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Involvement of toll-like receptors 3 and 7/8 in the neuropathogenesis of bovine herpesvirus types 1 and 5

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ABSTRACT

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are closely related alpha-herpesviruses. BoHV-5 is the causal agent of non-suppurative meningoencephalitis in calves. BoHV-1 causes respiratory disease, abortions, genital disorders and, occasionally, encephalitis in cattle. Both viruses are neurotropic and they share similar biological properties. Nevertheless, they differ in their ability to cause neurological disease. Toll-like receptors (TLRs) are involved in the innate immune response to pathogens. In this study, the variations in the expression levels of TLRs were evaluated in different regions of the bovine central nervous system during the acute infection and reactivation of BoHV-1 and BoHV-5- infected cattle. With the exception of TLR9, significant up-regulation of all TLRs was detected following primary infection of neural tissues by both bovine alpha-herpesviruses. Furthermore, the stages of acute infection and reactivation were characterized by a distinguishable TLR expression pattern. Important differences in TLR expression upon infection of the central nervous system by BoHV-1 or BoHV-5 were not detected. The striking differences in TLR mRNA levels during acute infection and reactivation provide evidence that the innate immune response may be involved in the clinical outcomes observed at each stage. Further research is required to analyze the mechanisms that initiate TLR activation and the signaling cascade mediated by each TLR to elucidate the precise role these receptors play in bovine herpesvirus encephalitis.

1. Introduction

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. A closely related alpha-herpesvirus, bovine herpesvirus type 1 (BoHV-1), causes several syndromes in cattle, including respiratory disease, abortions and genital disorders (Tikoo et al., 1995). BoHV-1 is also responsible for some cases of encephalitis in cattle (Silva et al., 2007; Rissi et al., 2008). However, these particular cases are not as frequent as those associated with BoHV-5. Both viruses are neurotropic and they share similar biological properties. Nevertheless, it is unknown why these alpha-herpesviruses differ in their ability to cause neurological disease in cattle.

Herpesviruses' life cycle is characterized by stages of acute infection, latency and reactivation. Sensory neurons of the trigeminal ganglion are the main site of latency of alpha-herpesviruses. Sporadic virus reactivation from latency can occur under conditions of natural stress or it can be experimentally induced by glucocorticoids administration. These episodes of reactivation are the main source of virus dissemination (Rock et al., 1992). BoHV-1 reactivation is subclinical. However, reactivation of BoHV-5 may occur in the presence of mild neurological signs (Pérez et al., 2002; Vogel et al., 2003).

In many viral infections, an inappropriate or exacerbated immune response may be responsible for pathological alterations. Particularly, it has been described that the inflammatory response mediated by toll-like receptors (TLRs) during virus infection can result in tissue damage (Carty et al., 2014). It is unknown whether the innate immune response plays a role in the development of meningo-encephalitis by alpha-herpesviruses in cattle. Furthermore, it has not been reported whether differences in the immune response might be associated with differences in the pathogenesis of BoHV-1 and BoHV-5.

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TLRs allow the recognition of pathogens by the innate immune system (Kawai and Akira, 2005; Akira et al., 2006). These receptors are transmembrane signaling proteins which mediate the recognition of pathogen associated molecular patterns (PAMPs). TLRs can also be activated by danger associated molecular patterns (DAMPs), which derive from the host after tissue injury (Lee et al., 2013). They are expressed by cells of the immune system or by those cells which are primary target for infectious agents (Zarember and Godowski, 2002). TLRs 3, 7, 8 and 9 are involved in the immune response against viral infections (Wang et al., 2006; Cargill and Womack, 2007) and they are located in the membranes of endosomic compartments (Latz et al., 2004). TLR3 recognizes double-stranded RNA, TLR7 and TLR8 are activated by single-stranded, GU-rich RNA and sequences of double-stranded DNA, rich in un-methylated CpG, are the stimulus for TLR9 activation (Borrow et al., 2010). TLRs stimulation induces a series of events that result in the production of pro-inflammatory cytokines and interferon and, finally, in the stimulation of the adaptive immune response (Smith et al., 2005; Rosenthal, 2006). For this reason, many TLR agonists may have therapeutic efficacy in the control of viral infections of the central nervous system (CNS). Nevertheless, research must be focused on determining whether the triggering of the innate immune response leads to the control of the infection or to the induction of pathology.

Previous studies by Marin et al. (2014a, 2014b) have shown that TLRs participate in the response to bovine herpesviruses infections *in vitro* and *in vivo*. The aim of this study was to evaluate whether TLR expression in the CNS of BoHV-1- and BoHV-5-experimentally infected calves was involved in the differences in the neuropathogenesis observed after infection of cattle with each alpha-herpesvirus.

2. Materials and methods

2.1. Cells

For viral stocks, virus isolation and *in vitro* tests, Madin-Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics were used. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.2. Virus

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 tissue culture infectious dose $(TCID)_{50}$/ml (Reed and Müench, 1938).

2.3. 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). Ten BoHV-1- and BoHV-5-free and seronegative cross-bred, 1 year-old calves were used. Calves were randomly assigned to one of three 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). In Group 2 (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. With this low dose inoculum, virus latency is established without the presence of clinical signs during the acute stage of infection, as previously demonstrated by Pérez et al. (2002). At 20 dpi they received an intravenous dose of 0.1 mg/kg bodyweight dexamethasone (DEX) (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 DEX treatment. Establishment of latency in these animals was confirmed by serology and absence of virus excretion in nasal and ocular swabs after the period of primary infection (data not shown). Group 3 (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 DEX using the same regime as the calves in Group 2. This calf was euthanased at 25 dpi.

2.4. Tissue samples

At necropsy, once the brain was removed, it was transversally sliced into eight 1–2 cm-thick sections. Samples from anterior cerebral cortex, including the olfactory and frontal cortex; posterior cerebral cortex (ectomarginal groove area) and cervical medulla were collected aseptically and individually for virus isolation and Real Time-PCR. Samples from posterior cortex were not available from calves in Group 2. Neural tissues were also placed in 10% neutral buffered-formalin and processed for histopathology.

2.5. Virus isolation and histopathology from nervous tissue

Tissue homogenates were prepared as a 10% solution in MEM with antibiotics. Homogenates were centrifuged at 1000 $\times g$ for 30 min at 4 °C. One hundred microliters of supernatant were inoculated into MDBK cells in 24 well-plates in duplicate. Cultures were observed daily for cytopathic effect and the supernatants were passaged every three days, for a total of three passages. Samples were evaluated by direct immunofluorescence using an anti-BoHV polyclonal antibody (VMRD). For histopathology, neural tissue sections were cut at 5 μm and stained with hematoxylin-eosin.

2.6. Nucleic acids extraction

Total DNA from tissue samples of experimentally-infected animals was extracted using a commercial kit (Dneasy Blood and Tissue kit, Qiagen Inc., Valencia, CA, USA), as recommended by the manufacturer. The DNA concentration was measured by absorbance at 260 nm using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). To test the efficiency of DNA extraction or the presence of inhibitors in the Real-Time PCR reaction, the detection of cytochrome B, a gene with constitutive expression in bovine tissues, was evaluated. The amplifications were carried out in a final volume of 20 μl using EvaGreen as intercalating fluorescent dye (KAPA HRM FAST Master Mix, Biosystems, Woburn, USA) and primers that amplify a 134 bp fragment of bovine cytochrome B DNA (Santaclara et al., 2007). The cycling program consisted of an initial denaturation of 2 min at 95 °C and 40 cycles of 10 s at 95 °C, 15 s at 56 °C and 20 s at 72 °C. Samples with bovine cytochrome B Cycle Threshold (Ct) values below 35 were considered suitable for further analysis. The melting temperature of bovine cytochrome B specific amplification fragment was 84 °C. All real-time PCR reactions were carried out in a Rotor Gene Q thermocycler and were performed in duplicate.

Total RNA from nervous tissue samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and digested with DNase I for 30 min at 37 °C to remove any contaminating genomic DNA (gDNA). The quality and quantity of the resulting RNA were determined using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). Complementary DNA (cDNA) was synthesized using a reaction mixture containing 1 μg of total RNA, random hexamers (12 ng/μl) (Promega, Madison, WI, USA) and Moloney murine leukaemia virus reverse transcriptase (10 U/μl) (Promega, Madison, WI, USA), following the procedures suggested by the manufacturer. Negative controls, omitting the RNA or the reverse transcriptase, were included.