Experimental infection of calves with a gI, gE, US9 negative bovine herpesvirus type 5

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Abstract

In this work, a role for the genes encoding glycoproteins I (gI) and E (gE) and the US9 protein of bovine herpesvirus type 5 (BHV-5) in neuropathogenicity and reactivation of latent infections was examined. Calves infected intranasally with a gI/gE/US9 deleted recombinant shed up to 10^{2.85} TCID_{50}/ml infectious virus in nasal secretions. Calves infected with the wild type BHV-5 parental virus shed up to 10^{5} TCID_{50}/ml virus. No signs of disease were observed in calves infected with the recombinant virus, whereas those infected with wild type virus displayed respiratory and neurological signs. The recombinant was only able to reach the basal portions of the central nervous system. In contrast, wild type virus was found widespread within the brain. Reactivation with dexamethasone 60 days post-infection resulted in reactivation of wild type virus, whereas the recombinant virus could not be reactivated. These studies demonstrate that genes gI, gE and US9 of BHV-5 are important for its neuropathogenicity and its ability to reactive from latency.

Keywords: Bovine herpesvirus 5; gI; gE; US9; Recombinant virus; Experimental infection

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Résumé

Le rôle des gènes codant pour les Glycoprotéines gI et gE et la protéine US9 de l’Herpès virus bovin type 5 dans la neuropathogénèse et dans la réactivation des infections latentes a été étudié.

Les veaux infectés par voie nasale avec un virus recombinant déleté des gènes gI, gE, US9 ont éliminé le virus dans les sécrétions nasales à des titres allant jusqu’à $10^{2.85}$ TCID$_{50}$/ml.

Les veaux inoculés avec la souche sauvage BHV5 ont excrété des virus à des titres allant jusqu’à $10^5$ TCID$_{50}$/ml

Il n’a pas été observé de signe d’infection chez les veaux inoculés avec la souche recombinante.

En revanche, les veaux inoculés avec la souche sauvage ont présenté des signes respiratoires et nurologiques.

La souche recombinante a seulement atteint les portions basses du système nerveux central alors que le virus sauvage a été retrouvé dans l’ensemble du cerveau.

Le traitement avec la dexaméthasone 60 jours après l’infection a induit une réaction de l’infection latente et l’excrétion de la souche sauvage mais pas celle de la souche recombinée. Ces travaux montrent que les gènes gI, gE et US9 du virus BHV 5 sont importants pour la neuropathogénèse et pour la réactivation des infections latentes.

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Mots Clés: Herpès virus bovin; gI; gE; US9; Virus recombinant; Infection expérimentale

1. Introduction

Bovine herpesvirus 5 (BHV-5), a member of the family Herpesviridae, subfamily Alphaherpesvirinae, is a highly neurovirulent virus that infects cattle [1,20]. The virus is able to replicate in cells of the nasal and pharangeal mucosa [1,18]. After infection of neural cells at peripheral sites, it is transported to the central nervous system (CNS) where severe encephalitis may ensue [1,18]. Animals that recover from primary BHV-5 infection develop a latent infection, mainly within neurons of the trigeminal ganglion [18].

BHV-5 neurovirulence is intriguing, particularly because its close relative, bovine herpesvirus 1 (BHV-1) is highly homologous antigenically and genomically to BHV-5 [7,22], yet BHV-1 does not display such marked affinity for neural tissues as BHV-5. Several genes have been examined in attempting to determine the basis for BHV-5’s neurovirulence in experimental models. The gene that codes for the envelope glycoprotein E (gE) seems to play a role in virus spread within the brain of experimentally infected rabbits. In addition, those infected with a gE-negative BHV-5 recombinant were less severely affected than wild type virus infected controls [4]. It was recently demonstrated that another BHV-5 transmembrane protein, US9, is important for viral transport from the olfactory receptor neurons to the second order neurons in the bulb of rabbits [5]. A US9-negative recombinant was avirulent and incapable of invading the CNS of intranasally inoculated rabbits [5].

For BHV-1, another glycoprotein, gI, was shown to play a role in residual virulence, since a gI-negative recombinant was significantly less virulent than the wild type [13]. For other herpesviruses such as pseudorabies virus (PRV), it was demonstrated that gI is involved in transneuronal transport and neurovirulence [8,14,17]. Besides, gE and gI form a noncovalently linked complex [26] which may indicate that the gE/gI complex represent a functional unit.
Since gI, gE and US9 genes may be involved in neurovirulence as well as in the spread of the BHV-5 within the CNS, this study was performed to examine the effect of a recombinant with a triple gene deletion (BHV-5 gI/gE/US9\(^{-}\)) on experimentally infected calves.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby bovine kidney cells (MDBK) were grown in Minimal Essential Medium (MEM) supplemented with 10% of fetal bovine serum (FBS) and 10 mg/ml of enrofloxacin (Bayer). Cells were maintained at 37 °C in an incubator under a 5% CO\(_2\) atmosphere. The wild type BHV-5 EVI 88/95 was isolated from an outbreak of meningoencephalitis in the central region of Brazil in 1995 and was characterized elsewhere [6,22]. This isolate was used to construct the recombinant BHV-5 gI/gE/US9\(^{-}\). The construction of the recombinant BHV-5 gI/gE/US9\(^{-}\) was described in detail [9]. Both parental virus and recombinant were multiplied in MDBK cells to prepare stocks with approximately 10\(^7\) 50% tissue culture infective doses per ml (TCID\(_{50}\)/ml). Virus stocks were clarified by centrifugation (2000 \(\times\) g) and stored at \(-70\) °C until use.

2.2. Experimental infection of calves

Fourteen, 6-month old calves of mixed beef breeds, devoid of neutralizing antibody to BHV-5 and BHV-1, were used for the experimental infections. After an adaptation period of two weeks for calves to get used to the experimental conditions, six calves were intranasally inoculated with 10 ml (5 ml per nostril) of a virus suspension with 10\(^8\) TCID\(_{50}\) of the BHV-5 gI/gE/US9\(^{-}\) recombinant (group A). Six other calves were inoculated with the same amount of the parental wild-type virus BHV-5 strain EVI 88/95 (group B). Two animals were inoculated with sterile cell culture medium and maintained as uninfected controls (group C). Animals of each group were housed in separate units.

Calves were monitored daily for clinical signs of disease. Rectal temperatures were recorded and nasal swabs collected from days 0 to 14 post-infection (pi). Serum samples were collected on days 0, 7, 11 and 14 pi. On day 14 pi, three animals of each of the two groups of inoculated calves were culled and CNS tissues submitted to virus isolation, PCR, immunohistochemical and histopathological examinations.

2.3. Attempts to reactivate latent infections

Three calves in each group were maintained to examine whether reactivation of potentially latent virus could be achieved. For that, on day 60 pi, calves on both groups A and B received a course of dexamethasone (Azium, Schering) injections, administered by the intramuscular route (0.1 mg per kg of body weight) for five consecutive days [23]. Subsequently, calves were examined clinically and nasal swabs collected daily from day 0 to 14 post-dexamethasone (pd), as for primary infection. Serum samples were
collected on days 0, 7 and 14 pd. All calves were killed on day 14 pd and CNS tissue samples were examined as below.

2.4. Virus isolation

Attempts to isolate virus were carried out on nasal swabs and CNS tissue suspensions. Nasal swabs were collected and eluted in 3 ml of MEM supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin and 5 µg/ml amphotericin B for 1 h at room temperature. Samples were stored at −70 °C until processing. Fragments of CNS tissues (olfactory, frontal, temporal, parietal and occipital cortices, diencephalon, mesencephalon, cerebellum, pons and medulla oblongata) were homogenized to 10% (W/V) with MEM supplemented with antibiotics and clarified at 2500 × g for 20 min at 4 °C. Volumes of 200 µl of each supernatant were used to inoculate MDBK monolayers in 24 well plates. Infected cultures were observed for the presence of cytopathic effects (CPE) for 7 days. All negative cultures were frozen at −70 °C and inoculated again on fresh MDBK monolayers to confirm the absence of infectious virus. BHV-5 positive samples were titrated according to the method of Spearman and Kárber [16]. Identification of BHV-5 infected cells was performed by immunoperoxidase on fixed monolayers as described below.

2.5. Immunoperoxidase monolayer assay (IPMA)

Supernatants of viral isolation were used to infect 96 well plates with preformed MDBK monolayers. After visualization of CPE, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) and the IPMA performed as described [15], using an anti-BHV-5 monoclonal antibody (MAb 2F9), specific to glycoprotein gC [19].

2.6. Immunohistochemistry and histopathology

To compare the neural spread of wild type and recombinant viruses, CNS tissue fragments were fixed in 10% buffered neutral formalin and examined for the presence of BHV-5 antigens and lesions. Sections were exposed to enzymatic digestion with Streptomyces griseus protease (1 mg/ml) as described [12] and incubated with the MAb 2F9 [19]. Specific binding was detected by a biotin labelled goat anti-mouse immunoglobulins conjugate (Dako) and a streptavidin-peroxidase complex (Dako). Sections were counterstained with haematoxylin for 10 s. For histological examinations, the same tissue samples were stained with haematoxylin and eosin using routine protocols.

2.7. Polymerase chain reaction (PCR) analysis

CNS tissues from calves were submitted to PCR in search of BHV-5 gG gene sequences essentially as described [10], with modifications as follows. Total DNA was extracted from homogenized brain tissues as described [24]. PCR primers were designed from conserved regions identified in presently available BHV-5 sequences (GenBank accession nos. NC 005261, X99755 and AF250038). In the first round of
amplification, primers were: 5'-CCGGCGATTACGAGGAGCAG-3' (forward) and 5'-TGCAGGACGACGGGCAGC-3' (reverse). These primers target a fragment of 590 base pairs (bp) on gene gG of BHV-5 (corresponding to nucleotides 120312–120904 of the BHV-5 genome). Second stage primers were: 5'-TACGGACTGCCGGATTACA-3' (forward) and 5'-TGCACCACCAACCGGCCCCACAT-3' (reverse). Those primers target a 222 bp fragment, corresponding to nucleotides 120376 to 120597 of the genome. The identities of the internal products were confirmed by nucleotide sequencing (data not shown).

PCRs were performed in a total volume of 20 μl containing 1% glycerol, 1% dimethyl sulfoxide (DMSO), 1.5 mM MgCl₂, 200 μM dNTPs, 0.2 μM of each primer and 1 μl (50 ng) of extracted DNA. After an initial denaturation step at 98 °C for 6 min, Taq DNA polymerase (0.5 U) and dNTPs were included and the amplification reactions carried out in 35 cycles with denaturation at 95 °C for 1 min, annealing at 61 °C (first round) or 57 °C (second round) for 1 min each and extension at 72 °C for 1 min. In all reactions, samples containing H₂O only were included as negative controls; DNA from BHV-5 strain EVI 88/95 was included as positive control. PCR products were run on 1% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator [21].

2.8. Virus neutralization (VN)

VN tests were performed by the constant virus-variable serum method [11], with modifications as follows. Serial twofold dilutions of each serum to be tested were prepared in 96 well microtitre plates. An equal volume of MEM (50 μl/well) containing 100 TCID₅₀ of virus (BHV-5 strain EVI 88/95) was added to each well. The mixtures were incubated at 37 °C for 1 h. Fifty microlitres of the MDBK cell suspension containing 1.5–3 × 10⁴ cells were added to each well and plates incubated at 37 °C under a 5% CO₂ atmosphere. Titres were expressed as the reciprocal of the highest dilution giving complete neutralization.

3. Results

3.1. Acute infection

3.1.1. Calves inoculated with the recombinant BHV-5 gI/gE//US9⁻ (group A)

Calves from group A shed recombinant virus in nasal secretions from day 1 to 4 pi with titres of up to 10².₈⁵ TCID₅₀/ml (Fig. 1). None of the calves developed fever or signs of either respiratory or neurological disease throughout the experiments. Infectious (recombinant) virus could be recovered only after a second passage, from tissues of the pons and mesencephalum in one calf of group A. Other CNS tissues had no detectable infectious virus. Virus-specific antigens were detected in tissues of the pons. Histopathological examinations revealed no detectable lesions. PCR amplification revealed the presence of BHV-5 DNA in medulla oblongata, pons, mesencephalum and trigeminal ganglia of this calf.

Neutralizing antibodies to BHV-5 were initially detected in calves by day 14 pi (Fig. 2). The animals from the uninfected control group (group C) remained BHV-5 seronegative.
throughout; no BHV-5 virus could be recovered from such calves. In addition, no clinical alterations were observed in controls calves throughout the experiments.

3.1.2. Calves inoculated with wild-type BHV-5 strain EVI 88/95 (group B)

Calves from group B shed virus from day 1 to 12 pi with significantly higher titres than those detected on group A (up to 10^5 TCID_{50}/ml Fig. 1). Calves showed moderate hyperthermia (40 °C) by days 3 and 4 pi. Four calves from this group showed mild signs of upper respiratory tract infection (nasal discharge, sneezing, coughing and forced respiration) from days 4 pi to 14 pi. Moreover, starting on day 10 pi three calves developed signs of neurological involvement characterized by apathy, hypersalivation, teeth chewing, incoordination, tremors, convulsions and difficulty to stand. In these calves, virus was isolated from all CNS tissues examined, to high titres (> 10^6 TCID_{50}/ml) from pons, mesencephalum and frontal, temporal and parietal cortices. In all calves infected with wild type, BHV-5 specific immunostaining could be observed in neurons in the CNS. Histopathological alterations were found widespread within the brain and characterized by diffuse gliosis, disruption of the neurophil and perivascular cuffs of mononuclear cells, neuronal satellitosis and neuronal necrosis.
Calves developed neutralizing antibodies to BHV-5 from day 14 pi. Neutralizing antibody titres were significantly higher in group B than in group A throughout these studies (Fig. 2).

3.2. Attempts to reactivate latent infections

3.2.1. Calves inoculated with the recombinant BHV-5 gI/gE/US9\(^{-}\) (group A)

After administration of dexamethasone, calves in group A did not show any clinical alterations. Such calves did not shed infectious virus in nasal secretions. Attempts to recover infectious virus from the CNS were unsuccessful. Likewise, all samples were negative at PCR, and histopathological examinations revealed no lesions in the CNS (data not shown). Moreover, the calves did not reveal any alteration in their levels of neutralizing antibodies (Fig. 2).

3.2.2. Calves inoculated with the parental wild-type virus BHV-5 strain EVI 88/95 (group B)

In group B virus was excreted in nasal secretions from day 1 up to day 12 pd (Fig. 3). Reactivation of infection was not followed by any evident clinical recrudescence. BHV-5 virus, BHV-5 antigens, BHV-5 DNA and lesions were not detected on samples of the CNS (data not shown). The inoculated calves did show neutralizing antibody titres increased following dexamethasone treatment (Fig. 2).

4. Discussion

This study deals with the effect of the intranasal inoculation of a triple BHV-5 deletion mutant (gI/gE/US9\(^{-}\)) in calves. During the experiments reported here, fever and signs of respiratory or neurological disease were not observed in calves inoculated with the recombinant virus. In contrast, the group of animals inoculated with wild type virus showed both respiratory and neurological signs. Following intranasal infection of calves,
the recombinant virus was able to replicate in the tissue at the site of inoculation, as detected by virus isolation from nasal swabs. However, the capacity of the BHV-5 gI/gE/US9⁻ to enter and spread in the CNS was strikingly reduced when compared to the parental wild type BHV-5, which was isolated widespread within the CNS. While the wild type virus could be recovered from CNS tissues to high titres, the recombinant virus was only able to reach the basal portions of the CNS (pons and mesencephalum), yet to low titres (as revealed by the need for at least two passages to identify infected cells). Analyses by IHC and PCR confirmed the absence of the recombinant virus in others parts of the brain. These results show that the recombinant was able to reach the CNS; however, it was not capable of spreading to deeper brain tissues.

Several mechanisms may be hypothetised in trying to explain the absence or substantially reduced levels of the BHV-5 gI/gE/US9⁻ in the CNS. The recombinant virus may infect a small number of first-order neurons at the peripheral site of inoculation. This might be supported by the low titres of the recombinant virus detected in nasal secretions. If this was the case, insufficient virus may have gained access to the CNS, thereby limiting neuronal infection. Another possibility is related to virus spreading. As commented previously, apparently the gE/gI complex plays a role in spreading through intercellular bridges and intra-axonal transport [8,26]. As the gE/gI complex is not expressed in this deletion mutant, this process may have been limited, resulting in impairment in cell-to-cell transmission. Consequently, even when sufficient virus can infect the neurons directly at the site of infection, the reduced virulence of BHV-5 gI/gE/US9⁻ may be due to a reduced ability to spread from peripheral neurons to CNS neurons.

Following dexamethasone treatment, the wild type virus was reactivated in calves from group B, as evidenced by virus isolation from nasal secretions. Other indications of viral reactivation like virus isolation or detection of viral DNA from brain sections were not observed among animals of group B. These results suggest that, in the present study, the latent infection induced by the wild type virus was probably restricted to the trigeminal ganglia. In contrast, other authors found a widespread distribution of BHV-5 DNA in the CNS of latently infected calves [18,25]. As marked differences in neuroinvasiveness have been reported among BHV-5 isolates [2,3], different patterns of distribution of viral DNA in the brain could be expected for different viral strains. In addition, different animal age and genetic background might also account for differences in the BHV-5 distribution in the brains of latently infected animals.

No recombinant virus could be recovered from nasal secretions of calves from group A, following dexamethasone treatment. This indicates that detectable amounts of infectious recombinant virus were not present in the nasal mucosa after the corticosteroid administration. The inability to reactivate from the latent state has been related to the role that gI, gE and US9 play in the anterograde axonal transport that has to take place to transport reactivated virus to the peripheral sites [8]. It is supposed that US9 is important because it promotes the movement of virion envelope components along axons [8]. Therefore, the recombinant evaluated here may not have the ability to reactivate, or may have such capacity somehow impaired. Another possible explanation for the failure of the recombinant virus to be reactivated from infected animals is that the deletion of gI, gE and US9 genes may result in a reduced chance of establishing latent infections.
Neutralizing antibody response to the recombinant BHV-5 was much less intense than the response observed in wild type virus infected controls. This would be expected, in view that the limited virus replication within the host would also induce a limited antibody response.

The phenotype of the recombinant virus was substantially different from the wild type BHV-5 when both viruses were inoculated in calves. Although no major unintended genomic alterations were observed when previous genomic characterization of the deletion mutant was performed, it should be considered that undetected genomic changes may have contributed to the observed phenotype of the BHV-5 deletion mutant in calves.

In conclusion, this study demonstrated that BHV-5 gI, gE and US9 genes do play a significant role in the virus’s neuropathogenicity as well as in its capacity to induce latency or reactivation of infection in calves. Further studies shall be conducted in order to examine the ability of this recombinant to induce latent infections.

References


