Mapping the sites of latency and reactivation by bovine herpesvirus 5 (BoHV-5) and a thymidine kinase-deleted BoHV-5 in lambs[1]

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ABSTRACT.- Cadore G.C., Marcon G., Brum M.C.S., Weiblen R. & Flores E.F. 2013. **Mapping the sites of latency and reactivation by bovine herpesvirus 5 (BoHV-5) and a thymidine kinase-deleted BoHV-5 in lambs.** *Pesquisa Veterinária Brasileira 33(12):1409-1415.* Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: eduardofurtadoflores@gmail.com

A thymidine kinase (*tk*)-deleted bovine herpesvirus 5 (BoHV-5*tk*Δ) was previously shown to establish latent infection and reactivate - even poorly - in a sheep model (Cadore et al. 2013). As TK-negative alphaherpesviruses are unlike to reactivate in neural tissue, this study investigated the sites of latency and reactivation by this recombinant in lambs. For this, groups of lambs were inoculated intranasally with the parental BoHV-5 strain (SV-507/99) or with the recombinant BoHV-5*tk*Δ. During latent infection (40 days post-inoculation, pi), the distribution of recombinant virus DNA in neural and non-neural tissues was similar to that of the parental virus. Parental and recombinant virus DNA was consistently detected by PCR in trigeminal ganglia (TGs); frequently in palatine and pharyngeal tonsils and, less frequently in the retropharyngeal lymph nodes. In addition, latent DNA of both viruses was detected in several areas of the brain. After dexamethasone (Dx) administration (day 40pi), the recombinant virus was barely detected in nasal secretions contrasting with marked shedding of the parental virus. In tissues of lambs euthanized at day 3 post-Dx treatment (pDx), reverse-transcription-PCR (RT-PCR) for a late viral mRNA (glycoprotein D gene) demonstrated reactivation of parental virus in neural (TGs) and lymphoid tissues (tonsils, lymph node). In contrast, recombinant virus mRNA was detected only in lymphoid tissues. These results demonstrate that BoHV-5 and the recombinant BoHV-5*tk*Δ do establish latent infection in neural and non-neural sites. Reactivation of the recombinant BoHV-5*tk*Δ, however, appeared to occur only in non-neural sites. In anyway, the ability of a tk-deleted strain to reactivate latent infection deserves attention in the context of vaccine safety.

INDEX TERMS: Bovine herpesvirus, BoHV-5, thymidine kinase, recombinant, latency, sheep.

RESUMO.- [**Mapeamento dos sítios de latência e reativação pelo herpesvírus bovino tipo 5 (BoHV-5) e por mutante deletado no gene da timidina quinase em ovinos.**] Um recombinante do herpesvírus bovino tipo 5 com deleção no gene da timidina quinase (BoHV-5*tk*Δ) foi capaz de estabelecer latência e reativar - embora ineficientemente - em modelo experimental em ovinos (Cadore et al. 2013). Como a reativação de alfaherpesvírus defectivos na TK em tecido neural é improvável, o presente estudo investigou os sítios de latência e reativação por esse recombinante em ovinos. Para isso, grupos de ovinos foram inoculados com a cepa de BoHV-5 parental (SV-507/99) ou com o recombinante BoHV-5*tk*Δ. Durante a infecção latente (dia 40 pós-infecção, pi) a distribuição do DNA do vírus recombinante no encéfalo de ovinos infectados experimentalmente foi similar ao do vírus parental (SV-507/99). O DNA de ambos os vírus foi detectado consistentemente por PCR nos gânglios trigêmeos (TGs), frequentemente nas tonsilas farín-

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geas e palatinas e, com menos frequência, nos linfonodos retrofaríngeos. Após administração de dexametasona (Dx), o vírus recombinante foi raramente detectado nas secreções nasais, contrastando com excreção abundante do vírus parental. RT-PCR para mRNA de um gene tardio (glicoproteína D) realizado em tecidos de animais eutanasiados 3 dias pós-Dx demonstrou reativação do vírus parental em tecido neural (TGs) e não-neural (tonsilas, linfonodo). Em contraste, a reativação do vírus recombinante ficou restrita ao tecido linfoide. Esses resultados demonstram que tanto o BoHV-5 parental quanto o recombinante estabelecem latência em sítios neurais e não-neurais. No entanto, o recombinante BoHV-5*tk*Δ parece reativar apenas nos tecidos não-neurais (linfoide). De qualquer forma, a capacidade do recombinante reativar a infecção latente deve ser considerada no contexto de segurança vacinal.

TERMOS DE INDEXAÇÃO: Herpesvírus bovino, BoHV-5, timidina quinase, recombinante, latência, ovinos.

INTRODUCTION

Bovine herpesvirus type 5 (BoHV-5) is the agent of non-suppurative, frequently fatal meningoencephalitis in cattle, a disease frequently described in Argentina (Perez et al. 2003) and Brazil (Salvador et al. 1998, Rissi et al. 2008). BoHV-5 is an enveloped DNA virus classified within the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Roizman et al. 1992). BoHV-5 is closely related to bovine herpesvirus 1 (BoHV-1), the agent of bovine infectious rhinotracheitis and vulvovaginitis/balanoposthitis (Kahrs 2001). Like other alphaherpesviruses, BoHV-1 and BoHV-5 establish latent infections in sensory nerve ganglia and can be reactivated spontaneously or by corticosteroid administration (Rock 1994, Vogel et al. 2003).

The BoHV-5 genome is a linear double-stranded DNA molecule of approximately 138 kb in length and encodes at least 70 gene products (Delhon et al. 2003). Approximately half of viral-encoded products are believed to be non-essential (NE) for virus replication in tissue culture (Delhon et al. 2003). Deletions of individual NE genes – especially the gene encoding the enzyme thymidine kinase (TK) - have been used to produce attenuated BoHV-1 strains for potential use in vaccines (Kit et al. 1985, Chowdhury 1996, Kaashoek et al. 1996). Herpesvirus-encoded TK is involved in the metabolism of deoxyribonucleotides (dNTPs), an enzymatic activity that is necessary for viral DNA synthesis and genome replication in neurons (Tenser 1994). Deletion or inactivation of *tk* gene leads to deficient virus replication in neurons and reduced neurovirulence of alphaherpesviruses (Coen et al. 1989, Mengeling 1991, Tenser 1994, Ferrari et al. 2000). TK-deleted BoHV-1 mutants have been shown to be attenuated to different levels (Kit et al. 1985, Chowdhury 1996, Kaashoek et al. 1996), and a *tk* deletion BoHV-5 mutant was attenuated for rabbits (Silva et al. 2010) and calves (Santos et al. 2011).

Although herpesvirus-encoded TK is required for virus replication in neurons, it is not necessary for the establishment of latent infection (Tenser et al. 1979, Coen et al. 1989, Volz et al. 1992, Kaashoek et al. 1996, Chen et al. 2004). In contrast, TK activity is required for efficient reactivation in neural tissue (Coen et al. 1989, Volz et al. 1992, Kaashoek et al. 1996, Ferrari et al. 1998, Chen et al. 2004). Thus, it is generally accepted that TK-defective alphaherpesviruses do not reactivate - or reactivate poorly - from sensory nerve ganglia (Tenser et al. 1979, Coen et al. 1989, Chen et al. 2004).

In a recent study, a *tk*-deleted recombinant BoHV-5 strain (BoHV-5*tk*Δ, Brum et al. 2010a) was demonstrated to establish latent infection in lambs, a proposed animal model. In addition, the recombinant was reactivated - even poorly - upon dexamethasone (Dx) administration, being shed in small amounts in nasal secretions. As truly TK-negative alphaherpesviruses are unlikely to reactivate in neural tissue, the present study aimed at investigating the sites of latency and reactivation by this recombinant in lambs.

MATERIALS AND METHODS

Experimental design

Lambs were inoculated intranasally (IN) with the parental virus (BoHV-5 SV-507/99, n=15) or with the recombinant (BoHV-5*tk*Δ, n=15) and submitted to clinical, virological and serological monitoring during acute infection. At day 40 post-inoculation (pi), 9 animals of each group were euthanized for tissue collection. Total DNA extracted from TGs was submitted to a nested-PCR for detection of latent viral DNA. The remaining lambs (n=6 from each group) were submitted to Dx treatment; three of each group were euthanized at day 3pDx for tissue collection and the remaining were monitored for virus shedding and seroconversion.

Viruses and cells

The recombinant virus containing a deletion of the tk gene (BoHV-5*tk*Δ) was constructed out of a well characterized Brazilian strain, BoHV-5 SV-507/99 (Brum et al. 2010a). The parental virus SV-507/99 was isolated from a cow with neurological disease in southern Brazil and has been submitted to nucleotide sequencing of the entire genome (Delhon et al. 2003). All procedures of virus multiplication, isolation and serological tests were performed in a MDBK-derived cell line named CRIB (ATCC-CRL 11883). Cells were maintained in minimum essential medium (MEM, Invitrogen, Brazil), supplemented with 10% fetal bovine serum (Nutricell, Brazil), 100 U/mL of penicillin and 100µg/mL of streptomycin (Nutricell, Brazil). The two viruses were used at passage # 6 in CRIB cells.

Animals, virus inoculation and monitoring

Thirty Pollwarth lambs of both genders, aging 4 to 6 months, were randomly allocated in two groups of 15 animals each and inoculated with either virus (parental or recombinant). Each animal received an inoculum of 2mL, divided in the two nostrils, containing a total dose of $10^{6.7}TCID_{50}$/mL. After inoculation, animals were monitored clinically on a daily basis and nasal swabs for virus isolation and quantification were collected up to day 15 pi; blood for serology was collected at days 0 and 40 pi. At that day, 9 animals of each group were euthanized for tissue collection. TGs were collected aseptically and stored at -80°C until use. The remaining lambs (n= 6 for each group) were then submitted to five daily intramuscular administrations of dexamethasone (Dx, 0.2 mg/kg/day; Decadronal®, Aché, Brazil). Three animals from each group were euthanized for tissue collection at day 3pDx. The remaining lambs were monitored as described for acute infection. Swabs for virus isolation and quantification were collected up to day 15 post Dx treatment (pDx) and blood for serology was collected at day 15 pDx.

All procedures of animal handling and experimentation were performed under veterinary supervision and according to recom-

mendations by the Brazilian Committee on Animal Experimentation (COBEA; law # 6.638 of May 8, 1979). The animal experiments were approved by the Institutional Ethics and Animal Welfare Committee (UFSM, approval # 96/2010 of January 18, 2011).

Sample processing

Viral isolation and quantification from nasal swabs were performed in CRIB cells according to standard protocols (Diel et al. 2007). Virus titers in nasal secretions were expressed as $\log_{10}$TCID$_{50}$/ml. Sera obtained at days 0 and 40 pi; and at day 15 pDx were submitted to a standard virus neutralizing assay (VN) for neutralizing antibodies, testing two-fold dilutions of sera against 100-200 TCID$_{50}$ of virus (Diel et al. 2007). Geometric mean titers (GMT) of neutralizing antibodies of each group were calculated according to Thrusfield (2005).

Tissue collection, DNA extraction and nested-PCR

Upon necropsy (40dpi), the brain was removed and the areas were collected individually to avoid contamination. The following areas were collected separately and processed for PCR: olfactory bulbs, olfactory cortex, cerebral cortex (anterior, temporal, occipital), thalamus, midbrain, pons, bulb, medulla and trigeminal ganglia (TG). In addition, retropharyngeal lymph nodes and tonsils (pharyngeal and palatine) were collected. After cleaning, tissues were minced with a razor blade and submitted to total DNA extraction using phenol-chloroform protocol (Vogel et al. 2003). DNA extraction of nasal swabs was performed using DNAzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After extraction, DNA was solubilized in Tris-EDTA (80 µL) and stored at -80 ºC until testing. DNA concentration was measured by ultra violet light (UV) absorbance at 260 nm. Total DNA was submitted to a nested PCR using two set of primers of the glycoprotein B (gB) amplified according Diel et al. (2007). External primers, used in first reaction were – forward: 5'-CCAGTCCAGGCAACCGTCAC-3' and reverse: 5'-CTCGAAAGCCGAGTACCTGCG-3'. The internal primers, used in second reaction were – forward: 5'-GTGGTGGCCTTTGACCGCGAC-3' and reverse: 5'-GCTCCGGCGAGTAGCTGGTGTG-3'. The first PCR reaction amplifying a 444 bp DNA fragment and the second reaction results in a 294 bp amplicon. The PCR products were added with 3µL GelRed® (Biotium, Inc., CA, USA) and analyzed under UV light after electrophoresis in an 1% agarose gel. Total DNA extracted from the brain of a control non-infected lamb, and from a calf with acute BoHV-5 infection was used as negative and positive controls, respectively.

For swabs, nasal secretions obtained from an uninfected lamb were tested in parallel as negative control. PCR for *tk* gene applied on nasal swabs used the following primers: forward: 5'-GACGTCGTGACCCTCGTGTTTG-3' and reverse: 5'-TAGGAAGGCGCACGTGTTCG-3'. The PCR amplifying a 285 pb DNA fragment and was carried out in a 25 µL volume containing 1X PCR buffer, 10mM dNTPs, 100ng of each primer, 2.5 units Taq polymerase, 1.5mM $MgCl_2$, 10% DMSO, and 100ng DNA as template. The PCR conditions consisted of initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, 60°C for 30 s and 72°C for 45 s, and final extension of 10 min at 72°C. The PCR to gB gene was the same as described previously.

RNA extraction, cDNA synthesis and reverse transcription PCR (rt-PCR)

The TGs, tonsils and retropharyngeal lymph node collected at intervals after Dx treatment (day 3pDx) were minced with a razor blade and submitted to total RNA extraction using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and eluted in 30µL of buffer, and stored at -80°C. The DNA was removed by digestion with 1 U of Deoxyribonuclease I, Amplification Grade (Invitrogen, Carlsbad, CA, USA) at room temperature for 15 min. The first-strand cDNA synthesis from 8µL of total RNA, extracted from TGs, retropharyngeal lymph node and tonsils, was carried out using a SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) using 100 µM oligo DT primers, according to the manufacturer's protocol. The rt-PCR step conditions primer sets were 50 min at 50°C and 5 min at 85°C. The complete absence of contaminating DNA within these RNA samples was verified by conducting PCR amplification on sample aliquots that were not subjected to reverse transcription.

Total RNA (1.5µL) extracted from TGs and lymphatic tissues were reverse transcribed with 100 U SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) using 100µM oligo DT primers, according to the manufacturer's protocol. The rt-PCR step conditions primer sets were 50 min at 50°C and 5 min at 85°C.

RESULTS

Virus replication during acute infection

Lambs inoculated with SV-507/99 shed virus in nasal secretions up to days 9 to 12 pi, with a mean period of excretion of 11.1 days (Table 1). Lambs inoculated with the recombinant BoHV-5*tk*Δ shed virus for a shorter period of time ($p<0.05$), up to days 6 to 12 (average of 8.5 days). Virus shedding peaked at day 1 pi (parental virus) and 2 pi (recombinant), and the highest titers were observed at day 4 pi for SV-507/99 ($10^{5.9}$TCID$_{50}$/ml) and 2 pi for BoHV-5*tk*Δ ($10^{5.1}$ TCID$_{50}$/ml) (not shown). Virus titers were also significantly higher in secretions of lambs inoculated with the parental virus, noticeably at day 1 pi and between days 4 at 9 pi ($p<0.01$). Most lambs inoculated with the parental and recombinant strains developed neutralizing antibodies at

Table 1. Virus shedding in nasal secretions during acute infection and virus neutralizing titers at day 40 post inoculation (pi) in lambs inoculated with parental bovine herpesvirus type 5 (BoHV-5) SV-507/99 and recombinant (BoHV-5*tk*Δ)

Group	Virus shedding in nasal secretions						Virus neutralizing antibody	
	Animal #	Day post inoculation (pi)					Day pi	
		2	4	6	8	10-14	0	40
SV-507/99	3	5.6[a]	4.0	2.1	0.9	-[b]	< 2	4
	4	4.0	3.5	4.1	1.0	-	< 2	2
	5	3.9	3.0	3.3	1.0	-	< 2	2
	7	4.0	3.0	2.5	1.8	-	< 2	8
	8	4.5	1.3	3.3	1.1	-	< 2	2
	9	4.6	4.0	4.1	1.0	-	< 2	8
	10	3.9	3.1	4.3	1.5	-	< 2	4
	11	3.1	4.1	4.0	1.5	-	< 2	4
	12	4.1	0.9	3.0	1.1	-	< 2	4
BoHV-5*tk*Δ	1	4.1	2.1	+[c]	1.5	-	< 2	2
	2	4.0	0.9	3.1	0.9	-	< 2	2
	6	3.0	1.8	+	1.0	-	< 2	4
	13	4.5	2.0	3.0	0.9	-	< 2	4
	14	4.7	2.0	2.7	1.0	-	< 2	4
	16	3.9	2.3	2.0	1.0	-	< 2	2
	17	3.3	1.9	2.5	-	-	< 2	4
	18	4.0	2.6	2.0	-	-	< 2	2
	19	5.1	1.9	1.0	1.3	-	< 2	4

[a] Positive sample; viral titers expressed as $\log_{10}$TCID$_{50}$/mL. [b] Negative sample after inoculation in cell culture. [c] Positive sample after second or third passage in cell culture (titer $<10^{0.9}$TCID$_{50}$/mL).

day 40 pi (Table 1). The geometric mean titer (GMT) of the parental group (2.36) was higher than the GMT of the group BoHV-5*tk*Δ (1.90). A few lambs inoculated with either virus presented a mild, transient serous nasal secretion between days 2 to 6 pi. No systemic signs were recorded. These results demonstrate that the recombinant BoHV-5*tk*Δ replicated to moderate titers in the nasal mucosa of lambs.

Distribution of latent viral DNA at day 40pi

Nested-PCR examination of total DNA extracted from tissues of lambs euthanized at day 40pi revealed the presence of viral DNA in the TGs, pharyngeal and palatine tonsils of virtually all inoculated animals, regardless the group (Table 2).

Retropharyngeal lymph nodes were also frequently positive in both groups. No infectious virus was detected upon inoculation and three passages of tissue homogenates into CRIB cells, confirming the status of latent infection. These results confirm our previous findings (Cadore et al. 2013) that the recombinant BoHV-5*tk*Δ retains its ability to establish latent infection in the TGs of inoculated lambs. In addition, both parental and recombinant viruses were shown to establish latency in regional lymphoid tissue.

In addition, both parental and recombinant viral DNA was detected by PCR in several sections of the brain (not shown). No major differences in frequency and distribution were evident between the groups, indicating that the recombinant retained its ability to invade and to replicate, to a certain extent, in the brain.

Table 2. Detection of viral DNA by PCR in neural and non-neural tissues of lambs inoculated with the parental bovine herpesvirus type 5 (BoHV-5) SV-507/99 or with the recombinant (BoHV-5*tk*Δ), at day 40 post-inoculation

Group	Animal #	Trigeminal Ganglia	Pharyngeal Tonsil	Palatine Tonsil	Retropharyngeal Lymph Node
SV-07/99wt	4	+[a]	+	+	-[b]
	5	+	+	+	+
	8	+	+	+	-
	7	+	+	+	+
	9	+	+	+	-
	11	+	+	+	+
	3	+	+	+	-
	10	+	+	+	+
	12	+	+	+	+
BoHV-5*tk*Δ	13	+	+	+	-
	17	+	+	+	-
	18	+	+	+	-
	2	+	+	+	-
	6	+	+	-	+
	16	+	+	+	+
	1	+	+	+	+
	14	+	+	+	-
	19	+	+	+	+

[a] Positive sample by nested-PCR. [b] Negative sample by nested-PCR.

Virus shedding upon Dx administration

Following Dx administration - starting at day 40pi -, all three lambs inoculated with parental strain and kept for monitoring shed infectious virus in nasal secretions (mean duration = 5 days [4-6]) (Table 3). Shedding was first detected at day 3 and lasted up to day 9 pDx in one lamb. The peak in virus titers was observed at days 5 and 6 pDx, with titers reaching $10^{4.1}$TCID$_{50}$/mL. In contrast, nasal secretions from BoHV-5*tk*Δ-inoculated lambs were negative for infectivity upon virus inoculation in cell cultures (Table 3). Swabs of lambs inoculated with the recombinant virus were positive for viral DNA by PCR (Table 3). Four (66.6%) and three (50%) lambs of the parental and recombinant virus group, respectively, showed a four-fold increase in VN titers after Dx treatment. Taken together, these results confirm our previous findings (Cadore et al. 2013) and demonstrate that the recombinant BoHV-5*tk*Δ was able to reactivate following Dx treatment.

Monitoring virus reactivation in TGs and lymphoid tissues

In order to investigate the sites/origin of reactivated virus, a search for viral mRNA by RT-PCR was performed in

Table 3. Virus shedding in nasal secretions and virus neutralizing antibodies after dexamethasone (Dx) treatment in lambs inoculated with parental bovine herpesvirus type 5 (BoHV-5) SV-507/99 and recombinant (BoHV-5*tk*Δ)

Group	Virus shedding in nasal secretions							Virus neutralizing antibody		
	Animal #	Day after dexamethasone administration (pDx)						Day pDx		
		0-2	3	4	5	6	7	0	3	7
SV-507/99wt	7	-[a]	†-					8	16	
	9	-	†-					8	16	
	11	-	†-					4	16	
	3	-	-	-	2.1[b]	1.7	-	4		32
	10	-	-	-	1.3	1.5	1.3	4		8
	12	-	+[c]	2.1	1.9	1.3	-	4		16
BoHV-5*tk*Δ	2	-	†(+)[d]					2	4	
	6	-	†-					4	4	
	16	-	†-					< 2	8	
	1	-	-	(+)	(+)	-	(+)	2		16
	14	-	-	-	(+)	(+)	-	4		32
	19	-	-	(+)	(+)	(+)	-	4		8

[a] Negative sample. [b] Positive sample. Viral titers expressed as $\log_{10}$ TCID$_{50}$/mL. [c] Positive sample after second or third passage in cell culture(titer <$10^{0.9}$TCID$_{50}$/mL). [d] Samples negative by virus isolation – positive by nested-PCR.

TGs and tonsils/lymph nodes of lambs submitted to euthanasia at day 3 after Dx treatment. Total RNA was submitted to a RT-PCR for mRNA of a late viral gene. As presented in Table 4, evidence of viral gene expression (and reactivation) was demonstrated in TGs of the parental virus group (2/3). No viral gene expression was detected in TGs of lambs inoculated with the recombinant virus. Evidence of reactivation was also detected in pharyngeal tonsils of both groups (2/3), palatine tonsils of parental (2/3) and recombinant (1/3) and retropharyngeal lymph node of parental virus group (1/3). These results indicate that reactivation of parental virus occurred in neural (TG) and non-neural tissues (tonsils, lymph node). Reactivation of the recombinant virus was not detected in TGs but seemed to occur in the tonsils, as demonstrated by detection of gB mRNA.

Table 4. Detection of mRNA of glycoprotein B gene of bovine herpesvirus type 5 (BoHV-5) by RT-PCR in neural and non-neural tissues of lambs inoculated with the parental BoHV-5 (SV-507/99) or with the recombinant (BoHV-5*tk*Δ), three days after dexamethasone administration

Group	Animal #	Trigeminal ganglia	Pharyngeal tonsil	Palatine tonsil	Retropharyngeal lymph node
SV507/99wt	7	+[a]	+	-[b]	-
	9	+	-	+	-
	11	-	+	+	+
BoHV-5tkΔ	2	-	-	-	-
	6	-	+	+	-
	16	-	+	-	-

[a] Positive sample by nested-PCR. [b] Negative sample by nested-PCR.

DISCUSSION

The results presented herein demonstrate that the recombinant BoHV-5*tk*Δ established latent infection with a similar distribution of the parental strain, in TGs and in regional lymphoid tissues. In addition, BoHV-5*tk*Δ DNA was detected in nasal secretions after Dx treatment, confirming that the recombinant retains its ability to reactivate. However, BoHV-5*tk*Δ reactivation seemed to be restricted to lymphoid tissue since no viral mRNA was detected in TGs following Dx treatment. In contrast, reactivation of the parental virus was demonstrated in neural and non-neural tissues. The ability of a tk-deleted strain to reactivate latency from lymphoid tissue - even poorly - deserves attention in the context of vaccine safety.

The recombinant replicated to lower titers than the parental strain in the nose during acute infection. However, it was still able to establish latent infection in all inoculated animals and with a similar distribution of the parental virus. In a previous experiment, qPCR analysis showed a 9.7-fold reduction in the amount of recombinant virus DNA in TGs compared to the parental strain (Cadore et al. 2013). Thus, *tk* deletion did not abolish the ability of the virus to establish latency, but resulted in reduced colonization of TGs with latent viral DNA.

Confirming our previous results (Cadore et al. 2013), BoHV-5*tk*Δ DNA was detected by PCR in nasal swabs after Dx treatment, indicating some level of reactivation. This is an unusual event for many *tk* negative alphaherpesviruses examined to date. Nevertheless, low levels of Dx-induced reactivation have been also demonstrated for this recombinant in calves (Santos et al. 2011) and lambs (Cadore et al. 2013). The absolute need of alphaherpesvirus-encoded TK activity for virus reactivation is controversial. A number of studies have shown that truly TK negative HSV mutants derived from laboratory strains do not reactivate in nerve ganglia (Tenser et al. 1987, Coen et al. 1989, Tenser et al. 1996, Chen et al. 2004), yet some mutants derived from clinical isolates might reactivate inefficiently (Horsburgh et al. 1998, Griffiths et al. 2003). Conflicting findings have also been reported for BoHV-1 and PRV *tk* deletion mutants, and both reactivation (Mengeling et al. 1992, Whetstone et al. 1992) and lack of reactivation have been reported (Mengeling 1991, Kaashoek et al. 1996, Ferrari et al. 2000). The general concept, however, is that herpesvirus TK activity is crucial for efficient reactivation since truly *tk*-null mutants do not reactivate - or reactivate very poorly - from nerve ganglia (Coen et al. 1989, Tenser et al. 1996, Chen et al. 2004). An aspect worth to consider is that most – if not all - studies reporting lack of reactivation by tk-negative HSV mutants were based on the absence of reactivation in explant cultures *in vitro* and not from *in vivo* studies (Coen et al. 1989, Tenser et al. 1996, Chen et al. 2004). In the same line, evidence of reactivation by tk-defective PRV, BoHV-1 and BoHV-5 was obtained *in vivo* (Mengeling et al. 1992, Whetstone et al. 1992, Santos et al. 2011). In the later studies, reactivation was demonstrated by recovery of infectious virus upon Dx treatment, yet the origin (neural versus non-neural) of reactivated virus was not determined. Thus, it should not be discarded that, in these cases, the origin of infectious virus was, in fact, reactivation in non-neural tissues.

We have previously hypothesized that shedding of the recombinant BoHV-5*tk*Δ upon Dx treatment would either reflect a residual ability of the virus to reactivate in neural tissue (an unexpected event for alphaherpesviruses) or, alternatively, virus reactivation from lymphoid tissue (Cadore et al. 2013). Regional lymphoid tissues, especially tonsils, have been shown to be secondary sites of latency/persistence by PRV (Wheeler & Osorio 1991), BoHV-1 (Inman et al., 2002) and also for BoHV-5 (Cadore, G., unpublished). The present study confirmed the presence of latent recombinant viral DNA in tonsils and lymph nodes, reinforcing this hypothesis. Further, latent BoHV-1 present in tonsils of latently infected calves was shown to reactivate and lead to productive virus replication upon Dx treatment and explant cultures *in vitro* (Inman et al. 2002, Perez et al. 2002). The data presented herein confirms latent infection and reactivation of BoHV-5 in lymphoid tissue, in addition to nerve ganglia. Although lymphoid cells and sensory neurons have different phenotypes, they are highly differentiated cells. Such differentiated cells might lack factors necessary for productive infection yet may present conditions for the establishment of latency. In this sense, the lack of transcription factors or the presence of cellular factors that repress viral transcription are important factors during establishment of latency (Winkler et al. 2000, Jones 1998). Moreover, the present findings strongly indicate that recombinant virus shedding observed after Dx treatment was indeed

derived from virus reactivation in lymphoid tissue rather than from reactivation in TGs. This hypothesis accommodates the general concept that TK-negative alphaherpesviruses are unlike to reactivate in neural tissue and support reactivation of recombinant virus in lymphoid tissue.

CONCLUSIONS

In summary, the results presented herein demonstrate that the recombinant BoHV-5*tk*Δ established latent infection in TGs and tonsils of lambs with a similar distribution of the parental virus.

Reactivation at low levels took place in lambs inoculated with the recombinant after Dx treatment. This reactivation, however, seemed to occur only in lymphoid tissues, in contrast with reactivation of the parental virus which occurred in neural and non-neural tissues.

The ability of tk-negative animal herpesviruses to reactivate latent infection *in vivo* should be further examined as to ascertain the safety of use of these recombinants in vaccines.

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