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Latency of bovine herpesvirus type 5 (BoHV-5) in tonsils and peripheral blood leukocytes

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ABSTRACT

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) can both establish latency in the trigeminal ganglion. Non-neural sites of latency have been described for BoHV-1 but not for BoHV-5. The aim of this study was to determine whether peripheral blood leukocytes and tonsils are targets for BoHV-5 infection and to establish whether all stages of that virus's infectious cycle can occur in those cell types. Comparisons with BoHV-1 infection of these tissues were also made in order to better understand the pathogenesis of both viruses.

BoHV-1 and BoHV-5 were isolated from tonsils of acutely-infected calves. BoHV-5 was also isolated from a tonsil homogenate after dexamethasone-induced reactivation. During latency, infectious virus was recovered from a tonsil explant of one BoHV-5-infected calf. The genomes of BoHV-5 and BoHV-1 were detected in tonsils from acutely-infected calves although were not detected in tonsils from latently-infected calves or from calves treated with dexamethasone. Virus DNA was intermittently detected in leukocytes.

The study has shown that BoHV-5 can establish latency in bovine tonsils and peripheral white blood cells, and that it can be reactivated from latently-infected tonsils, which might contribute to viral transmission. The titres of BoHV-1 and BoHV-5 in tonsils were similar, suggesting that replication at this site is a common feature for both viruses.

Introduction

Bovine herpesvirus type 1 (BoHV-1) is an alpha-herpesvirus which causes several syndromes in cattle, including respiratory disease, abortions and genital disorders. Acute infection of the respiratory tract by BoHV-1 causes immunosuppression, leading to secondary bacterial infections, pneumonia and death (Tikoo et al., 1995). Another closely-related alpha-herpesvirus, bovine herpesvirus type 5 (BoHV-5), is the causal agent of non suppurative meningoencephalitis in calves (Pérez et al., 2002), a condition which is highly prevalent in South America, particularly Argentina and Brazil. Meningoencephalitis in calves is usually fatal (Rissi et al., 2008). However, mild to moderate (Del Médico Zajac et al., 2010) or subclinical (Cascio et al., 1999) BoHV-5 infections have been described. Additionally, BoHV-5 has recently been associated with genital infection (Kirkland et al., 2009), demonstrating that cell types other than neural tissue might be targeted during infection.

Infection by alpha-herpesviruses is characterized by acute infection, latency and reactivation cycles. The trigeminal ganglion is the main site of latency for both alpha-herpesviruses. Latent herpesvirus infections can be reactivated by natural or glucocorticoid-induced stress and virus shedding during reactivation is responsible for virus transmission (Rock et al., 1992). In contrast to the subclinical reactivation observed in most herpesvirus infections, BoHV-5 reactivation is frequently accompanied by mild clinical signs (Caron et al., 2002; Pérez et al., 2002).

Latency of BoHV-1 in peripheral blood leukocytes (PBLs) (Mweene et al., 1996; Wang et al., 2001), spleen (Mweene et al., 1996), tonsils (Winkler et al., 2000; Pérez et al., 2005), inguinal and sacral lymph nodes (Vogel et al., 2004) has been demonstrated. Unlike BoHV-1, BoHV-5 can establish latency in several areas of the central nervous system (Vogel et al., 2003) although non-neural sites of latency have not been reported for BoHV-5 infection.

Tonsils are lympho-epithelial, immunocompetent tissues, located in strategic anatomical areas of the oral-pharynx and naso-pharynx

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and are the portal of entry for many viruses (Griebel et al., 1990). Winkler et al. (2000) and Pérez et al. (2005) demonstrated that BoHV-1 can infect and persist in the tonsils. In contrast, there is no information available regarding BoHV-5 infection of bovine tonsils and PBLs.

The objectives of the present study were to determine whether PBLs and tonsils were targets for BoHV-5 infection and to establish whether all stages of the virus infectious cycle (primary acute infection, latency and reactivation) could take place in these cell types. In addition, comparisons were made with the infection of lymphoid cells by BoHV-1.

Materials and methods

Viruses

Cooper (BoHV-1) and 97–613 (BoHV-5) strains were used for animal inoculations. BoHV-5 97–613 was isolated from the brain of a 2-year-old cow with neurological signs (Pérez et al., 2002). Titres of virus stocks were determined by the end-point titration method and expressed as $TCID_{50}$/mL (Reed and Müench, 1938).

Cell cultures

For viral stocks, virus isolation and in vitro tests, Madin–Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Internegocios), L-glutamine (10 μL/mL) (L-glutamine Sigma-Aldrich), penicillin (100 μg/L) (penicillin G sodium salt, Sigma-Aldrich) and streptomycin (200 μg/L) (streptomycin sulfate, Sigma-Aldrich) were used. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Experimental design

All procedures for animal handling and experimentation were performed according to the Animal Welfare Committee of the University of the Center of Buenos Aires Province (Res. 087/02). BoHV-1- and BoHV-5-free and seronegative crossbred, 1-year-old calves were used. Calves were randomly assigned to one of four groups. In group 1 (primary acute infection; $n = 4$) two calves were intranasally inoculated with a high dose ($10^{6.3}$ $TCID_{50}$ in 10 mL) of BoHV-1 and the other two with $10^{6.3}$ $TCID_{50}$ (in 10 mL) of BoHV-5. These calves were euthanased at 6 days post-infection (dpi).

Group 2 (latency; $n = 4$) contained two calves that were intranasally inoculated with a low dose (10^3 $TCID_{50}$ in 10 mL) of BoHV-1 and two with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-5. Calves in this group were euthanased at 24 dpi. In group 3 (reactivation; $n = 4$), two calves were intranasally inoculated with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-1 and two with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-5. At 20 dpi they received an intravenous dose of 0.1 mg/kg bodyweight dexamethasone (Dexametona, Schering Plough) followed by two intramuscular doses 24 and 48 h later (Inman et al., 2002). Calves in this group were euthanased at 25 dpi, 2 days after finishing dexamethasone treatment. Group 4 (mock-infected; $n = 2$) calves were intranasally inoculated with 10 mL MEM as placebo. One calf was euthanased at 6 dpi and the other treated with dexamethasone using the same regime as the calves in group 3. This calf was euthanased at 25 dpi.

Preparation of PBLs

PBLs were separated from 10 mL of unclotted blood (anticoagulant: sodium citrate/EDTA, pH 6.5). Blood samples were obtained from all inoculated groups and mock-infected calves at 6 dpi. Additional blood samples were obtained at 9, 13, 20 and 24 or 25 dpi from calves in groups 2 and 3 and from one mock-infected calf in group 4. Blood samples were centrifuged at 2000 *g* for 15 min and the buffy coat was transferred to tubes containing 10 mL of cold ammonium chloride. The cell pellet obtained after centrifugation at 1000 *g* for 7 min at 4 °C was re-suspended in 1 mL of phosphate-buffered saline solution (PBS) and centrifuged at 10,000 *g* for 2 min. The supernatant was discarded and the cell pellet was stored at –20 °C.

Virus isolation from tonsil homogenates and explants

Tonsil homogenates were prepared as a 10% solution in MEM with antibiotics, namely, penicillin (200 μg/L; penicillin G sodium salt, Sigma-Aldrich) and streptomycin (400 μg/L; streptomycin sulfate, Sigma-Aldrich). Homogenates were centrifuged at 1,000 *g* for 30 min at 4 °C. One hundred microlitres of supernatant was inoculated into MDBK cells in 24-well plates in duplicate. Cultures were observed daily for cytopathic effect (CPE) and the supernatants were passaged every 3 days, for a total of three passages.

Explants were prepared from the tonsils of latently-infected calves (collected at 24 dpi) as well as from latently-infected and mock-infected calves that received dexamethasone treatment (collected at 25 dpi). To prepare the explants, tonsils were minced into 1 mm^3 pieces, washed in PBS and two to three pieces were inoculated into six-well plates. Explants were overlaid with 3 mL MEM with antibiotics (penicillin and streptomycin, as indicated for tonsil homogenates) and 10% FCS. MDBK cells were used as indicator cells for co-cultivation. The culture medium was replaced every 3–4 days, and cultures were observed daily for CPE. Samples were evaluated by direct immunofluorescence using an anti-BoHV polyclonal antibody (VMRD). Virus titres in the supernatants from tonsil homogenates were determined by the end-point titration method, as previously described.

DNA extraction and PCR from PBLs and tonsils

DNA extraction from white blood cell pellets and tonsils was performed by the phenol-chloroform method. Nested PCRs for BoHV-1 and BoHV-5 as described by Wang et al. (2001) and Mayer et al. (2006), respectively, were used for genome amplification. PCR products were run in 1.2% agarose gels stained with SYBRsafe (Life Technology) and visualized under ultraviolet light.

Histopathology

At necropsy, tonsils were collected from each calf, placed in 10% neutral buffered-formalin and processed for histopathology. Sections were cut at 5 μm and stained with haematoxylin–eosin.

Statistical analysis

Virus titres were analyzed under a completely randomized design by ANOVA with repeated measurement (R Core Team, 2013). The experimental unit was the calf. Data are presented as least-squares means ± standard error of the mean.

Results

Primary (acute) infection, latency and reactivation of experimentally-inoculated calves

Virus shedding in nasal and/or ocular secretions was detected until 6 dpi (time of euthanasia) for calves in group 1. Calves in groups 2 and 3 shed virus until 9 dpi. At 24 dpi, BoHV-5-inoculated calves in group 2 (latency) had neutralizing antibody titres of 1:128 (calf 7) and 1:256 (calf 10). For BoHV-1, antibody titres were 1:8 and 1:128 (calves 12 and 13, respectively). Viral DNA was detected by PCR in the trigeminal ganglion of all calves. For calves in group 3, virus re-excretion was detected after dexamethasone administration. Control calves remained seronegative during the experiment and virus shedding was not detected.

Pathological findings

Gross lesions were not observed in mock-infected calves (Fig. 1A). In BoHV-5-acutely-infected calves (group 1), haemorrhages and petechiae were observed in retro-pharyngeal lymph nodes and tonsils, respectively. Retro-pharyngeal lymph node haemorrhages were observed at necropsy of calves inoculated with a high dose inoculum of BoHV-1. Macroscopic lesions in the tonsils of these calves were not seen (Figs. 1B–E). Similarly, gross lesions in lymphoid tissues were not evident in animals in groups 2 and 3 (data not shown).

At 6 dpi, tonsils from BoHV-1- and BoHV-5-infected calves had hyperplastic lymphoid follicles, with large, pale germinal centers (Fig. 2A). Furthermore, BoHV-5-infected tonsils collected at the same time had a moderate number of cells infiltrating the tonsil epithelium (Fig. 2B). For both BoHV-1- and BoHV-5-inoculated calves, tonsils collected during latency or after dexamethasone-induced reactivation had no evident microscopic abnormalities or variable degree of enlargement of the germinal centers, either for BoHV-1- or BoHV-5-inoculated calves (Figs. 2C–F). Microscopic alterations were not detected in mock-infected tonsils (Fig. 2G).