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Recent developments in animal models for human herpesvirus 6A and 6B

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Progress in the identification of suitable animal models for human herpesvirus (HHV)-6A and HHV-6B infections has been slow. Recently, new models have been established, mainly for HHV-6A, which reproduce some pathological features seen in humans. Neuroinflammatory signs were observed in infected marmosets and CD46-transgenic mice; although viral replication was not prominent, persistence of viral DNA and specific immunologic responses were detected, suggesting an immune-mediated pathogenic mechanism. Pig-tailed macaques showed robust viral replication concomitant with acute-phase symptoms, and provided a model to study the effects of HHV-6A on AIDS progression. In humanized mice, viral replication was less evident, but infection led to T-cell alterations. Altogether, these recent developments have opened new perspectives for studying the pathogenic role of HHV-6A in humans.

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Introduction

HHV-6A and HHV-6B are members of the betaherpesvirus subfamily, and humans are their only known natural host. HHV-6A and HHV-6B share many properties with other herpesviruses, including the establishment of a persistent latent infection characterized by highly restricted viral gene expression, and the ability to reactivate from latency to produce infectious virus. Although originally categorized as two variants, HHV-6A and HHV-6B were recently re-classified as independent viruses based upon differences in epidemiology, tropism, and disease associations [1]. CD46 serves as the main cellular receptor for HHV-6A [2], while CD134 was recently identified as a novel receptor for HHV-6B [3]. *In vivo* tropism of these viruses includes $CD4^+$ T cells, epithelial cells in salivary glands and liver, endothelial cells, and cells of the central nervous system (CNS) [4]. HHV-6A replicates in neural cells in culture more efficiently than HHV-6B and is thought to be overall more neurotropic [5].

HHV-6B infection is very common in the human population worldwide, with a very high seroprevalence (>90%) by age two [6]. Acute primary HHV-6B infection can result in exanthem subitum [7], a childhood febrile disease accompanied by a rash and, in rare cases, by febrile convulsions. No disease association has been firmly established for HHV-6A, although evidence suggests a role in hematopoietic stem cell and solid organ transplant complications [8], graft-versus-host disease [9], and multiple sclerosis [10,11]. Disease manifestations by both HHV-6A and HHV-6B are often correlated with host immunosuppression, which may promote viral reactivation from latency. The prevalence of HHV-6A infection is still largely undefined due to a lack of serological assays that can clearly distinguish between HHV-6A and HHV-6B infections.

The lack of animal models that efficiently support HHV-6A or HHV-6B replication has long hindered studies of viral pathogenesis. The focus of this review is on recent work aimed at developing new animal models that sustain HHV-6A and/or HHV-6B replication, which may help to better understand the pathogenic mechanisms of these viruses in humans.

New animal models of neuropathology

Marmoset model

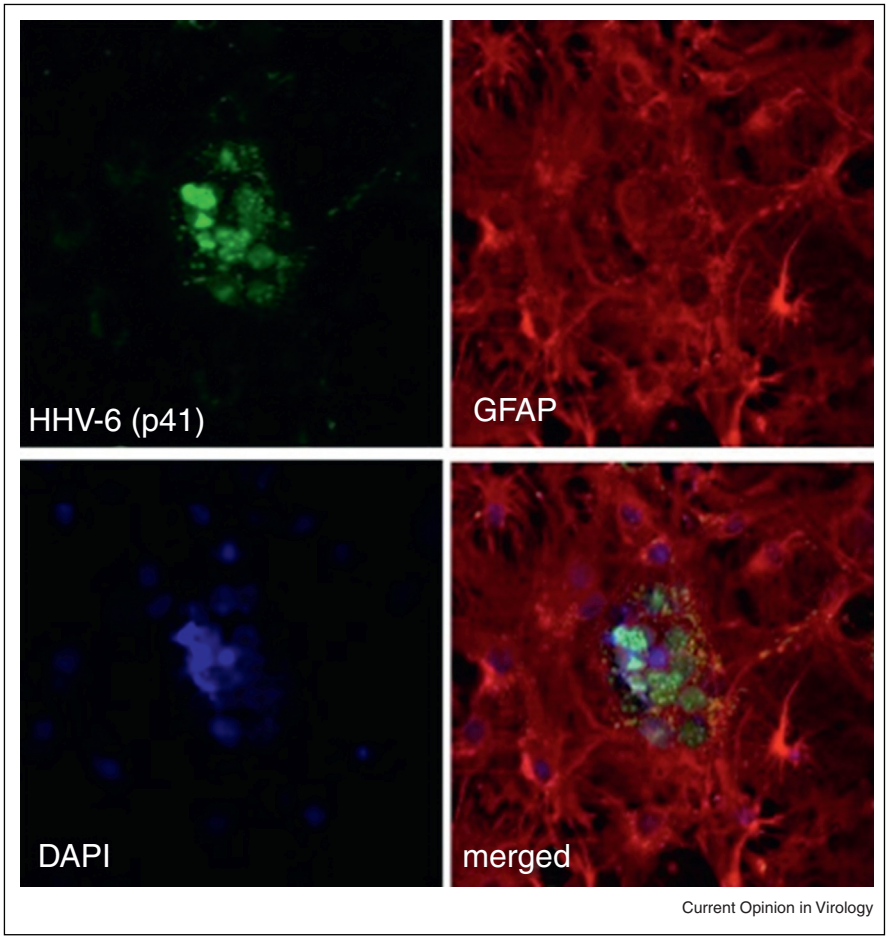
Recently, a marmoset (*Callithrix jacchus*) model was developed to study HHV-6A and HHV-6B infections [12••]. Marmosets that received multiple intravenous injections of HHV-6A developed neurological symptoms, including motor weakness and sensory abnormalities, associated with the development of virus-specific antibody responses and with the presence of histopathological lesions in the CNS, primarily microgliosis. Viral DNA was detected in the brain of HHV-6A-infected and HHV-6B-infected animals, confirming the neurotropism of both viruses. However,

while HHV-6A infection led to evident neurological symptoms, infection with HHV-6B remained asymptomatic. Surprisingly, HHV-6A infection through the intranasal route also remained completely asymptomatic and elicited limited, if any, antibody responses despite detectable levels of plasma viremia. The correlation between the development of neurological signs and the elicitation of virus-specific humoral immune responses in this model suggests a possible immune-mediated pathogenic mechanism rather than a direct neuropathic effect of HHV-6A infection. This study provided the first conclusive *in vivo* evidence that HHV-6A infection is able to trigger neurological disease.

CD46 transgenic mouse model

Since monkey experiments are often limited by ethical constraints and elevated costs, efforts were made in last few years to develop mouse models of HHV-6-associated neuropathology. The human transmembrane complement regulatory protein CD46 was identified as the receptor for HHV-6A entry into host cells [2], opening novel possibilities to develop humanized murine models of HHV-6A infection. Recently, it has been demonstrated that intracranial and intraperitoneal infection of CD46 transgenic mice with HHV-6A results in long-term persistence of viral DNA in the brains of infected animals, followed by lymphocyte infiltration and upregulation of the chemokine CCL5/RANTES, in the absence of clinically apparent signs of disease [13••]. In the presence of HHV-6A-infected human lymphocytes, transgenic murine primary brain cultures were shown to produce viral proteins and develop syncytia (Figure 1); however, viral RNA and proteins have not been detected *in vivo* in mice. Infection with HHV-6B did not yield any signs of viral replication in transgenic murine CD46 transgenic cells either *in vitro* or *in vivo*, probably due to the main utilization of another

Figure 1



HHV-6A infection of primary murine glial brain culture from CD46 trangenic mice. Primary murine brain glial cells generated from CD46-transgenic mice were co-cultured with HHV-6A-infected HSB2 cells as described [13••]. Seven days after the establishment of the co-culture, supernatants and non-adherent cells were removed, and adherent cells were fixed and analyzed for the presence of viral antigens by confocal microscopy. Cells were stained with antibodies against HHV-6 proteins (green) p41 (A-C) or gp116 (D), and glial fibrillary acidic protein (GFAP) antibody (red) and cell nuclei were stained with DAPI (blue).

recently identified entry receptor, CD134 [3]. The secretion of a panel of chemokines was increased after HHV-6A infection of transgenic primary murine brain glial cultures and the induced chemokine expression was inhibited when TLR9 signaling was blocked. These results described the first murine model for HHV-6A-induced brain infection and highlighted the potential importance of the TLR9 pathway in HHV-6A-initiated neuroinflammation, opening novel perspectives for the study of virus-associated neuropathology.

New animal models of immunomodulation and immunodeficiency

Pig-tailed macaque model

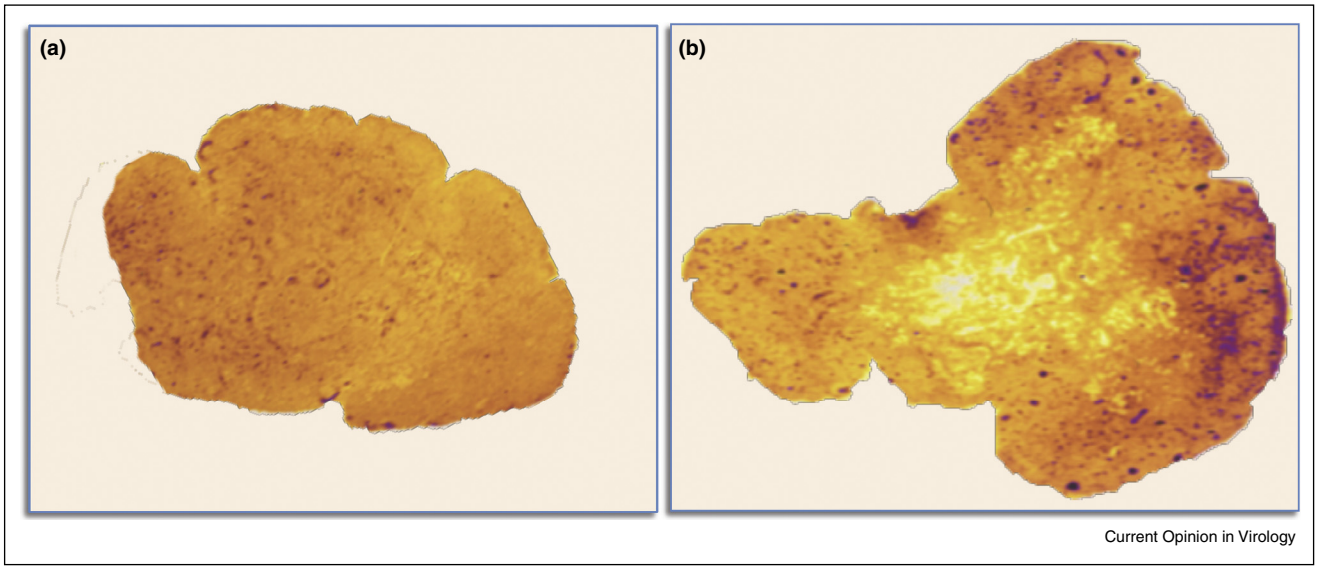
Various non-human primate species have been studied in the past for their susceptibility to HHV-6A, HHV-6B and HHV-7 infections with limited success [14] and (Lusso *et al.*, unpublished), reflecting the inefficient *in vitro* replication of these viruses in primary lymphocytes from the same animals [15]. However, the pig-tailed macaque (*Macaca nemestrina*) was singled out for its ability to sustain HHV-6A replication with human-like efficiency both *in vitro* [16] and, more recently, *in vivo* [17••]. Intravenous inoculation of HHV-6A into naïve pig-tailed macaques resulted in a rapid appearance of plasma viremia and viral RNA transcription in lymph nodes, associated with transient clinical manifestations such as fever, lymphadenopathy and, in one animal, cutaneous rash; IgG antibody seroconversion ensued after approximately 3 weeks of inoculation [17••]. After the acute phase, HHV-6A infection entered a clinically latent state, analogous to observations in healthy children, and no long-term clinical or immunological alterations were detected, except for the occasional finding of transient, low-level plasma viremia. These results reproduced the main virological, immunological, and clinical features of acute HHV-6B infection in humans (to date, no definitive data are available for acute HHV-6A infection), suggesting that pig-tailed macaques may represent a reliable experimental model for these viruses.

The pig-tailed macaque model was also instrumental for investigating the *in vivo* interactions between HHV-6A and simian immunodeficiency virus (SIV), the monkey homologue of human immunodeficiency virus (HIV). Animals co-infected with HHV-6A and SIV showed a dramatic acceleration of SIV-disease progression toward full-blown AIDS, associated with early depletion of both CD4+ and CD8+ T cells and increased SIV expression in lymph nodes (Figure 2); interestingly, as seen in immunodeficient humans, frequent HHV-6A plasma viremia was observed in co-infected animals, concomitant with a progressive deterioration of the host immunologic defenses [17••]. These results provided the first *in vivo* evidence for an accelerating effect of HHV-6A on AIDS progression.

RAG-hu mouse model

Humanized mice are an attractive model for the study of human viral pathogens because they produce human

Figure 2



Enhanced replication of SIV in lymph nodes from HHV-6A-co-infected pig-tailed macaques. *In situ* hybridization in lymph node tissues from macaques singly infected with SIVsmE660 **(A)** or dually infected with SIV and HHV-6A **(B)**. In tissue from the animal singly infected with SIV, the overall architecture is conserved and low levels of SIV RNA (purple signal) are visible throughout the parenchyma, with little, if any, specific signal within reactive germinal centers. In tissue from the dually infected animal, a florid follicular hyperplasia is visible with an intense SIV RNA signal. Co-infection with HHV-6A induced a dramatic acceleration of disease progression toward full-blown AIDS.

target cells and can generate human anti-viral immune responses. In the humanized Rag2$^{-/-}\gamma c^{-/-}$ mouse (RAG-hu) model, human CD34^{+} hematopoietic stem cells are extracted from cord blood or fetal liver and injected into neonatal immunodeficient mice. Engrafted animals produce a variety of human lymphoid and myeloid cells, including CD4^{+} T cells, which are major target cells for HHV-6A.

Recent data show that RAG-hu mice can be infected with HHV-6A following intraperitoneal injection of either cell-free or cell-associated virus, with persistence of viral DNA in blood and lymphoid organs [18••]. Viral DNA was detected only sporadically in plasma and blood cells, possibly due to inefficient replication and establishment of latent infection. The bone marrow was positive for viral DNA in all animals tested at 1 week post-infection. Brain infection has not yet been examined, although human immune cells have been detected in the brain of humanized mice, accompanied by HIV-1 penetration, after peripheral HIV-1 inoculation [19]. Human thymocyte populations were modified after peritoneal inoculation of HHV-6A, indicating cytopathic effects in that organ. The CD3^{+}CD4^{-} and CD3^{-}CD4^{+}CD8^{-} populations were depleted in infected animals (Figure 3). Interestingly, depletion of the CD3^{-}CD4^{+}CD8^{-} thymocyte population had previously been observed in a SCID-hu thy/liv humanized mouse model where HHV-6A or HHV-6B was injected directly into the thymic organoid [20]. A possible contributing mechanism is CD3 downregulation, which has previously been reported in peripheral blood T cells [21] and in cells extracted from lymphoid tissues [22] and is likely mediated by the viral U24 protein [23]. An unusual finding in infected RAG-hu mice was an elevated proportion of CD3^{+}CD4^{+}CD8^{+} T cells in blood, as compared to mock-infected animals. While the origin of these cells is still unclear, HHV-6A infection can promote expression of CD4 on cells that do not normally express it [24]. Thus, it is possible that these cells were either infected with HHV-6A, or triggered to exit the thymus prematurely (most CD4^{+}CD8^{+} cells reside in thymus).

Taken together, the findings in infected RAG-hu mice suggested that HHV-6A has a natural tropism for the human thymus and bone marrow, and that infection leads to alteration of T lymphocyte subpopulations. Depletion and/or alteration of specific thymocyte subsets may play an important role in HHV-6A-induced immunomodulation and the ability of this virus to persist in the host.

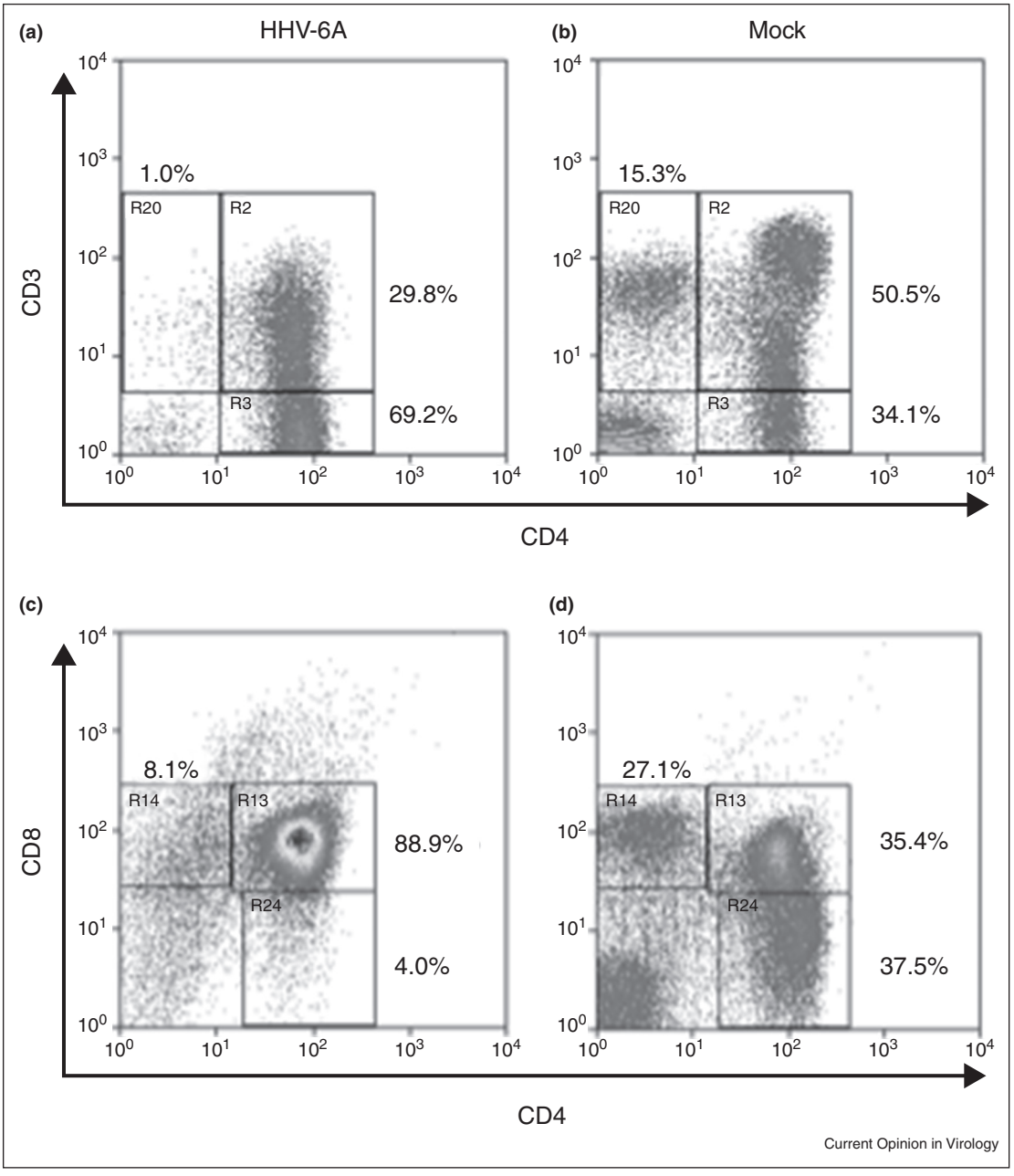
Conclusions and future perspectives

The predominantly latent nature of human roseolovirus infections and our ignorance of the mechanisms that control such viruses *in vivo* have made studies in animal models particularly challenging. Nevertheless, new animal models to study pathology induced by HHV-6A and HHV-6B have been developed during the last decade (Table 1). Although they do not mimic entirely all aspects of the human infections, these models have provided some important insights into the neurological and immunological disorders associated with these viruses. Work in the marmoset model showed neurological symptoms in association with HHV-6A infection. A correlation between HHV-6A infection and multiple sclerosis (MS) has been noted for some time, specifically with increased detection of viral nucleic acids and anti-viral antibody responses in MS patients [25,26]. Further work in the marmoset model may yield additional insights into the role of HHV-6A in this disease whose etiology remains poorly understood. The CD46 transgenic mouse model has further illustrated the potential for neurologic disease associated with HHV-6A in humans by demonstrating that various proinflammatory chemokines are upregulated after infection both *in vivo* and *in vitro* and that immune cells respond to HHV-6A infection in the brain. The TLR9 pathway was identified as a pathway responsible for chemokine upregulation and has been implicated in a different animal model of MS [27], thus providing additional evidence that HHV-6A may be linked to the development of MS.

The recent findings in RAG-hu mice have provided *in vivo* evidence to support a role for HHV-6A in immunosuppression associated with alterations of thymocyte populations. Since the thymus is responsible for T cell development, this may represent a novel mechanism for viral persistence by manipulating T cells before they become functional. The ramifications of thymocyte depletion are currently unclear, but could promote generalized immunosuppression. In addition, macaque studies have provided *in vivo* evidence to support the hypothesis that HHV-6A co-infection leads to more rapid AIDS progression in HIV-infected individuals. Further studies are required to firmly establish a role for HHV-6A in human immunosuppression *in vivo*; however, if a role for this virus in AIDS progression is confirmed, HHV-6A may represent an important new drug target for AIDS treatment.

More animal models have been described for HHV-6A than for HHV-6B infection, possibly reflecting the conservation and ubiquitous distribution of the main HHV-6A receptor, CD46. The recent identification of the immunoregulatory molecule CD134 (OX40), which is expressed predominantly on activated human T cells, as a novel receptor for HHV-6B [3] will certainly lead to the development of additional models for this virus, including transgenic mice. The absence of CD134 expression on CNS cells may explain the apparently lower neurotropism of HHV-6B, compared to HHV-6A; whether and to what

Figure 3



Alteration of thymocyte populations in humanized mice after HHV-6A infection. RAG-hu mice were infected with HHV-6A (or mock infected) and thymocytes were collected at 7.5 wpi and analyzed by flow cytometry. The CD3+CD4− population is depleted in HHV-6A infected animals **(A)** but not in mock infected animals **(B)**. Gating in panels A/B was on lymphocytes, and data were normalized to the sum of gates R2/R3/R20. The CD4+CD8− population is depleted in HHV-6A infected animals (C) but not in mock infected animals (D). Data in panels C/D were not gated and were normalized to the sum of gates R13/R14/R24. Although changes in the CD4−CD8+ and CD4−CD8− populations can be seen in this representative case, these findings were not statistically significant for the entire group of animals.

extent at least some strains of HHV-6B can also utilize CD46 as a receptor, as previously reported [2], remains uncertain. The availability of suitable animal models, especially murine models for which a wide array of experimental tools are available, should facilitate further studies of virus–host interactions and pathogenesis and open novel perspectives for devising effective therapeutic and preventive approaches for HHV-6A and HHV-6B.

Table 1

New animal models recently established to study HHV-6A and HHV-6B pathogenesis

Model [reference]	Virus(es) studied	Route of infection	Major pathologic findings	Disadvantages
Pig-tailed macaques [17••]	HHV-6A	Intravenous	Acute-phase symptoms, robust viral replication, antibody responses, accelerated AIDS progression	Costly, ethical constraints
Marmosets [12••]	HHV-6A and HHV-6B	Intravenous	CNS pathology (HHV-6A only), antibody responses	Low viral replication, costly, ethical constraints
Humanized $Rag2^{-/-}\gamma c^{-/-}$ mice [18••]	HHV-6A	Intraperitoneal	Viral DNA persistence in blood, antibody responses, alteration of human thymocyte and T cell populations	Low viral replication
huCD46-transgenic mice [13••]	HHV-6A	Intracranial + intraperitoneal	Long-term viral DNA persistence in CNS, antibody responses, CNS production of pro-inflammatory cytokines	Low viral replication

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- • of special interest
- •• of outstanding interest

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