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Short Communication

Comparison of inflammatory infiltrates in trigeminal ganglia of cattle infected with wild-type Bovine herpesvirus 1 versus a virus strain containing a mutation in the LR (latency-related) gene

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During latency, the bovine herpesvirus 1 (BHV-1) latency-related (LR) RNA is abundantly expressed in neurons within trigeminal ganglia (TG). A LR mutant virus that does not express two LR proteins is unable to reactivate from latency following dexamethasone treatment. Increased infiltration of inflammatory cells occurs in TG of calves acutely infected with the LR mutant virus. Throughout acute infection, wild-type BHV-1 DNA is detected in neurons surrounded by mononuclear infiltrates and in non-neuronal cells comprising the infiltrate. Conversely, LR mutant DNA is only detected in neurons near the end of acute infection, suggesting LR gene products promote virus spread in TG. *Journal of NeuroVirology* (2006) **12,** 392–397.

Keywords: bovine herpesvirus 1 (BHV-1); infiltrating lymphocytes; latency; trigeminal ganglia

Bovine herpesvirus 1 (BHV-1) is an important pathogen that causes respiratory and genital disease, abortion, conjunctivitis, and multisystemic infection in neonate calves (Tikoo *et al*, 1995). Like other members of the *alphaherpesvirinae* subfamily, BHV-1 establishes latent infection in ganglionic neurons of the peripheral nervous system after initial infection of mucosal surfaces. Viral reactivation and spread to other susceptible animals can occur after glucocorticoid-induced stress (Jones, 1998, 2003; Rock *et al*, 1992). During latency, viral gene expression is restricted to the latency-related (LR) gene (Jones, 1998, 2003; Rock *et al*, 1992) and ORF-E gene (Inman *et al*, 2004). The LR gene encodes at least two proteins (Hossain *et al*, 1995, Jiang *et al*, 2004) whose expression correlates with inhibiting apoptosis (Ciacci-Zanella *et al*, 1999; Lovato *et al*, 2003), and reactivation from latency (Inman *et al*, 2001, 2002).

To study the role of LR gene products during the latency-reactivation cycle, a BHV-1 LR mutant virus strain containing three stop codons at the 5′ terminus of the LR transcript was constructed (Inman *et al*, 2001, 2002). The LR mutant, wild-type (wt) BHV-1 or the LR-rescued virus have similar growth characteristics in cell culture. However, calves infected with the LR mutant virus have reduced ocular shedding and diminished clinical symptoms compared to calves infected with wt BHV-1 or the LR-rescued virus (Inman *et al*, 2001; Perez *et al*, 2005). Lower levels of infectious virus were also detected in trigeminal ganglia (TG) homogenates from the LR mutant–infected calves, and less viral DNA is present in TG of calves latently infected with the LR mutant (Inman *et al*, 2002). Finally, the LR mutant virus does not reactivate from latency after dexamethasone treatment (Inman *et al*, 2002).

Following infection of calves with BHV-1, foci of infiltrating lymphocytes are detected during acute infection (Winkler *et al*, 2002). Several studies have

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concluded that a cell-mediated immune response persists in TG of mice latently infected with herpes simplex virus (HSV)-1 (Cantin *et al*, 1995; Halford *et al*, 1996; Liu *et al*, 1996; Shimeld *et al*, 1995, 1996, 1997) or rodents (mice or guinea pigs) latently infected with HSV-2 (Milligan *et al*, 2005). In human TG that harbor latent HSV-1, there is also a chronic inflammatory response, which appears similar to what is seen in rodents latently infected with HSV-1 or HSV-2 (Theil *et al*, 2003). Persistence of immune cells in TG is important for maintaining latency because CD8+ T cells that produce interferon-γ can prevent reactivation from latency in sensory neurons of mice latently infected with HSV-1 (Liu *et al*, 2001, 2000). Two independent studies have also concluded that interferon-α and interferon-γ gontrol recurrent herpetic lesions (Cunningham and Mikloska, 2001; Mikloska and Cunningham, 2001). Collectively, these studies suggest that establishment of latency by α-herpesviruses results in chronic infiltration of immune cells within the peripheral nervous system. Because the BHV-1 LR gene is abundantly expressed during latency, we hypothesized that the LR gene may regulate the inflammatory response to ensure that latency is established and/or maintained.

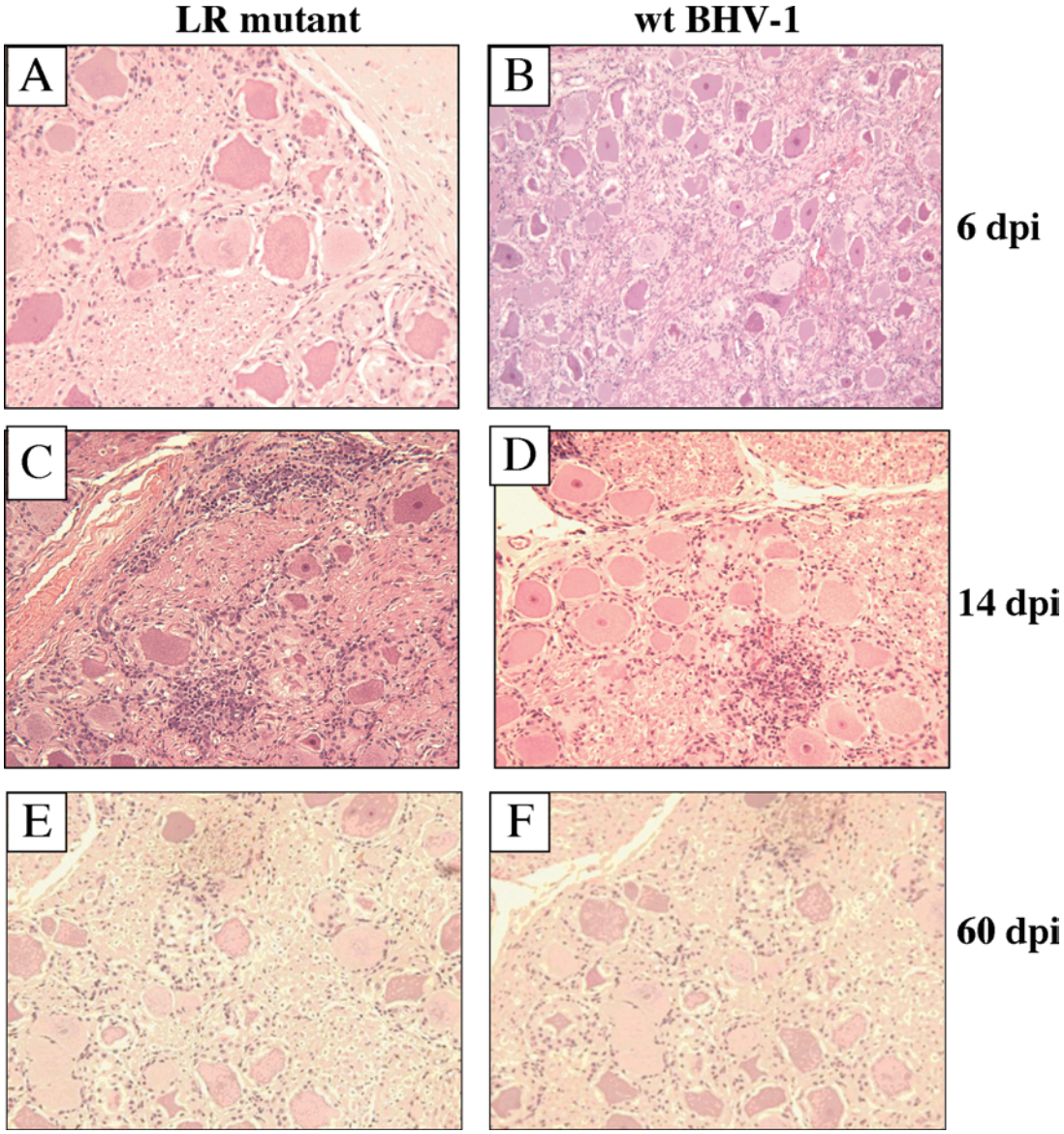

Figure 1 Mononuclear cell infiltrates in TG of wt BHV-1– and LR mutant–infected calves. Thin sections were prepared and stained with H&E as described previously (Winkler *et al*, 2002). **A** and **B**: TG sections from LR mutant and wt BHV-1–infected calves at 6 days post infection (dpi), respectively (magnification of 200× and 100×, respectively). **C** and **D** (magnification 200×): TG sections from LR mutant and wt BHV-1–infected calves at 14 dpi, respectively. **E** and **F** (magnification 200×): TG sections prepared from calves latently infected (60 dpi) with the LR mutant or wt BHV-1. The results for wt BHV-1 and the LR-rescued virus were identical (data not shown). The respective panels are representative of many sections that contain inflammatory infiltrates.

To test whether the LR gene influenced infiltration of inflammatory cells into TG, we compared TG of calves infected with the LR mutant virus versus wt BHV-1 using hematoxylin eosin (H&E) staining. At 6 days post infection (dpi), small foci of infiltrating lymphocytes were detected in wt BHV-1–infected TG, but not in TG from LR mutant–infected calves (Figure 1A and B). At 14 dpi, TG prepared from calves infected with the LR mutant contained large foci of inflammatory cells (Figure 1C). It was also noted that more foci were typically found in thin sections prepared from TG of calves infected with the LR mutant virus relative to calves infected with wt BHV-1 (Figure 1C and D). At 60 dpi (latency), the inflammatory response had diminished, and there was little difference between samples obtained from calves infected with the LR mutant (Figure 1E) versus wt BHV-1 (Figure 1F). Regardless of the virus used for

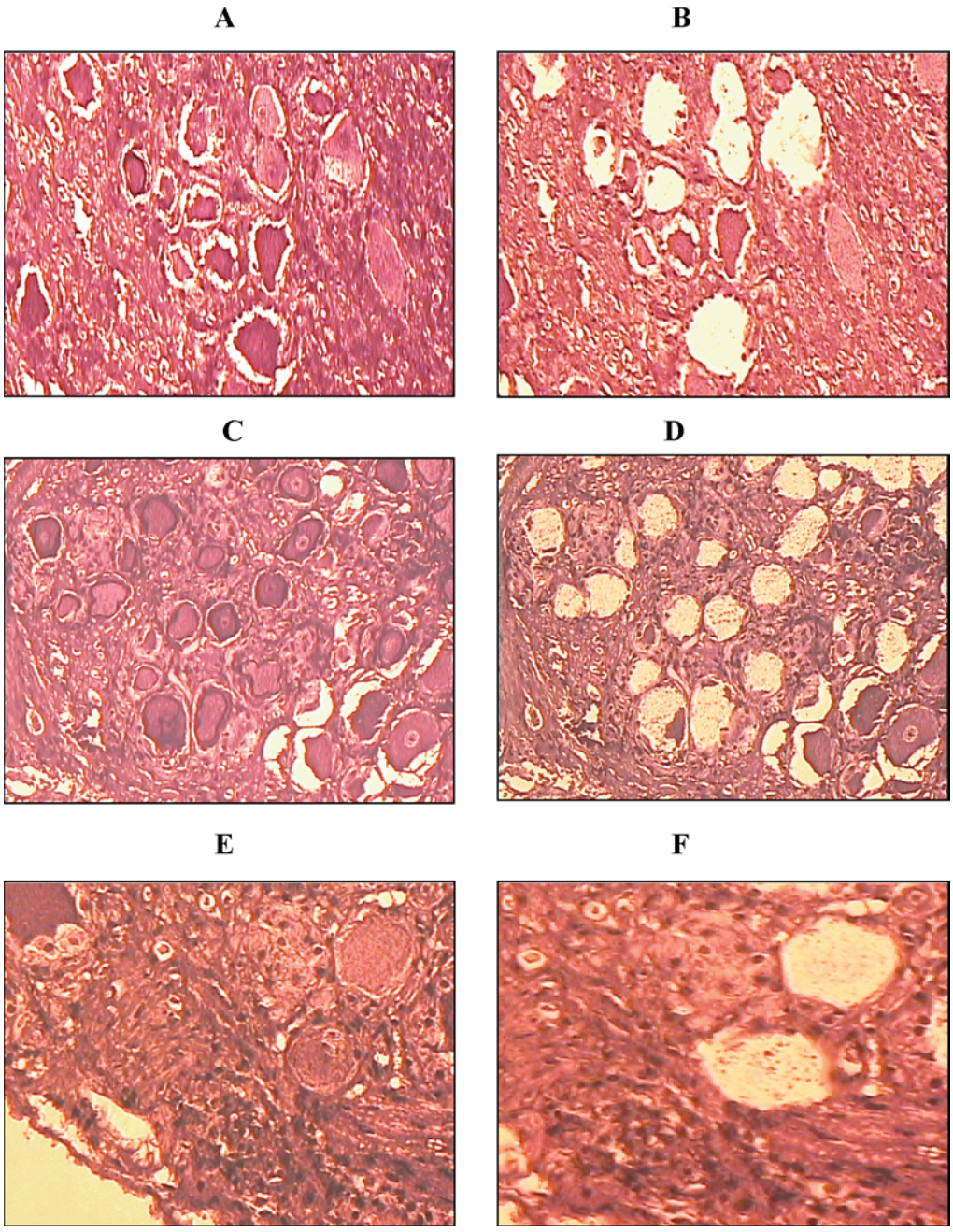

Figure 2 LCM of neurons from wt BHV-1– and LR mutant–infected TG. **A** and **B**: Sections from TG of wt BHV-1–infected calves at 6 days post infection (6 dpi) before (**A**) and after (**B**) LCM of neurons. **C–E**: Sections from TG of LR mutant–infected calves at 14 dpi before (**C** and **E**) and after (**D** and **F**) LCM of neurons. Microdissection of cells was performed using a PixCell apparatus (Arcturus Engineering, MountainView, CA).

infection, high levels of apoptotic cells were present in foci of infiltrating cells at 6 or 14 dpi (data not shown). In general, infiltration of inflammatory cells in TG of calves infected with wt virus correlates with viral gene expression in TG (Schang and Jones, 1997). With respect to calves acutely infected with the LR mutant, infiltration of inflammatory cells did not correlate with viral gene expression or production of infectious virus in TG.

A previous study demonstrated that lower levels of viral DNA were detected in TG following infection with the LR mutant virus (Inman *et al*, 2002). Although this study suggested that the LR gene influenced establishment of latency, it did not provide information about differences between the LR mutant virus versus wt BHV-1 in TG of acutely infected calves. For example, if the LR gene were important for establishing latency, one would predict that viral replication or spread would be different. Because we were unable to detect BHV-1+ neurons in TG of calves acutely infected with the LR mutant using *in situ* hybridization (Inman *et al*, 2002), laser capture microdissection (LCM) was used to isolate neurons surrounded by inflammatory infiltrates, and then polymerase chain reaction (PCR) performed with viral specific primers. Procedures were developed in which we could reproducibly "pick" neurons from sections that were hematoxylin stained, and remove these neurons from the slide (for representative before LCM and after LCM, see Figure 2). DNA was then extracted from the cells picked by LCM, and PCR performed. For these studies, we used gC-specific primers to detect viral DNA, and bovine growth hormone primers to verify that samples contained DNA (for representative PCR results, see Figure 3).

LR mutant DNA was not detected in neurons or non-neural cells comprising the inflammatory infiltrate at 2, 4, or 6 dpi (Table 1 and Figure 3A). However, sensory neurons from TG collected at 10 dpi consistently contained viral DNA. In calves infected with the LR mutant, viral DNA was not readily detected in non-neuronal cells surrounding infected neurons at any time point tested (Table 1 and Figure 3A). In TG prepared from calves infected with wt BHV-1, viral DNA was detected in neurons and surrounding cells at 4, 6, and 10 dpi (Figure 3B). In non-neuronal cells, viral DNA was detected at 2, 4, 6, or 10 dpi.

Table 1 Summary of the detection of wild-type (wt) BHV-1 or LR mutant DNA in bovine TG cells by LCM

	wt BHV-1		*LR mutant*	
Days post infection	*N*	*SC*	*N*	*SC*
2	−	+	−	−
4	+	+	−	−
6	+	+	−	−
10	+	+	+	−
14	−	−	−	−

Note. Tissue sections were processed and stained as described for Figures 1 to 3. Detection of viral DNA was performed with BHV-1-gC–specific primers, as described in Figure 3. +: presence of viral DNA; −: absence of viral DNA. N refers to neurons and SC to surrounding cells. For these studies, two calves were used for each time point (LR mutant virus or the wt virus). From 2 to 6 dpi, four slides were examined/calf. For 10 and 14 dpi, two slides were examined for each calf.

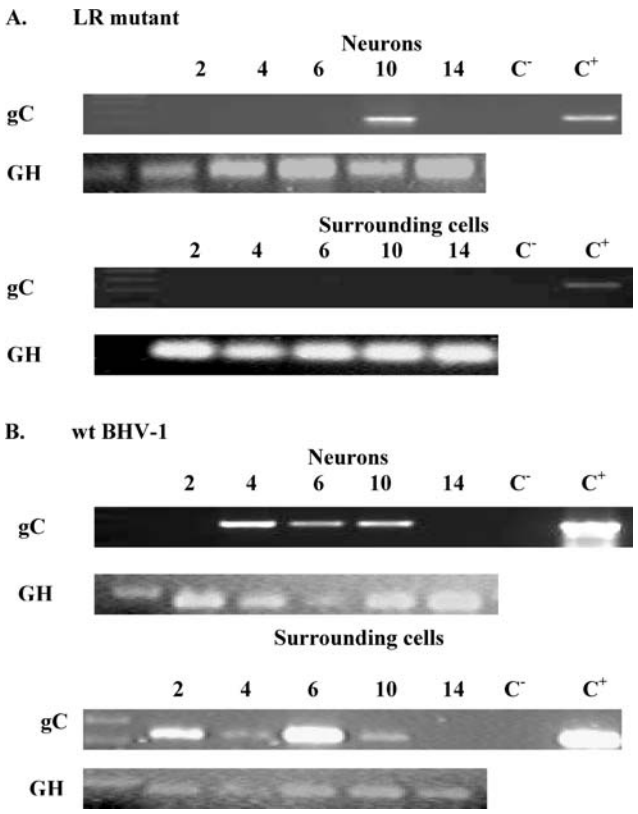

Figure 3 Detection of viral DNA in neurons and cells surrounding neuronal bodies in trigeminal ganglia (TG) of cattle acutely infected with wt BHV-1 or the LR mutant strain. As described in Figure 2, LCM was used to collect TG cells. Twenty-five to 50 neuronal cells surrounded by inflammatory infiltrates were selected and captured on thermoplastic caps (CapSure Macro; Arcturus Engineering) using a laser beam 5 to 15 μm in diameter. When less than 25 neurons were present in the area of infiltration, neuronal cells distant from these areas were also collected. Cells surrounding neurons were captured in a separate cap. Because inflammatory infiltrates were not readily evident at 2 dpi, neuronal cells were randomly selected for capture. Caps with cells were then placed in Eppendorf tubes containing 55 μl of lysis buffer (PicoPure DNA extraction kit; Arcturus Engineering). Samples were incubated overnight at 65°C. The reaction was stopped by incubation at 98°C for 8 min. PCR for detection of viral DNA was performed using BHV-1 gC-specific primers (+5′-GAGCAAAGCCCCGCCGAAGGA-3′and–5′-TACGAACAGCAGCACGGGCGG-3′). Bovine growth hormone (GH) was used as an internal control for the presence of amplification products. The primers for GH were (+ 5′-GCTTTCGCCCTGCTCTGCC and–5′-TCCTGCCTCCCCACCCCTA-3′). PCR was performed as previously described (Perez, 2005). **A**: PCR for detection of LR mutant DNA in TG neurons and cells surrounding neuronal bodies captured by LCM. **B**: PCR for detection of wt BHV-1 DNA in TG neurons and cells surrounding neuronal bodies captured by LCM. Numbers above gels indicate days post infection. DNA from BHV-1– and mock-infected MDBK cells were used as positive (C^+) and negative (C^-) controls, respectively, for PCR reactions.

By 14 dpi, neurons or cells present around neuronal bodies did not contain detectable levels of wt or LR mutant DNA (Table 1 and Figure 3). However, DNA prepared from total TG contained detectable levels of viral DNA regardless of the virus used to infect calves (Inman *et al*, 2002).

The results in this study suggested that following infection of calves with wt BHV-1, neurons surrounded by infiltrating immune cells and adjacent non-neuronal cells were consistently infected throughout acute infection. It was not possible to discern whether viral DNA in non-neuronal cells was present in infiltrating immune cells and/or satellite cells. In contrast, we only detected viral DNA in TG neurons at 10 dpi following infection with the LR mutant. These findings were consistent with the previous observation demonstrating that lower levels of viral DNA were detected in TG of calves latently infected with the LR mutant virus (Inman *et al*, 2001, 2002). Surprisingly, viral DNA was detected in non-neuronal cells, but not in neurons, at 2 dpi following infection with wt BHV-1. Although one could argue that the non-neuronal cells were contaminated with neurons, there is precedence that BHV-1 can infect non-neural cells in TG. For example, we have detected an occasional non-neuronal cell during latency that is BHV-1 positive (Winkler *et al*, 2002).

In general, it is believed that infiltration of immune cells into TG following acute infection with BHV-1 or HSV-1 inhibits viral replication, and consequently promotes establishment of latency (Khanna *et al*, 2004; Khanna *et al*, 2003; Liu *et al*, 1996, 2000, 2001; Prbhakaran *et al*, 2005; Winkler *et al*, 2002). These studies also indicated that infiltration of immune cells into TG is a result of viral gene expression and replication. Because the LR mutant virus produces less infectious virus and viral DNA in TG (Inman *et al*, 2002), the results we obtained were not expected if infiltration of lymphocytes correlates exclusively with viral gene expression and replication. In the absence of LR protein expression, we predict that a cycle of increased cell death and lymphocyte infiltration occurs in TG during late stages of acute infection (establishment of latency). Enhanced proapoptotic signals induced by the LR mutant virus in TG (Lovato *et al*, 2003) would likely increase infiltration of lymphocytes, in part, because certain caspases have inflammatory properties (Martinon and Tschopp, 2004). Furthermore, following a significant number of cells undergoing cell death, proapoptotic signals and "eat me" signals are released to surrounding cells (Lauber *et al*, 2004), which would also increase the inflammatory response. Taken together, these observations suggest that enhanced cell death induced by the LR mutant virus leads to enhanced inflammatory responses near the end of acute infection. Consequently, the number of infected neurons is reduced, resulting in a decrease in the amount of viral DNA in TG, and a dramatic reduction in the frequency of reactivation from latency. Additional studies will be necessary to understand the mechanism by which LR gene products interfere with infiltration of lymphocytes into TG during acute infection.

For these studies, we chose to perform LCM versus *in situ* hybridization because, in general, the levels of viral DNA in non-neuronal cells is low, and thus difficult to detect. Although LCM is useful for examining specific regions of TG that contain inflammatory infiltrates, there are complications and limitations to this procedure. For example, we had problems consistently detecting viral DNA in single neurons following hematoxylin staining. It was also difficult to isolate individual satellite cells without contaminating these samples with inflammatory cells in infected TG. In spite of these current limitations, this technique is useful for examining virus-host interactions at the cellular level in specific tissue.

References

Cantin EM, Hinton DR, Chen J, Openshaw H (1995). Gamma interferon expression during acute and latent nervous system infection by herpes simplex virus type 1. *J Virol* **69:** 4898–4905.

Ciacci-Zanella J, Stone M, Henderson G, Jones C (1999). The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. *J Virol* **73:** 9734–9740.

Cunningham AL, Mikloska Z (2001). The Holy Grail: immune control of human herpes simplex virus infection and disease. *Herpes* **8(Suppl 1**): 6A–10A.

Halford WP, Gebhardt BM, Carr DJ (1996). Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. *J Immunol* **157:** 3542–3549.

Hossain A, Schang LM, Jones C (1995). Identification of gene products encoded by the latency-related gene of bovine herpesvirus 1. *J Virol* **69:** 5345–5352.

Inman M, Lovato L, Doster A, Jones C (2001). A mutation in the latency-related gene of bovine herpesvirus 1 leads to impaired ocular shedding in acutely infected calves. *J Virol* **75:** 8507–8515.

Inman M, Lovato L, Doster A, Jones C (2002). A mutation in the latency related gene of bovine herpesvirus 1 interferes with the latency-reactivation cycle of latency in calves. *J Virol* **76:** 6771–6779.

Inman M, Zhou J, Webb H, Jones C (2004). Identification of a novel transcript containing a small open reading frame that is expressed during latency, and is antisense to the latency related gene of bovine herpes virus 1 (BHV-1). *J Virol* **78:** 5438–5447.

Jiang Y, Inman M, Zhang Y, Posadas NA, Jones C (2004). A mutation in the latency related gene of bovine herpesvirus 1 (BHV-1) inhibits protein expression of a protein from open reading frame 2 (ORF-2) and an adjacent

reading frame during productive infection. *J Virol* **78:** 3184–3189.

Jones C (1998). Alphaherpesvirus latency: its role in disease and survival of the virus in nature. *Adv Virus Res* **51:** 81–133.

Jones C (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16:** 79–95.

Khanna KM, Bonneau RH, Kinchington PR, Hendricks RL (2003). Herpes simplex virus-specific memory CD*+ T cells are selectively activated and retained in latently infected sensory ganglia. *Immunity* **18:** 593–603.

Khanna KM, Lepisto AJ, Decman V, Hendricks RL (2004). Immune control of herpes simplex virus during latency. *Curr Opin Immunol* **16:** 463–469.

Lauber K, Blumenthal SG, Waibel M, Wesselborg S (2004). Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell* **14:** 277–287.

Liu T, Khanna KM, Carriere BN, Hendricks RL (2001). Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. *J Virol* **75:** 11178–11184.

Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL (2000). CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *J Exp Med* **191:** 1459–1466.

Liu T, Tang Q, Hendricks RL (1996). Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection. *J Virol* **70:** 264–271.

Lovato L, Inman M, Henderson G, Doster A, Jones C (2003). Infection of cattle with a bovine herpesvirus 1 (BHV-1) strain that contains a mutation in the latency related gene leads to increased apoptosis in trigeminal ganglia during the transition from acute infection to latency. *J Virol* **77:** 4848–4857.

Martinon F, Tschopp J (2004). Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117:** 561–574.

Mikloska Z, Cunningham AL (2001). Alpha and gamma interferons inhibit herpes simplex virus type 1 infection and spread in epidermal cells after axonal transmission. *J Virol* **75:** 11821–11826.

Milligan GN, Meador MG, Chu C, Young CG, Martin TL, Bourne N (2005). Long-term presence of virus-specific plasma cells in sensory ganglia and spinal cord following intravaginal inoculation of herpes simplex virus type 2. *J Virol* **79:** 11537–11540.

Perez S, Inman M, Doster A, Jones C (2005). Latency-realated gene encoded by bovine herpesvirus 1 promotes virus growth and reactivation from latency in tonsils of infected calves. *J Clin Microbiol* **43**, 393–401.

Prbhakaran K, Sheridan BS, Kinchington PR, Khanna KM, Decman V, Lathrop K, Hendricks RL (2005). Sensory neurons regulate the effector functions of CD8+ T cells in controlling HSV-1 latency ex vivo. *Immunity* **23:** 515–523.

Rock D, Lokensgard J, Lewis T, Kutish G (1992). Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. *J Virol* **66:** 2484–2490.

Schang LM, Jones C (1997). Analysis of bovine herpesvirus 1 transcripts during a primary infection of trigeminal ganglia of cattle. *J Virol* **71:** 6786–6795.

Shimeld C, Whiteland JL, Nicholls SM, Grinfeld E, Easty DL, Gao H, Hill TJ (1995). Immune cell infiltration and persistence in the mouse trigeminal ganglion after infection of the cornea with herpes simplex virus type 1. *J Neuroimmunol* **61:** 7–16.

Shimeld C, Whiteland JL, Williams NA, Easty DL, Hill TJ (1996). Reactivation of herpes simplex virus type 1 in the mouse trigeminal ganglion: an in vivo study of virus antigen and immune cell infiltration. *J Gen Virol* **77:** 2583–2590.

Shimeld C, Whiteland JL, Williams NA, Easty DL, Hill TJ (1997). Cytokine production in the nervous system of mice during acute and latent infection with herpes simplex virus type 1. *J Gen Virol* **78:** 3317–3325.

Theil D, Derfuss T, Paripovic I, Herberger S, Meinl E, Schueler O, Strupp M, Arbusow V, Brandt T (2003). Latent herpesvirus infection in human rigeminal ganglia causes chronic immune response. *Am J Pathol* **163:** 2179–2184.

Tikoo SK, Campos M, Babiuk LA (1995). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. *Adv Virus Res* **45:** 191–223.

Winkler MT, Doster A, Sur JH, Jones C (2002). Analysis of bovine trigeminal ganglia following infection with bovine herpesvirus 1. *Vet Microbiol* **86:** 139–155.