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Bovine herpesvirus-5 infection in a rabbit experimental model: Immunohistochemical study of the cellular response in the CNS

Gisele F. Machado [a,*], Fernanda Bernardi [b], Fernando Y.M. Hosomi [b], Juliana R. Peiró [a], Rudi Weiblen [c], Paulo M. Roehe [d], Antonio C. Alessi [e], Guilherme D. Melo [a], Adriano T. Ramos [f], Paulo C. Maiorka [b]

[a] UNESP – Univ Estadual Paulista, Faculdade de Medicina Veterinária, Departamento de Clínica, Cirurgia e Reprodução Animal, Araçatuba, SP, Brazil
[b] Univ de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Patologia, São Paulo, SP, Brazil
[c] Univ Federal de Santa Maria, Departamento de Medicina Veterinária Preventiva e Departamento de Microbiologia e Parasitologia, Santa Maria, RS, Brazil
[d] Univ Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia, Porto Alegre, RS, Brazil
[e] UNESP – Univ Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, Departamento de Patologia Veterinária, Jaboticabal, SP, Brazil
[f] Univ Federal de Santa Catarina, Curso de Medicina Veterinária, Curitibanos, SC, Brazil

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ABSTRACT

Since little information is available regarding cellular antigen mapping and the involvement of non-neuronal cells in the pathogenesis of bovine herpesvirus type 5 (BHV-5) infection, it were determined the BHV-5 distribution, the astrocytic reactivity, the involvement of lymphocytes and the presence of matrix metalloproteinase (MMP)-9 in the brain of rabbits experimentally infected with BHV-5. Twelve New Zealand rabbits that were seronegative for BHV-5 were used for virus inoculation, and five rabbits were used as mock-infected controls. The rabbits were kept in separate areas and were inoculated intranasally with 500 µl of virus suspension (EVI 88 Brazilian isolate) into each nostril (virus titer, $10^{7.5}$ TCID50). Control rabbits were inoculated with the same volume of minimum essential medium. Five days before virus inoculation, the rabbits were submitted to daily administration of dexamethasone. After virus inoculation, the rabbits were monitored clinically on a daily basis. Seven rabbits showed respiratory symptoms and four animals exhibited neurological symptoms. Tissue sections were collected for histological examination and immunohistochemistry to examine BHV-5 antigens, astrocytes, T and B lymphocytes and MMP-9. By means of immunohistochemical and PCR methods, BHV-5 was detected in the entire brain of the animals which presented with neurological symptoms, especially in the trigeminal ganglion and cerebral cortices. Furthermore, BHV-5 antigens were detected in neurons and/or other non-neural cells. In addition to the neurons, most infiltrating CD3 T lymphocytes observed in these areas were positive for MMP-9 and also for BHV-5 antigen. These infected cells might contribute to the spread of the virus to the rabbit brain along the trigeminal ganglia and olfactory nerve pathways.

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1. Introduction

Bovine herpesvirus type 5 (BHV-5), a member of the family *Alphaherpesvirinae*, is a major etiological agent of meningoencephalitis in cattle [1,2]. The disease has been characterized by tremors, nystagmus, tooth grinding, circling, ataxia, recumbence, paddling, and death [3,4]. Following replication in the nasal mucosa, BHV-5 invasion into the brain is thought to occur mainly through the olfactory pathway and sensory neurons of the trigeminal ganglion (TG) [5].

Acute BHV-5 infection and disease have been successfully reproduced in rabbits [6–9] and sheep [10]. These experimental models have been used for biological studies to understand the molecular basis of BHV-5 acute and latent infection [11,12].

In contrast to many other animal herpesvirus infections, BHV-5 reactivation is frequently accompanied by a recrudescence of clinical disease, both in the natural host and in a rabbit model. In animals surviving after acute infection, BHV-5 establishes a lifelong latent infection that can be reactivated under certain natural or induced stimuli [4,11].

Lesions related to central nervous system (CNS) infection have been well-described in cattle [4], rabbits [7,9] and sheep [10]; these descriptions have primarily focused on aspects of neuronal

* Corresponding author. Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária, Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Rua Clóvis Pestana, 793 Araçatuba, SP, CEP 16050-680, Brazil. Tel.: +55 18 3636 1420; fax: +55 18 3636 1401.
E-mail addresses: giselem@fmva.unesp.br, di.melo@uol.com.br (G.F. Machado).

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degeneration and inflammatory response. The distribution of BHV-5 DNA during the acute and latent phases of infection in experimentally infected rabbits and calves has already been determined [12,13].

In rabbits, the trigeminal nerve and the olfactory pathway are important routes for BHV-5 access to the CNS [5,14]. However, other pathways for BHV-5 spread must be considered, such as leukocyte-facilitated entry across the cerebral barriers or via the choroid plexus and meninges [15,16]. A key component for leukocyte migration is matrix metalloproteinase 9 (MMP-9, gelatinase B) because MMP-9 degrades type IV collagen, the primary component of the basal membrane [17,18].

Little information is available regarding cellular antigen mapping or the possible participation of non-neuronal cells in the pathogenesis of BHV-5 infection in rabbits or calves. Also, other cellular types that can serve as a virus keeper and transporter must be better established. Therefore, the purpose of this study was to examine BHV-5 distribution, astrocytic reactivity, involvement of the main populations of lymphocytes (T and B) and the presence of MMP-9 in several areas of the brain from rabbits experimentally infected with a Brazilian isolate of BHV-5.

2. Materials and methods

2.1. Animals

Seventeen New Zealand white male rabbits provided by the College of Veterinary Medicine and Animal Science – University of São Paulo (FMVZ-USP) that were seronegative for BHV-5, five to six weeks-old, and weighed 0.8–1.0 kg were used in this experiment. All rabbits were group-housed (three rabbits per cage). Before any experimental manipulation, the rabbits were allowed to acclimatize to laboratory conditions for one week. Food and water were provided *ad libitum*. Twelve rabbits were used for virus inoculation (infected group), and five animals were used as controls (control group).

2.2. Virus, BHV-5 intranasal infection and dexamethasone treatment

The Brazilian BHV-5 isolate EVI-88 was obtained from Dr. Paulo M. Roehe (UFRGS - Rio Grande do Sul – Brazil). The virus that was used in all experiments was propagated and titrated in Madin–Darby bovine kidney (MDBK) cells. Rabbits were anesthetized by intramuscular (i.m.) injection of ketamine (5 mg/kg) plus xylazine (35 mg/kg). The rabbits were inoculated intranasally with $10^{7.5}$ $TCID_{50}$ (median tissue culture infectious dose; which is the amount of a pathogenic agent that will produce pathological change in 50% of cell cultures) of BHV-5 in 500 μL of minimum essential medium (MEM). Control rabbits were inoculated with the same volume of MEM. Five days before inoculation (b.i.), the rabbits were submitted to daily administration of dexamethasone (Dx; 0.5 mg/kg/day, i.m.) in order to promote infection [7,11,12]. After Dx treatment and virus inoculation, the rabbits were monitored clinically on a daily basis for 23 days. Animals were sacrificed as they exhibited neurological symptoms and all the other rabbits were euthanized at day 23 *post* inoculation (p.i.).

2.3. Postmortem sampling

The rabbits were sacrificed, necropsies were performed, and macroscopic lesions were recorded. Each brain was removed and sagitally sliced; one hemisphere was fixed in 10% neutral buffered formalin and paraffin-embedded for histopathology and immunohistochemistry. The other hemisphere was frozen and stored at −80 °C for DNA extraction and PCR.

2.4. Immunohistochemistry

For immunohistochemistry, endogenous peroxidase activity was blocked after dewaxing by incubating the sections in 2% (v/v) hydrogen peroxide (30 vol.) diluted in 50% (v/v) methanol for 30 min. Pre-treatments for antigen retrieval were performed according to the specifications for the primary antibodies (Table 1). Non-specific binding was blocked with 3% (w/v) nonfat dry milk in PBS (phosphate-buffered saline) pH 7.2 for 30 min. The sections were incubated with the primary antibodies (Table 1) for 18–22 h at 4 °C in a humidified chamber. The slides were washed in PBS and incubated with a biotinylated secondary antibody and a streptavidin-HRP complex (LSAB+ Kit, K0690, Dako) according to the manufacturer's instructions. The reaction was developed with 3,3′-diaminobenzidine (K3468, Dako). The slides were counterstained with Harris's hematoxylin, dehydrated, cleared, and mounted with coverslips. Negative control sections were prepared by replacing the primary antibody with 1% (v/v) bovine serum albumin (BSA). The tissue samples were examined by light microscopy in the following areas of the cerebral hemisphere: (1) cerebellum, (2) cerebral cortex (frontal, temporal, occipital, parietal and piriform), (3) hippocampus, (4) midbrain, (5) olfactory bulb, (6) pons-medulla oblongata, (7) sub-ependymal zone, (8) thalamus, and (9) trigeminal ganglion.

The intensity of glial fibrillary acidic protein (GFAP) labeling was scored semiquantitatively on a four-point scale of 1–4. A score of 1 represented mild GFAP labeling; a score of 2, moderate; 3, intense; and a score of 4 represented very intense GFAP labeling. The quantification of $CD3^+$ and $CD79^+$ cells was performed by counting the number of positive cells/mm^2 [19] in the pre-established brain areas. The evaluation of BHV-5 and MMP-9 labeling consisted of semiquantitative mapping according to the labeled cell and to the brain area. All analyses were "blind", i.e., the researchers were denied knowledge of the experimental groups.

2.5. DNA extraction and PCR

Fragments from seven different brain areas were obtained: (1) brainstem, (2) cerebellum, (3) frontal cortex and olfactory bulb, (4) temporal cortex, (5) parietal and piriform cortices, (6) midbrain and

Table 1
Panel of antibodies used in this study to characterize astrocytes, T and B lymphocytes, MMP-9 and BHV-5 infected cells in the brain of rabbits.

Antibody/lectin	Specificity	Dilution	Pre-treatment	Source
Monoclonal anti-BHV-5	BHV-5 antigen	1:1000	Proteinase K 37 °C for 10 min	Dr. R. Weiblen, UFSM, Brazil [39]
Polyclonal anti-human GFAP (glial fibrillary acidic protein)	Astrocytes	1:500	Trypsin 37 °C for 30 min	Sigma–Aldrich, G9269
Polyclonal anti-human CD3	T lymphocytes	1:200	Citrate pH6.0 in steamer for 30 min	Dako, A0452
Monoclonal anti-human CD79αcy, clone HM57	B lymphocytes	1:50	Citrate pH6.0 in steamer for 30 min	Dako, M7051
Polyclonal anti-human MMP-9	MMP-9 (latent and active forms)	Ready-to-use	Tris-EDTA-Tween pH9.0 in steamer for 30 min	NeoMarkers, RB-9234-R7

diencephalon, (7) trigeminal ganglion. The extraction of DNA was performed using proteinase K and phenol-chloroform. The primers forward (GD1: 5′-GCCCGCAGTTTCCCCTACC-3′) and reverse (GD2: 5′-CGCACCCGCTCTCAATCTT-3′) were designed to amplify one region of the viral glycoprotein D, resulting in an amplicon of 564 bp [20]. The PCR conditions were: 95 °C for 3 min, followed by 40 cycles of 95 °C for 45 seg, 61 °C for 45 seg, and 72 °C for 45 seg, and then 72 °C for 10 min. The PCR products were analyzed on ethidium bromide-stained agarose gels.

2.6. Statistical analysis

For $CD3^+$ and $CD79^+$ cells, significant differences between groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. A value of $P < 0.05$ was considered statistically significant. Data are expressed as mean ± standard deviation (SD). All statistical analyses were performed using Prism software (GraphPad, California, USA).

2.7. Ethics

All animal handling procedures and experimentation were performed according to the recommendations of the Brazilian College on Animal Experimentation, and all experiments were approved by the institutional Ethics and Animal Welfare Committee (CEEA – Comissão de Ética e Experimentação Animal - UNESP, process #08/05).

3. Results

3.1. Clinical signs

Seven animals (rabbit identification numbers 4, 6, 7, 9, 12, 14, and 15) displayed nasal and ocular discharges from day 12 to day 23 p.i. Rectal temperatures were not notably different between infected and control rabbits. Neurological symptoms were observed only in rabbits 4, 6, 7, and 14, and they were characterized by slight apathy to depression, seizures and grinding of teeth. Because the remaining animals were euthanatized at day 23 p.i., whether neurological signs would have developed after this period is unknown.

3.2. Postmortem findings

Gross cerebral lesions were observed in 3 rabbits (numbers 6, 7, and 14). These lesions were composed of softening of the parenchymal tissue and hemorrhagic foci in the olfactory bulbs and frontal cortex. The lesions were not symmetrical and did not have a well-defined pattern of distribution. No macroscopic lesions were seen in the CNS of the control rabbits.

3.3. CNS histopathology

Microscopic examination revealed histological changes of varied severity in several areas of the brain, but mostly in the cerebral cortex (frontal, temporal, occipital, parietal and piriform) of rabbits that died or were sacrificed with neurological symptoms (rabbits 4, 6, 7, and 14). These changes were characterized by the presence of mononuclear perivascular cuffs, neuronophagia, satellitosis, focal and diffuse gliosis, congestion, hemorrhages, neuronal degeneration and necrosis. Non-suppurative leptomeningitis was present in 91.7% (11/12) of the animals in the olfactory bulb and frontal/temporal cortex, but was less frequent in caudal encephalic areas, such as the pons, medulla oblongata and cerebellum. Inclusions bodies were not observed in neurons or astrocytes.

3.4. BHV-5 detection in the brain of rabbits

The infected rabbits that exhibited neurological symptoms (neurological, $n = 4$) presented a different pattern of viral distribution in the brain than the infected rabbits without neurological involvement (non-neurological, $n = 8$).

BHV-5 was detected by means of PCR in all infected rabbits. Regarding the viral distribution across the brain, BHV-5 could be detected in all the selected areas in the neurological rabbits. On the other hand, in the brains of the non-neurological rabbits, BHV-5 was detected in an inconstant manner (Fig. 1).

In regards to BHV-5 antigen detected by means of IHC, the findings were similar to those seen with PCR. The viral antigen was detected principally in cortical neurons, but also in ependymal cells, endothelial cells, the hippocampus, diencephalon and midbrain. A small number of positive neurons were observed in the cerebellum and in the pons/medulla oblongata. Trigeminal ganglion cells and inflammatory mononuclear cells with positive staining were also detected (Fig. 2a–f). Whereas the BHV-5 antigen was detected all over the brains of neurological animals, the distribution of the virus in the brains of non-neurological animals was noted primarily in the cranial areas, periventricular regions and the olfactory and trigeminal tracts (Fig. 2g).

3.5. Immunohistochemical evaluation of astrocyte alterations during BHV-5 infection in rabbits

Reactive astrocytes were observed, particularly in cortical areas, the hippocampus, and the subependymal layer; these reactive astrocytes were detected in the same locations as the viral antigen in the neurons of some of the animals (Fig. 3). Astrocytosis, gemistocytic astrocytes and astrogliosis were also observed. Alterations in astrocyte morphology were more intense within the inflamed areas, and the astrocytes tended to present few numbers of

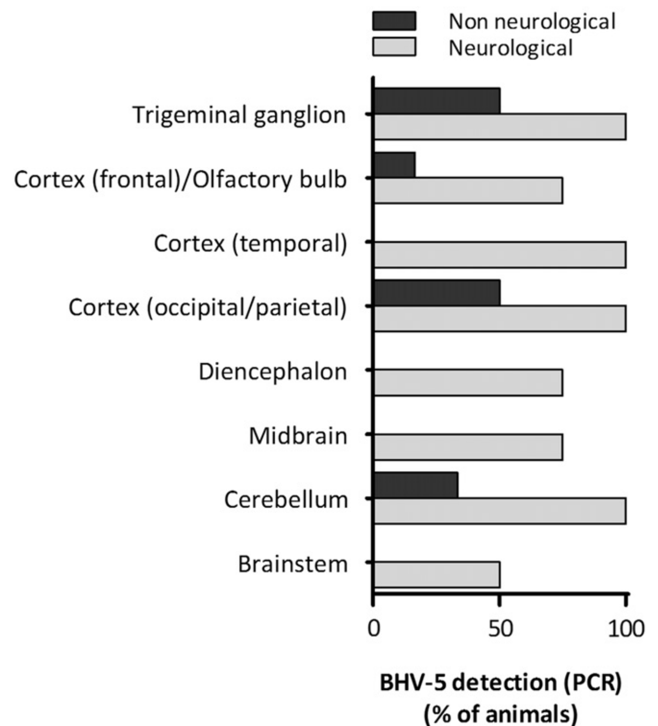

Fig. 1. Detection of bovine herpesvirus type 5 (BHV-5) in different brain areas of experimentally infected rabbits that exhibited neurological symptoms (neurological, $n = 4$) or did not exhibit symptoms (non-neurological, $n = 8$).

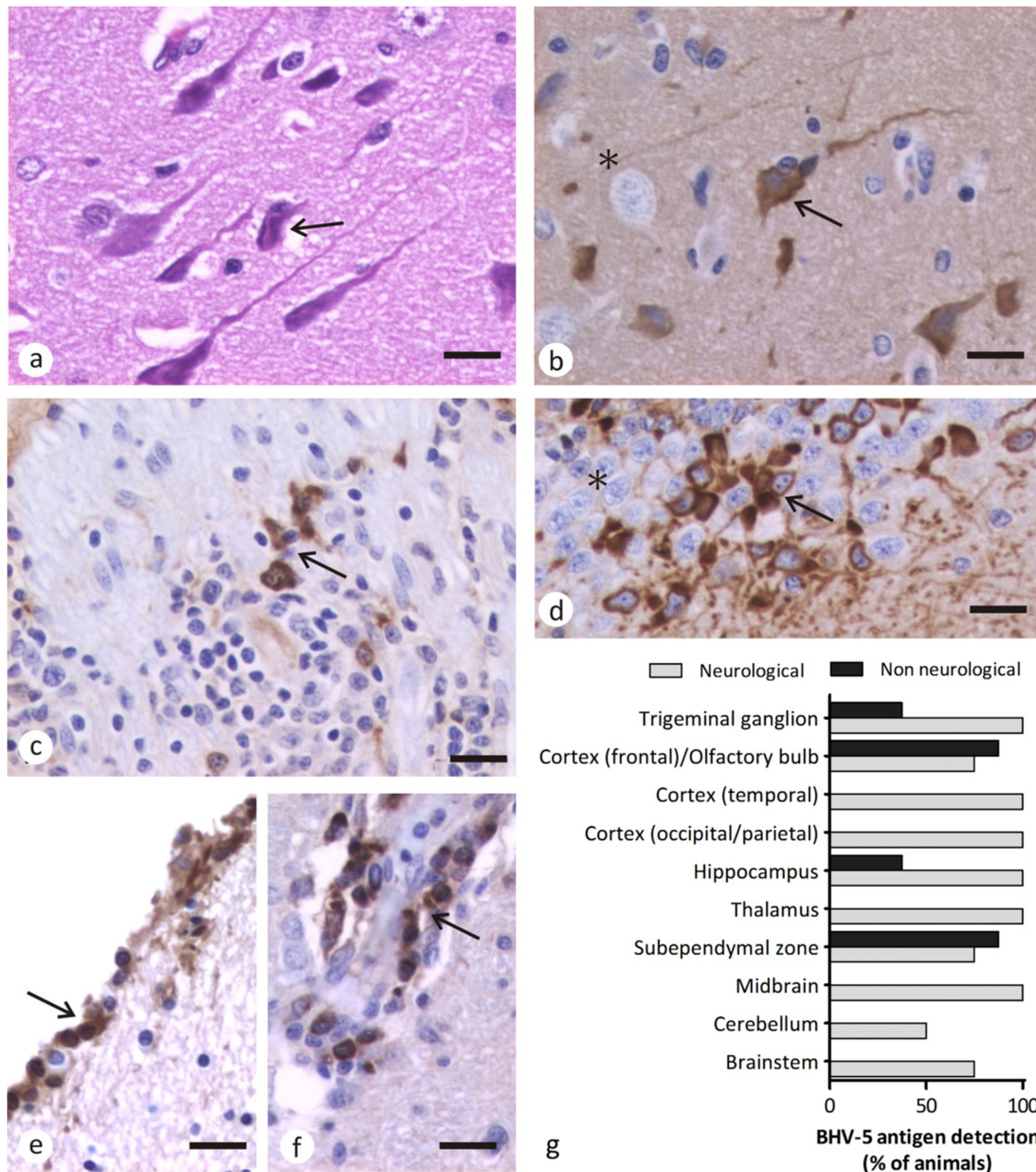

Fig. 2. Distribution of bovine herpesvirus type 5 (BHV-5) antigen in the brain of experimentally infected rabbits. a: Presence of dark neurons in the frontal cortex. b: Cortical neurons presenting intense cytoplasmic staining for BHV-5 (arrow). Note the absence of staining in adjacent neurons (*). Also observe the similarity between the immunostained neurons and the dark neurons shown in a. c: Positive staining in interstitial cells in the trigeminal ganglion. d: Hippocampal neurons presenting strong cytoplasmic staining for BHV-5 (arrow). As observed in B, there is also an absence of staining in other nearby neurons (*). e: Ependymal cells presenting strong cytoplasmic staining for BHV-5 (arrow). f: Positive mononuclear cells forming a perivascular cuff in the temporal cortex. a (HE); b–f (streptavidin–biotin complex). Scale bar = 25 μm g: Frequency of BHV-5 antigen detection in different brain areas of experimentally infected rabbits that exhibited neurological symptoms (neurological, $n = 4$) or did not exhibit symptoms (non-neurological, $n = 8$).

processes, thickening of the processes, loss of processes, and large swollen nuclei. In the infected group, an increase in GFAP labeling was observed in the cerebral cortices, the hippocampus and the thalamus.

3.6. Immunohistochemical analysis of lymphocytes and MMP-9 in the brain of rabbits upon BHV-5 infection

Inflammatory infiltrates constituted by $CD3^+$ T lymphocytes were detected in all of the pre-established encephalic areas of the infected rabbits, including the perivascular cuffs, leptomeninges and in areas of malacia. A strong association between $CD3^+$ T lymphocytes and BHV-5 was detected since the $CD3^+$ cells were also positive for BHV-5 antigen (Fig. 4). $CD3^+$ T lymphocytes predominated in the brain of the infected rabbits with neurological symptoms ($P = 0.0010$), which presented 4.56 (±1.93) $CD3^+$ cells/mm^2. In contrast, only 1.71 (±0.57) $CD3^+$ cells/mm^2 were found in the infected rabbits without neurological symptoms and 1.39 (±0.39) $CD3^+$ cells/mm^2 were detected in the control group. Regarding B lymphocytes, the infected rabbits with neurological symptoms presented 0.32 (±0.11) $CD79^+$ cells/mm^2, the infected rabbits without neurological symptoms showed 0.15 (±0.13) $CD79^+$ cells/mm^2, and the control rabbits presented 0.19 (±0.11) $CD79^+$ cells/mm^2, with no significant difference among the groups ($P = 0.0509$) (Fig. 5).

MMP-9 staining was observed diffusely in the neuropil and in different cells within the brain of the infected rabbits, such as neurons, astrocytes, and endothelial cells, but it was particularly

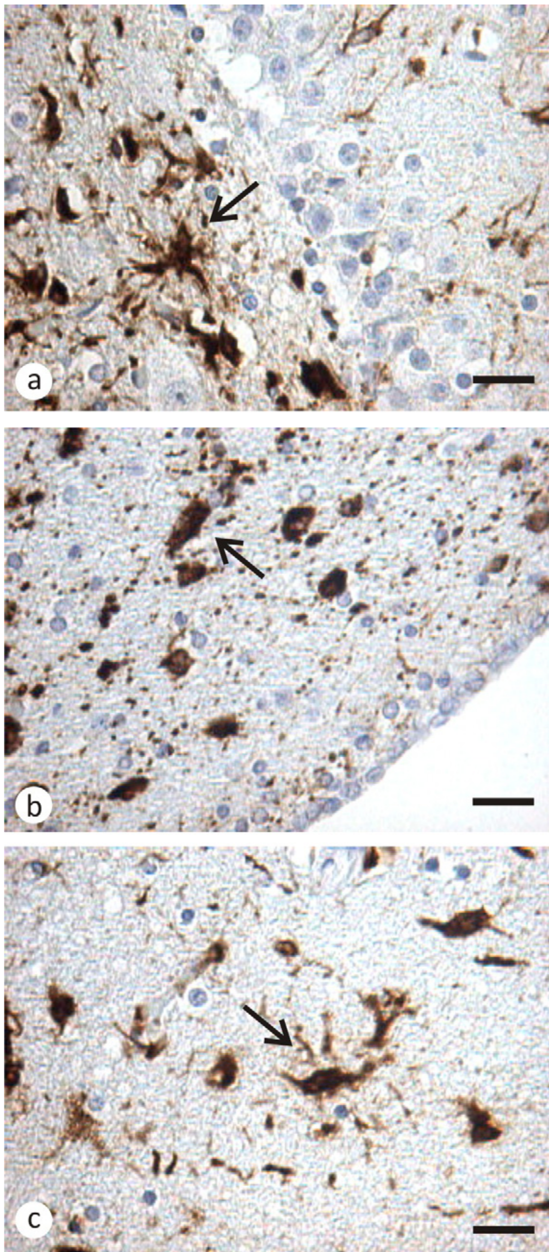

Fig. 3. Glial fibrillary acidic protein (GFAP) staining in astrocytes in the brains of BHV-5 experimentally infected rabbits. a: Strongly stained protoplasmatic astrocytes (arrow) in the dentate gyrus. b: Intensely stained gemistocytes (arrow) in the subependymal zone of the lateral ventricle. c: Fibrous astrocytes with thick processes (arrow) and intensely stained in low numbers. Streptavidin-biotin complex. Scale bar = 40 μm.

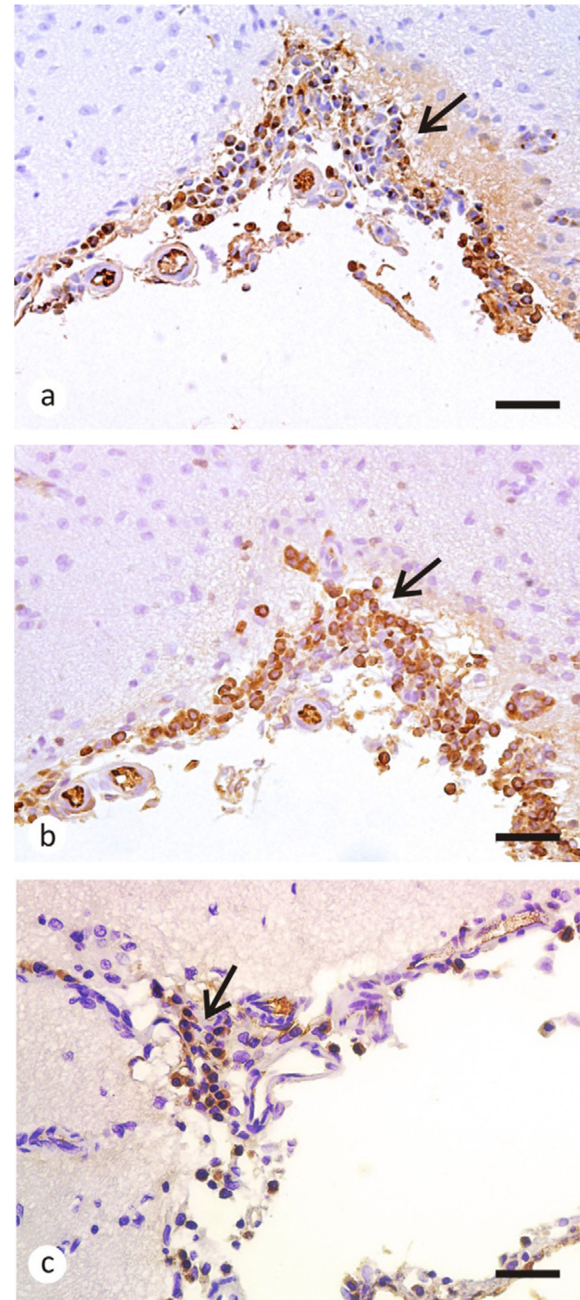

Fig. 4. Bovine herpesvirus type 5 (BHV-5), T lymphocyte and matrix metalloproteinase-9 (MMP-9) detection in the brain of BHV-5 experimentally infected rabbits. a: Mononuclear cells positive for the BHV-5 antigen (arrow) in the leptomeninges. b: T lymphocyte infiltration in the same area as in a (arrow). Note the positive CD3 cytoplasmic staining and the correlation with the BHV-5-positive cells shown in a. c: Positive MMP-9 mononuclear cells (cytoplasmic staining) around a meningeal blood vessel and in the adjacent extracellular area. Streptavidin-biotin complex. Scale bar = 40 μm.

present in the mononuclear cells of the inflammatory infiltrates, especially in the leptomeninges and in parenchymal perivascular cuffs. Furthermore, the pattern of MMP-9 labeling was very similar to that of the $CD3^+$ cells; in these cases, MMP-9 labeling was positive in the $CD3^+$ cells and in the extracellular area adjacent to these cells (Fig. 4).

4. Discussion

Rabbit models have been used to address some questions concerning the immunopathogenesis of BHV-5 infection. Although this model has provided important information regarding some aspects of virus infectivity and pathogenesis, the participation of blood-derived immune cells and glial cells during this viral meningoencephalitis model must still be fully elucidated. Herpesvirus reactivation usually occurs in the absence of obvious clinical signals because a reduced number of neurons are involved and virus production is limited during reactivation [21].

The p.i. onset of clinical signs in the rabbits was rather divergent from the times related in the literature. Differences in the time of respiratory and neurological signs presented after inoculation observed by Chowdhury et al. [7] and Silva et al. [9] may be related with the age of animals and/or inoculation method. Furthermore,

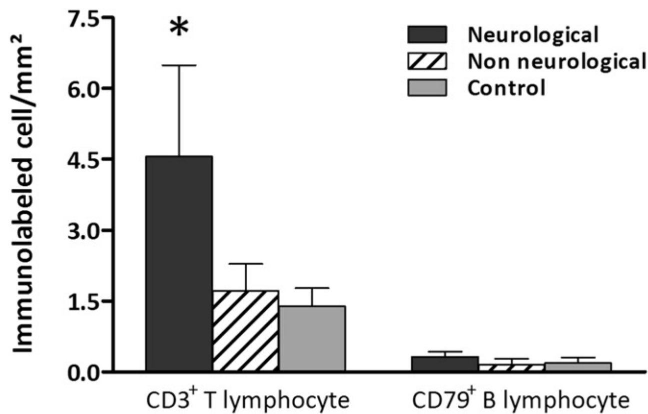

Fig. 5. Number of $CD3^+$ T lymphocytes and $CD79^+$ B lymphocytes detected in the brains of the experimentally infected rabbits that exhibited neurological symptoms (neurological, $n = 4$) or did not exhibit neurological symptoms (non-neurological, $n = 8$), and uninfected rabbits (control, $n = 5$). Bars indicate the mean (±standard deviation). The mean value was statistically significant by the Tukey's Multiple Comparison Test ($^*P < 0.05$).

Chowdhury et al. [7] used a different viral strain, which can result in different response to infection as observed by Silva et al. [9] with the Argentinean isolate A663.

Arachnoid lymphocytic infiltrate (leptomeningitis) was observed in an elevated percentage of encephalons, even in asymptomatic rabbits, indicating the involvement of lymphocytes in the pathogenesis of BHV-5 infection. One of the most distinctive viral encephalitis lesions, the perivascular cuffs, was most apparent in the symptomatic rabbits. The presence of lymphocytic leptomeningitis and/or perivascular cuffs demonstrated the progression of sub-acute to acute inflammatory changes in the brain and was well-correlated with BHV-5 antigen detection. Similar lesions have previously been demonstrated in acutely infected rabbits [7,9,22] and calves [4].

Trigeminal ganglionitis has been described relatively early in calves [4] and less frequently in rabbits [9]. We observed inflammatory cells in the trigeminal ganglion in only three rabbits (25%), while antigen detection by immunohistochemistry occurred in 58.3% (7/12) of infected animals.

The intranasal route of infection directly introduces a neurotropic virus to a mucosal surface with distinct neuronal pathways to the CNS. In this study, we demonstrated through immunohistochemistry that the antigen was distributed over the entire brain of the rabbits after BHV-5 infection. In rabbits with neurological symptoms, the viral antigen was detected mostly in the hippocampus, parietal/occipital cortex, diencephalon, piriform cortex and midbrain, in accordance with the results found with PCR. The pattern of BHV-5 immunoreactivity supports the idea that following infection of the olfactory bulb glomeruli, the virus spreads via both ventricular surfaces and by retrograde transport within axons through structures indirectly connected to the olfactory pathway [22]. We verified that viral dissemination was compatible with the description of inflammatory/degenerative observed lesions. Most ischemic ("dark") neurons observed by HE exhibited positive staining for the BHV-5 antigen on serial sections. We did not detect the viral antigen in astrocytes, even though these cells showed remarkable changes in their morphology and areas of astrogliosis were detected where BHV-5 was observed.

In general, latency and reactivation are considered critical strategies for herpesviruses to survive in nature [23]. The major sites of latent infection in human (i.e., herpes simplex virus type 1 – HSV-1) and animal α-herpesviruses (BHV-5, BHV-1, and pseudorabies virus -PRV) are the sensory nerve ganglia that innervate the site of primary viral replication [24–26]. Typically, most cells infected by either α- or β-herpesviruses support lytic replication, while only specific subsets of cells normally harbor latent virus.

The administration of corticosteroids is used in the BHV-5 infection model to mimic stress conditions and latent infection reactivation [7,11]. Corticosteroids also may facilitate BHV-5 replication inside lymphocytes, since in a study with BHV-1, the authors detected no differences in lymphocytes population comparing latently infected calves and calves treated with dexamethasone, and concluded that $CD4^+$ T lymphocytes can be infected by BHV-1 [27]. Furthermore, despite the spread of the virus through the trigeminal ganglion and the olfactory bulb, infected leukocytes may contribute to viral dissemination into the rabbit brain. We detected BHV-5 positive $CD3^+$ T lymphocytes, suggesting that these cells can harbor the virus and facilitate their dissemination across the cerebral barriers. Not uncommonly, some α-herpesviruses infect and replicate in peripheral blood mononuclear cells, including BHV-1, PRV and equine herpesvirus type 1 (EHV-1) [28–31]. In addition, BHV-1 DNA has also been detected in $CD4^+$ T lymphocytes in the tonsils [27,32].

In further support of the premise that leukocytes facilitate the entry of BHV-5 into the CNS, we also detected the presence of MMP-9 in the inflammatory infiltrates that were composed of primarily $CD3^+$ T lymphocytes. The involvement of MMP-9 has already been correlated with the pathogenesis of other herpesviruses infection, such as Epstein–Barr virus (EBV) and HSV-1 [33,34]. Since MMPs, especially MMP-2 and MMP-9, are key to leukocyte influx into the brain [35], this study provides evidences of BHV-5-infected T lymphocytes migration into the nervous milieu. Further, despite the anti-inflammatory effects that dexamethasone presents in the brain [36,37], the treatment with this drug in our model seems not to have influenced MMP-9 production by T lymphocytes, as previously described [38].

In conclusion, these results provide important contributions to the understanding of BHV-5 neuropathogenesis in a rabbit model. The data presented herein suggest a new additional pathway for CNS invasion, in which BHV-5 spread is mediated by infected leukocytes. T lymphocytes, astrocytes and MMP-9 clearly play roles in the pathogenesis of BHV-5 infection. Nevertheless, further studies are needed to confirm the CNS invasion pathways and to identify other cells and molecules involved in this process.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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