Experimental infection of sheep with bovine herpesvirus type-5 (BHV-5): acute and latent infection

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Abstract

We demonstrated that sheep are susceptible to acute and latent infection by bovine herpesvirus type-5 (BHV-5). Lambs inoculated intranasally with two South American BHV-5 isolates replicated the virus with titers up to $10^{7.1}$ TCID\textsubscript{50}/ml for up to 15 days and showed mild signs of rhinitis. Four lambs in contact with the inoculated animals acquired the infection and excreted virus for up to seven days. One lamb developed progressive signs of neurological disease and was euthanized in extremis. Clinical signs consisted of tremors of the face, bruxism, ptyalism, incoordination, lateral flexion of the neck and head, circling, walking backwards, recumbency and paddling. The virus was detected in the anterior and posterior cerebrum, dorso- and ventro-lateral cortex, cerebellum, pons, midbrain and olfactory bulb. Viral nucleic acids were demonstrated in neurons and astrocytes of the anterior and ventro-lateral cortex by in situ hybridization. Histological changes consisting of non-suppurative meningitis, perivascular mononuclear cuffing, focal gliosis, neuronal necrosis and intranuclear inclusions were observed in the anterior cerebrum, ventro-lateral cortex and midbrain. Dexamethasone treatment at Day 50 pi resulted in reactivation of the latent infection and virus shedding in 13/16 (81%) of the lambs. Together with previous reports of BHV-5 antibodies in sheep, these findings show that sheep are fully susceptible to BHV-5 suggesting that infection by BHV-5 in sheep may occur naturally.

Keywords: Bovine herpesvirus type-5; Experimental infection; Sheep-viruses; Latency; Encephalitis

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1. Introduction

Bovine herpesvirus type-5 (BHV-5), also known as bovine encephalitis herpesvirus (BEHV), is an alphaherpesvirus associated with fatal meningoencephalitis in young cattle (Studdert, 1989; Roizman, 1992). Antigenically, genetically and biologically, BHV-5 is closely related to the respiratory and genital BHV-1 strains (Bagust, 1972; Metzler et al., 1986; Bulach and Studdert, 1990; Bratanich et al., 1991). The major difference between BHV-5 and BHV-1 is the distinct ability of the former to cause neurological disease in cattle (Bagust, 1972; Studdert, 1989; Belknap et al., 1994). Isolation of BHV-1 has been largely restricted to cases of respiratory and genital disease, whereas BHV-5 has been almost exclusively associated with meningoencephalitis (Studdert, 1989). Clinical signs of BHV-5-associated neurological disease include tremors, circling, bruxism, incoordination, nystagmus, recumbency followed by convulsions, paddling and inevitably death (Hall et al., 1966; Bagust and Clark, 1972; Carrillo et al., 1983).

The natural history, serology and geographic distribution of BHV-5 infections are largely unknown, partially due to the historic unprecise taxonomic classification of the virus. Only recently, serologic and virologic means of identifying and differentiating BHV-5 from BHV-1 on a field scale have been developed (Lindner et al., 1993a, b; d’Offay et al., 1995). Severe outbreaks of meningoencephalitis by BHV-5 have been frequently described in well-defined geographical locations, mainly in Argentina and southern Brazil (Carrillo et al., 1983; Schudel et al., 1986; Riet-Correa et al., 1989; Weiblen et al., 1989, 1996; Roehe et al., 1997; Odeon, 1998; Salvador et al., 1998). Elsewhere, BHV-5-associated neurological disease has been only sporadically reported (Johnston and McGavin, 1962; Bartha et al., 1969; Eugster et al., 1974). Cross-protection by naturally occurring or vaccine-induced BHV-1 antibodies has been suggested as a possible explanation for the rare occurrence of BHV-5-associated disease in BHV-1 endemic areas (d’Offay et al., 1995). The rare occurrence and high fatality of BHV-5-induced neurological disease have led to speculations about the origin of the virus. It has been suggested that BHV-5 might be a virus naturally infecting zebu cattle or another closely related ruminant species (Studdert, 1989; Bulach and Studdert, 1990).

In this study, we investigated the susceptibility of sheep to infection with two South American BHV-5 isolates. Our results demonstrate that sheep are fully susceptible to acute and latent BHV-5 infection and may even develop neurologic disease. Taken together with recent reports of BHV-5 antibodies in sheep (Lindner et al., 1993a, b), these findings suggest that natural infections by BHV-5 in this species may occur.

2. Material and methods

2.1. Experimental design

Two inoculations were performed independently to evaluate the susceptibility of sheep to BHV-5 infection. Lambs were inoculated intranasally with either of two South American BHV-5 isolates (A663 and EVI-88) and submitted to clinical and virological monitoring. In the first experiment (isolate A663), nine lambs were inoculated and three
remained as non-inoculated controls. In the second experiment (isolate EVI-88), 12 lambs were inoculated and four non-inoculated lambs were maintained in close contact for monitoring virus transmission. Fifty days after inoculation, the lambs from both experiments were treated with dexamethasone during five days and monitored thereafter. Nasal swabs collected after virus inoculation and dexamethasone treatment were examined for infectivity. Necropsy was performed in one animal that developed neurological signs and was euthanized in extremis. Tissue samples were submitted to virus detection and quantitation, histological examination and in situ hybridization.

2.2. Cell culture and viruses

The BHV-5 strain A663 was isolated in Argentina and has been extensively characterized (Carrillo et al., 1983; Schudel et al., 1986; Bratanich et al., 1991). The BHV-5 isolate EVI-88 was isolated from calves with clinical meningoencephalitis in Brazil and characterized by Roehe et al. (1997). Pestivirus-free Madin-Darby bovine kidney cells (MDBK, American Type Culture Collection, CCL-22) were used for all procedures of virus multiplication, quantitation and isolation from nasal swabs and tissues. Cells were routinely maintained in Eagle’s minimal essential medium (MEM) containing penicillin (1.6 mg/l), streptomycin (0.4 mg/l), supplemented with fetal calf serum (Gibco, BRL). Lambs were inoculated with $5 \times 10^6$ TCID$_{50}$ of isolate A663 or $5 \times 10^7$ TCID$_{50}$ of isolate EVI-88.

2.3. Animals, virus inoculation and dexamethasone treatment

Two to three-month-old, BHV-5 seronegative, recently weaned Corriedale lambs (weighing approximately 10–12 kg) were used for experimental inoculation. Lambs were maintained in collective pens (3–4 animals each) (Experiment No. 1) or in a yard with native grass (Experiment No. 2). The animals were inoculated with 5 ml of viral suspension divided in the two nostrils, with the help of a cotton swab. Beginning at Day 50 pi, the animals were treated with dexamethasone (Azium, Schering) intramuscularly during five days (2 mg per animal per day) and monitored thereafter.

2.4. Animal monitoring, sample collection and processing

Lambs were monitored clinically and nasal swabs for viral isolation were collected on a daily basis up to Day 21 post-inoculation (dpi) and Day 15 post dexamethasone treatment (dpt). Nasal swab specimens (0.5 ml) were inoculated onto MDBK cells grown in 24-well plates and monitored for cytopathic effect (CPE) for 5 days. Negative samples were further inoculated onto fresh MDBK cell monolayers and monitored for five additional days. Samples positive for CPE were subsequently quantitated by inoculating ten-fold dilutions onto MDBK cells cultivated in 96-well plates. Virus titers were calculated according to Reed and Muench (1938) and expressed as log$_{10}$TCID$_{50}$/ml. Blood for serology was collected from all inoculated lambs before virus inoculation, at Days 15 and 30 pi and 15 days after DX treatment. Serum samples were submitted to a standard microtiter virus-neutralizing (VN) assay (Bratanich et al., 1991), using two-fold
dilutions of serum against a fixed dose of virus (100–200 TCID$_{50}$/well). Tissue samples for viral isolation, histologic examination and ISH were collected at the necropsy of the animal which developed neurological disease (lamb No. 60). Different sections of the CNS (Table 2) were aseptically collected and submitted to virus isolation. Tissue samples for virus isolation were processed by preparing a 10% (w/v) homogenized suspension and inoculated onto MDBK cell monolayers. Monitoring of CPE and quantitation of infectivity were performed as described above. Tissues for histopathologic examination were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin–eosin (HE) using routine methods. In situ hybridization (ISH) for BHV-5 nucleic acids was performed in formalin-fixed tissue sections essentially as described by Chowdhury et al. (1997). Briefly, sections were deparaffinized, rehydrated, treated with proteinase K (Sigma; 20 mg/ml in PBS) and hybridized with a 1.7 kb DNA probe corresponding to the BHV-5 gC coding region (Dr. S. Chowdury, Kansas State University). The probe was obtained by digestion of the plasmid pGEM3Z with BamHI/AfIII. The digestion fragment was gel-purified and labelled by nick translation with digoxigenin-dUTP (DIG; Boehringer Mannheim, Indianapolis, IN). The hybridized DNA probe was detected with the DIG-alkaline phosphatase conjugate and colour substrate nitroblue tetrazolium salt (Boehringer Mannheim) as previously described (Sur et al., 1996).

3. Results

3.1. Acute infection

Lambs inoculated with BHV-5 shed virus in nasal secretions from Day 1 to Day 16 pi (Table 1). Peaks of virus shedding were observed between Days 3 and 5 pi, declining progressively thereafter (data not shown). Maximum individual titers reached $10^{7.1}$ and $10^{5.9}$ TCID$_{50}$/ml for isolates EVI-88 and A663, respectively. Some lambs inoculated with isolate EVI-88 shed virus in titers above $10^6$ TCID$_{50}$/ml for two to three days. The extent of virus shedding was capable of spreading virus to lambs maintained in close contact: all four non-inoculated contact controls (sentinels) acquired the infection and shed virus from Day 2 pi up to 7 days (Table 1). Most lambs inoculated with isolate A663 did not show any clinical alteration. Only two lambs showed very mild signs of upper respiratory tract infection, characterized by mild hyperemia of the nasal mucosa. Nine lambs (out of 12) inoculated with the isolate EVI-88 and two in-contact controls showed transient and moderate hypertermia, mild to moderate hiperemia of the nasal mucosa and serous to mucopurulent nasal discharge. These signs lasted for three to four days and spontaneously subsided. Starting at Day 10 pi, one lamb (No. 60) showed intermittent neurological signs consisting of depression, bruxism, tremors of the face, ptyalism, incoordination, circling, backwards walking, lateral flexion of the neck and recumbency. These signs were progressive, in both intensity and frequency, and this animal was euthanized in extremis at Day 13 pi. All 13 inoculated lambs kept for latency studies seroconverted to BHV-5 after acute infection, with VN titers ranging from $1 : 4$ to $1 : 128$ at Day 30 pi.
Infectious BHV-5 was recovered from several regions of the brain of the lamb No. 60. The distribution and levels of infectivity detected in the brain of this animal are shown in Table 2. The highest virus titers were demonstrated in the cerebral cortex, mainly in the dorso- and ventro-lateral hemispheres and anterior cerebrum. Nonetheless, other areas of the brain also harbored moderate levels of infectivity (Table 2). Infectivity was not detected in non-neural tissues such as lungs, bronchial lymph nodes, kidneys and liver.

### 3.2. Histopathology

No gross lesions were observed in the brain of this lamb. Microscopic examination revealed histological changes of varied severity in several areas of the brain, but mostly in the cerebral cortex (anterior, ventro- and dorso-lateral). These changes were characterized by mononuclear perivascular cuffing, focal gliosis, occasional neuronal degeneration, mononuclear meningitis (Table 2 and Fig. 1). Large intranuclear inclusions were observed in neurons and astrocytes of the anterior cerebrum (Fig. 2).

### 3.3. In situ hybridization

In situ hybridization using a BHV-5 gC-specific DNA probe revealed positive staining in neurons and astrocytes of the anterior cerebrum and ventro-lateral cortex (Fig. 1). The number of reactive cells was significantly higher in the anterior cerebrum, agreeing with the highest viral titers and the more severe histological changes observed in this location. No reactive cells were observed in brain sections of a BHV-5 seronegative animal obtained in a BHV-5 seronegative lamb used as negative control (not shown).

### 3.4. Latent infection

Administration of dexamethasone to the lambs, starting at Day 50 pi, was followed by reactivation of latent infection and viral shedding in 13 out of 16 (81%) BHV-5-infected
Table 2
Histological changes and infectivity in the brain of the lamb that developed neurological disease following intranasal inoculation with bovine herpesvirus type-5 (BHV-5)

<table>
<thead>
<tr>
<th>Location</th>
<th>Infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mononuclear meningitis</th>
<th>Perivascular cuffing</th>
<th>Gliosis</th>
<th>Neuronal necrosis</th>
<th>Intranuclear inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior cerebrum</td>
<td>4.67 R</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Ventro-lateral Cortex</td>
<td>3.88 L</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Dorso-lateral Cortex</td>
<td>3.81 R</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Midbrain</td>
<td>4.52 L</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Posterior cerebrum</td>
<td>5.88 R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pons</td>
<td>4.50 L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>3.31 R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.50 L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>2.88 R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.01 L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.88 R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.11 L</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Right.
<sup>b</sup> Left.
<sup>c</sup> Infectivity expressed as log<sub>10</sub>TCID<sub>50</sub>/g of tissue.
<sup>d</sup> Histological changes: severe (+++); moderate (++); mild (+); no histological alteration (-).
Fig. 1. Anterior cerebrum of lamb No. 60. In situ detection of viral nucleic acids in the cerebral cortex. Black foci represent virus-infected neurons and astrocytes detected by in situ hybridization with a BHV-5 glycoprotein gC coding region probe. Methyl green counterstain. (×40).

Fig. 2. Anterior cerebrum of lamb No. 60. Intranuclear inclusions in astrocytes of the cerebral cortex (arrows). HE. ×320.
lambs, including the in-contact controls (Table 1). The animals excreted virus in nasal secretions from day four post-treatment up to 11 days. Reactivation of infection was not followed by any evident clinical recrudescence. Viral shedding was intermittent in many animals and occurred at lower titers and for a shorter period than in acute infection. Nine inoculated lambs showed an increase (two- and four-fold) in the VN titers when tested at Day 15 post-dexamethasone treatment. Four inoculated lambs plus the in-contact controls did not show detectable increase in the VN titers following dexamethasone treatment.

4. Discussion

The present study was conducted to investigate the susceptibility of sheep to infection with bovine herpesvirus type-5 (BHV-5). Recent studies have reported the natural occurrence of BHV-5 antibodies in sheep (Lindner et al., 1993a, b). In many regions, including some of those experiencing frequent outbreaks of meningoencephalitis due to BHV-5, cattle and sheep are raised together, sharing pastures and facilities. These mixed herds may provide conditions for interspecies transmission of pathogens. Under such conditions, if they were susceptible to BHV-5, sheep could be naturally infected and potentially serve as occasional reservoirs of the virus.

Our results demonstrated that sheep are fully susceptible to BHV-5 acute and latent infection and may even develop neurological disease upon experimental inoculation. To our knowledge, these are the first direct evidences of the susceptibility to BHV-5 of a ruminant species different than cattle. Previous reports of BHV-5 infection in this species were limited to detection of positive serology in sheep flocks (Lindner et al., 1993a, b). In our study, intranasal inoculation of seronegative lambs with two South American BHV-5 isolates was followed by efficient virus replication in the nasal mucosa. The duration and levels of virus shedding during acute infection were even superior to values reported in some experimental infections with BHV-5 in cattle (Hall et al., 1966; Belknap et al., 1994) and with BHV-1 in sheep (Shankar and Yadav, 1987; Giuliani and Sharma, 1995; Hage et al., 1997). Most lambs inoculated with the isolate EVI-88 developed mild signs of upper respiratory tract infection. Mild signs of rhinitis have also been reported in sheep experimentally inoculated with respiratory BHV-1 strains (Shankar and Yadav, 1987; Giuliani and Sharma, 1995; Hage et al., 1997) and in calves inoculated with BHV-5 (Hall et al., 1966; Belknap et al., 1994). These results demonstrated that seronegative lambs are susceptible to BHV-5 infection following intranasal inoculation. Moreover, the extent and duration of virus shedding was capable of spreading the virus to other animals. All the in-contact controls were infected, indicating that transmission of the virus among sheep during acute infection can occur. The ability of sheep to transmit the virus to cattle was not investigated in this experiment, yet this will be necessary as to ascertain the potential of sheep-to-cattle transmission of BHV-5.

Although the isolate EVI-88 was isolated from cases of meningoencephalitis in cattle (Rohe et al., 1997), this virus has subsequently demonstrated a limited ability to reproduce the neurological disease in calves upon experimental inoculation (Rohe, P.M., personal communication). In addition, no previous report of neurological disease caused by BHV-5 in a ruminant non-bovine species had been published to date. For these
reasons, the development of neurological disease by lamb No. 60 was somewhat unexpected. Nevertheless, the clinical, histological and virological findings associated with this clinical case were very similar to the observations described in natural and experimental BHV-5 infections of cattle (Bagust and Clark, 1972; Schudel et al., 1986; Riet-Correa et al., 1989; Belknap et al., 1994).

In order to investigate whether acute BHV-5 infection in sheep is followed by latent infection, infected lambs were treated with dexamethasone at Day 50 pi and monitored thereafter. Since the ability to establish and reactivate latent infections is crucial for the perpetuation of alphaherpesviruses in nature, a potential role for sheep in the transmission of BHV-5 should necessarily include their ability to harbor the virus in a latent state. Dexamethasone administration was followed by reactivation of latent infection and viral excretion in 13 out of 16 lambs, with virus shedding lasting up to 11 days in some animals. These results are consistent with previous observations of BHV-5 latent infection in calves (Belknap et al., 1994) and of BHV-1 latent infection in sheep and goats (Shankar and Yadav, 1987; Tolari et al., 1990; Hage et al., 1997). Moreover, these are the first observations of BHV-5 latent infection in sheep. Further studies are underway to ascertain the capacity of BHV-5 latently infected sheep to spread virus to other sheep and cattle due to virus shedding occurring upon reactivation of latent infection.

Evidence of natural infection by BHV-5 in a non-bovine ruminant species have been limited to positive serology in sheep (Lindner et al., 1993a, b). Nevertheless, natural infections by a closely related alphaherpesvirus, BHV-1, either by detection of antibodies or by direct identification of the virus, have been reported in several domestic and wild ruminants (Mohanty et al., 1972; Taylor et al., 1977; Legrottagile and Mancianti, 1982; Tolari et al., 1990; Clark et al., 1993; Rosadio et al., 1993; Suresh and Suribabu, 1993; Frolich, 1996; Pituco and Del Fava, 1998). Likewise, experimental studies have demonstrated the susceptibility of sheep to acute and latent infection by BHV-1 and sheep-to-sheep and sheep-to-cattle transmission of the virus (Shankar and Yadav, 1987; Giuliani and Sharma, 1995; Hage et al., 1997). In a study by Rosadio et al. (1993), the prevalence of antibodies cross-reacting with BHV-1 in sheep and goats was found to be higher in herds where these species were raised together with BHV-1 seropositive cattle. Although the epidemiological significance of these observations remains uncertain, these findings are at least suggestive that cattle may not be the only natural host for BHV-1 and that natural infections in other species may occur. As a consequence, a potential role for these species in the epidemiology of BHV-1 should not be discounted (Shankar and Yadav, 1987; Tolari et al., 1990; Suresh and Suribabu, 1993). The potential implication of other ruminants in the epidemiology of this closely related virus, in addition to the recent reports of BHV-5 positive serology in sheep (Lindner et al., 1993a, b), led us to consider a possible involvement of sheep in the transmission of BHV-5 in nature. In this sense, the epidemiological meaning of our findings that sheep are susceptible to BHV-5 infection should be subject of further investigation.

In summary, our results demonstrated that sheep are fully susceptible to acute and latent BHV-5 infection. These findings represent the first direct evidence of the susceptibility of a ruminant species other than cattle to infection with BHV-5. In addition, these results may serve to reinforce the hypothesis that other ruminant species may occasionally participate in the transmission of BHV-5 in nature. Indeed, the degree of
susceptibility taken together with previous reports of BHV-5 antibodies in sheep (Lindner et al., 1993a, b) are suggestive that natural infections by BHV-5 in this species possibly occur. Since raising cattle and sheep together is a common practice in many regions, opportunities for interspecies transmission of BHV-5 in the field are likely to occur. If so, a potential role for sheep in the epidemiology of BHV-5 infection should be considered. In addition, the susceptibility of sheep to BHV-5 may also render this species a suitable animal model in which to characterize BHV-5 infection and disease.

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