Exacerbation of experimental autoimmune encephalomyelitis in rodents infected with murine gammaherpesvirus-68

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Viral infections have long been suspected to play a role in the pathogenesis of multiple sclerosis. In the present study, two different rodent models of experimental autoimmune encephalomyelitis (EAE) were used to demonstrate the ability of murine gammaherpesvirus-68 (γHV-68) to exacerbate development of neurological symptoms. SJL mice received UV-inactivated γHV-68 or intranasal γHV-68, followed by immunization against proteolipid-protein peptide 139–151. Infected mice became moribund within 10 days post-immunization, whereas mice exposed to UV-inactivated γHV-68 recovered. In the second model, Lewis rats were exposed to UV-inactivated γHV-68 or to γHV-68, followed by passive transfer of encephalitogenic T lymphocytes specific for myelin basic protein. Consistently, infected rats had higher clinical scores, and this result was observed during acute or latent γHV-68 infection. It is unlikely that this γHV-68-induced exacerbation was due to significant viral replication within the central nervous system since nested PCR, viral plaque assays, and infectious-centers assays demonstrated no detectable virus in spinal cords or brains of infected rodents undergoing EAE. Taken together, these studies demonstrate increased clinical symptoms of EAE in rodents infected by a gammaherpesvirus that has a limited ability to invade the central nervous system.

Received	14/5/02
Revised	7/4/03
Accepted	24/4/03

Key words: Gammaherpesvirus / Experimental autoimmune encephalomyelitis / Autoimmune disease

1 Introduction

It has been suggested that multiple sclerosis (MS) develops in patients, who are genetically predisposed to this demyelinating disease, following exposure to environmental factors [1–3]. There is striking epidemiological evidence to suggest that environmental factors play a role in the development of MS [4]. These factors include geographic variation in prevalence [3], altered susceptibility following relocation [5, 6], and discordance for clinical disease in a majority of monozygotic twins [6, 7]. However, it has not been possible to define exactly which environmental factor(s) in association with a genetic predisposition result in the development of clinical disease [1, 3].

[DOI 10.1002/eji.200323148]

Abbreviations: CNS: Central nervous system **EAE:** Experimental autoimmune encephalomyelitis **γHV-68:** Murine gammaherpesvirus 68 **MS:** Multiple sclerosis **PFU:** Plaque-forming units **PLP:** Proteolipid protein

At the forefront of the list of possible environmental factors that might exacerbate the development or severity of MS are viral infections. In fact, Theiler's-virus infection of the central nervous system (CNS) has been widely used as a mouse model of experimental autoimmune encephalomyelitis (EAE) that consistently reproduces the symptomology of human disease [8]. A coronavirus — murine hepatitis virus — has also been shown to cause a demyelinating disease of the CNS [9]. Recent studies have suggested an association between infection with the human gammaherpesvirus, EBV, and the risk of MS. In one prospective epidemiological study, anti-EBV antibody levels were elevated in women with MS, suggesting that EBV might be involved in the etiology of this autoimmune disease [10]. This recent work supports previous studies that demonstrated increased anti-EBV antibody titers in patients with MS [11]. In addition, a particular T cell receptor from an MS patient recognized both a myelin-basic-protein peptide and a peptide derived from an EBV protein [12]. These studies suggest a mechanism of molecular mimicry that might facilitate development of MS at some time after EBV infection.

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A direct relationship between viral infection and the development of MS has been difficult to prove because of the reliance on human autopsy material or material taken from blood and spinal fluid. To circumvent such limitations, rodent models of MS have been developed and have contributed greatly to our understanding of the cellular and molecular events that occur during demyelinating diseases of the CNS. Relapsing EAE is a T-helper-cell- and macrophage-mediated disease, characterized by inflammation and then demyelination within the CNS. The SJL mouse is often used as a model for such a disease, and active immunization with the immunodominant peptide of proteolipid protein (PLP 139–151) induces relapsing EAE in these mice [13]. EAE can also be induced by passive transfer of $CD4^+$ encephalitogenic T lymphocytes into certain strains of mice [14] and rats [15]. Such passive transfer of antigen-specific T helper cells obviates the need to actively immunize mice, and allows studies of post-immunization events in developing EAE. Passive transfer of encephalitogenic T lymphocytes into Lewis rats provides a reproducible non-relapsing form of EAE that has been used to investigate the acute form of the disease [16].

In the present study, immunization-induced EAE in SJL mice and passive-transfer-induced EAE in Lewis rats were used as models to address whether infection with the murine gammaherpesvirus-68 (γHV-68) could exacerbate clinical disease. γHV-68 has been used as a mouse model for EBV and human herpesvirus-8 infections [17–21], and is the only well-characterized rodent model for studying gammaherpesviruses. In studies presented here, rodents exposed to UV-inactivated γHV-68 or infected with γHV-68 and undergoing EAE were scored for severity of disease. Results from these studies clearly demonstrate that infection with this murine gammaherpesvirus exacerbates the clinical symptoms associated with EAE without substantial entry of the virus into the CNS.

2 Results

2.1 SJL mice infected with γHV-68 show increased clinical scores, increased weight loss, and increased CNS inflammation during immunization-induced EAE

Following immunization of SJL mice with PLP peptide 139–151, a neurodegenerative disease ensues that can be followed by scoring clinical symptoms. As shown in Fig. 1A (closed squares), 11 days following the initiation of immunization, groups of SJL mice that had previously been exposed to UV-inactivated γHV-6 began to show signs of CNS disease, which peaked by day 14. The kinetics and magnitude of this response were very similar to those previously reported by others [22]. In contrast, clinical scores in SJL mice that had been infected with γHV-68 were dramatically different in both their kinetics and magnitude (Fig. 1A, closed circles), when compared with groups of mice exposed to UV-inactivated γHV-68. Clinical symptoms of infected mice began 4 days earlier and resulted in all mice being moribund by day 10 post-immunization. Clearly mice infected with γHV-68, and then immunized with PLP peptide 139–151, demonstrated a marked exacerbation of clinical symptoms when compared with controls.

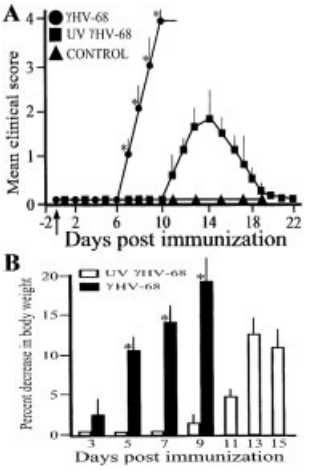

Fig. 1. SJL mice infected with γHV-68 show increased clinical scores and weight loss during immunization-induced EAE. (A) Groups of SJL mice were exposed to UV-inactivated γHV-68 (closed squares, n=8) or infected with γHV-68 (closed circles, n=10), 2 days prior to immunizing with PLP peptide 139–151. Control mice (closed triangles, n=4) were γHV-68 infected, but immunized with an irrelevant peptide. An arrow indicates the day of immunization (*i.e.* day 0). Following immunization, mice were scored daily for the presence of clinical symptoms. Results are presented as mean clinical scores (± standard deviations) at the indicated times post-immunization. (B) Mice were weighed daily and the percent decrease in body weight determined. Results are presented as mean body weights (± standard deviations). Asterisks indicate significant differences ($p<0.01$) when comparing γHV-68-infected mice *versus* mice treated with UV-inactivated γHV-68. These studies are representative of three separate experiments.

As controls to demonstrate the antigen-specific nature of EAE induction, several groups of mice were used. These included groups of mice that were infected with γHV-68 and given complete Freund's adjuvant alone, or given pertussis toxin alone, or immunized with an irrelevant peptide. None of the mice in any of the control groups showed neurological deficits during the course of these studies (*e.g.* Fig. 1A, closed triangles).

As an additional assessment of clinical disease, decreased body weight was followed in mice exposed to UV-inactivated γHV-68 or infected with γHV-68 and then immunized to induce EAE. Fig. 1B shows an early and exacerbated weight loss in mice infected with the virus when compared with mice exposed to UV-inactivated γHV-68.

In addition, histological analyses were performed to indicate the level of inflammation in the spinal cord and cerebellum of infected *versus* uninfected mice undergoing EAE. On day 9 post-immunization, groups of mice were killed and spinal cord and cerebellum stained for scoring of infiltrating leukocytes. Mice infected with γHV-68 had significantly more ($p<0.05$) inflammation (mean score 1.30±0.27, $n=5$) than did mice that were exposed to UV-inactivated γHV-68 (mean score 0.25±0.50, $n=4$), whereas control mice that were infected, but not immunized had no detectable inflammation in the CNS (mean score 0.0±0.0, $n=3$).

2.2 Absence of detectable γHV-68 viral DNA in the CNS of SJL mice undergoing clinical EAE

One possible explanation for γHV-68-induced exacerbation of EAE would be the replication of virus within the CNS, directly causing neuronal death or causing a destructive inflammatory response. If γHV-68 did gain access to the CNS during clinical EAE, the presence of virus should not be difficult to detect. To address whether γHV-68 was present in the CNS, a sensitive, nested PCR procedure was performed to detect the presence of the DNA encoding γHV-68 gp150 in the spleens and spinal cords of infected mice, and infected mice undergoing clinical EAE. Fig. 2 shows the results of one such PCR where viral genomes were easily detectable in peripheral lymphoid organs (*i.e.* spleen), but not in the spinal cord of infected mice. This was true even when nested PCR was performed for 20 + 25 cycles, whereas such viral DNA sequences were abundant in the spleen using only 20 + 20 PCR cycles. Although qualitative, these differences in viral genome presence in the CNS *versus* peripheral lymphoid organs suggested that minimal viral replication or latency occurs in the CNS.

To more directly address the presence of replicating virus in the spleens and CNS of γHV-68 infected mice undergoing clinical EAE, viral-plaque assays were performed. A plaque-forming assay consistently failed to demonstrate the presence of lytic virus in tissue homogenates of brain and spinal cord from infected mice undergoing clinical EAE, regardless of the time-point chosen for analysis. In addition, an infectious-centers assay was used to quantify latent virus present in the spleens of infected SJL mice. Fig. 3 shows similar numbers of infectious centers in splenic leukocytes from γHV-68-infected mice, whether EAE was induced or not. However, we were unable to detect latent γHV-68 in cells isolated from the spinal cord of the same mice (Fig. 3). Taken together, the results in Fig. 2 and 3 strongly argue for the presence of a significant viral burden in peripheral tissues, but not in the CNS.

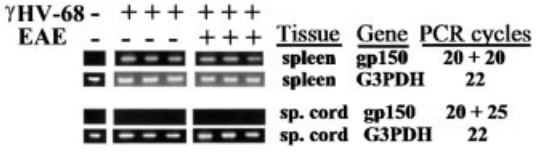

Fig. 2. Absence of detectable γHV-68 viral DNA in the CNS of SJL mice undergoing clinical EAE. Groups of SJL mice were exposed to UV-inactivated γHV-68 (γHV-68 –) or infected with γHV-68 (γHV-68 +), and non-immunized (EAE –) or immunized to induce EAE (EAE +) as indicated. Mice that were exposed to UV-inactivated γHV-68 or infected with γHV-68, but non-immunized ($n=3$), were killed at day 15 post-infection, which represents the peak of leukocytosis. Mice infected and immunized to induce EAE ($n=3$) were killed at day 10 post-infection, when mice had clinical scores between 2 and 3. After mice were killed, tissue from the spleen and spinal cord was taken and DNA extracted for PCR to amplify the genes encoding γHV-68 gp150 or G3PDH. The number of PCR cycles used for each amplification is indicated. Results are presented as amplified PCR products electrophoresed on ethidium-bromide-stained agarose gels. These studies were performed twice with similar results.

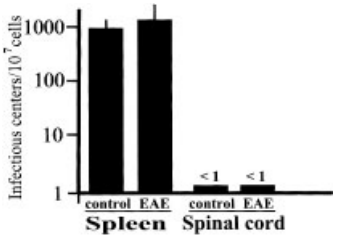

Fig. 3. Absence of detectable latent γHV-68 virus in the CNS of SJL mice undergoing clinical EAE. Groups of SJL mice were infected with γHV-68, and non-immunized (control, $n=4$) or immunized (EAE, $n=4$) as indicated. Mice were killed at day 10 post-infection, when mice had clinical scores between 2 and 3, and tissue from the spleen and spinal cord were taken. Cells were isolated from each tissue and used to quantify the amount of latent virus using an infectious-centers assay. Results are presented as mean virus counts per 10^7 cells (± standard deviations). A score of <1 indicates that the presence of any latent virus was below the level of detectability of this assay. These studies were performed twice with similar results.

2.3 γHV-68 infection induces leukocytosis and establishes viral latency in Lewis rats

Passive transfer of encephalitogenic T lymphocytes into Lewis rats was also used as a model to investigate the ability of this gammaherpesvirus to exacerbate an autoimmune disease of the CNS. However, it was first important to demonstrate that this strain was susceptible to viral infection, since no reports to date have demonstrated γHV-68 infection in rats. Following intranasal inoculation of groups of Lewis rats with 15,000 plaque-forming units (PFU) of virus, a characteristic splenomegaly and leukocytosis, with establishment of latent virus, was observed in all infected rats. Typically spleens from γHV-68 infected rats were increased in size up to three-fold compared with rats exposed to UV-inactivated γHV-68 (389±88 mg *versus* 150±23 mg, respectively), with splenic leukocyte numbers peaking by day 15 post-infection (Fig. 4A).

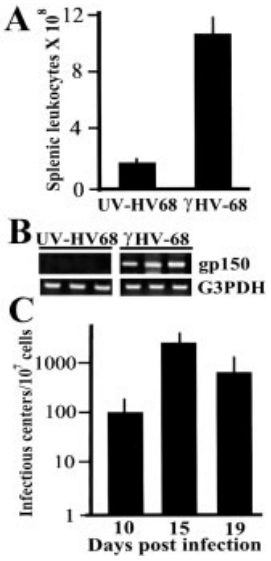

Fig. 4. γHV-68 infection induces leukocytosis and establishes viral latency in Lewis rats. Groups of Lewis rats were exposed to UV-inactivated γHV-68 (UV-HV68, *n*=5) or infected with γHV-68 (γHV-68, *n*=6). (A) At the peak of leukocytosis (day 15 post-infection), rats were killed and the number of splenic leukocytes quantified. Results are presented as mean values (± standard deviations). (B) DNA was also isolated and PCR performed to detect the presence of the genes encoding viral gp150 or G3PDH. Results are presented as amplified PCR products electrophoresed on ethidium-bromide-stained agarose gels. (C) Groups of Lewis rats (*n*=4) were infected with γHV-68 and, at the indicated days post-infection, the presence of latent virus in splenic leukocytes was quantified. Results are presented as mean infectious centers (± standard deviations). These studies were performed three times with similar results.

The presence of viral genomes was easily detectable in γHV-68-infected rats using a nested PCR for gp150 (Fig. 4B), but such amplified products were absent in control rats. In addition, viral latency was established in the γHV-68-infected rats, with highest levels of viral burden being observed at day 15 post-infection (Fig. 4C). As previously observed by our laboratory [23] and others [24, 25], infectious centers were due to the presence of latent virus and not that of lytic or replicating virus as mechanical disruption of splenocytes produced no PFU when incubated with a permissive monolayer of NIH-3T3 cells. Taken together, these results demonstrate that the murine gammaherpesvirus-68 does infect rats and induces the symptoms of a mononucleosis-like disease, similar to that seen in mice, with the hallmarks of leukocytosis and the establishment of latency.

2.4 Lewis rats infected with γHV-68 show increased clinical scores and increased weight loss during passive-transfer-induced EAE

Having established the characteristics of γHV-68 infection in Lewis rats, we induced EAE in these animals using passive transfer of encephalitogenic T lymphocytes. For these studies, groups of rats were exposed to UV-inactivated γHV-68 or infected intranasally with γHV-68. After infection, 3×10^6 encephalitogenic T lymphocytes, specific for myelin basic protein, were given intravenously, and rats were scored daily for the development of clinical symptoms. Consistently, rats infected with γHV-68 had increased clinical scores when compared with animals that had been exposed to UV-inactivated γHV-68 (Fig. 5). It should be noted that there was no significant difference in clinical EAE scores in groups of untreated rats compared with rats treated with UV-inactivated γHV-68 after both groups received encephalitogenic T lymphocytes (*e.g.* Fig. 5, experiment #1).

As controls to demonstrate the antigen-specific nature of EAE induction, several groups of rats were used. These included groups that were infected with γHV-68 but received no encephalitogenic T lymphocytes, and γHV-68-infected rats given normal, Con-A-activated T lymphocytes (*e.g.* Fig. 5, experiment #2). No detectable neurological symptoms were observed in these control groups.

As an additional assessment of clinical disease, decreased body weight was followed in rats exposed to UV-inactivated γHV-68 or infected with γHV-68 and then administered encephalitogenic T lymphocytes to induce EAE. Fig. 6 shows increased weight loss at two time-points post-infection when compared with rats exposed to UV-inactivated γHV-68.

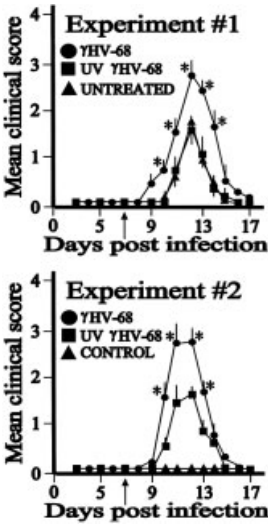

Fig. 5. Lewis rats infected with γHV-68 show increased clinical scores during passive-transfer-induced EAE. Groups of Lewis rats were exposed to UV-inactivated γHV-68 (closed squares, *n*=7) or infected with γHV-68 (closed circles, *n*=8). At 7 days post-infection (as indicated by the arrow), rats received 3×10^6 encephalitogenic T lymphocytes by passive administration via tail-vein injection. Following administration of encephalitogenic T lymphocytes, rats were monitored daily in a blinded fashion for the presence of clinical symptoms. Results are presented as mean clinical scores (± standard deviations) at the indicated times post-immunization. Asterisks indicate significant differences ($p<0.01$) when comparing γHV-68-infected rats *versus* rats exposed to UV-inactivated γHV-68. For comparison, the panel for experiment #1 shows induction of EAE in rats given encephalitogenic T lymphocytes but not treated with γHV-68 or UV γHV-68 (closed triangles, *n*=4). In addition, the panel for experiment #2 shows a lack of clinical symptoms inγHV-68-infected rats given normal, Con-A-activated T lymphocytes (closed triangles, *n*=3). The two experiments shown are representative of four separate studies.

The severity of clinical symptoms can be manipulated in the Lewis rat model by varying the number of encephalitogenic T lymphocytes that are injected. Therefore, in some studies, groups of rats were exposed to UV-inactivated γHV-68 or infected with γHV-68 and given 3×10^7 encephalitogenic T lymphocytes. Fig. 7 shows that animals given this higher number of encephalitogenic T lymphocytes progressed rapidly to a moribund state. However, once again, rats that were infected with γHV-68 had higher initial scores than those animals that were exposed to UV-inactivated γHV-68 (Fig. 7).

Thus, regardless of the protocol used, the ability of γHV-68 to exacerbate clinical disease was consistent. Using a protocol that allowed all rats to recover (Fig. 5) or using a protocol where rats rapidly progressed to a moribund state (Fig. 7) showed that γHV-68-infected rats had significantly higher clinical scores during EAE.

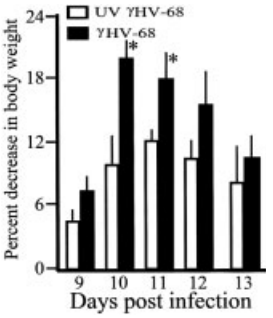

Fig. 6. Lewis rats infected with γHV-68 show increased weight loss during passive-transfer-induced EAE. Groups of Lewis rats were exposed to UV-inactivated γHV-68 (*n*=7) or infected with γHV-68 (*n*=8). At 7 days post-infection, rats received 3×10^6 encephalitogenic T lymphocytes by passive administration via tail-vein injection. Following administration of encephalitogenic T lymphocytes, rats were weighed on a daily basis. Results are presented as mean percent decreases in body weight (± standard deviations) at the indicated times post-immunization. Asterisks indicate significant differences ($p<0.01$) when comparing γHV-68-infected rats *versus* rats exposed to UV-inactivated γHV-68.

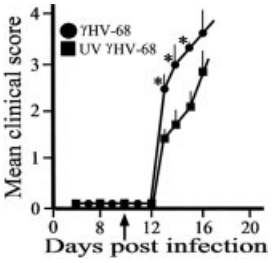

Fig. 7. Lewis rats infected with γHV-68 show increased clinical scores following high-dose administration of encephalitogenic T lymphocytes. Groups of Lewis rats were exposed to UV-inactivated γHV-68 (closed squares, *n*=6), or infected with γHV-68 (closed circles, *n*=6). At 10 days post-infection (as indicated by the arrow), rats received 3×10^7 encephalitogenic T lymphocytes by passive administration via tail-vein injection. Following administration of encephalitogenic T lymphocytes, rats were monitored daily in a blinded fashion for the presence of clinical symptoms. Results are presented as mean clinical scores (± standard deviations) at the indicated times post-immunization. Asterisks indicate significant differences ($p<0.01$) when comparing γHV-68-infected rats *versus* rats exposed to UV-inactivated γHV-68.

2.5 Lewis rats that are latently infected with γHV-68 show increased clinical scores during passive-transfer-induced EAE

In the SJL mouse (Fig. 1) and the Lewis rat (Fig. 5 and 7) models, the experimental design to demonstrate γHV-68-induced exacerbation of EAE had rodents beginning a leukocytosis as clinical symptoms progressed. However, upon resolution of the mononucleosis-like acute-phase of the disease, splenomegaly resolves and γHV-68 remains latent in leukocytes. We questioned whether rats with latent γHV-68 infection also had increased clinical scores following passive administration of encephalitogenic T cells. For these studies, groups of rats were exposed to UV-inactivated γHV-68 or infected with γHV-68, and allowed to recover from acute infection and splenomegaly. By this time post-infection, rats had viral genomes present and latent virus in splenic leukocytes, but no detectable plaque-forming virus in tissue homogenates. On day 41 post-infection, 3×10^6 encephalitogenic T lymphocytes were given intravenously, and rats were scored daily for the development of clinical symptoms. Consistently, rats with latent γHV-68 infection had increased clinical scores when compared with animals exposed to UV-inactivated γHV-68 (Fig. 8).

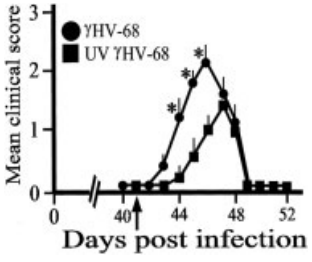

Fig. 8. Lewis rats latently infected with γHV-68 display increased clinical scores during passive-transfer-induced EAE. Groups of Lewis rats were exposed to UV-inactivated γHV-68 (closed squares, *n*=6), or infected with γHV-68 (closed circles, *n*=6). At 41 days post-infection (as indicated by the arrow), rats received 3×10^6 encephalitogenic T lymphocytes by passive administration via tail-vein injection. Following administration of encephalitogenic T lymphocytes, rats were monitored daily in a blinded fashion for the presence of clinical symptoms. Results are presented as mean clinical scores (± standard deviations) at the indicated times post-immunization. Asterisks indicate significant differences ($p<0.01$) when comparing γHV-68-infected rats *versus* rats exposed to UV-inactivated γHV-68. These studies were performed twice with similar results.

2.6 Absence of detectable γHV-68 viral DNA in the CNS of Lewis rats undergoing clinical EAE

Once again we questioned whether the cause for γHV-68-induced exacerbation of EAE might be the presence of a significant viral infection within the CNS. If present, replicating virus might directly result in neuronal damage or cause a deleterious inflammatory response. If γHV-68 did gain access to the CNS, during clinical EAE, the presence of virus would not be difficult to detect.

To address this possibility, a sensitive, nested PCR procedure was performed to detect the presence of the DNA encoding γHV-68 gp150 in the spleens and spinal cords of infected rats and infected rats undergoing clinical EAE. Fig. 9 shows the results of one such PCR where viral genomes were easily detectable in peripheral lymphoid organs (*i.e.* spleen), but not in the spinal cord of infected rats. When nested PCR was performed for 20 + 25 cycles, no γHV-68 gp150 DNA could be detected in spinal cord, whereas such viral DNA sequences were abundant in the spleen using only 20 + 15 PCR cycles. Similar results were found when nested PCR was performed using frontal cortex and cerebellum (data not shown).

Although qualitative, these differences in viral-genome presence in the CNS *versus* peripheral lymphoid organs

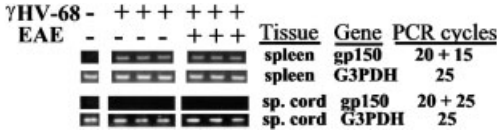

Fig. 9. Absence of detectable γHV-68 viral DNA in the CNS of Lewis rats undergoing clinical EAE. Groups of Lewis rats were exposed to UV-inactivated γHV-68 (γHV-68 –) or infected with γHV-68 (γHV-68 +) as indicated. Within the latter group, some rats were non-treated (EAE –) or some rats were administered encephalitogenic T cells to induce EAE (EAE +) as indicated. Rats that were exposed to UV-inactivated γHV-68 or infected with γHV-68, without EAE induction (*n*=3), were killed at day 15 post-infection, which represents the peak of leukocytosis. Rats infected and administered encephalitogenic T cells to induce EAE (*n*=3) were killed at day 12 post-infection, when rats had clinical scores between 1 and 2. After the rats were killed, tissue from the spleen and spinal cord was taken and DNA extracted for PCR to amplify the genes encoding γHV-68 gp150 or G3PDH. The number of PCR cycles used for each amplification is indicated. Results are presented as amplified PCR products electrophoresed on ethidium-bromide-stained agarose gels. These studies were performed twice with similar results.

suggested that minimal viral replication occurred in the CNS. In addition, a plaque-forming assay [23, 26] consistently failed to demonstrate the presence of lytic virus in tissue homogenates of brain and spinal cord from infected rats undergoing clinical EAE, regardless of the time-point chosen for analysis. Together these results strongly suggest that γHV-68-induced exacerbation of EAE in the Lewis rat model was not due to significant replicative virus in the CNS.

3 Discussion

Recent epidemiological studies [10] and previous investigations [11] have postulated an association between the development and/or severity of MS and infection with the gammaherpesvirus, EBV. The necessity to rely on analyses using anti-EBV antibodies in patients' sera, or on CNS tissue obtained at autopsy, has provided intriguing correlations, but no definitive results. In the present study, two very different rodent models of EAE were used to assess the ability of a gammaherpesvirus infection of rodents to exacerbate neurological symptoms. Using immunization-induced EAE in SJL mice (Fig. 1) or passive-transfer-induced EAE in Lewis rats (Fig. 5, 7, and 8), the overall result was, qualitatively, the same. Rodents infected with γHV-68 had significantly more-severe clinical disease than rats exposed to UV-inactivated γHV-68.

The limited ability of γHV-68 to enter the CNS (Fig. 2 and 9) and the lack of replicative and latent virus there (Fig. 3) provides an important focus for beginning to understand the mechanisms involved during virus-induced exacerbation of EAE. Certain viruses, including Theiler's virus [8] and murine hepatitis virus [9], that are injected directly into the CNS of mice can cause inflammation and demyelination that resembles an MS-like disease. Thus there is precedence for certain viruses to replicate within the CNS and to induce the pathology and clinical symptomology described as EAE.

However, it is highly unlikely, based on results presented here and on the results of others, that γHV-68 directly invades the CNS to a significant extent following a peripheral infection [27]. Of the numerous laboratories that have used γHV-68 as a model to investigate in great detail the pathophysiology of gammaherpesvirus infections, none has reported neurological symptoms during the acute or latent phases. Thus, by itself, γHV-68 infections in the periphery do not result in EAE or in any discernable clinical symptomology. Additionally, viral genomes were not found within the CNS of infected rodents even when a very sensitive, nested PCR procedure was used (Fig. 2 and 9). The lack of a significant viral burden in the CNS argues strongly against γHV-68-induced destruction of neurons or glial cells.

If γHV-68 does not enter the CNS to any significant extent, then the mechanisms required for exacerbation of EAE must occur in peripheral tissues. Recent discoveries suggest some intriguing possibilities for understanding how gammaherpesviruses might contribute to the severity of autoimmune diseases. Molecular mimicry between viral protein sequences and encephalitogenic epitopes has been proposed for the gammaherpesvirus, EBV. One study [28] found that the viral protein, BSLF1, could activate T lymphocytes that are specific for myelin basic protein, in SJL mice. Another recent study found that a particular T cell receptor from an MS patient recognized both a myelin-basic-protein peptide and a peptide derived from an EBV protein [12]. Theoretically, such molecular mimicry could lead to exaggerated induction, activation, and/or differentiation of encephalitogenic T cells, thereby augmenting the development or severity of MS.

However, investigating the contribution of molecular mimicry to development of autoimmune diseases during EBV infections will be a challenging problem. The inherent limitations in human experimentation, and the fact that encephalitogenic epitopes might differ between rodents and men, will make it difficult to conclusively prove molecular mimicry as a mechanism for EBV-exacerbated MS. Fortunately, the γHV-68 genome has been sequenced [29]. Therefore it should be possible to exploit this information to identify likely encephalitogenic epitopes, and, more importantly, to use experimental models to definitively prove whether such sequences are involved in inducing or increasing the severity of EAE.

A second mechanism that could contribute to the observed γHV-68-induced exacerbation of EAE would be one that does not depend upon antigenic cross-reactivity of epitopes. Gammaherpesvirus cause a unique dysregulation of the host immune response that has been characterized as "immunological dissonance". The expansion of $CD4^+$, $CD8^+$, and B lymphocytes that occurs during the mononucleosis-like phase of the disease is largely not specific for viral antigens [24, 30]. In fact, T lymphocytes are not infected by γHV-68, therefore the mechanisms that lead to T cell expansion must require interaction or input from other cell populations. It is possible that mechanisms involved in this dysregulated host T lymphocyte response following viral infection are also involved in augmenting the number or activity of encephalitogenic T lymphocytes present during developing EAE.

It should be noted, however, that it was not necessary for rats to be experiencing peak leukocytosis for there to be

a γHV-68-induced exacerbation of clinical EAE (Fig. 8). Rats with latent γHV-68 have very low levels of replicating virus, have little splenomegaly, and have relatively normal numbers and percentages of T lymphocytes. When encephalitogenic T lymphocytes were passively transferred into such rats, exacerbated clinical scores were still observed. This result is significant since essentially all patients who develop MS have latent EBV infections [11, 31], not acute mononucleosis. Ongoing studies are aimed at defining whether the encephalitogenic events that result in the development of EAE can also serve as a stimulus to reactivate latent γHV-68 to a productive, peripheral infection. If this is the case, then suppression of viral replication prior to, or during, clinical disease might prove effective in limiting the severity or duration of damage in the CNS.

It is highly unlikely that gammaherpesviruses can directly cause MS or EAE. Greater than 80% of the world's population is latently infected with EBV [11, 31], and only a small percentage of these individuals develop clinical MS. Therefore the assertion that EBV is an etiologic agent for this neurodegenerative disease has met with much skepticism. A more likely possibility, that could help explain the epidemiological and experimental data, would be the role of EBV and γHV-68 as environmental factors that may exacerbate disease in those patients or rodents predisposed for developing autoimmune-mediated demyelination. Whether such virus-induced exacerbation involves molecular mimicry or dysregulated leukocyte function is not clear. However, the studies presented here clearly demonstrate that peripheral infection with γHV-68 is capable of augmenting an inflammatory response in the CNS. This rodent model of viral infection should be extremely valuable in dissecting the mechanisms that are responsible for gammaherpesvirus-mediated exacerbation of autoimmune disease.

4 Materials and methods

4.1 Virus isolation, propagation and intranasal inoculation with γHV-68

γHV-68 was propagated and isolated as previously described [23, 26, 32]. Intranasal inoculations were performed on female SJL mice (Jackson Laboratories, Bar Harbor, ME, USA) or female Lewis rats (Charles River Laboratories, Wilmington, MA, USA) as previously described [23, 24, 33].

4.2 Immunization-induced EAE in SJL mice

Groups of SJL mice were exposed to UV-inactivated γHV-68 or infected with γHV-68, 2 days prior to initiating the immunization protocol. The immunization protocol is similar to that previously reported [22, 34], and involved an initial injection of 200 μg of PLP peptide 139–151 emulsified in complete Freund's adjuvant (containing mycobacteria). In addition, mice received 200 ng of *Bordetella pertussis* toxin (Research Biochemicals International, Natick, MA, USA) at the time of immunization and at day 3 post-immunization. Mice exposed to UV-inactivated γHV-68 or infected with γHV-68 were coded and assessed daily for the development of EAE in a blinded fashion using the following scale: 0, no evidence of disease; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, moribund.

Several control groups of SJL mice were infected with γHV-68 and given complete Freund's adjuvant alone, or *B. pertussis* toxin alone, or immunized with an irrelevant peptide (amino acid sequence HSFNCGGEFFY) using an immunization scheme identical to the one described above.

4.3 Passive-transfer-induced EAE in Lewis rats

The passive administration of encephalitogenic T lymphocytes into Lewis rats was used as a second rodent model of EAE and was performed as previously described [16, 35]. Encephalitogenic T cell lines were established by immunizing Lewis rats with an encephalitogenic peptide of myelin basic protein as previously described [35–37]. Groups of Lewis rats were untreated, exposed to UV-inactivated γHV-68, or infected intranasally with 15,000 PFU of γHV-68. At the indicated times post-infection, EAE was induced in these rats by tail-vein injection of the indicated number (3×10^6 or 3×10^7) of encephalitogenic T lymphocytes. Animals exposed to UV-inactivated γHV-68 (<1 PFU per rat) were used as controls. Rats exposed to UV-inactivated γHV-68 or γHV-68-infected rats were coded and assessed daily for the development of EAE in a blinded fashion using the following scale: 0, no evidence of disease; 1, flaccid tail; 2, hind limb paralysis; 3, lower-body paralysis; 4, moribund. To confirm the antigen-specific nature of the model, control groups of γHV-68-infected rats received 3×10^6 Con-A-activated leukocytes expanded from a non-immune Lewis rat.

4.4 PCR amplification of γHV-68 gp150 or G3PDH DNA from the spleen, spinal cord, or brain

The presence of γHV-68 DNA encoding gp150 in the spleens, spinal cord, or brains of rodents was detected by a sensitive nested PCR protocol as previously described [23, 26, 32].

4.5 Quantification of lytic γHV-68 in tissue homogenates and latent γHV-68 in leukocytes

The presence of lytic virus was quantified as previously described [23, 26, 32] using a plaque-forming assay. The

presence of latent virus was quantified using an infectious-centers assay as previously described [23, 26, 32].

4.6 Histological analysis of CNS for inflammation

On day 9 post-immunization, groups of mice that were infected with γHV-68 or exposed to UV-inactivated virus were killed. Five-micrometer sections of spinal cord and cerebellum were stained using hematoxylin and eosin, and scored for inflammation as previously described [38].

4.7 Statistical analysis to determine significant differences in mean clinical scores

The significance of differences in mean scores was assessed using the Mann-Whitney *t*-test of non-parametric values, or the Student's *t*-test, as appropriate. Results were determined to be significantly different at $p<0.05$.

Acknowledgement: This work was supported by PHS grant NS40307.

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