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Toll-like receptor expression in the nervous system of bovine alpha-herpesvirus-infected calves

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ABSTRACT

In this study, the expression levels of viral Toll-like receptors (TLRs) in the nervous system of bovine herpesvirus type 5 (BoHV-5)-infected calves were investigated. A significant increase in the expression of TLRs 3 and 7–9 was found in the anterior cerebral cortex during acute infection and viral reactivation. In the trigeminal ganglia, only TLR9 expression was significantly affected. The magnitude of the increase was lower in BoHV-1-infected calves, suggesting that a restricted immune response might protect against exacerbated inflammatory responses in the brain. This work describes, for the first time, the involvement of TLRs 3 and 7–9 in the recognition of BoHV in the bovine nervous system, indicating that the expression of these receptors might be associated with the development of neurological disease. Modulation of the signalling pathways mediated by TLRs might provide an effective approach to control the neuro-immune response to BoHV-5, which may be responsible for neurological lesions.

1. Introduction

Alpha-herpesviruses are capable of causing neurologic disease in cattle. Bovine herpesvirus type 1 (BoHV-1) and type 5 (BoHV-5) are neuroinvasive. Whereas encephalitis caused by BoHV-1 has been reported occasionally (Roels et al., 2000; Silva et al., 2007), BoHV-5 has a marked neurotropism and is the causative agent of necrotising meningoencephalitis in young cattle (Pérez et al., 2002). Both BoHV-1 and BoHV-5 are genetically and antigenically related. BoHV-1 has a worldwide distribution, whereas BoHV-5 has a more limited geographic distribution, as it is most frequently reported in southern Brazil and Argentina (Carrillo et al., 1983; Salvador et al., 1998; Weiblen et al., 1989). Like other alpha-herpesviruses, BoHV-1 and BoHV-5 establish a lifelong latent infection primarily in the neural ganglia of animals that survive acute infection (Meyer et al., 2001; Pérez et al., 2002). The reactivation of a latent infection may occur under certain natural or induced stimuli, and this phenomenon provides adequate means for virus transmission and spread (Del Médico Zajac et al., 2010). Unlike BoHV-1 reactivation, natural or pharmacologically induced reactivation of BoHV-5 is frequently accompanied by the recrudescence of neurological disease (Pérez et al., 2002; Vogel et al., 2003). The mechanisms underlying the clinical manifestations after an infection caused by BoHV, especially encephalitis, have not been well-defined. However, it is likely that the immune system plays an important role in the development of this neurological condition (Abril et al., 2004).

Toll-like receptors (TLRs) are a broad family of evolutionarily conserved innate immune receptors that recognise pathogen-associated molecular patterns (PAMPs) from diverse organisms (Mogensen, 2009). Among the TLRs, TLRs 3, 7, 8 and 9, which are expressed in intracellular vesicles, recognise microbial nucleic acids, particularly of viral origin, and constitute a powerful sensor system that detects viral invasion. Double-stranded RNA (dsRNA) is recognised by TLR3, single-stranded RNA (ssRNA) is detected by TLR7/8, and TLR9 recognises the unmethylated CpG dinucleotides in DNA molecules. These components are often present in the viral genome, and they are generated during the replication of many viruses (Borrow et al., 2010). The activation of TLRs by pathogens and pathogen-derived products induces the expression of pro-inflammatory mediators and anti-microbial effector molecules (Mogensen, 2009). Efficient inflammatory responses mediated by TLRs are required for the host defence against invading pathogens and tissue repair. However, exaggerated or chronic inflammation induced by the activation of these receptors may also lead to tissue damage and the development of disease. In the central nervous system (CNS), TLRs are expressed in various cell types, including microglia, astrocytes, neurons and cerebral vascular cells (Kong and Le, 2011). Accumulating evidence supports a model in which TLRs play a major role in brain infection and injury. Presently, investigation is aimed at demonstrating how the outcome of TLR

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involvement can lead to the resolution of infection, neurodegeneration or neural repair, depending on the context (Konat et al., 2006; Ransohoff and Brown, 2012).

Previous research on the neuropathogenesis of BoHV-5 has focused on the role of envelope glycoproteins (Chowdhury et al., 2000, 2002; Kaashoek et al., 1998), and investigations addressing the involvement of other viral products or host factors are lacking. Mechanisms mediated by TLRs have been recognised as key factors in many human infectious diseases. However, no information about the participation of these receptors in viral diseases of cattle is available. Thus, the aim of this work was to determine whether variations in the expression levels of viral TLRs might be detected at different stages of the infectious cycle of BoHV-5 in the nervous system of its natural host. Understanding the mechanisms that govern TLR signalling during a BoHV-5 infection in the nervous system will undoubtedly facilitate the design of effective therapeutic or preventive measures for the control of BoHV infection.

2. Materials and methods

2.1. Animals

Thirty-five crossbred calves, 6–8 months old, were used in this experiment. All of the animals were free of detectable antibodies to BoHV-1, BoHV-5 and bovine viral diarrhoea virus.

2.2. Cells and virus

Madin–Darby Bovine Kidney (MDBK) cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used for this study. MDBK cells were propagated in Minimum Essential Medium (Eagle) with Earle salts (E-MEM) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% foetal bovine serum (Bioser, Buenos Aires, Argentina), certified free from adventitious viruses and antibodies, and with antibiotic–antimycotics (Gibco, Langley, OK, USA) at a concentration of 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate and 0.025 μg/ml amphotericin B. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

The reference strain Los Angeles 38 (LA38) (BoHV-1.1) and the Argentinean BoHV-5 field strain (identified as 97/613), both in the eighth passage, characterised and provided by the Specialised Veterinary Diagnostic Service, INTA Balcarce (Argentina), were used for animal challenges. The isolate 97/613 was recovered from the brain of a 2-year-old cow with necrotising encephalitis. The virus identification was confirmed by isolation in cell culture followed by direct immunofluorescence using a polyclonal antibody against BoHV (American BioResearch, Sevierville, TN, USA) and *nested*-PCR (Campos et al., 2009). Viral inocula were propagated in MDBK cells in T-175 flasks (Greiner Bio-one, Frickenhausen, Germany) for 24 h. Supernatants were harvested and stored at –80 °C until use. Virus titres were determined by the endpoint titration method and expressed as tissue culture infective doses ($TCID_{50}$) according to Reed and Müench (1938).

2.3. Experimental design and BoHV challenge

A total of 35 BoHV-1- and BoHV-5-free and seronegative British cross-bred, 1-year-old calves were used in this study. To evaluate the expression levels of viral TLRs at different stages of the BoHV-5 infectious cycle, 23 bovines were randomly assigned to two main experimental groups: **Group 1** (12 calves) for the study of BoHV-5 acute primary infection, and **Group 2** (11 calves) for the study of BoHV-5 reactivation. An additional group was designed to evaluate the expression levels of TLRs during BoHV-5 latency: **Group 3** (two calves). Furthermore, to establish comparisons of TLR expression levels in neural tissues from BoHV-1-infected calves, animals were also assigned to the following groups: **Group 4** (two calves) for BoHV-1 acute primary infection, and **Group 5** (two calves) for BoHV-1 latency and reactivation. Additionally, **Group 6**, consisting of six mock-infected calves, was also included.

Calves were challenged intranasally by aerosolisation with 25 ml of inoculum, evenly distributed in both nostrils. The virus inoculum for calves in Group 1 and Group 4 contained $10^{6.3}$ $TCID_{50}$ of the respective virus to induce acute infection. The virus inoculum for calves in Groups 2, 3 and 5 contained 10^{3} $TCID_{50}$ to induce a latent infection. Mock-infected calves were intranasally inoculated with 25 ml of E-MEM.

Calves from each treatment group were maintained in isolated pens and fed on grass hay, commercial concentrate foodstuff and water *ad libitum*.

To induce reactivation from latency, at 3 months after the primary inoculation (PI), all calves in Groups 2 and 5 and three calves in Group 6 were given dexamethasone (DXM; Dexametasona Vet, Schering Plough, Sanidad Animal, Argentina) at a dose of 0.1 mg/kg/day intravenously for 5 consecutive days. Animals in Groups 1 and 2 were sequentially killed, one calf per day, on days 6–17 PI and on days 6–16 after DXM treatment (day post-reactivation [PR]), respectively. Calves in Group 3 were killed on day 54 PI. The euthanasia of calves in Group 4 was performed on day 7 PI and for calves in Group 5 on day 6 PR. Mock-infected calves were killed on days 6, 10, and 16 PI (three control calves for the study of BoHV-1 and BoHV-5 acute infection) and on the same day PR (three control calves for the study of BoHV-1 and BoHV-5 latency and reactivation). To minimise the use of animals according to the Institutional Committee for Care and Use of Experimental Animals (CICUAE) of INTA, which is where the experiments were held, and because the only objective of this group is to obtain uninfected tissues, the organs of mock-infected calves killed during the acute infection are used as controls for the study of BoHV-5 latency. Calves were deeply anaesthetised and killed according to the regulations of the CICUAE of INTA, Argentina.

2.4. Sample collection

After euthanasia, the brain was removed and transversally sliced into eight 1- to 2-cm-thick sections. Different sections of the CNS were collected aseptically and individually for viral isolation and viral DNA detection. The following areas were evaluated: anterior cerebral cortex (three samples: olfactory cortex, frontal cortex and dorsolateral cortex), posterior cerebral cortex (two samples: marginal groove area and ectomarginal groove area), cerebellum (one sample), medulla and pons (three samples: cervical medulla, medulla oblongata and pons) and diencephalon (one sample). Trigeminal ganglia (TG) were also collected. Samples from the anterior cerebral cortex, where microscopic lesions of nonsuppurative meningoencephalitis are usually observed (Pérez et al., 2002), and the TG were selected for TLR expression studies by real-time RT-PCR.

2.5. Virus isolation and identification

Tissue samples were homogenised in 1X Hank's solution (10% w/v), and the suspensions were centrifuged at 1000 × g for 15 min at 4 °C. Fifty microlitres of supernatant were inoculated in duplicate into monolayers of MDBK cells in 96-well plates (Greiner Bio-one) and incubated at 37 °C. Samples were passaged every 3 days, for a total of four to six times, and monitored daily for the presence of cytopathic effects. At the end of each passage, the samples were tested by direct immunofluorescence using a polyclonal antibody against BoHV conjugated with fluorescein isothiocyanate (American BioResearch).