Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis

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A general association of human and primate lymphotropic herpesviruses (γ-herpesviruses) with the development of lymphomas, as well as other tumors, especially in immunocompromised hosts, has been well documented. The lack of relevant small animal models for human γ-herpesviruses has impeded progress in understanding the role of these viruses in the development of chronic disease. Recent research characterizing infection of inbred strains of mice with a murine γ-herpesvirus, gamma-herpesvirus 68 (γHV68), is providing insights into viral and host factors involved in the establishment and control of chronic γ-herpesvirus infection.

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Abbreviations

EBV	Epstein-Barr virus
γHV68	gamma-herpesvirus 68
HVS	herpesvirus saimiri
IFN	interferon
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
KSHV	Kaposi's sarcoma-associated herpesvirus
ORF	open reading frame
PEC	peritoneal exudate cell
pfu	plaque-forming units

Introduction

The human lymphotropic γ-herpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV8), are closely associated with the development of several human cancers, particularly in immunosuppressed individuals such as AIDS patients [1–4]. Outstanding questions remain about the cellular and transcriptional basis of *in vivo* latency and tumor induction by these viruses, and the specific mechanisms by which the immune system controls latency and tumor development. In part, the incomplete nature of our understanding of these issues relates to the difficulties inherent in analyzing pathogenesis, tumor induction, and mechanisms of immunity in primate models. Fortunately, murine gamma-herpesvirus 68 (γHV68; also referred to as MHV68) appears closely related to the γ2-herpesviruses KSHV and herpesvirus saimiri (HVS), and serves as a viable model to understand general strategies involved in γ-herpesvirus pathogenesis.

This review focuses on recent advances in our understanding of the sites of γHV68 latency, the identification of candidate viral genes associated with latent infection, and the role of specific components of the host immune system in controlling γHV68 infection

γHV68 infection of mice as a model system

γHV68 was initially judged to be an alpha-herpesvirus since it appeared neurotropic and caused fatal encephalitis in neonatal mice [5–7]. Adult mice, however, showed no neurologic disease upon γHV68 infection, instead they showed a severe exudative pneumonia and hematogenous dissemination of γHV68 to multiple organs including the trigeminal ganglia [6,7]. γHV68 infects numerous organs but curiously, as discussed further below, the virus exhibits a pronounced tropism for vascular smooth muscle cells with infection leading in some cases to a dramatic large vessel arteritis. Another common feature is splenomegaly that is frequently observed during acute infection, and is associated with a two- to three-fold increase in the number of $CD4^+$ T cells, $CD8^+$ T cells and B cells in the spleen [8–11].

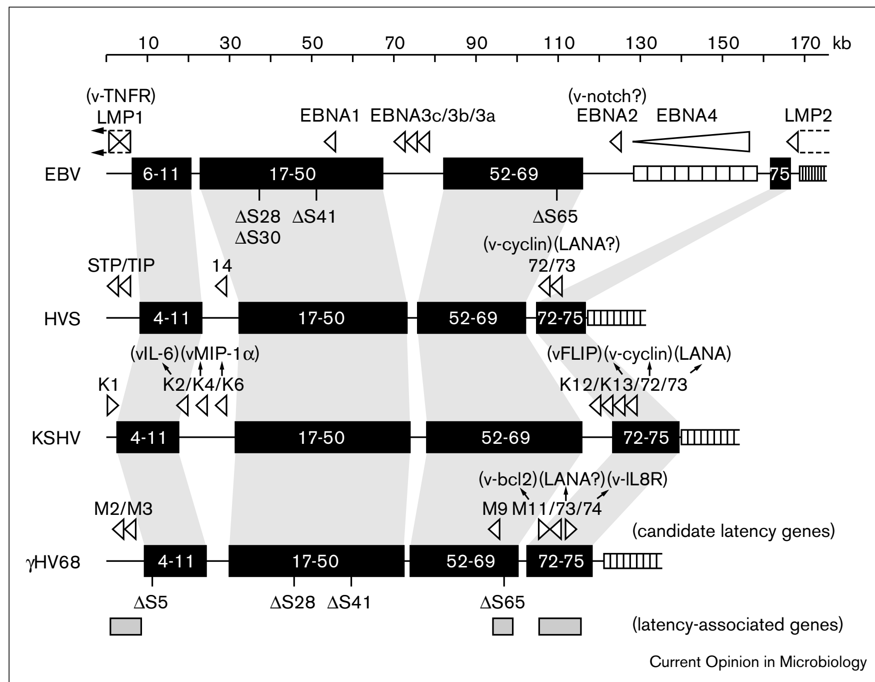
On the basis of organization of the viral genome, and limited sequence analysis, γHV68 was reclassified as a γ-herpesvirus [12,13]. This assignment was confirmed when the entire genome was sequenced and analyzed in 1997 [14]. Comparison of candidate genes encoded by γHV68 to the known γ-herpesviruses indicated that γHV68 is most closely related to the γ2-herpesviruses, HVS and KSHV, and is more distantly related to the γ1-herpesvirus, EBV [14] (Figure 1). Notably, all the γ-herpesviruses share blocks of conserved genes, which are interspersed with genes that appear to be largely virus specific (i.e. no significant homology to genes present in other γ-herpesviruses) (Figure 1). As discussed below, a number of these virus-specific genes may be involved in regulating viral latency.

Consistent with the classification of γHV68 as a γ-herpesvirus, an association of γHV68 with lymphoproliferative disease or high-grade lymphomas in γHV68-infected immunocompetent mice has been observed, and the frequency of disease was significantly enhanced by administration of the immunosuppressant cyclosporin A [15]. The latter is very consistent with the increased risk of developing KSHV and EBV associated malignancies upon immunosuppression, and emphasizes the importance of the host immune system in controlling chronic γ-herpesvirus infection.

Sites of γHV68 latency

Latency is the presence of the γHV68 genome in an infected cell without the presence of ongoing production of progeny virions. The latent γHV68 genome can be reactivated (i.e. emerge from latency), but little is known about how latent reservoirs are maintained or what signals trigger

Figure 1



Gamma-herpesvirus latency-associated and transforming genes. The map positions of viral genes implicated in latency and/or cellular transformation by the human and primate γ-herpesviruses EBV, KSHV and HVS are shown. The blocks of conserved viral genes present in the genomes of all, or most, γ-herpesviruses are indicated by black rectangles. Genes missing from these blocks are designated as ΔS below the black rectangles. In addition, the candidate γHV68 latency genes identified by RT-PCR analysis of latently infected PECs and splenocytes are shown. See [33••] and references therein for details of the identification and characterization of EBV, KSHV and HVS gene transcription in latently infected cells and associated tumors.

reactivation from latency. Chronic γHV68 infection differs from chronic infection with lymphocytic choriomeningitis virus (LCMV) or HIV in which continuous productive infection occurs. This fundamental difference very likely has a significant impact on mechanisms of immunity involved in controlling γ-herpesvirus infection; for example, antigens expressed in latent cells might stimulate an immune response. Intermittent reactivation from latency, perhaps at discreet anatomical sites, probably occurs [16••] providing a potential source of chronic stimulation with lytic viral antigens.

Detection and quantification of γHV68 latently infected cells

To date, γHV68 latency has primarily been assessed by determining the frequency of cells that reactivate γHV68 from latently infected tissue upon *ex vivo* culture (growth in tissue culture). A critical control for all reactivation analyses is a quantitative assessment of the level of preformed infectious virus present in the samples being analyzed, since this can dramatically alter the interpretation of the results obtained. Notably, latency and persistent productive infection can co-exist in the same animal, and, therefore, an assay is required to distinguish between the two types of infection. For example, B cell deficient mice have demonstrable latency in spleen and peritoneal exudate cells (PECs) [10,17••,18••], while persistent productive infection has been clearly demonstrated in lung [19••] and in the aortic wall ([20]; A Dal Canto, SH Speck, HW Virgin, unpublished data). Initial studies of γHV68 latency used plaque assays to monitor the presence of preformed infectious virus, while viral latency was assessed by plaque formation after 5–7 days in an infectious center assay [8,21–25]. The plaque assays and infectious center assay, however, have not been standardized to demonstrate that they have equivalent sensitivity. Thus the absence of detectable preformed infectious virus by plaque assay does not assure that the infectious centers observed solely reflect reactivation of latently infected cells. The limitation of plaque assays for detecting preformed infectious virus has been demonstrated for γHV68 [10,19••,26•], as well as murine cytomegalovirus (MCMV) [27–29].

Limiting dilution analysis provides a different assay for simultaneously quantifying both preformed infectious virus and cells that reactivate latent γHV68 [10,17••,18••]. To distinguish preformed infectious virus from latent virus, cells are mechanically disrupted (latent virus cannot reactivate from killed cells) and diluted in parallel with live cells (i.e. not mechanically disrupted) onto permissive monolayers, and the presence of infectious virus is detected by cytopathic effect on the monolayer over 2–4 weeks in culture. Importantly, controls have demonstrated that preformed infectious γHV68 is not inactivated during tissue preparation or by the mechanical disruption procedure employed [10,17••,18••].

Identification of cell types latently infected by γHV68

The fact that herpesviruses exhibit different genetic programs, depending on the cell type infected, raises the question 'What cell types harbor latent γHV68?' γHV68 can establish a latent or persistent infection in the cells of the spleen [10,22]. The pool of latently infected cells that reactivate γHV68 upon *ex vivo* culture expands over the

first two weeks of infection and then decreases in size over the ensuing several weeks [10,17••,21,25]. The mechanism(s) responsible for this decrease is not clear. In addition to the spleen, latent γHV68 can be detected in the bone marrow [17••], and the presence of latently infected cells in the lung has been postulated [19••]. Notably, resident peritoneal cells are to date the reservoir with the highest frequency of γHV68 latently infected cells [17••]; 9–10 days after mice were infected with HV68, ~1 in 100 peritoneal cells were shown to carry latent γHV68 [17••,18••].

B lymphocytes have been implicated as the major hematopoietic reservoir of persistent and/or latent virus in infected mouse spleens [22]. We have demonstrated, however, that γHV68 efficiently establishes a latent infection in B-cell-deficient mice (MuMT) [10], and independent analyses reported the persistence of viral DNA in the lungs of B-cell-deficient mice [19••,25]. These studies provided compelling evidence that mature B cells are not required for γHV68 latency, and raised the possibility that γHV68 might be able to establish latency in another cell type(s). We have fractionated PECs from infected C57BL/6 mice and demonstrated that macrophages are the predominant cells harboring the γHV68 genome, and that these viral genome positive macrophages can reactivate γHV68 indicating that they represent a bona fide latency reservoir [18••]. In addition, our analysis of latently infected PECs revealed that ~10% of the viral genome positive cells were B cells, consistent with the previous identification of B cells as a reservoir for latent γHV68 [22]. Further analysis of viral infection in the lungs has provided evidence that lung epithelial cells may also harbor latent γHV68 [19••]. Additional characterization of these lung epithelial cells that harbor viral genome will be required to demonstrate that they do not shed low levels of virus, and that γHV68 can reactivate from these cells. Notwithstanding the latter issues, it is clear that γHV68 is capable of establishing a latent infection in multiple cell types; the significance of specific γHV68 latency reservoirs remains to be elucidated.

Candidate γHV68 latency-associated genes

An understanding of the molecular biology of γHV68 latency is hindered by the lack of tissue culture cell lines that harbor latent γHV68. The availability of EBV, HVS and KSHV latently infected cell lines has greatly facilitated the identification of viral genes expressed during latency. Although a γHV68 infected B lymphoma line has been established (S11 cell line), this cell line exhibits high levels of productively infected cells (i.e. lytic infection) [30]. In the absence of a tissue culture model that exhibits tight viral latency, attempts to identify viral genes expressed in latently infected tissue have been carried out.

γHV68 encodes eight tRNA-like genes, which are clustered near the left end of the viral genome [14,31]. It is notable that the known γ-herpesviruses all encode small noncoding RNAs [32]. γHV68 viral t-RNA transcripts are abundantly expressed during lytic infection ([31]; K Weck, A Dal Canto, J Gould, HW Virgin, SH Speck, unpublished data). In addition, the continued presence, detected by *in situ* hybridization, of viral t-RNA positive cells for prolonged periods post-infection (up to 70 days post-infection [16••]) within the germinal centers of latently infected spleens has been reported [31] suggesting that the viral t-RNAs are also expressed during latent infection. Further analysis of a panel of 20 candidate γHV68-encoded genes using *in situ* hybridization of latently infected mouse spleens detected expression of the M3 open reading frame (ORF) (detected up to day 21 post-infection). The latter study is difficult to interpret since M3 is known to be abundantly expressed during the viral lytic cycle [33••,34•] and detection until day 21 might represent residual lytic gene expression in some population of splenocytes (which may not be productively infected). Notably, the M3 ORF has recently been shown to encode a secreted protein that is abundantly expressed in lytically infected fibroblasts [34•].

We have identified candidate γHV68 latency-associated genes by developing sensitive reverse transcriptase (RT)-PCR assays for a panel of known lytic genes and potential latency-associated genes [33••]. Candidate latency-associated genes were selected on the basis of known locations of latency- or tumor-associated genes encoded by the primate and human γ-herpesviruses (see Figure 1). With the exception of the region containing genes 72–74, which is moderately well conserved between HVS, KSHV and γHV68, it is apparent that the other candidate latency genes are virus-specific (i.e. encoded within the regions of the viral genomes that are not conserved among the γ-herpesviruses) (see Figure 1) [14].

To critically assess expression of particular candidate lytic and latency genes, nested-PCR assays were developed for 19 candidate genes and demonstrated to have approximately one-copy sensitivity [33••]. Because the presence of virus replication would obscure the ability to distinguish latency-associated transcripts from lytic-cycle-associated viral transcripts, cDNA was prepared from latently infected splenocytes and PECs isolated from MuMT mice, and was then exhaustively analyzed for the presence of known lytic transcripts. This analysis revealed little or no lytic gene expression; however, the presence of transcripts that amplified with primers to several candidate latency-associated genes were detected [33••]. Employing cDNA generated from RNA of latently infected PECs, transcripts were detected that amplified with primers to the M2, M11 (v-bcl-2 homolog), gene 73 (latency associated nuclear antigen homolog; LANA) and gene 74 (v-G protein coupled receptor homolog; v-GCR) regions of the viral genome in greater than 50% of PCR reactions. With cDNA prepared from RNA of latently infected spleen cells, transcripts were amplified with primers to the M2, M3, and M9 ORFs. Notably, primers within gene 72, which encodes the γHV68 cyclin D homolog (v-cyclin), only sporadically gave rise to a

product from latently infected PEC and spleenoctye cDNA. We have recently demonstrated that the γHV68 v-cyclin is an oncogene [35••], and thus is a strong candidate gene to be involved in the genesis of γHV68-associated lymphomas and lymphoproliferative disease. Because our analysis was restricted to screening viral gene expression from latently infected tissue harvested 42–46 days post-infection, it is possible that the v-cyclin is expressed at a different stage of γHV68 latency (e.g. during establishment or reactivation), or that our RT-PCR analysis was not sensitive enough to detect v-cyclin transcripts. It is noteworthy that the transcription pattern in latently infected PECs and splenocytes appeared distinct, suggesting perhaps the presence of different latency programs. This analysis represents the first step towards identifying viral genes expressed in latently infected tissue.

Immune control of γHV68 infection

Role of CD8$^+$ T cells

Several reports analyze the role of immunity following acute infection. Mice lacking lymphocytes (either SCID or RAG1$^{-/-}$ mice) die of acute γHV68 infection when infected with as little as 10 plaque-forming units (pfu) of virus ([36]; A Dal Canto, SH Speck, HW Virgin, unpublished data). Similarly, athymic mice or mice depleted of both CD4$^+$ and CD8$^+$ T cells succumb to γHV68 infection [9,26•,36]. CD8$^+$ T lymphocytes appear more important for controlling acute infection [9] than chronic infection. Thus, β2$^{-/-}$ mice (i.e. CD8$^+$ T cell deficient) remain persistently infected for weeks after primary infection [10,26•], and depletion of CD8$^+$ T cells in γHV68 infected normal mice results in the expansion of latently infected cells in the spleen [24,26•]. The latter studies, however, could be confused by the presence of ongoing virus replication and immune-mediated changes in reactivation efficiency. In BALB/c mice, γHV68 infection after CD8$^+$ T cell depletion can be lethal [9,26•], although this is not the fate of γHV68 infected C57BL/6 mice depleted of CD8$^+$ T cells [26•]. These studies demonstrate the inherent difficulties in interpreting experiments carried out on different genetic backgrounds.

Role of CD4$^+$ T cells

Although it has been reported that CD4$^+$ T cells are required for maintenance of an effective CD8 response over time [24], more recent data demonstrate that γHV68-specific CD8$^+$ T cells are maintained in CD4-deficient mice [37••]. Depletion of CD4$^+$ T cells appears to have little effect on clearance of acute virus replication from the lung and spleen [9], although the presence of CD4$^+$ T cells is required for the appearance of γHV68-induced splenomegaly [9,11,24]. Similar results were obtained upon infection of MHC class II knockout mice (CD4$^+$ T cell deficient) [24]. In B-cell-deficient mice (MuMT) depleted of CD8$^+$ T cells, however, acute virus replication in the lungs was controlled after intranasal (i.n.) inoculation [38••]. This study indicates that CD4$^+$ T cells can control acute virus replication when the need arises. CD4$^+$ T cell control of acute γHV68 infection is probably mediated by interferon-γ (IFNγ) [38••]. CD4$^+$ T cells are the principal splenic cell type that produces IFNγ in γHV68 infected mice [39•], and IFNγ depletion in conjunction with CD8$^+$ T cell depletion resulted in severe disease in B-cell-deficient mice [38••]. In MHC class II knockout mice approximately three weeks post-infection there is a reported increase in latently infected cells in the spleen and the presence of virus replication in the lung was noted [24]. These mice eventually succumb to viral infection. CD4$^+$ T cells are also apparently required for the significant hypergammaglobulinemia that occurs upon γHV68 infection [40•,41••]. Notably, γHV68 induction of serum IgG does not appear to involve stimulation of already existing memory B cells [41••]. Interestingly, infection of spleen cell cultures *in vitro* results in striking activation of B cells, although, unlike the situation *in vivo*, CD4$^+$ T cells are not required for this activation [41••].

Role of B cells

Infection of B-cell-deficient mice (MuMT), which lack mature B cells due to a disruption in the exon encoding the transmembrane domain of the immunoglobulin μ heavy chain [42], results in significant perturbations in acute and latent γHV68 infection. Acute virus replication in the spleen is not detected in MuMT mice after either i.n. or intraperitoneal (i.p.) infection [10,17••,25], although the virus replicates normally in the lungs of MuMT infected mice [25]. Although there is no detectable acute replication in the spleen, we have observed efficient establishment of latency in the spleens of MuMT mice [10,17••]. This suggests that acute virus replication in the spleen is not required for the establishment of latency in this organ, and might indicate seeding of latently infected cells to the spleen from another tissue (e.g. bone marrow) [17••,18••].

Initial analysis of viral latency in MuMT mice and normal control mice (C57BL/6) demonstrated an ~100-fold higher frequency of MuMT spleen cells reactivating γHV68 [10]. Subsequent analysis of this phenomenon revealed that both MuMT and C57BL/6 mice harbor very similar frequencies of γHV68 genome positive cells in the spleen and PECs [17••]. Early post-infection (10–12 days), the frequency of PECs or splenocytes reactivating γHV68 was nearly equivalent in cells harvested from infected MuMT and C57BL/6 mice [17••]. The frequency of MuMT PECs and splenocytes reactivating γHV68 remained fairly constant for >100 days post-infection, whereas the frequency of C57BL/6 PECs and splenocytes that reactivated γHV68 dropped sharply over the first 3–4 weeks post-infection [17••]. Thus, the higher frequency of cells reactivating γHV68 from MuMT mice compared with C57BL/6 control mice appears to reflect an enhanced reactivation efficiency and not an increase in the establishment of viral latency. It remains, however, to be determined whether the γHV68 genome positive cells that do not reactivate virus from C57BL/6 mice represent bona fide latently infected cells since one of the key criteria for latency is the capacity to

reactivate. Mixing latently infected MuMT PECs with latently infected C57BL/6 control PECs did not inhibit the enhanced γHV68 reactivation frequency of MuMT latently infected cells, largely ruling out the presence of an inhibitor of either viral reactivation or detection of reactivation (e.g. production of neutralizing antibody) in the C57BL/6 reactivation cultures [17••].

The analysis of latency in MuMT mice suggests a fundamental difference in γHV68 latency established in MuMT versus C57BL/6 mice, perhaps indicative of a disregulation in the ability to control γHV68 latency. Consistent with this hypothesis is the reported observation of continued virus production in the lungs of MuMT mice [19••]. Furthermore, the majority of infected MuMT mice die between day 100 and 200 post-infection, whereas no death was observed in γHV68 infected C57BL/6 control mice [17••]. Importantly, the analysis of viral latency in B-cell-deficient mice underscores the importance of quantifying the frequency of viral genome positive cells, the frequency of cells that reactivate virus, and the amount of preformed infectious virus present in samples.

Role of cytokines

Not surprisingly, the type I interferons are critical for controlling acute γHV68 infection, since mice lacking either the IFNα/β receptor or the transcription factor STAT-1 die very rapidly (3–4 days) after i.p. inoculation with 10^6 pfu of γHV68 [20]. IFNγ and interleukin (IL)6 are abundantly produced during acute infection [43], but neither IFNγ [20,21,23] or IL-6 [44] is required for recovery from acute γHV68 infection. One study, however, reported that approximately one third of $IFN\gamma^{-/-}$ mice infected with 10^6 pfu of γHV68 i.n. died during the acute phase of infection, and also reported significant mortality in inducible nitric oxide synthetase (iNOS) knockout mice [36], suggesting that iNOS may be an important mediator of IFNγ action. Lack of IL-6 was reported not to affect the level of latent virus, although the possible presence of preformed infectious virus was not ruled out [44].

IFNγ unresponsive mice developed two unique pathologies: a striking large vessel arteritis [20], and splenic fibrosis [20,23]. The latter which appears to be dependent on the presence of both $CD4^+$, and $CD8^+$ T cells [23]. The arteritis that occurs in IFNγ unresponsive mice is restricted to the large elastic arteries and involves ongoing virus replication in the media of these vessels (assessed by the presence of both viral nucleic acid and lytic viral antigens) ([20]; A Dal Canto, SH Speck, HW Virgin, unpublished data). Thus, acute γHV68 replication is cleared from the visceral organs of IFNγ unresponsive mice, whereas virus replication apparently cannot be controlled in the elastic arteries (unpublished data). Notably, we have also observed milder forms of γHV68 induced elastic vessel arteritis in infected B-cell-deficient mice (MuMT) and newborn 129 mice [20]. The latter result indicates that this pathology does not require ongoing immunocompromise, since by the time lesions are observed in 129 mice they should have a functionally intact immune system. The analysis of γHV68-induced vasculitis promises to reveal fundamentally important principles relevant to the question of how virus infection of the large elastic arteries is (or fails to be) controlled.

Conclusions

The analysis of γHV68 pathogenesis in mice provides a manipulable small animal model for addressing critical issues in establishing a chronic infection, and potentially the role of viral and host factors in the generation of tumors associated with γ-herpesvirus infection. The studies to date clearly demonstrate that γHV68 readily establishes a lifelong latent infection in mice. Notably, our analysis of long-term latency indicated that the frequency of viral genome positive cells remains fairly constant, suggesting that the latency reservoirs are stably maintained. Future studies will need to determine the relationship between viral genome positive cells, and those capable of reactivating virus. Identification of reagents that either inhibit or enhance virus reactivation would be very useful for addressing the latter issue. In addition, using targeted ablation of specific viral genes, those genes involved in establishment and maintenance of γHV68 latency, as well as those involved in either suppressing or inducing viral reactivation will be identified. The studies to date have demonstrated that host regulation of γHV68 infection is complex. Future studies will need to address the interplay between lymphocyte populations involved in regulating viral replication, latently infected cells, and viral reactivation. As further insights into the role of specific immune mechanisms in controlling γHV68 infection in mice are gained, it may be feasible to assess the general importance of these mechanisms in controlling EBV and KSHV infection in humans.

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