Early Establishment of γ-Herpesvirus Latency: Implications for Immune Control[1]

Emilio Flaño[2],[*], **Qingmei Jia**[†], **John Moore**[*], **David L. Woodland**[*], **Ren Sun**[†], and **Marcia A. Blackman**[3],[*]

[*]Trudeau Institute, Saranac Lake, NY 12983

[†]Department of Molecular and Medical Pharmacology, AIDS Institute, Jonsson Comprehensive Cancer Center, Dental Research Institute, and Molecular Biology Institute, University of California, Los Angeles, CA 90095

Abstract

The human γ-herpesviruses, EBV and Kaposi's sarcoma-associated herpesvirus, infect >90% of the population worldwide, and latent infection is associated with numerous malignancies. Rational vaccination and therapeutic strategies require an understanding of virus-host interactions during the initial asymptomatic infection. Primary EBV infection is associated with virus replication at epithelial sites and entry into the circulating B lymphocyte pool. The virus exploits the life cycle of the B cell and latency is maintained long term in resting memory B cells. In this study, using a murine γ-herpesvirus model, we demonstrate an early dominance of latent virus at the site of infection, with lung B cells harboring virus almost immediately after infection. These data reinforce the central role of the B cell not only in the later phase of infection, but early in the initial infection. Early inhibition of lytic replication does not impact the progression of the latent infection, and latency is established in lymphoid tissues following infection with a replication-deficient mutant virus. These data demonstrate that lytic viral replication is not a requirement for γ-herpesvirus latency in vivo and suggest that viral latency can be disseminated by cellular proliferation. These observations emphasize that prophylactic vaccination strategies must target latent γ-herpesvirus at the site of infection.

The oncogenic human γ-herpesviruses, EBV and Kaposi's sarcoma-associated herpesvirus (KSHV),[4] persist in the host because of their ability to establish and maintain latent infection using a complex strategy of immune evasion and immune exploitation. Because of their prevalence in the population and association with a wide variety of malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and Kaposi's sarcoma, prophylactic vaccines to prevent the establishment of γ-herpesvirus latency are

[3]Address correspondence and reprint requests to Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983. mblackman@trudeauinstitute.org..
[2]Current address: Center for Vaccines and Immunity, Columbus Children's Research Institute, Columbus, OH 43205.

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[4]Abbreviations used in this paper:

KSHV	Kaposi's sarcoma-associated herpesvirus
MHV-68	murine γ-herpesvirus 68
γHV68	γ-herpesvirus 68

eagerly sought. To design rational vaccine strategies, it is important to understand virushost interactions during the initial infection. However, events associated with primary human γ-herpesvirus infections are not well characterized because of the asymptomatic nature of the initial infection. The murine γ-herpesvirus, MHV-68 or γHV68, is a γ-herpesvirus closely related to EBV and KSHV (1,2) and provides an important in vivo experimental model for elucidating fundamental principals of γ-herpesvirus infection, immune mechanisms of control, and vaccination strategies.

The γ-herpesviruses are B lymphotrophic viruses that establish and maintain latent infection exploiting the host B lymphocyte life cycle (3–8). Analysis of the early events in EBV infection has been limited to in vitro studies or characterization of the infectious mononucleosis response, which is manifest well after viral colonization of B lymphocytes. According to the classic model, virus infection and proliferation in oropharyngeal epithelial cells leads to secondary latent infection of B lymphocytes (9). However, other models favor the B lymphocyte playing a central role in both primary and persistent infection (4,10,11). The current study exploits the mouse model to characterize the early stages of γHV68 infection of B lymphocytes. Following intranasal administration of low doses of virus to mice, γHV68 induces an acute respiratory infection, which is cleared, followed by the establishment of lifelong latency (12). We demonstrate, first, that γHV68 localizes in B lymphocytes as early as day 1 after infection and that latently infected cells constitute a large component of the viral reservoir in the lung during the acute infection. Second, we show that early inhibition of viral replication has no major kinetic or quantitative effect on the establishment of splenic latency. Third, using a replication-deficient virus, γHV68 ORF31STOP (13), we also demonstrate that γ-herpesviruses do not require viral replication for the establishment of latency in lymphoid organs after i.p., but not intranasal, infection. Altogether, the data indicate that B cells play a central role in the initial viral infection and that establishment of latency occurs early after infection and is not dependent on lytic viral replication. Furthermore, the data suggest that the dissemination of latency can be mediated by cellular proliferation. These insights into early events in the establishment and dissemination of latency have important implications for therapeutic and vaccination strategies against γ-herpesviruses.

Materials and Methods

Animal procedures and virus infection

γHV68, clone WUMS, was propagated and titered on monolayers of NIH-3T3 fibroblasts. A replication-deficient transgenic virus, γHV68 ORF31STOP, was generated using the γHV68 BAC system (13). C57BL/6J mice were purchased from Taconic Farms and housed under specific pathogen-free conditions in BL3 containment. The Institutional Animal Care and Use Committee at the Trudeau Institute approved all studies described here. Mice were anesthetized with 2,2,2-tribromoethanol and inoculated with 4×10^2–7.5×10^5 PFU of virus in PBS. To prevent viral replication, mice were i.p. inoculated daily beginning on day 3 following infection with 125 μg of cidofovir (kindly supplied by Gilead Sciences) diluted in PBS, which effectively inhibits lytic γHV68 replication in vivo (Refs. 14 and 15; our unpublished data).

Viral assays

Plaque assay—To determine the titer of infectious virus, lungs obtained at various times after infection were stored frozen and mechanically homogenized. The cells were broken by three quick successive cycles of freezethawing. The lytic virus concentration of the lung homogenates was determined in a standard plaque assay on NIH-3T3 fibroblasts. The next

day the monolayers were overlaid with carboxymethyl cellulose (Sigma-Aldrich). After 6 days of culture, plaques were quantitated after methanol fixation and Giemsa staining

Limiting dilution-nested PCR—The number of cells containing the γHV68 genome was determined by a combination of limiting dilution analysis and nested PCR (16). To isolate lung cells, mice were perfused with 50 ml of PBS and the lungs were treated with 5 mg/ml collagenase D (Roche) and 5 mM PBS/EDTA as described elsewhere (16). The total number of cells isolated per organ was determined and the cells were serially diluted in uninfected NIH-3T3 fibroblasts in 96-well plates, lysed, and DNA amplified by nested PCR as described previously (16,17) using primers specific for γHV68 *ORF50*. This procedure was able to consistently detect a single copy of the target sequence. Twelve replicates were assessed for each cell dilution, and linear regression analysis was performed to determine the reciprocal frequency (95% degree of confidence) of cells positive for γHV68 DNA. As controls of nested PCR, 10^4 NIH-3T3 cells/well with and without plasmid DNA containing the γHV68 *ORF50* gene were included in each 96-well plate.

Infectious center assay—To determine the number of latently and lytically γHV68-infected cells, we used a modification of the infective center assay as previously described (16). Single-cell suspensions from spleen and lung were obtained as described above. Duplicates of the cell sample were mechanically disrupted using a Minibeadbeater 3110BX (Biospec Products) in the presence of 0.5-mm zirconia/silica beads. This procedure involves cell membrane cracking rather than high shear (manufacturer specifications). The complete killing of the cells (>98%) without destruction of the cellular integrity was confirmed by propidium iodine staining and flow cytometry analysis. The intact (total infected cells) and disrupted (lytically infected cells) cell samples from each organ were plated in triplicate onto monolayers of NIH-3T3 cells in serial 10-fold dilutions in 12-well plates. The monolayers were overlaid and the plaques were quantitated as described above. The number of latently infected cells was calculated as the difference between total and lytically infected cells.

In vivo infections are nonsynchronous, and thus any cell committed to viral replication that has not yet produced infectious viral particles will score as latently infected in an infectious center assay. Therefore, a conventional infectious center assay underestimates the frequency of lytically infected cells. To overcome this limitation of the assay, we have designed a modified infectious center assay to determine the relative contribution of cells committed to the lytic cycle and of true latently infected cells to the total pool of infected cells. A single-cell suspension from lung obtained as described above was maintained at 37°C under continuous rotation in tissue culture medium to allow lytically infected cells to complete their lytic replication cycle in vitro. Cells were sampled at different intervals (0, 8, and 12 h), washed to eliminate viral particles in the supernatant, counted, and a duplicate of the samples was mechanically disrupted as described above. The intact (total infected cells) and disrupted (lytically infected cells) cell samples were plated in triplicate onto permissive monolayers as described above. This modification of the conventional infectious center assay will allow infected cells 12 h to complete the lytic cycle and form infectious particles in vitro. This will ensure that we are not erroneously scoring lytically infected cells as latently infected because they are in an early stage of lytic infection and fail to form plaques.

Cell purification

Lung B and stromal cells were purified on a FACSVantage SE/Diva sorter (BD Biosciences) as described elsewhere (16). Briefly, pooled lung cell suspensions from four mice obtained as described above were stained with Abs against CD11c (HL3), CD11b (M1/70), CD5 (53-7.3), and CD19 (1D3) (BD Pharmingen). B cells were sorted as CD19+ and negative for the other markers with a mean purity of 90%. A population of stromal cells (97% purity)

was sorted as negative for all of the markers used, therefore lacking macrophages, granulocytes, NK cells, T and B lymphocytes, and dendritic cells.

Gardella gel electrophoresis

The resolution of episomal and linear γHV68 genomes in lung B cells purified as described above was done by Gardella gel analysis (18,19). The lysis and separating gels were prepared as described previously (19). Purified B cells were resuspended in loading buffer containing 20% Ficoll (Sigma-Aldrich) and 0.01% bromphenol blue and then loaded onto the gels. The sample was overlaid with lysis buffer containing 5% Ficoll, 1% SDS, and 1 mg/ml self-digested Pronase (Calbiochem). The gels were run at 4°C at 40 V for 3 h and then at 160 V for 16 h. The gels were then sliced at 0.5-cm intervals, the agarose was digested, and the DNA was extracted using β agarase (Promega) and ethanol precipitation following the manufacturer's instructions. The presence of γHV68 DNA in the samples was determined by PCR in a 25-μl reaction containing primers (6.25 μmol each) specific for γHV68 (5′-GATGGAAACAGAAAACGAGCCC-3′ and 5′-TCGCTTGTTTCTGGGGAGGTTT-3′, product 425 bp), 1 U of TaKaRa Ex Taq (Takara Biomedicals), 2.5 μl of 10× Ex Taq buffer (Takara Biomedicals), and 3 μl of dNTP mixture (Takara Biomedicals). Amplification was for 45 cycles (94°C, 60 s; 67°C, 60 s; 72°C, 30 s), followed by a 7-min extension at 72°C. The γHV68-containing B cell line S11, which has been shown to harbor both episomal and linear γHV68 DNA (20), was used as a control for the Gardella gel analysis.

Results

Latently infected cells constitute a large fraction of the infected cell pool during acute primary infection

Intranasal infection with γHV68 results in a lytic infection in the lung, which is cleared by the host immune system. Despite this, the virus sneaks through, and latency is established. The numbers of latently infected cells peaks at day 14 in the spleen and latency is localized primarily in activated, germinal center B cells, as well as macrophages and dendritic cells. Over the long term, the virus persists in a latent state predominantly in resting memory B cells in the spleen, respiratory tract, and other anatomical sites (6,16,20). We had previously shown that there were high frequencies of latently infected B cells in the lung at 14 days after infection, but it was not clear whether latency was initiated in the spleen and latently infected cells migrated back to the lung or whether lung B cells were latently infected in situ. To determine when latency in the lung was first established, we monitored lytic and latent virus in the lung at various time points after intranasal infection. As expected, the titer of lytic (infectious) viral particles in lung tissue homogenates as determined by a standard plaque assay peaked between days 5 and 9 and was cleared by day 14 (Fig. 1*a*). However, determination of the number of cells harboring viral genome using a limiting dilution-nested PCR assay showed a different pattern, in that the number of cells carrying γ-herpesvirus DNA remained stable even after infectious cell-free virus could no longer be detected (Fig. 1*b*). In addition, the number of infected cells was 10- to 100-fold higher than the absolute number of plaques produced by lytic virus. These data suggested that many of the cells harboring viral genome in the lung during the first 2 wk of infection are latently infected, consistent with the possibility that lytic and latent infection coexist in the respiratory tract from the beginning of the infection.

To quantitate productively infected cells, we used an infective center assay (16). This assay detects virally infected cells by their ability to form plaques on a susceptible monolayer following in vitro incubation. Lytically infected cells form plaques directly, whereas latently infected cells only form plaques in vitro following spontaneous reactivation. Although the

reactivation assay (Fig. 1*c*) is less sensitive than the PCR assay (Fig. 1*b*), the total numbers of γHV68-infected cells detected by both assays showed a similar pattern of infection, in that the infected cells remained high at a time when lytic virus was being cleared. Since reactivation of latent virus requires the presence of live cells, cells carrying latent virus are determined by subtracting the plaques determined by analysis of killed cells (lytic) from the total plaques (lytic plus latent). The data (Fig. 1*d*) show that most of the γ-herpesvirus-reactivating cells scored as latently infected in this assay.

Although the data suggest that a high proportion of the cells harbor latent virus, it is possible that the in vitro reactivation assay overestimated the number of latently infected cells. In vivo infections are nonsynchronous and thus any γHV68-infected cell committed to viral replication but not yet producing infectious viral particles will score as latently infected in an infectious center assay. Therefore, to assess the contribution of cells in the early stages of lytic replication to the latent pool, we modified the infectious center assay to allow infected cells to complete their lytic cycle in vitro. Thus, a lung cell suspension from acutely infected mice was sampled at different intervals (0, 8, and 12 h) during in vitro incubation and assayed in the infectious center assay. If the cells assessed by this assay to be latent were really in the early stages of lytic infection, the apparent latent titers would be expected to fall with increasing in vitro incubation, and the lytic titers would increase. However, the data show that substantial numbers of latently infected cells were detected at all three time points analyzed (Fig. 2). Altogether, our results support the idea that viral latency constitutes a large component of the acute primary γHV68 infection.

Latency is established in lung B cells immediately after respiratory infection

The previous data indicate the presence of latently infected cells in the lung using viral quantification assays. We also used a molecular approach to identify latent and replicative forms of viral DNA early during lytic infection. Latent γ-herpesvirus DNA is episomal (covalently, closed circular) whereas lytic, replicating virus is in a linear conformation (20,21). Gardella gels differentiate latent episomal from lytic linear viral DNA on the basis of electrophoretic mobility (18). Therefore, to demonstrate the presence of latent virus at early time points, we determined the conformational state of the viral DNA harbored in sort-purified lung B cells at day 3 following intranasal infection by Gardella gel analysis. The migration properties of linear and circular DNA were confirmed by analysis of DNA from S11 cells, a B cell line known to harbor both lytic and latent virus (20,22). The data show that B cells isolated from the lung 3 days after γHV68 infection harbor both circular and linear viral genomes (Fig. 3*a*). Episomal DNA indicates the presence of latent virus. The presence of both circular and linear DNA in the purified B cells is consistent with the pattern of DNA observed following in vitro infection of B cells with EBV (23). Cells initially contained linear genomes, some of which migrated to the nucleus. Circular DNA in that synchronous infection was first detected by 20 h after infection, but only a small amount of the total EBV DNA within infected cells assumed a circular conformation. The possibility that the coexistence of circular and linear γHV68 DNA in lung B cells in our experiments reflects the presence of replicative intermediates of the lytic infection which occurs by a rolling circle mechanism, rather than latent virus, is unlikely, as the replicative θ form of the virus would be a very minor component of virus in a cell that is undergoing lytic replication. The failure to detect circular DNA in cells undergoing lytic infection was confirmed by Gardella gel analysis of DNA from lytically infected 3T3 cells (Fig. 3). The Gardella gel analysis, taken along with the viral assays, strongly supports the interpretation that latency is established early during the lytic infection.

Next, we kinetically analyzed γHV68 infection in FACS-purified lung B lymphocytes and stromal cells (nonlymphoid and non-myeloid cells). The data show that the virus could be detected in both cell subsets as early as day 1 after infection (Fig. 3*b*). These results,

demonstrating that B lymphocytes harbor γHV68 almost immediately after respiratory infection and that latency is established as early as 3 days after infection, support the hypothesis that viral infection of B lymphocytes is a concurrent event with the ongoing lytic infection of the mucosal epithelium. The continued high levels of virus in the stromal cells after clearance of infectious virus at day 11 (Fig. 1*a*) may be partly attributable to latency in the epithelial cells contained within the stromal cells (20) or attributable to the persistence of viral nucleic acid after the elimination of virally infected cells, as described previously (24–26).

γ-Herpesvirus latency can be established in the absence of viral replication

The finding that γ-herpesvirus latency is established in lung B cells very early after mucosal infection prompted us to assess the relationship between lytic and latent infection. Mice were infected with γHV68 and then, 3 days later, were treated daily with cidofovir, which has been shown to halt ongoing γHV68 replication in vitro and vivo (14,15,27,28). By day 3, latent infection has already been initiated in lung (Fig. 3). Thus, if the latency pool expands after the initiation of the cidofovir treatment, it would indicate that this process is independent of viral replication. Cidofovir-treated and control mice were then analyzed on day 14, the peak of viral latency in the spleen (29). The data show that the number of latently infected cells in both the lung and spleen of cidofovir-treated mice are not significantly different from the control mice (Fig. 4). Successful inhibition of γHV68 replication in the lungs was confirmed by analysis of lytic virus in the drug-treated mice on day 7, at the peak of acute infection. The cidofovir-treated animals showed a 10,000-fold reduction in the number of infectious viral particles per lung compared with nontreated controls (Fig. 4*c*). These results suggest that once the primary infection is initiated, the establishment of latency can proceed indepen-dently of the lytic infection by cellular proliferation of the latently infected cells.

To formally determine whether the establishment of latency absolutely depends on lytic viral replication, we infected mice using a replication-deficient γHV68 virus (γHV68 ORF31STOP). This virus was generated by inserting a stop codon to disrupt expression of open reading frame 31 (13). Open reading frame 31 is conserved among the β- and γ-herpesviruses, does not have a mammalian homologue, and is of unknown function. Importantly, disruption of ORF31 expression renders the virus completely incapable of in vitro replication and the virus can only be grown by *trans* rescue with either γHV68 or KSHV ORF31 (13). We inoculated mice both intranasally and i.p. with γHV68 ORF31STOP and wild-type virus and monitored splenic latency 14 and 30 days later (Fig. 5). After i.p. inoculation of the replication-deficient virus, latency was established in spleen cells, albeit at lower levels than after inoculation with wild-type γHV68. At 14 days after infection, the normal peak of latency, the reciprocal frequency of cells harboring viral genome after i.p. infection with ORF31STOP was 1 in 5800 cells compared with the frequency after i.p. infection with the wild-type virus of 1 in 177 cells. As expected, the latency frequency of wild-type virus declined to 1 in 1897 at 30 days after infection, whereas the frequency of cells latently infected with the replication-deficient virus remained relatively constant. The recombinant virus was constructed with a *Bam*HI restriction site to differentiate the actual transgenic virus from wild-type virus (13). Restriction enzyme analysis of PCR-amplified viral DNA extracted from spleen cells isolated from mice infected i.p. with γHV68 ORF31STOP confirm that the virus harbored by the latently infected cells at both 14 and 30 days after i. p. infection is the replication-deficient mutant virus (Fig. 5*c*). These data are the first conclusive demonstration that the establishment of γ-herpesvirus latency in vivo is independent of lytic viral replication.

In contrast to the results with i.p. infection, intranasal inoculation with a comparable viral dose of γHV68 ORF31STOP was not capable of establishing latent infection at either 14 or

30 days after infection (Fig. 5). That the ability of a replication-defective virus to establish latency is dependent on the site of inoculation supports the possibility that although lytic replication is not required for the establishment of latency per se, it plays another as yet undefined essential role, such as facilitating viral access to its host target cells. Altogether, the data indicate that lytic viral replication is not a requirement for γ-herpesvirus latency once the virus has penetrated the mucosal barrier and that latency can be disseminated by cellular proliferation.

Discussion

In the current study, we have examined the early events following mucosal infection of mice with γHV68. The data show that latent infection can be established very early during the acute response and that B lymphocytes in the lung harbor latent virus as early as 3 days after infection. Remarkably, not only can latency proceed after inhibition of the lytic cycle early after infection, but latency can also be established in the complete absence of lytic replication following in vivo infection with a replication-deficient virus. On the basis of these data, we propose a model of γ-herpesvirus infection (Fig. 6) in which lytically and latently infected cells coexist at the mucosal site of infection, and in which latently infected cells constitute a major component of the early infection at the entry site. These observations provide new insight into early γ-herpesvirus infection and have important implications for understanding immune control of the virus and for developing prophylactic vaccination strategies.

These studies enhance our understanding of the relationship between lytic and latent infection. A direct correlation between lytic virus and the latent load has been shown for CMV, a β-herpesvirus (30), but not HSV, an α-herpesvirus (31). Recent data for γHV68 suggested that establishment of γ-herpesvirus latency was similarly independent of the infectious dose beyond the level required to infect 100% of the mice (32). Relative independence of latency on lytic load was also demonstrated by studies of a mutant γHV68, lacking thymidine kinase. Despite severe attenuation of lytic cycle replication in vivo, the virus still established latency (33). The current study provides the first demonstration that γ-herpesvirus latency can be established in vivo after infection with a completely replication-deficient virus.

The ability to establish latency in the absence of lytic infection has relevance for understanding the mechanisms by which latency is disseminated throughout the host. A common characteristic of the γ-herpesviruses is that they exploit the host B lymphocyte life cycle to establish and maintain long-term latency (3–8). The finding that γ-herpesvirus latency is established in lung B cells as early as 3 days after respiratory infection reinforce the central role of the B cell not only in the later phase of infection, but early in the initial infection. Following the early infection of B cells, amplification of the infected pool can apparently occur by cellular proliferation rather than lytic viral spread and subsequent latent infection, thus avoiding immune detection. The observation that establishment of latency is independent of lytic virus suggests that the spread of latency is accomplished by cellular proliferation. That latency can be established by migration and proliferation of latently infected B cells has been reported (20). More recently, analysis of a gp150-deficient virus led to the conclusion that in vivo host colonization is independent of virion release, suggesting that cell-cell virus spread and cellular proliferation of latently infected cells are essential for the establishment of latency (34). Moreover, our results blocking viral replication with the drug cidofovir also demonstrate that once latent infection has been initiated, the lack of viral replication does not impact the dissemination of latency. In agreement with this result, it was previously shown that establishment of latency was delayed, but not prevented, after pretreatment with acyclovir (35).

Although B cells are the major target of γ-herpesvirus latency (9), other cell subsets have been identified as γHV68 reservoirs in various anatomical sites, including the lung (16,20,36,37). In the current manuscript, we have focused on early B cell latency. However, as part of a separate study, we have evidence to suggest that lung dendritic cells are also an early reservoir of latency (E. Flaño and M. A. Blackman, unpublished observations). In addition, the persistence of viral genome in lung stromal cells after the clearance of lytic virus in this report (Fig. 3b) raises the possibility that latency is established in lung epithelial cells within 2 wk of intranasal infection. We have not yet examined macrophages as an early reservoir of latency. Taken together, the data suggest that latency is established early in multiple cellular reservoirs in the lung. An important issue for future investigation is the contribution of each of these early reservoirs to the spread and maintenance of latency in the host.

If latency can be effectively established after infection with a replication-deficient virus, is the only role for lytic replication to allow the virus to be efficiently transmitted to another host? The finding that the replication-deficient virus only established latency after i.p. infection indicates that lytic replication also plays an essential role in the establishment of latency when the epithelial barrier is intact. One likely possibility is that viral replication is essential for the virus to cross the epithelial barrier and gain access to the leukocyte pool. An alternative possibility is that lytic replication is necessary to induce an inflammatory response in the lung, so that a sufficient pool of (activated?) B cells is available in situ for efficient latent infection. Further experimentation is necessary to distinguish these possibilities.

The ability to establish latent infection in vivo in the absence of lytic replication also provides insight into mechanisms of immune evasion. Our data raise the possibility that the lytic infection may play an important role in the establishment of latency by eliciting strong immunity and thus diverting the host immune response away from the latent infection. This possibility is supported by the observation that the early immune response to EBV is largely directed toward lytic viral epitopes, and T cells specific for latent epitopes are infrequent or absent during infectious mononucleosis (38,39). In the γHV68 system, T cells responding to the only known latent epitope are not detectable in the lung, mediastinal lymph node, or spleen until 19 days after infection (40). Herpesviruses establish a delicate equilibrium with the host: they depend on immune control for their host's survival (and hence their own), yet must impair immunity to avoid eradication (41). The demonstration of an early latent infection in the apparent absence of a T cell response directed at latent Ags supports the possibility that γ-herpesviruses accomplish this equilibrium in part by directing most of the immune resources toward controlling an ongoing lytic infection while allowing the establishment of lifelong latency. Viruses have evolved a number of strategies to evade immune responses (42). In this sense, γ-herpesviruses are highly adapted to counterattack host control interfering with Ag presentation, cell cycle and survival as well as chemokine and cytokine activity among other strategies (43,44). In addition, they shut down viral genes and establish lifelong latency, exploiting host mechanisms of cellular homeostasis (9,45). In this study, we propose another immune evasion mechanism, that the strongly immunogenic lytic phase of the infection serves to distract the immune system and allows the latent virus to establish a foothold. Confirmation of this hypothesis awaits detailed characterization of the immune response elicited by the replication-deficient ORF31STOP virus.

Our findings have important implications for the design of vaccine strategies against γ-herpesviruses. γHV68 provides an experimental model in which fundamental principles of vaccination for the γ-herpesviruses can be tested. A common approach has been to target early lytic replication based on the general notion that the temporal lag between the lytic and latent stages of infection would allow successful interference with the establishment of

latency. Early results with vaccination protocols that targeted the lytic cycle were shown to reduce the overall viral load in the lung and the early peak of latency in the spleen, but had no impact on long-term latency (46–49). The failure of this approach can be explained by our current finding that latency is established early after infection at the mucosal site of entry and is not dependent on sustained viral replication. Thus, effective γ-herpesvirus vaccination strategies must target not only infectious virus, but also lytically and latently infected cells in the oral mucosa and respiratory tract. Importantly, the ability of a replication-deficient virus to establish latency after i.p. inoculation points out the risks associated with attenuated vaccine strains and emphasizes the importance of epitope-based vaccines. In conclusion, analysis of the early events in γHV68 infection in an in vivo model has enhanced our understanding of mechanisms for the establishment and dissemination of latency in an immunocompetent host. The γHV68 system provides a valuable in vivo experimental model for testing "proof of principal" vaccination strategies.

Acknowledgments

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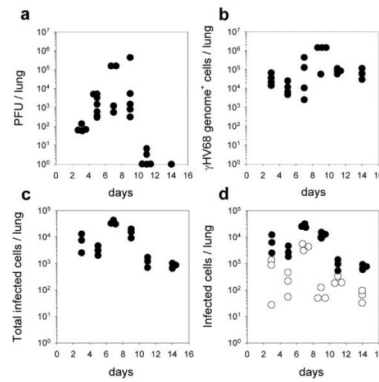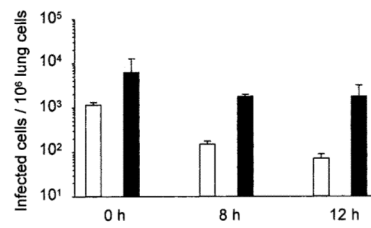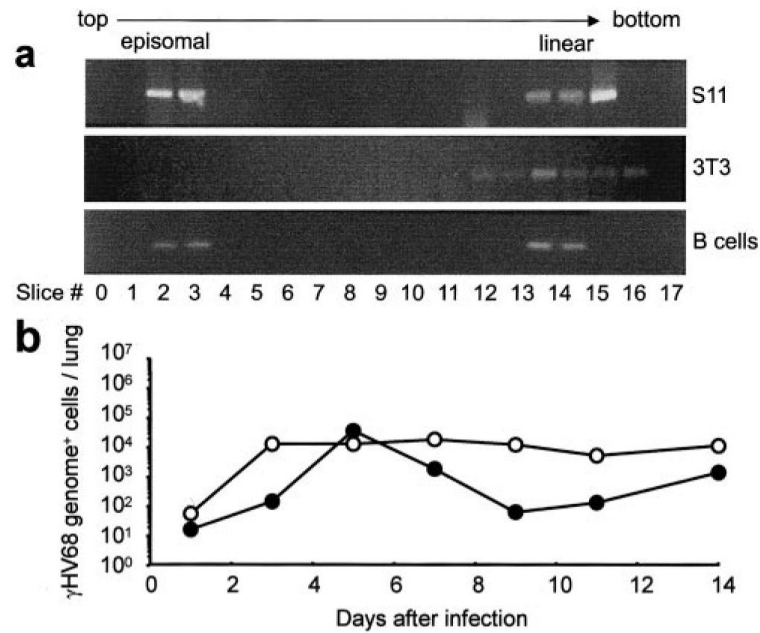
FIGURE 1.

γ-Herpesvirus latently infected cells constitute a large fraction of the cellular reservoir during primary lung infection. *a*, Preformed virus peaks between days 7 and 9 and is not detected by day 14. Free infectious virus was determined by plaque assay of lung homogenates. *b*, The number of cells carrying γHV68 DNA remained stable 2 wk after infection. Cells harboring viral genome were determined by a limiting dilution-nested PCR assay. *c*, The total number of infected cells remained stable at day 14. An infectious center assay was used to identify infected cells based on the ability of the infected cells to reactivate in vitro and to form plaques on a susceptible monolayer. *d*, The cells harboring latent virus constitute a large fraction of the infected cells during primary respiratory infection. The number of lytically infected cells (○) was determined by analysis of a suspension of killed lung cells in an infectious center assay The number of latently infected cells (●) was determined by subtracting the number of lytically infected cells from the absolute number of infected cells. Each symbol represents analysis of an individual animal (*n* = 3– 6).
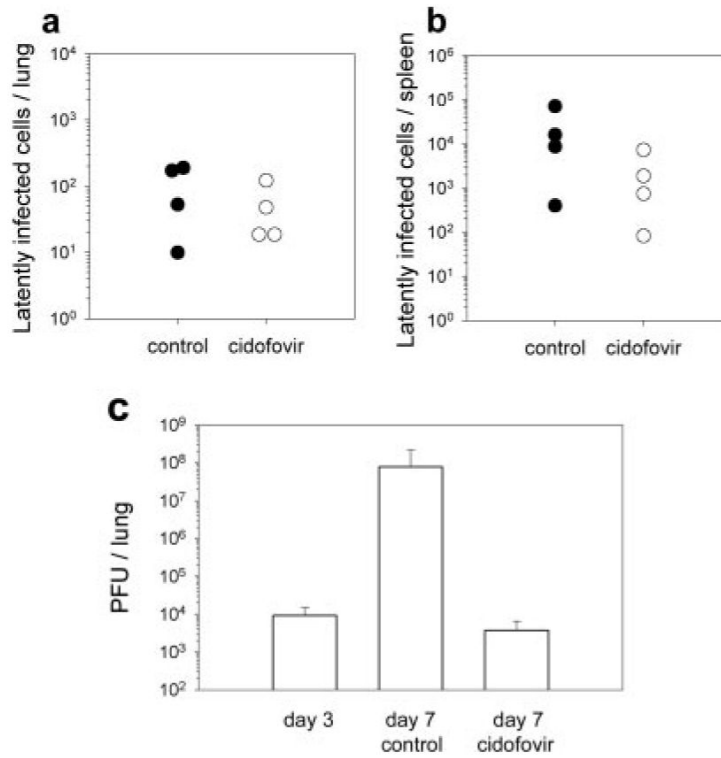
FIGURE 2.
The number of γHV68 latently infected cells in the lung does not decrease after in vitro incubation. A single-cell suspension from acutely infected lungs was incubated at 37°C to allow lytically infected cells to complete the viral replication cycle. Cells were sampled at 0, 8, and 12 h and processed as described in *Materials and Methods*. The number of lytically (○) and latently (●) infected cells was determined using an infectious center assay. Lung cells were pooled from six lungs at day 7 after infection. Error bars represent SD.

FIGURE 3.
γHV68 establishes latency in lung B cells very early following respiratory infection. *a*, Lung B cells contain both latent (episomal) and lytic (linear) virus at day 3 after infection. Gardella gel analysis of DNA from S11 cells, a γHV68 latently infected B cell tumor line that has been shown to contain both episomal and linear genomes (20) (*top panel*), acutely infected 3T3 fibroblasts (*middle panel*) and of FACS-sorted CD19$^+$ B cells from lungs 3 days after γHV68 infection (*bottom panel*). The gel photograph shows PCR for viral DNA of sequential individual gel slices, starting from the *top* of the gel (*left* to *right*). The slower migrating episomal DNA indicates latent viral genome and the faster migrating linear DNA represents lytic viral genome. *b*, γHV68 can be detected both in stromal and B cells as early as day 1 after respiratory infection. The numbers of FACS-purified lung stromal cells (CD19$^-$CD11c$^-$CD11b$^-$CD5$^-$) (○) and lung CD19$^+$ B cells (●) harboring viral genome at indicated times after intranasal infection were determined by limiting dilution-nested PCR.

FIGURE 4.
Inhibition of viral replication once early latency has been established has no impact on the latent viral load. Mice were i.p. inoculated daily with cidofovir (○) or PBS (●) starting 3 days after γHV68 infection. Fourteen days after infection, latent viral load was assayed in lung (*a*) and spleen (*b*). Each symbol represents analysis of an individual animal (*n* = 4). The lack of significant differences was determined by Student's *t* test, lung *p* = 0.17, spleen *p* = 0.31. *c*, Plaque assay showing the number of infectious viral particles in cidofovir-treated and nontreated mice at day 7 after infection (day 4 of cidofovir treatment) and at the initiation of the treatment. Each bar represents the mean value of four to five individual mice analyzed, and error bars represent SD.
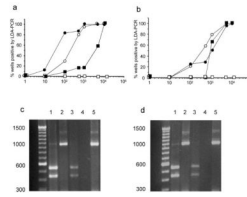
FIGURE 5.
A replication-defective γ-herpesvirus is capable of establishing splenic latency. The percentage of spleen cells harboring viral genome after intranasal (○, □) and i.p. (●, ■) infection with wild-type γHV68 (○, ● or γHV68 ORF31STOP (□, ■) was assessed by limiting dilution assay (LDA)-PCR assay 14 days (*a*) and 30 days (*b*) after infection. *Bam*HI restriction enzyme analysis of PCR-amplified γHV68 DNA from 14 days (*c*) and 30 days (*d*) after infection. *Lane 1*, Positive control, DNA extracted from BHK-21 cells transfected with 31STOP Bac plasmid. *Lane 2*, DNA extracted from spleens of mice intranasally infected with 400 PFU of wild-type γHV68. *Lane 3*,DNA extracted from spleens of mice i.p. infected with 7×10^5 PFU of ORF31STOP γHV68. *Lane 4*, DNA extracted from spleens of mice intranasally infected with $\sim 3 \times 10^5$ PFU of ORF31STOP γHV68. *Lane 5*,DNA extracted from spleens of mice i.p. infected with 10^6 PFU of wild-type γHV68.
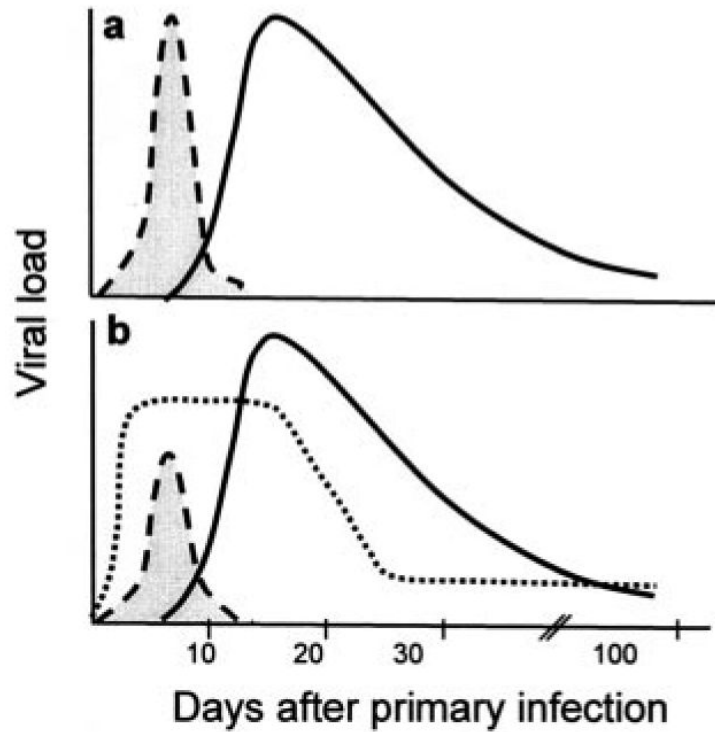
FIGURE 6.
Kinetics of γHV68 primary infection. *a*, Classic model of infection. Following intranasal inoculation, there is a sharp rise in lytic virus, which is cleared by host immune mechanisms. Latent virus is first detected at ~6 days after infection in the spleen and draining lymph nodes, peaks at approximately day 14, and declines to a low, stable level. *b*, New model of infection. Latent virus dominates the acute phase of infection and latently infected B cells are detectable in the lung as early as day 3 after infection. Latency can be established in the absence of lytic replication. Lytic virus in the lung is indicated by the dashed line (shaded peak), latent virus in the spleen is indicated by the solid line, and latent virus in the lung is indicated by the dotted line.