day:			
			6	8	10	12
I (pharmacologically immunosuppressed)	16	BTV-3	—	1:1	1:100	1:1,000
	18	BTV-3	—	1:1	1:10	1:1,000
	19	BTV-3	—	—	1:10	1:100
	24	BTV-3	1:1	1:10	1:1,000	>1:1,000
	20	BTV-11	—	1:1	1:100	>1:1,000
	23	BTV-11	—	—	—	1:100
	28	BTV-11	—	1:1	1:100	1:1,000
	29	BTV-11	—	—	1:100	1:1,000
II (lentivirus induced immunosuppression)	22	BTV-3	—	1:1	1:10	1:10
	525	BTV-3	1:1	1:10	1:1,000	>1:1,000
	1416	BTV-3	—	—	1:10	>1:1,000
	9336	BTV-3	1:1	1:100	1:100	>1:1,000
	14	BTV-11	—	1:1	1:10	1:100
	487	BTV-11	—	1:1	1:100	1:1,000
	3678	BTV-11	—	1:100	1:1,000	>1:1,000
	6858	BTV-11	—	1:10	1:10	1:100
III (immunocompetent, clinically normal)	13	BTV-3	1:1	—	1:10	1:100
	17	BTV-3	—	1:100	1:100	1:100
	25	BTV-3	—	1:1	1:100	>1:1,000
	27	BTV-3	—	1:1	1:10	1:100
	21	BTV-11	—	1:10	1:1,000	>1:1,000
	26	BTV-11	1:1	1:10	1:100	1:100
	30	BTV-11	1:1	1:100	1:100	>1:1,000
	31	BTV-11	—	—	1:100	1:1,000

^a Animals were infected by subcutaneous injection of 10⁵ TCID₅₀ of BTV-3 and BTV-11.

^b Serial 10-fold dilutions of sera were analyzed for antibody to the BTV inner capsid antigen VP7. Results are presented as the maximal titer to which antibody could be detected by ELISA. —, antibody not detectable. No antibody was detectable in any animal on day 4.

Impairment of the immune system by drugs (33, 40), environmental contaminants (20, 33), or UV radiation (27) or by pathogens such as human immunodeficiency virus (43) and measles virus (50, 52) are just a few examples of conditions that are known to contribute to the severity of the disease state. In this study, we investigated the effects of pharmacological and retrovirus-induced immunosuppression in sheep as a mechanism of orbivirus persistence and disease induction. We demonstrated massive covert infection of vascular endothelium and cells of monocyte lineage and propose that widespread dissemination of virus during early stages of infection is augmented by immunosuppression and provides another mechanism for virus persistence and acute disease.

MATERIALS AND METHODS

Experimental design. Age (4.1 ± 0.2 years)- and breed (Rambouillet)-matched BTV-seronegative ewes were segregated into three groups (Table 1) and housed separately in barrier-maintained biocontainment level 3 facilities for 2 weeks prior to treatment and/or infection. Animals in group 1 (*n* = 8) were healthy and had immune responses, as described below, within normal ranges. These animals received immunosuppressive doses of dexamethasone (40 mg intramuscularly; Phoenix, St. Joseph, Mo.), azathioprine (Immunan; 200 mg orally; Glaxo Wellcome, Research Triangle Park, N.C.), and cyclophosphamide (Cytosan; 50 mg/m² orally; Bristol-Meyers Squibb, Princeton, N.J.) for 8 weeks prior to experimental BTV infection. Drug dosages for dexamethasone and cyclophosphamide were estimated based on veterinary formulary indexes for sheep and/or cattle. The dose of azathioprine was derived from that used for humans and adjusted for body surface area. It is important to note that these categories of drugs are not commonly used in food animals, and rumen metabolism may affect their absorption. During this time, animals were monitored for

clinical and laboratory indicators of drug-related toxicities that could be confused with BTV-mediated disease, including erythrocyte and platelet counts. Animals in group 2 (*n* = 8) were seropositive for ovine lentivirus (OvLV), and all showed classic signs of chronic lentivirus infection, including severe emaciation and interstitial pneumonia (7, 9, 10). Animals in group 3 (*n* = 8) were clinically normal and seronegative for OvLV, and they were not treated prior to experimental BTV infection. Half of the animals in each group were infected by subcutaneous inoculation with BTV (10⁵ 50% tissue culture infective doses [TCID₅₀]) serotype 3 (BTV-3), a Central American isolate with unknown pathogenicity in North American livestock. The remaining animals were inoculated with BTV serotype 11 (BTV-11), a serotype common to North America and an isolate obtained during an outbreak in Colorado (44, 46).

BTV infection in animals was determined by a rise in serum antibody and by detection of erythrocyte-associated viral RNA by using nested reverse transcription (RT)-PCR (14). Animals were monitored for fever and clinical signs of BTV infection which included edema, cyanosis, congestion, and/or icterus of mucous membranes, as well as hemorrhage from oral, nasal, rectal, and urogenital cavities (signs associated with BTV-induced vasculitis). Those with severe clinical signs were euthanized by intravenous overdose of pentobarbital (100 mg/kg of body weight). The remainder of animals were sacrificed at peak pyrexia and/or viremia or allowed to resolve clinical signs and then assessed for duration of virus persistence. All animals were kept in accordance with the guidelines prepared by the committee on the Care and Use of Laboratory Animals, National Research Council.

Clinical samples. Tissue biopsy samples, including oral mucocutaneous skin (obtained by punch biopsy), bone marrow from the iliac crest (obtained by needle aspiration), prescapular lymph node (obtained by tru-cut biopsy), and peripheral blood were collected at 2-day intervals for the first 2 weeks following infection and every 4 days thereafter until death. All procedures were performed under sedation and local anesthesia. Tissues were also collected at the time of death and included peripheral blood, skin (coronary band and oral mucocutaneous juncture), skeletal muscle (tongue), right frontal cerebral cortex, midbrain, spinal cord (second cervical vertebra), right caudal lung lobe (including pulmonary artery), trachea, tonsil, heart, spleen, kidney, liver, urinary bladder, lymph nodes (submandibular, mediastinal, prescapular, supramammary, and mesenteric), rumen, abomasum, and small and large bowel. Citrated blood samples were obtained from all animals prior to inoculation and following infection, and plasma was separated from cells by centrifugation and stored frozen at -70°C. Mononuclear leukocytes, which included purified monocytes (discussed below), and erythrocytes were added to an equal volume of buffered lactose peptone (50 mM Na₂PO₄, 10 mM NaH₂PO₄, 0.2% peptone, 10% lactose) and stored at 4°C for preservation of virus infectivity. Tissues were fixed for 48 h in 4% deionized paraformaldehyde. Paraffin-embedded sections were examined for viral RNA by *in situ* hybridization and RT-*in situ* PCR. Tissues were also snap-frozen in OCT (optical cutting temperature) compound (Miles Inc., Elkhart, Ind.) for detection of viral antigens by immunohistochemistry.

Measurements of immunosuppression. A variety of tests were used collectively to assess lentivirus- and drug-induced immunosuppression, including (i) complete blood cell counts, (ii) plasma immunoglobulin G (IgG) and IgM concentrations, (iii) peripheral blood lymphocyte (PBL) proliferative response to phytohemagglutinin (PHA-P; Sigma Chemical, St. Louis, Mo.), (iv) PBL CD4/CD8 ratios, and (v) quantitative expression of interleukin-2 receptor (IL-2R) (CD25) and major histocompatibility class II (MHC-II) DR, DQ antigens in PHA-stimulated PBL. Immunological and hematological tests were performed in all animals (groups 1 to 3) prior to BTV infection (Table 1) and at 8 weeks following drug treatment (group 1). Plasma immunoglobulin concentrations were determined by radial immunodiffusion using commercial kits (Bethel Laboratories, Montgomery, Tex.). Peripheral blood mononuclear cells were separated on Histopaque (specific gravity, 1.077; Sigma), and adherent cells were depleted by adhesion to fibronectin-coated (20 µg/ml; Gibco, Grand Island, N.Y.) plastic surfaces (8). Greater than 94% of nonadherent cells were shown to be PBL based on reactivity with monoclonal antibodies (MAbs) to T cells (clone SBU-T1; University of Melbourne, Victoria, Australia) and B cells (clone B-B2; Veterinary Medical Research and Development [VMRD], Pullman, Wash.). Conversely, adherent cells were >96% CD14 positive (clone M; VMRD) and were designated monocytes. PBL (10⁶/ml) were stimulated with PHA (5 µg/ml) for 24 and 48 h, and IL-2R (clone A5/IL-2R; VMRD) and MHC-II (clone 28.1; VMRD) expression was determined by flow cytometry (EPICS V; Coulter Corp., Hialeah, Fla.). The peripheral blood CD4/CD8 ratio was determined similarly, using MAbs to the ovine CD4 (SBU-T4 clones 44.38 and 44.97; University of Melbourne) and CD8 (SBU-T8 clone 38.65; University of Melbourne) human homologs. Lastly, lymphocyte proliferation in response to PHA stimulation was assessed by [³H]thymidine (³HT; Amersham Corp., Arlington Heights, Ill.) incorporation and as described previously (25). Briefly, 2 × 10⁶ PHA-stimulated (24 and 48 h of stimulation) and unstimulated PBL were incubated with 1 µCi of ³HT (18 h, 37°C, 5% CO₂). Cell proliferation was a measurement of ³HT uptake and was assessed by liquid scintillation beta particle emission (Packard Instrument Co., Grove, Ill.).

Viruses. Animals were infected by subcutaneous injection with 10⁵ TCID₅₀ of BTV-3 (Central American strain) or BTV-11 (Colorado front-range strain) (Table 1). BTV-3 is exogenous to North America but found commonly in other parts of the world and for purposes of this study was isolated from *Culicoides insignis*

in Central America (41). Unlike BTV-11, which was isolated from sheep during a disease outbreak in North America, the pathogenesis of BTV-3 in North American livestock is not known. The virus inocula were prepared in baby hamster kidney (BHK) cells, and first-passage cell culture supernatants were used for animal and cell culture inoculation. The concentration of viruses used for animal inoculations was based on previous animal infection studies (46).

Animals with classical signs of lentivirus infection were shown to harbor OvLV by methods described previously, including virus isolation (8) and *in situ* hybridization (9, 10). To assess the effects of lentivirus infection on BTV replication, monocytes were infected *in vitro* with OvLV strain 85/34 (37) and then later coinoculated with BTV-3 or BTV-11. All animal inoculations and laboratory procedures utilizing foreign viruses were performed in a biocontainment level 3 facility.

Plasma antibody. Plasma was assessed for BTV and OvLV antibodies by indirect enzyme-linked immunosorbent assay (ELISA) (Veterinary Diagnostic Technology, Inc., Wheat Ridge, Colo.). In addition, serial twofold dilutions of thawed plasma starting with a 1:100 dilution were used to estimate antiviral antibody concentrations to BTV-3 and BTV-11 nonstructural (NS1, NS2, and NS3) and major structural (VP3, VP7, VP2, and VP5) proteins by immunoblot analysis using methods described previously (8). Antibody titers were expressed as the reciprocal of the highest dilution of a sample which revealed the specific band of the viral polypeptide.

***In vitro* analysis of viral coinfection.** Peripheral blood was obtained from retrovirus- and orbivirus-free sheep (21-95, 22-95, and 23-95). Monocytes were isolated and purified as described earlier and applied at 10^6 cells per well in fibronectin-coated eight-quadrant chamber slides. The cells were treated with phorbol 12-myristate 13-acetate (1 ng/ml; Sigma) and then maintained in RPMI 1640 medium (Gibco) supplemented with 100 U of penicillin, 100 μ g of streptomycin, and 0.25 μ g of amphotericin B per ml, 2 mM L-glutamine, 25 mM HEPES buffer, 5 μ M 2-mercaptoethanol, 10% fetal calf serum, and 10% conditioned medium (F0165 monocyte/macrophage maintenance medium; Pan Data Systems, Rockville, Md.). Under these conditions, monocyte-derived macrophages were shown to maintain viability for at least 21 days.

Approximately 20 ng of viral capsid antigen (OvLV at a multiplicity of infection [MOI] of ~0.02) derived from OvLV strain 85/34 was applied to cultures of 10^6 ovine monocytes. After 2 h of absorption, the cultures were washed and incubated for another 48 h, and infection was verified by immunocytochemistry as described previously (7, 10). Monocyte cultures were then inoculated with 10^5 TCID₅₀ of BTV-3 or BTV-11. Following 2 h of incubation with BTV, the cultures were washed, and virus infection and replication were assessed at 24-h intervals by end-point RT-PCR (11) and antigen capture ELISA (37, 39), respectively. The lower limits of detection were 10 virus copies per 50- μ l sample by RT-PCR and 0.3 ng of viral capsid antigen per ml by capture ELISA.

Gross and microscopic pathology. Animals were euthanized at the height of clinical disease and/or peak viremia, and gross lesions were identified. Paraformaldehyde-fixed, paraffin-embedded tissues were sectioned (5 μ m) by routine methods, stained with hematoxylin and eosin, and examined by incident light microscopy.

Virus burden in blood monocytes and tissues. Experiments were performed to estimate the virus burden in peripheral blood monocytes and selected tissues from sheep naturally infected with OvLV (group 2) and those experimentally infected with BTV-3 and BTV-11 (Table 1, groups 1 to 3).

(i) **Virus isolation.** Isolation of BTV from peripheral blood erythrocytes and monocytes and from biopsy tissues of bone marrow, skin, and prescapular lymph node at sequential time points following infection was attempted. Tissues were also collected at postmortem for virus isolation and included brain, cervical spinal cord, heart, lung, mediastinal lymph node, spleen, kidney, liver, rumen, and small intestine. Monolayer cultures (25-cm² tissue culture flasks) of BHK cells were inoculated with lysates of bone marrow and erythrocytes (10^7 cells), solid tissues (0.5 cm³), or serial fourfold dilutions of monocytes (starting at 8×10^6 cells). The cultures were maintained in RPMI 1640 medium as described earlier. The monolayers were observed daily for cytopathic effects, passaged at 6-day intervals, and maintained for a minimum of 18 days or until cytopathic effects were observed. The cells were then harvested (1% trypsin and sodium EDTA) and cytocentrifuged to glass microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, Pa.). The slides were treated with 100% acetone for 10 min at 4°C, air dried, and then reacted with digoxigenin (DIG)-labeled RNA probes to BTV-10 as described previously (11).

(ii) **Solution-based RT-PCR.** Serial fourfold dilutions of peripheral blood erythrocytes and monocytes (starting with 8.0×10^6 cells in triplicate) were prepared as lysates by incubating the diluted cells in 20 μ g of proteinase K followed by 1 $\times$ PCR buffer containing 0.45% Tween 20. Viral RNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1; United States Biochemical, Cleveland, Ohio), and nested RT-PCR was performed as described previously (11). Briefly, double-stranded viral RNA was denatured by using 50% formamide (United States Biochemical) and heat (70°C, 10 min). Sample RNA was added to the RT reaction mix (RT-PCR kit; Perkin-Elmer, Norwalk, Conn.) containing both the antisense (BT10N1-1164C)- and sense (BT10N1-107)-strand primers (see below) and then incubated at 42°C for 1 h followed by 95°C for 5 min. The oligonucleotide primers used in the RT reaction and PCR were designed based on conserved sequence within the BTV gene segment 6 (49). The conditions of the first PCR were 95°C for 0.5 min, 52°C for 1 min, 72°C for 2 min,

and 5 min in the final cycle, with a total of 40 cycles. The second PCR consisted of 25 cycles under the same conditions of the first PCR. Cloned (pGEM vector kit; Promega, Madison, Wis.), and purified cDNA from gene segment 6 of BTV-10 was used as a positive control. Additional negative controls consisted of sterile water, erythrocyte lysates from sheep negative for BTV by serology, and plasmid vector DNA. Amplification products were resolved by electrophoresis in 2% agarose gels, blotted to Gene-Screen Plus nylon membranes (DuPont NEN, Wilmington, Del.), and hybridized with BTV-specific DIG-11-dUTP-labeled DNA probes designed to be internal to the amplification product. BTV-10-infected and uninfected BHK cells and DIG-labeled plasmid DNA (pGEM; 1 kb) were used as controls for all hybridization reactions.

(iii) **Immunocytochemistry.** MAbs to conserved epitopes to capsid proteins of BTV VP7 (50 μ g/ml; 10D4.90, IgG1) and OvLV p25 (50 μ g/ml; 3F, IgG1) were reacted with chamber slide preparations of monocytes and snap-frozen cryostat-sectioned tissues. The procedures utilized an avidin-biotin-peroxidase complex technique with diaminobenzidine as the chromogen (ABC Vectastain kit; Vector Laboratories, Burlingame, Calif.), performed by methods described previously (7, 8). Irrelevant MAbs (IgG1) to bovine herpesvirus 1 glycoprotein, BTV-11-infected and uninfected BHK cells, OvLV strain 85/34-infected and uninfected goat synovial cells, and a variety of tissues from sheep that were negative for BTV and OvLV by serology and PCR were used as controls.

(iv) ***In situ* hybridization.** A full-length copy of the coding region of gene segment 6 from BTV-10 (1,769 bp; GenBank accession no. Y00422) was cloned into a transcription vector under the control of T7 and SP6 RNA polymerase promoters (PCR II; Invitrogen, San Diego, Calif.). The construct was linearized and used as a template for transcription using T7 and SP6 polymerase and DIG-labeled ribonucleotides (DIG RNA labeling mix; Boehringer Mannheim, Indianapolis, Ind.). The resulting transcripts were precipitated with ethanol and quantified spectrophotometrically. The specificity and working concentrations of the final products were determined by dot blot analysis (11). Probes derived from BTV-10 were shown to react with BTV-3 and BTV-11 with high affinity. Experiments were also performed to evaluate the sensitivity of BTV-specific RNA probes. Briefly, BHK cells were infected with BTV-10 at MOIs of 0.1, 1, and 10 for 1, 4, 12, and 24 h. Cytocentrifuged preparations of fixed (Permeafix) cells containing known copy numbers of virus were then hybridized to BTV-specific riboprobes. Using this technique, cells containing ≥ 20 virus copies could be detected. In addition, full-length cDNA from the OvLV p25^{gus} (visna virus strain 1514) was labeled with ³⁵S by random priming (Boehringer Mannheim). Probe construction and hybridization conditions, including controls, were as described previously (9, 10). OvLV probe sensitivity approximated 20 virus copies.

Tissue sections were deparaffinized, digested with proteinase K (20 μ g/ml; 60 min at 37°C; Sigma), rinsed in diethyl pyrocarbonate-treated water, coverslipped, and then heated to 70°C, and BTV RNA probes (sense and antisense) were applied at a final concentration of 5 ng in a solution containing hybridization mix and formamide (1:1). Hybridization was performed overnight at 42°C in a humidified chamber. Following incubation, the slides were rinsed and blocked with a commercial reagent (Boehringer Mannheim) supplemented with 5% horse and 5% sheep serum. Alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) was diluted 1:100 (1,500 mU/ml) in phosphate-buffered saline and applied to tissues for 30 min at room temperature. The slides were then reacted with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate for 10 to 30 min. The presence of viral nucleic acid was indicated by a purple cell-associated precipitate. In addition, combined *in situ* hybridization for OvLV, using ³⁵S-labeled DNA probes, and immunohistochemistry for ovine monocytes/macrophages (CD14; VMRD) was performed on selected tissues as described previously (9, 10). Cytospin preparations of BTV-10-infected and uninfected cultured BHK cells, OvLV-infected and uninfected goat synovial membrane cells, and tissue from sheep that were negative for BTV and OvLV by PCR were used as controls. DIG-labeled plasmid DNA and antisense RNA probes from epizootic hemorrhagic disease virus serotype 2 (gene segment 6) were used as negative controls for all hybridizations.

(v) **PCR-driven RT *In Situ* hybridization.** Following deparaffinization, tissue sections were rehydrated, washed in diethyl pyrocarbonate-treated water, and treated overnight at 37°C in a RNase-free DNase I solution (Boehringer Mannheim). The sections were then treated with the RT mix according to the manufacturer's recommendations (RT-PCR kit; Perkin-Elmer). Conditions for *in situ* PCR were similar for both BTV and OvLV. Briefly, a solution containing 10 $\times$ PCR buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3]), 4 mM MgCl₂, 0.01% gelatin, 200 μ M deoxynucleoside triphosphates, 50 pM each primer, and *Taq* polymerase (0.15 U/ μ l) was made. The PCR primers were specific for BTV gene segment 6 (BT10N1-107, 5'-3' [TCACCACATGGACTTGACGTCAC]; BT10N1-1164C, 3'-5' [CGACGCCGGTACAGAGTCTACA]) and OvLV p25^{gus} gene (VVgpr.i3, 5'-3' [ACATAGGGAAGTTTACCCTATTGTG]; VVgpr.i4, 3'-5' [TCTATCCCAGGCATCATAACCAATAC]). Both resulted in amplification products of about 300 bp. The PCR mixture was then added to tissue sections in volumes that ranged from 40 to 60 μ l, depending on the size of the section, and coverslipped. Coverslips were anchored with nail polish, and edges were covered with mineral oil to prevent evaporation. The slides were then placed directly on the aluminum block of a thermocycler (OmniGene model HB-OS-BB; Hybaid, Woodbridge, N.J.). After 30 cycles, each consisting of denaturation at 94°C for 1 min and annealing at 55°C for 2 min, followed by polymerization for 2 min at 72°C, the slides were removed and treated for 5 min

FIG. 1. Localization of OvLV in chronic lymphocytic inflammatory lesions (lung; bars = 100 μ m). (A) Viral transcripts localize to interstitial macrophages (arrows) adjacent to lymphoid aggregates (f) in lesions of lymphocytic interstitial pneumonia (animal 9336, in situ hybridization [35 S-DNA]); (B) cellular localization of OvLV proviral DNA by in situ PCR (arrows) to areas adjacent to lesions of proliferative lymphocytic hyperplasia (f) (animal 525, anti-DIG-alkaline phosphatase).

with xylenes to remove mineral oil and for 5 min in 100% ethanol and then air dried. Amplified DNA was detected by hybridization with DIG-labeled RNA probes specific for full-length BTV gene segment 6 or DIG-labeled DNA probes specific for full-length OvLV p25^{gag} (as for in situ hybridization). Both probes were internal to the amplification product, and the BTV RNA probe was transcribed in sense orientation. The remainder of the procedure, including controls, was as described for in situ hybridization.

Data analysis. Data are presented as the mean $\pm$ standard error of the mean. Differences between two independent groups were evaluated by using the Mann-Whitney *U* test. Differences between more than two independent groups were evaluated by using the Kruskal-Wallis rank-sum test. Statistical analysis was performed with StatView 4.0 (Abacus Concepts, Berkeley, Calif.) software and a Macintosh Centris 650 computer. *P* values of <0.05 were considered significant.

RESULTS

Measurements of immunosuppression. Treatment groups are identified in Table 1. Animals treated with immunosuppressive drugs demonstrated minor elevations in peripheral blood neutrophils ($9.6 \pm 1.4 \times 10^3/\mu$ l), a slight decrease in lymphocytes ($1.6 \pm 0.6 \times 10^3/\mu$ l), and lowered serum cortisol concentrations ($1.2 \pm 0.4 \mu$ g of baseline cortisol/dl) within 2 months following drug treatment (all values were less than the reported standard mean). The leukogram and biochemical profiles of lentivirus-infected sheep, including serum cortisol, were within normal limits, although there was an inversion of the normal CD4 to CD8 ratio (22.3% CD4/43.6% CD8) in three (525, 9336, and 3678) of six OvLV-seropositive sheep. Both drug-treated and lentivirus-infected animals had depressed lymphocyte proliferative responses to PHA ($1,593 \pm 304$ cpm) compared to untreated animals ($45,314 \pm 1,230$) ($P < 0.001$). Similarly, immunosuppressed animals showed reduced expression of the cell surface antigens IL-2R (23.4 ± 5.3) and MHC-II (54.8 ± 9.7) compared to uninfected animals (38.5 ± 6.9 and 74.6 ± 11.2 , respectively) ($P < 0.05$). Plasma IgM and IgG concentrations were similar between all groups.

Characterization of chronic-productive OvLV infection. In addition to inverted CD4/CD8 ratios and depressed lymphocyte proliferative responses, all OvLV-seropositive animals had clinical signs of emaciation, lymphadenopathy, and respiratory distress. Postmortem histopathological findings included widespread lymphocytic infiltrative and proliferative lesions: lesions characteristic of chronic OvLV infection and suggestive of immune dysfunction (9). OvLV RNA localized in

high copy number to CD14-bearing cells (monocytes/macrophages) within the pulmonary interstitium (Fig. 1A) and within the prescapular and mediastinal lymph nodes. In situ PCR revealed OvLV DNA in high numbers of monocytes/macrophages (CD14), which were found in greatest density within the pulmonary interstitium and proximal to lymphoid aggregates (Fig. 1B).

The prescapular lymph node was biopsied at sequential time points and examined histologically for the effects of concurrent OvLV infection on BTV replication and disease severity in situ. Although both OvLV and BTV infect monocytes, there was no evidence that OvLV and BTV colocalized to mononuclear cells in situ. Similarly, when primary monocyte cultures were coinfecting with OvLV and BTV, there was no evidence of interference with the replication of either virus as assessed by capture ELISA and no evidence of viral coinfection by combining immunocytochemistry with in situ hybridization (data not shown).

Effects of immunosuppression on BTV pathogenesis. Peripheral blood and biopsy tissues were collected at sequential time points following BTV infection and then assessed for viral nucleic acid, viral proteins, and infectious virus. Because we found no difference when comparing the input virus (BTV-3 and BTV-11) and concentrations of BTV RNA in erythrocytes and monocytes ($P < 0.05$), results are presented as a summation of both virus serotypes within the specific treatment group (Fig. 2 and 4). In contrast, there were differences in BTV-3 and BTV-11 replication in monocytes; thus, these data are presented independently (Fig. 5).

Kinetics of infection in sequential blood and biopsy samples. The times from subcutaneous inoculation to first detection of virus in peripheral blood, and concentrations of virus in blood, were compared between different treatment groups. The presence of erythrocyte-associated viral RNA (Fig. 2) and erythrocyte-associated infectious virus (Fig. 3) was determined. Because viral RNA was found in peripheral blood, in most cases within 24 h of inoculation, initial detection was not dependent on amplification within regional lymph nodes. Although erythrocyte-associated BTV RNA was rapidly detected in the blood of animals in all experimental groups, viral RNA was detected at highest concentrations in animals that received

FIG. 2. Detection of peripheral blood erythrocyte-associated BTV RNA following experimental BTV infection. Viral RNA was extracted from endpoint dilutions of peripheral blood erythrocytes that were collected at sequential time points and then assessed for cell-associated BTV RNA by RT-PCR. Erythrocyte-associated viral nucleic acid was detected in most animals from all treatment groups within 24 h following subcutaneous inoculation. The numbers of erythrocytes yielding viral RNA were significantly less at all time points in animals that had received immunosuppressive drugs ($P < 0.05$), suggesting that these animals had higher virus concentrations in blood. Animals were organized by treatment groups, and results represent mean $\pm$ standard error of the mean of combined BTV serotypes.

immunosuppressive drugs (Fig. 2; $P < 0.05$). Infectious virus was also bound to erythrocytes but was not detected for at least 2 to 3 days following infection and was most abundant in animals that had been treated with immunosuppressive drugs or had chronic OvLV infection (Fig. 3).

Peripheral blood monocytes were isolated at sequential time points following infection and assessed for BTV RNA by RT-PCR (Fig. 4), and infectious virus was analyzed by cocultivation with an indicator cell line (Fig. 3 and 5). The kinetics of BTV infection and virus burden in monocytes was compared between treatment groups and between animals infected with BTV-3 (Fig. 5A) and BTV-11 (Fig. 5B). Monocyte-associated BTV was detected earlier and in higher cell numbers (at all time points) in immunosuppressed animals. As many as 1 in 970 monocytes revealed either BTV RNA (Fig. 4) or infectious virus (Fig. 5) at peak viremia (6 to 8 days following infection), compared to ≤ 1 in 10^5 monocytes from untreated clinically normal sheep ($P < 0.01$). Immunosuppressed animals that were infected with BTV-3 had higher numbers of monocytes yielding infectious virus (Fig. 5A) at all time points compared

FIG. 3. Time at which infectious BTV was first recovered from clinical samples. Peripheral blood erythrocytes (RBC), blood monocytes, and biopsy tissues were collected at 2-day intervals following experimental BTV infection and cocultivated with BHK cells as an indicator cell line. Infection was determined in cytoplasmic preparations of BHK cells by in situ hybridization to DIG-labeled BTV-specific riboprobes. Animals were organized by treatment groups, and results represent mean $\pm$ standard error of the mean of combined BTV serotypes.

FIG. 4. Quantification of BTV RNA in peripheral blood monocytes. Viral RNA was extracted from endpoint dilutions of CD14⁺ monocytes collected at sequential time points following experimental infection and then assessed by RT-PCR. The numbers of monocytes yielding BTV RNA were significantly less ($P < 0.05$) at all time points in animals that had received immunosuppressive drugs and at most time points in animals with concurrent lentivirus infection. BTV burden declined sharply around the time virus-specific antibody was first detected (*). Animals were organized by treatment groups and results represent mean $\pm$ standard error of the mean of combined BTV serotypes.

to those infected with BTV-11 (Fig. 5B) ($P < 0.05$). Activated lymphocytes have also been reported as target cells for BTV (24), although our preliminary studies did not confirm these findings. BTV RNA was rarely detected by PCR in cultured

FIG. 5. Quantification of infectious BTV in peripheral blood monocytes of animals infected with BTV-3 (A) and BTV-11 (B). PBL expressing the CD14⁺ surface antigen were isolated, quantified by limiting dilution, and then cocultivated with an indicator cell line. Infection was first determined by in situ hybridization with DIG-labeled BTV RNA to cytoplasmic preparations of BHK cells. It took lesser numbers of monocytes to recover infectious BTV from animals treated with immunosuppressive drugs and those with concurrent lentivirus infection compared to untreated animals ($P < 0.05$). Immunosuppression resulted in an increased virus burden at all time points and occurred with both BTV serotypes. However, immunocompromised animals that were infected with BTV-3 maintained a higher virus burden throughout the course of infection. Asterisks indicate when BTV-specific antibody was first detected in plasma. Results represent mean $\pm$ standard error of the mean.

lymphocytes (cultures with >99% CD2⁺ cells), and infectious virus was never isolated from this cell population in assays using the same indicator cell line as for monocytes.

The kinetics of BTV infection, including time from infection to first detection in target tissues, virus replication, and tissue distribution of virus, were investigated in biopsy samples from bone marrow, lymph node, and skin collected at 2-day intervals following infection (Fig. 3). Erythrocytes act as carrier cells for BTV, and they were the first cell type from which infectious virus was isolated (presumably by attachment to the cell surface). Virus was next detected in peripheral blood monocytes, followed by prescapular lymph node and bone marrow, and then much later in skin. There were no differences between animals infected with BTV-3 and BTV-11 in the ability of the virus to target specific tissues ($P > 0.05$) and no differences between treatment groups with respect to isolation of virus from erythrocytes and monocytes ($P > 0.05$). However, there were differences between treatment groups in the ability to isolate infectious virus from bone marrow, lymph node, and skin. Virus was detected earlier in immunosuppressed animals (both groups 1 and 2) than in those left untreated. Interestingly, BTV was never detected in the skin of untreated (immunocompetent) animals.

Serological and pathological features. Plasma antibody to BTV could be detected as early as 8 days following infection in most animals. The concentration of specific antibody and antibody reactivity to specific viral proteins did not vary between treatment groups or between animals infected with different BTV serotypes ($P > 0.05$ [Table 1]). Clinical signs were generally mild and sporadic and included lethargy, fever, and lameness. Gross pathological changes included widespread edema and erythema of mucous membranes and surrounding skin (mouth, anus, and urogenital tract), findings suggestive of acute vascular crises. Although gross postmortem lesions were generally mild and nonspecific, several animals (19, 24, 525, 3678, and 9336) showed mild petechial hemorrhages on the pleural surface of the lung, base of the pulmonary artery, and/or serosal surface of the rumen and reticulum, lesions characteristic of acute orbivirus infection. In contrast, histological lesions were common (Fig. 6) and were most severe in animals that had received immunosuppressive therapy and in sheep infected with BTV-3. We identified a wide variety of microscopic lesions, including acute endothelial hypertrophy associated with vascular stasis (Fig. 6A; most infected animals), pulmonary interstitial congestion and intra-alveolar hemorrhage (Fig. 6B; sheep 16, 24, 525, 1416, and 3678), and hemorrhage into the myocardium (Fig. 6C; sheep 16, 24, 525, and 9336) in association with vascular thrombosis and necrosis (Fig. 6D). Multifocal hemorrhage was also present in most lymph nodes (Fig. 6E; most immunosuppressed animals) and was found throughout the central nervous system (CNS), including the cervical spinal cord, midbrain, and cerebrum (Fig. 6F; sheep 18, 24, 525, 1416, 3678, and 9336). Surprisingly, none of the animals with CNS lesions showed gross neurological deficits. Hepatic (Fig. 6G) and renal (Fig. 6H) hemorrhage and/or congestion were found in most animals in all treatment groups. Again, signs of hepatic or renal failure were not observed. Of the few animals that were allowed to recover from acute infection, four (22, 24, 1416, and 9336) of eight had chronic inflammatory lesions in the myocardium (Fig. 7A) and/or skin at mucocutaneous junctures (Fig. 7B), and all four were immunosuppressed.

Localization of BTV proteins and transcripts. Cells and tissues from which BTV was isolated by cocultivation did not express the VP7 capsid antigen by immunochemistry. From these same samples, cell-associated viral RNA was rarely de-

tected by in situ hybridization and, when present, was found only in endothelial and mononuclear cells within lymphoid tissues (e.g., spleen, lymph nodes, palatine tonsil, and Peyer's patches) and only from animals with concurrent OvLV infection or those that had received immunosuppressive drugs. Furthermore, tissues from animals containing detectable viral RNA (i.e., ≥ 20 virus copies) were all infected with BTV-3. Interestingly, monocyte-derived macrophages expressed high levels of VP7 capsid antigen (Fig. 8A) following 48 h in culture. Collectively, these findings suggest that viral gene expression is restricted in the host, including animals with measurable immunosuppression.

Localization of BTV RNA by RT in situ PCR. In contrast to in situ hybridization, viral RNA was detected by RT-in situ PCR in vast numbers of vascular endothelium, particularly in thin-walled vessels, in the myocardium (Fig. 8B), pericardial sac, lung, CNS (cerebrum, midbrain, and spinal cord) and most lymphoid tissues from both immunosuppressed and, to a lesser extent, clinically normal animals. Infection of endothelium was frequently associated with hemorrhage attributable to microvascular degeneration. Viral transcripts were also localized within the cytoplasm of mononuclear leukocytes with morphological characteristics of monocytes/macrophages (Fig. 8C, inset) within the submandibular lymph node (Fig. 8C), palatine tonsil (Fig. 8D), spleen (Fig. 8E), Peyer's patches of the ileum (Fig. 8F), pulmonary interstitium (Fig. 8G), and bone marrow (Fig. 8H) and to cells lining hair follicles in areas of chronic inflammation, ulceration, and vesicle formation (Fig. 7B, inset). Viral RNA was also detected, although infrequently, in histologically normal tissues in association with vascular endothelium and/or resident mononuclear leukocytes.

Staining was predominantly extranuclear (Fig. 8C, inset), as would be expected with an RNA virus thought to have only a cytoplasmic replication cycle. Omitting PCR resulted in a much reduced or absent hybridization signal. Mispriming events, nonspecific hybridizations, and other potential causes of false reactions were discounted, because control samples gave predicted results, including cytoplasmic localization of viral RNA. Histologic sections from all tissue samples that were positive by RT-in situ PCR were subjected to protease-digestion prior to RNA extraction, and the presence of BTV RNA was confirmed by solution-based RT-PCR. Experiments using BHK cell culture infected with a specific MOI of BTV-3 and -11 showed that cells containing ≥ 20 genomic copies could be detected by in situ hybridization, and as few as one virus copy was detected by RT-in situ PCR. Collectively, these results show that RT-in situ PCR was specific for BTV and that virus burden was generally very low (i.e., < 20 virus copies per cell), yet infection was widespread and high numbers of individual cells were infected.

DISCUSSION

Because of the wide range in individual response to BTV infection, we investigated the effects of immunosuppression on virus pathogenesis in breed- and age-matched sheep experimentally infected with antigenically distinct BTV isolates. Erythrocyte-associated BTV RNA was detected by solution-based PCR usually within 24 h following infection, several days before infectious virus was first isolated, and remained at high levels for the duration of the study. The association of BTV with erythrocytes does not progress beyond virus adsorption. Instead, virions persist within invaginations of the cell membrane (36, 49). Presumably because of this virus-cell association, viremia (35 to 42 days) and antigenemia (160 days) are prolonged (2, 31). In contrast, peripheral blood monocytes can

FIG. 6. Representative histopathologic lesions in sheep experimentally infected with BTV following immunosuppressive therapy. (A and B, lung) Arteriole with fibrin thrombus (l) and hypertrophy of vascular endothelium (sheep 16, BTV-3) (A) and interstitial congestion and intra-alveolar (a) hemorrhage (sheep 24, BTV-3) (B). (C and D, heart) Intrafascicular myocardial hemorrhage (sheep 24, BTV-3) (C) and myocardial infarction associated with vascular thrombosis (l) and necrosis of the vessel wall (w) and surrounding cardiac muscle (sheep 24, BTV-3) (D). (E) Subcapsular (c) hemorrhage (arrow) of submandibular lymph node (sheep 20, BTV-11). (F) Hemorrhagic foci in cerebral cortex (sheep 18, BTV-3). (G) Severe hepatic congestion (sheep 23, BTV-11). (H) Severe intertubular hemorrhage and renal tubular necrosis (sheep 18, BTV-3). Hematoxylin and eosin; bars = 100 μ m.

FIG. 7. Chronic inflammation of the myocardium (A; sheep 24, BTV-3) and skin of the lower lip (B; sheep 29, BTV-11) 30 days following experimental infection with BTV-3. The inset in panel B shows BTV RNA localized to cells lining hair follicles (f) (anti-DIG-alkaline phosphatase). Hematoxylin and eosin; bars = 100 μ m.

be infected with BTV, as shown both *in vitro* (1, 24, 55) and *in vivo* (2, 17). However, the extent of infection in immunocompetent animals is thought to be minimal: usually less than 1 in 10^5 peripheral blood mononuclear cells harbors infectious BTV (17). Thus, the significance of monocyte infection in the pathogenesis of BTV is still largely unknown. Our results showed that monocytes are permissive to BTV infection, and animals with impaired immunity demonstrated significantly higher monocyte-associated virus burdens for a longer period of time. These findings suggest that immunosuppression could play a role in the natural history of orbivirus infection, allowing for increased virus persistence. Interestingly, differences were not observed when viral serotypes and RNA concentrations in monocytes were compared; yet BTV-3 replicated to a significantly higher titer in primary monocytes than did BTV-11. This finding suggests that both serotypes can enter monocytes but differ in capacity to replicate. Phenotypic differences in viral strains are common. Lentiviruses with “rapid-high” and “slow-low” growth potential have been described (37).

After an initial burst of virus replication in monocytes, replication ensued in regional lymph nodes, followed by release and systemic spread of virus to resident mononuclear leukocytes in the lung, CNS, and lymphoid and hematogenous tissues and to vascular endothelium in a wide variety of tissues. The profound differences in disease expression observed in different ruminant species following orbivirus infection are thought to be related to the extent of virus infection of endothelial cells (16, 35). Our findings support these earlier studies by demonstrating massive covert infection of vascular endothelium and mononuclear leukocytes, particularly in animals with impaired immunity. Widespread dissemination of BTV during early stages of infection may also provide a mechanism for accelerated disease and increased viral persistence by evasion of host immunity. Furthermore, because there was a difference between immunosuppressed and untreated animals in erythrocyte- and monocyte-associated virus burden and because these differences could not be accounted for by antigen-specific immune mechanisms, nonspecific mechanisms of immunity are likely to be important in controlling BTV in early infection. BTV infection of endothelium results in the rapid release of a variety of cytokines including gamma interferon (16, 26). Drug (33, 40)- and retrovirus (28)-induced immunosuppression is known to down-regulate cytokine gene expression and, in turn, may contribute to BTV persistence and/or pathogenesis.

Severe fluctuations in temperature, UV radiation, prolonged

transportation, and overcrowding are known to have an adverse affect on the ruminant immune system (42) by impeding the production of arachidonic acid and synthesis of leukotrienes and prostaglandins. Exogenous corticosteroids such as dexamethasone block cell membrane-associated phospholipase A_2 and prevent the production of arachidonic acid, which in turn affects leukopoiesis and leukocyte circulation. Alternatively, azothioprine is a purine analog that is quickly metabolized to the toxic derivative, 6-mercaptopurine. Its action affects predominantly T cells, although its onset of action is slow, often requiring greater than 1 month to take effect. In contrast, cyclophosphamide is a powerful alkylating agent. It causes rapid and severe immunosuppression by preferentially suppressing B cells. When these drugs are used in combination, as in the present study, a significant state of immunosuppression can be achieved. Animals treated with immunosuppressive drugs or those with chronic lentivirus infection, including those receiving high doses of cyclophosphamide, were shown to have normal antibody responses to BTV; however, most showed decreased lymphocyte proliferative responses to mitogens, decreased expression of cell surface antigens associated with cellular activation (MHC-II and IL-2R), and/or inverted CD4/CD8 lymphocyte ratios, all indicators of cellular immune dysfunction. Immunosuppression of mice by cyclophosphamide was shown to change the course of herpes simplex virus infection. Treatment affected dendritic cells and T cells, which normally prevent viral spread to the pancreas, CNS, and lymphoreticular organs (5). Similarly, immunosuppressed mice infected with murine herpesvirus had 2- to 3.5-times-higher viral burdens, and greater tissue distribution of virus-infected cells, and virus was recovered for longer periods of time (40).

OvLV infection is common in North America and has been associated with immune dysfunction (7, 9, 10). Infection has been reported to result in inverted CD4/CD8 T-lymphocyte ratios (32), decreased numbers of mature plasma cells within hyperplastic lymph nodes (21), decreased lymphocyte-generated IL-2 (22), decreased concanavalin A-induced suppressor cell activity (23), depressed cutaneous delayed-type hypersensitivity responses (47), decreased responses of PBL and bronchoalveolar lavage cells to mitogens (4), and increased pulmonary opportunistic infections (7). Although there was no evidence that OvLV altered BTV replication in blood monocytes infected *in vitro*, animals with chronic OvLV infection showed weakened cellular immunity and, in addition to having higher BTV burdens in monocytes and higher numbers of BTV-infected cells in tissues, had a wider range of BTV-asso-

FIG. 8. Cellular localization of BTV antigens (A) and viral nucleic acids (B to H). Arrows denote virus-infected cells. (A) Cytospin preparations of peripheral blood mononuclear cells cultivated for 48 h in the presence of phorbol 12-myristate 13-acetate and then reacted with MAbs to BTV inner capsid antigen VP7 (sheep 18, BTV-3). High numbers of monocytoid cells expressed the VP7 antigen ex vivo. (B to H) RT-PCR-driven in situ hybridization demonstrated BTV infection of capillary endothelia within the myocardium (B; sheep 24, BTV-3) and BTV RNA within the cytoplasm of large mononuclear leukocytes (C, inset) from the submandibular lymph node C; sheep 525, BTV-3), palatine tonsil (D; sheep 525, BTV-3), spleen (E; sheep 28, BTV-11), Peyer's patches of the ileum (F; sheep 3678, BTV-11), pulmonary interstitium (G, sheep 24, BTV-3), and bone marrow (H, sheep 16, BTV-3). Anti-DIG-alkaline phosphatase; bars = 100 μ m.

ciated lesions. Proliferative and infiltrative lesions associated with chronic OvLV infection may provide the optimal micro-environment for BTV replication and dissemination. In addition to their immunosuppressive properties, lentiviruses are known to have bidirectional interaction with other viruses (28). They can interact and alter and/or accelerate the disease course. For instance, HIV up-regulates herpesvirus genome expression and promotes transmissibility. This has been demonstrated *in vitro* through experiments showing transactivation, CD4 up-regulation, Fc receptor induction, pseudotype formation, cytokine production, and antigen presentation (15, 28, 34, 38).

Vascular degeneration and hemorrhage were present in a wide variety of tissues, mostly from animals treated with immunosuppressive drugs or with concurrent OvLV infection. The lesions were acute (lacked evidence of inflammation or a reparative process) and nonexpansive and were found exclusively in immunosuppressed animals, mostly those infected with BTV-3. Studies in mice have shown that intracranial BTV infection typically results in cerebral hemorrhage and necrosis (53). Others have shown that neurovirulence is attributed to genomic differences in viral serotypes (13) and the ability of viruses to gain access to the CNS (54). We observed high numbers of vascular endothelial cells in the CNS of immunocompromised animals to be infected with BTV-3, yet viral gene expression remained low. Furthermore, there was no evidence of neuronal or glial cell infection. These findings suggest that BTV-3 may have an increased tropism for the CNS, at least under circumstances of impaired immunity. In a related study, we have shown that deer infected with epizootic hemorrhagic disease virus, an orbivirus closely related to BTV, had significant CNS involvement manifest by widespread cerebral hemorrhage and massive infection of vascular endothelium (11).

Our results show that widespread dissemination of BTV occurs during early stages of infection and is heightened in animals with impaired immunity. This may provide a mechanism for accelerated disease and, for animals that survive, increased viral persistence. These findings also suggest that in addition to virulence factors that define viral serotypes, immunosuppression could play a role in the natural history of orbivirus infection, allowing for higher virus burden, increased virus persistence, accelerated disease, and greater potential for acquisition of virus by the arthropod vector.

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