Differential γ-Herpesvirus Distribution in Distinct Anatomical Locations and Cell Subsets During Persistent Infection in Mice[1]

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Murine γ-herpesvirus 68 (MHV-68) provides an important experimental model for analyzing γ-herpesvirus latent infection. After intranasal infection with MHV-68, we analyzed the distribution of the virus in different anatomical locations and purified populations of cells. Our data show that long-term latency is maintained in a variety of anatomical locations and cell populations with different frequencies. Importantly, we demonstrate that although latency in the lung is established in a variety of cell subsets, long-term latency in the lung is only maintained in B cells. In contrast, splenic latency is maintained in macrophages and dendritic cells, as well as in B cells. In blood, isotype-switched B cells constitute the major viral reservoir. These results show that the cell subsets in which latency is established vary within different anatomical sites. Finally, we demonstrate that long-term latency is accompanied by a low level of infectious virus in lung and spleen. These data have important implications for understanding the establishment and maintenance of latency by γ_2-herpesviruses. ***The Journal of Immunology*****, 2003, 170: 3828–3834.**

The γ-herpesviruses are oncogenic viruses associated with a number of malignancies. After clearance of the initial acute infection by the immune system, they establish lifelong latency in asymptomatic carriers. This persistent infection is the result of delicate balance between immune control, viral latency, viral reactivation, and persistent replication. Only a minor proportion of the infected hosts eventually develop malignancies due to either immune breakdown or tumor cell escape as a consequence of phenotypic changes. The γ-herpesviruses have developed multiple evolutionary mechanisms to evade immune surveillance, including the capacity to establish latency at very low frequencies in specific cell types. The human γ-herpesviruses, EBV and Kaposi's sarcoma-associated herpesvirus (KSHV),[3] establish latent infection in B lymphocytes (1, 2) and persistently replicate in different anatomical locations (1, 3–8). In addition, KSHV establishes persistent infection in cell types other than B cells, including epithelial cells, dendritic cells, and monocyte/macrophages (reviewed in Ref. 2). Because of the generally asymptomatic characteristics of the initial infection in man, the establishment of EBV and KSHV latency during the early stages of infection cannot be directly assessed in vivo. The maintenance of EBV long-term latency has been extensively analyzed in tonsillar samples from asymptomatic carriers (reviewed in Ref. 9), whereas little is known about the maintenance of KSHV latency. To better understand the role of the immune system in controlling latency and persistent replication, it is necessary to fully characterize the viral reservoirs during long-term latent infection.

Murine γ-herpesvirus 68 (MHV-68), a γ_2-herpesvirus, is structurally and biologically similar to its human counterparts and provides an important experimental in vivo model of γ-herpesvirus infection in the natural host (10–14). Intranasal infection of mice with MHV-68 causes an acute respiratory infection that is rapidly resolved, followed by the establishment of splenic latency, which peaks at 14 days after infection (15). Analogous to EBV infection, B cells are a principal reservoir of latent MHV-68 (16–18). MHV-68 exploits the B cell biology to establish and maintain latency in splenic germinal center and memory B cells (18, 19). However, latency is also established in splenic macrophages and dendritic cells (18) and in peritoneal B cells and macrophages (20).

The current experiments were initiated to identify the predominant cellular and anatomical reservoirs of persistent MHV-68 other than splenic B cells during long-term infection of immunocompetent mice. Characterization of the reservoirs for this class of viruses is not fully defined and is essential for understanding the balance between latent infection and immune control. The data show that latent infection is differentially established and maintained in diverse anatomical locations and cell subsets, with lung and spleen being the major organ reservoirs during long-term latency. Low levels of infectious virus are also observed in lung and spleen cells during long-term latency in immunocompetent mice.

Materials and Methods

Virus and mice

MHV-68, clone WUMS (21), was propagated and titered on monolayers of NIH-3T3 fibroblasts (ATCC CRL1568). C57BL/6J mice were purchased from Taconic Farms (Germantown, NY) and housed 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 intranasally with 400 PFU of MHV-68 in 30 μl of PBS.

Cell purification

Isolation of all cell populations was done using a FACSVantage SE/Diva sorter (BD Biosciences, Mountain View, CA) and Abs were obtained from BD PharMingen (San Diego, CA), except where indicated. Pooled organs from 3 to 10 mice were used in all experiments and erythrocytes were lysed in hemolytic Gey's solution.

Splenic macrophages and dendritic cells were isolated as described elsewhere (18), with minor modifications. Spleens were treated with collagenase D (5 mg/ml; Roche, Basel, Switzerland) for 45 min. to obtain a single-cell suspension. Cells were next incubated with 5 mM PBS/EDTA for 10 min at room temperature to disrupt multicellular complexes. B and T cells were depleted using a mixture of anti-B220 and anti-Thy-1.2 cell culture

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Received for publication October 30, 2002. Accepted for publication February 6, 2003.

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[1] This work was supported by Grant AI42927 from the National Institutes of Health (to M.A.B.) and the Trudeau Institute.

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[3] Abbreviations used in this paper: KSHV, Kaposi's sarcoma-associated herpesvirus; MHV-68, murine γ-herpesvirus 68; LDA, limiting dilution assay; v, viral.

0022-1767/03/$02.00

supernatants and magnetic beads (Dynal Biotech, Great Neck, NY). The remaining cells were Fc blocked and stained with fluorochrome-conjugated Abs specific for CD11c (HL3), CD11b (M1/70), $\alpha\beta$TCR (H57-597; Caltag Laboratories, Burlingame, CA), and B220 (RA3-6B2). Macrophages were sorted as $CD11b^+$ and negative for other markers; dendritic cells were sorted as $CD11c^+$ $CD11b^{+\text{ or }-}$ and negative for other markers.

To isolate lung cells, mice were perfused with 50 ml of PBS. Lungs were injected with collagenase D, minced, and incubated for 45 min at 37°C. At 10-min intervals during the incubation, the tissue was mechanically disrupted with a 3-ml syringe and the final single-cell suspension was obtained using a cell strainer. The cells were incubated with PBS/EDTA as described for the spleen. Next, cells were incubated with Fc-block and then stained with Abs against CD11c, CD11b, CD5 (53-7.3), and CD19 (1D3). B cells were sorted as $CD19^+$ and negative for the other markers. Macrophages were sorted as $CD11b^+$ and negative for the other markers. Dendritic cells were sorted as $CD11c^+$ $CD11b^{+\text{ or }-}$ and negative for the other markers. A population of "null" cells was sorted as negative for all of the markers used; therefore, lacking most macrophages, T, B, and dendritic cells.

To analyze peripheral blood cells, pooled heparinized blood was incubated with Fc-block and stained with Abs against IgD (11-26c.2a), IgM (R6-60.2), CD5, cell surface Igs indicative of isotype-switching (IgA (C10-1), IgG_1 (A85-1), IgG_{2ab} (R2-40), IgG_3 (R40–82), and CD19. CD5-positive cells were gated out to avoid T and B-1 cell contamination. Naive B cells were sorted as $CD19^+IgD/M^+$ $(IgAIgG_1IgG_{2ab}IgG_3)^-$ and isotype-switched B cells as $CD19^+(IgAIgG_1IgG_{2ab}IgG_3)^+IgD/M^-$.

Ex vivo assay for lytic virus

To detect the presence of infectious virus in cell samples during long-term infection, we modified an infective center assay (18). Single-cell suspensions from spleen, lung, and PBLs obtained as described above were mechanically disrupted in DMEM in the presence of 0.5-mm zirconia/silica beads (17). The complete killing of the cells after the bead beater procedure was ensured by propidium iodide staining and flow cytometry analysis. The total yield of disrupted cells from each whole organ or blood sample was plated onto monolayers of NIH-3T3 cells in 12-well plates with a maximum of 10^6–10^7 cell equivalents/well. The next day the monolayers were overlaid with carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO). After 7 days of culture, plaques were quantitated after methanol fixation and Giemsa staining. Control experiments have shown that the limit of detection of the infective center is 1 PFU in 10^7 cells. The bead beater procedure induces a 24% reduction in lytic virus titers. Therefore, the limit of detection of our assay for lytic virus is 1.24 PFU in 10^7 cells.

Limiting dilution-nested PCR

The frequency of cells containing the MHV-68 genome was determined by a combination of limiting dilution analysis and nested PCR (LDA-PCR) (22, 23). Sorted cells were serially diluted in uninfected NIH-3T3 fibroblasts in 96-well plates, lysed, and DNA amplified by PCR as described previously (24) using primers specific for MHV-68 *ORF50.* A 2-μl aliquot of the product was then reamplified using nested primers and the final product was analyzed by ethidium bromide staining of DNA following electrophoresis in a 3% agarose gel. 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 MHV-68 DNA. As controls of nested PCR, 10^4 NIH-3T3 cells/well with and without plasmid DNA containing the MHV-68 *ORF50* gene were included in each 96-well plate. At least three independent experiments were used to determine the mean reciprocal frequency and standard deviation of MHV-68 DNA-positive cells in each sorted cell population.

DNA PCR

The organs were removed after perfusion with 50 ml of PBS from mock-infected mice or from MHV-68-infected mice at 60 days after infection. Fifty milligrams of each tissue was minced, directly lysed, and processed for DNA isolation using the DNeasy Tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions. One hundred nanograms of DNA was used for amplification of the MHV-68 *ORF50* gene by nested PCR as described above. Mouse *β actin* was amplified as a control for the DNA quality in the samples. As PCR controls, DNA extracted from the MHV-68-infected B cell line, S11, was used as a positive control and H_2O as a negative control.

Results

MHV-68 has a systemic distribution during long-term latency

Previous studies on the distribution of latent MHV-68 have been focused on the spleen, lung, and peritoneal cavity. However, we have little information on the distribution of the virus in other anatomical sites. To address this issue, we monitored the presence of viral DNA in different tissues after intranasal infection using two different approaches. First, we isolated genomic DNA from several tissues 60 days after infection and used nested PCR to detect the presence of viral DNA. MHV-68 DNA is broadly distributed in different anatomical locations and there is high variability between individual mice (Fig. 1). Second, we used LDA-PCR to determine the frequency of cells carrying MHV-68 in different tissues. These studies showed that the frequency of genome-positive cells was 1 of 120,000 in the bone marrow, 1 of 63,000 in the nasopharyngeal-associated lymphoreticular tissue, 1 of 21,000 in the lung, and 1 of 7,700 in the spleen 3 mo after infection. The high viral frequencies in spleen and lung indicate that these two organs are important reservoirs of MHV-68 during long-term latency.

Long-term latency is maintained in splenic macrophages and dendritic cells

Whereas a major reservoir of splenic latency is the B cell (16–19), we previously reported the establishment of MHV-68 latency in splenic macrophages and dendritic cells at the peak of latent infection (18). In addition, we have shown that long-term latency is maintained in B cells (19). However, it is not known to what extent splenic macrophages and dendritic cells contribute to long-term

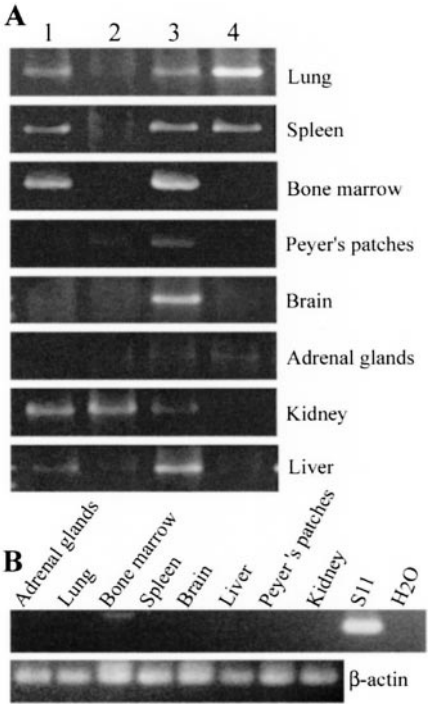

FIGURE 1. MHV-68 is distributed systemically during the latency phase of infection. *A*, Genomic DNA was isolated from different tissues 60 days after infection and subjected to nested PCR to detect MHV-68 *ORF50.* Each sample represents an individual mouse. *B*, The following controls are shown: amplification of MHV-68 *ORF50* and mouse *β actin* from DNA isolated from mock-infected mice; amplification of MHV-68 ORF50 from DNA isolated from the MHV-68-infected B cell line S11 and H_2O control.

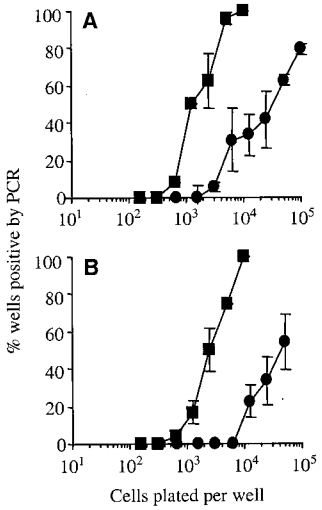

FIGURE 2. MHV-68 establishes and maintains long-term latency in splenic macrophages and dendritic cells. Spleen cells were sorted at 14 days (■) and 3 mo (●) after MHV-68 infection as dendritic cells (*A*) and macrophages (*B*). The frequency of genome-positive cells was determined by LDA-PCR assay. Shown are the percentages of PCR that scored positive as a function of the number of cells analyzed. Data represent the average of three independent experiments; error bars represent SD.

viral persistence. Therefore, we examined whether splenic macrophages and dendritic cells maintain long-term infection by determining their viral frequency 3 mo after MHV-68 intranasal infection.

To analyze the presence of viral DNA in macrophages ($CD11b^+$ $CD11c^-B220^-$) and dendritic cells ($CD11c^+CD11b^{+\text{ or }-}B220^-$), spleen cells isolated from MHV-68-infected mice were FACS sorted into purified populations and analyzed by LDA-PCR. The analysis was performed at two different time points. The establishment of latency was analyzed 14 days after infection, when latent virus is at peak levels and lytic virus is largely cleared (15, 18, 22, 25). Specifically, the frequency of lytic virus is 1 of 132,000 in splenic dendritic cells, too low to determine a frequency in macrophages, and not detectable in B cells (18). The maintenance of long-term latency was analyzed 3 mo after infection. At this time point, lytic virus was not detected in sorted subsets of splenic B cells, macrophages, and dendritic cells by limiting dilution in vitro reactivation (Ref. 18 and data not shown). Therefore, the ability to detect cells harboring viral genome in the absence of preformed infectious virus indicates cells infected with latent MHV-68. Data were plotted as percentage of positive PCR (Fig. 2), and linear regression analysis was used to determine the frequency of cells carrying viral DNA (Table I). MHV-68 was detected in macrophages and dendritic cells with similar reciprocal frequencies (Table I). The frequencies obtained with LDA-PCR at the peak of viral latency ($1–3 \times 10^3$, Table I) were very close to those previously determined by assessing the levels of latent reactivatable virus 14 days after infection using a LDA in vitro reactivation assay (18). During long-term latency, MHV-68 was still detected in macrophages and dendritic cells 3 mo after infection, although the frequency values dropped ~15- to 30-fold (Fig. 2 and Table I).

Latency in the lung is established in a variety of cell subsets; however, long-term latency is maintained in B cells

Infectious virus in the lung is normally cleared by days 11–14 after intranasal infection with MHV-68 (15, 25). Nevertheless, MHV-68 persists in the lung and lung epithelial cells have been reported to be a major reservoir of viral persistence (26). However, it is not known whether MHV-68 establishes latency in other cells types in the lung. Therefore, we analyzed MHV-68 infection in different lung cell populations 14 days after infection, when lytic virus is cleared, and 3 mo after infection, during long-term latency.

To determine the frequency of viral DNA in lung B cells, macrophages, and dendritic cells, cells isolated from the lung of MHV-68-infected mice were FACS sorted into purified populations of B cells ($CD19^+CD5^-$), macrophages ($CD11b^+CD11c^-CD19^-$ $CD5^-$), and dendritic cells ($CD11c^+CD11b^{+\text{ or }-}CD19^-CD5^-$) (Fig. 3). A population of "null" cells containing non-T, non-B, non-Mac-1^+, nonintegrin α_x-chain$^+$ cells ($CD19^-CD5^-$ $CD11b^-$ $CD11c^-$) was also purified. Because these cells constitute between 50 and 60% of the total lung parenchyma, it is likely that they contain high numbers of epithelial and stroma cells. The analysis of lung cells 14 days after infection indicated that viral DNA was present in every cell subset analyzed (Fig. 4) at reciprocal frequencies in the range of 500-1700 genome-positive cells (Table II). The analysis 3 mo after infection showed a dramatic decrease in the number of cells carrying MHV-68 (Fig. 4). Frequency analysis showed that the only population with a significant pool of MHV-68-infected cells during long-term latency in the lung was the B cell subset (1 in 17917 cells, Table II). All of the other populations

Table I. *MHV-68 frequencies within splenic macrophages and dendritic cells during the establishment and maintenance phases of latent infection*

Cells	Reciprocal Frequency of Genome-Positive Cells[a] (SD)	% of Total Spleen[b]	Total No. of Cells[c]	Latently Infected Cells[d]
14 days postinfection				
Dendritic cells[e]	1,239 (913)	7.5	1.5×10^7	1.2×10^4
Macrophages[f]	3,316 (339)	3	6×10^6	1.8×10^3
3 mo postinfection				
Dendritic cells	34,138 (29,854)	3.2	1.9×10^6	57
Macrophages	48,012 (12,702)	3	1.8×10^6	37

[a] Frequencies with 95% confidence limits were determined by linear regression analysis of LDA-PCR data. Data are the mean of three independent experiments, each analyzing pooled spleens from three to five mice. SD is shown in parentheses.

[b] Percentage of each subset of total spleen cells was determined by FACS analysis.

[c] Total number of each cell subset per spleen based on estimate of 2×10^8 total cells/spleen at 14 days after infection and 6×10^7 cells/spleen at 3 mo after infection.

[d] Number of latently infected cells based on the frequency of viral genome-positive cells within each cell type and the estimated total number per spleen.

[e] Dendritic cells were sorted as $B220^-CD11b^{+\text{ or }-}CD11c^+$. The sorted populations were 92.86 ± 3.47% pure and the contaminating fractions were 1.88 ± 0.94% macrophages, 0.01 ± 0.01% B cells, and 5.18 ± 3.36% nonstained cells.

[f] Macrophages were sorted as $B220^-CD11b^+CD11c^-$. The sorted populations were 96.34 ± 2.64% pure and the contaminating fractions were 0.35 ± 0.30% dendritic cells, 0.01 ± 0.02% B cells, and 3.28 ± 2.59% nonstained cells.

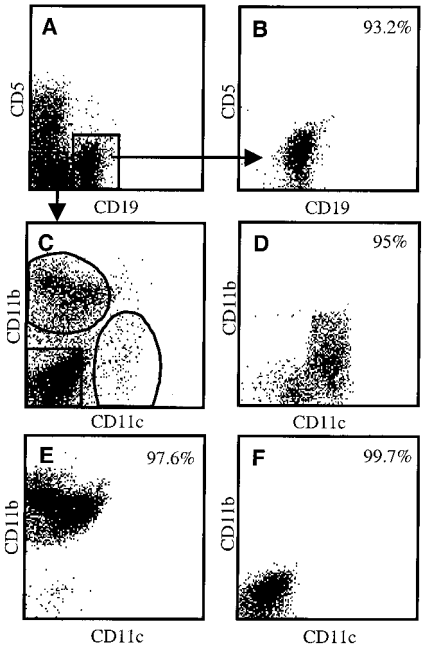

FIGURE 3. Flow cytometry analysis of lung cells at 14 days and 3 mo after infection. *A,* Lung cells were separated into B cells and $CD19^-CD5^-$ cells. *B.* B cells were sorted as $CD19^+$ and $CD5^-CD11c^-CD11b^-$. *C,* The $CD19^-CD5^-$ fraction was further separated into dendritic cells, macrophages, and null cells. *D,* Dendritic cells were sorted as $CD11c^+CD11b^{+\text{ or }-}$ and $CD19^-CD5^-$. *E,* Macrophages were sorted as $CD11b^+$ and $CD19^-CD5^-CD11c^-$. *F,* A population of null cells was sorted as negative for all of the markers used ($CD19^-CD5^-CD11b^-CD11c^-$), therefore lacking most of the APCs and T cells. Purities of the sorted cell populations are indicated.

analyzed, macrophages, dendritic cells, and null cells, had a reciprocal frequency value lower than 2×10^5 genome-positive cells, and each cell type contributed <10 infected cells to the latency pool (Table II).

MHV-68 latency remains relatively stable in circulating B cells, with isotype-switched B cells being the major reservoir

Circulating memory B cells are the major reservoir of the human γ-herpesvirus EBV (6, 27). However, whether MHV-68 establishes latency in circulating cells is largely unknown. Therefore, we analyzed the establishment and maintenance of viral latency in PBLs to determine the relative contribution of circulating cells to MHV-68 latency. Preliminary results showed that viral DNA was mainly located in the B cell subset during long-term latency and that the numbers of purified $CD11b^+$ and $CD11c^+$ cells were too low to determine the frequency, although viral DNA could be detected in the $CD11c^+$ subset (data not shown). Therefore, we limited our analysis to the B cell subset.

To further analyze the presence of viral DNA in circulating B cells, PBLs isolated from MHV-68-infected mice were FACS sorted into total B cells ($CD19^+$), naive B cells (IgD/M^+CD19^+), and isotype-switched B cells ($(IgAIgG_1IgG_{2ab}IgG_3)^+CD19^+$). As shown in Fig. 5, viral DNA was detected in all three purified populations. Linear regression analysis showed that isotype-switched cells constituted the major viral reservoir in blood both during the establishment and during long-term latency (Table III). Nevertheless, virus was also constantly detected in the circulating naive B cell compartment. It is surprising that the drop in viral frequency values in total and isotype-switched B cells, respectively, between 14 days and 3 mo (2- to 3-fold, Table III) is not as marked as the

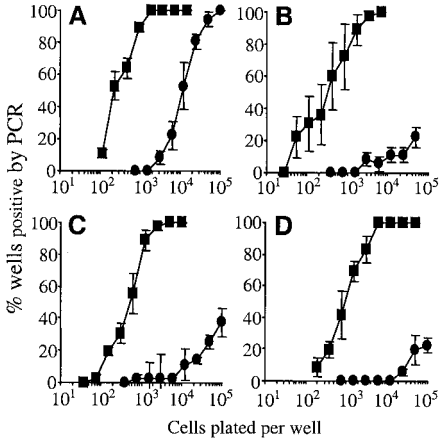

FIGURE 4. MHV-68 establishes and maintains long-term latency in lung cell subsets. Lung cells were sorted at 14 days (■) and 3 mo (●) after MHV-68 infection as B cells (*A*), dendritic cells (*B*), macrophages (*C*), and null cells (*D*). The frequency of genome-positive cells was determined by LDA-PCR assay. Shown are the percentages of PCR that scored positive as a function of the number of cells analyzed. Data represent the average of three independent experiments; error bars represent SD.

10-fold decrease previously observed in spleen and lung (Tables 1 and 2 and Ref. 19).

Preformed infectious MHV-68 is detected in spleen and lung during long-term latent infection

MHV-68, as all γ-herpesviruses, establishes lifelong latency in the host. In the case of EBV, persistent lytic replication takes place in the oropharynx, contributing to an active process of infection in the tonsils and the release of infectious viral particles into the saliva (reviewed in Refs. 9 and 28). How these processes take place in other γ-herpesvirus infections is largely unknown, and we and others have consistently failed to detect lytic MHV-68 late after infection in immunocompetent mice (17–19, 22).

To analyze the presence of preformed infectious virus in long-term infected immunocompetent mice, we modified the standard infectious center assay (29). Since MHV-68 infects all APCs (18), the organs were enzymatically and mechanically digested to maximize cell recovery. Next, cells were mechanically disrupted with beads to avoid potential loss of viral viability linked to freeze-thaw cycles (17, 29). Virus was then assayed in a 1-wk plaque assay in 12-well plates. It has previously been shown that 1 wk of in vitro culture is sufficient to detect lytic virus (15, 17). Using this approach, we were able for the first time to detect low levels of infectious MHV-68 (<100 PFU/tissue) in spleen and lung during long-term latent infection in four of eight mice analyzed (Fig. 6). This finding represents the first direct evidence that persistent replication (production of infectious virus during the chronic phase of infection) occurs at a low level and is detectable in immunocompetent mice throughout long-term latency.

Discussion

The current analysis of MHV-68 latency in different cellular and anatomical reservoirs has shown that latent virus is maintained long term in B cells, macrophages, and dendritic cells, but that the distribution and frequencies differ among spleen, lung, and blood. In the spleen, B cells (19), macrophages, and dendritic cells were reservoirs of latency. In the lung, despite the finding that virus was harbored in a variety of cell types early during the establishment of latency, only B cells contained MHV-68 during long-term infection. In the blood, isotype-switched B cells were the major viral

Table II. *MHV-68 frequencies within lung cell subsets during the establishment and maintenance phases of latent infection*

Cells	Reciprocal Frequency of Genome-Positive Cells[a] (SD)	% of Total Lung[b]	Total No. of Cells[c]	Latently Infected Cells[d]
14 days postinfection				
B cells[e]	812 (597)	6.1	3.41×10^5	421
Dendritic cells[f]	517 (379)	5.7	3.19×10^5	617
Macrophages[g]	719 (399)	36.5	2.04×10^6	2,837
Null cells[h]	1,685 (892)	52.9	2.96×10^6	1,757
3 mo postinfection				
B cells	17,917 (7,513)	22.3	1.24×10^6	69
Dendritic cells	223,172 (101,592)	3.9	2.18×10^5	1
Macrophages	252,934 (134,682)	13.9	7.78×10^5	3
Null cells	420,168 (193,395)	59.2	3.31×10^6	8

[a] Frequencies with 95% confidence limits were determined by linear regression analysis of LDA-PCR data. Data are the mean of three independent experiments, each analyzing pooled lungs from three mice.
[b] Percentage of each subset of total lung cells was determined by FACS analysis.
[c] Total number of each cell subset per lung based on an estimate of 5.6×10^6 cells/lung.
[d] Number of latently infected cells based on the frequency of viral genome-positive cells within each cell type and the estimated total number per lung.
[e] B cells were sorted as $CD19^+CD5^-$; purity was 93.21 ± 5.13%.
[f] Dendritic cells were sorted as $CD11c^+CD11b^{+\text{ or }-}CD19^-CD5^-$; purity was 95.01 ± 3.71%.
[g] Macrophages were sorted as $CD11b^+CD11c^-CD19^-CD5^-$; purity was 97.63 ± 1.63%.
[h] Sorted as $CD5^-CD19^-CD11b^-CD11c^-$; purity was 99.72 ± 0.44%.

reservoir both early and late during latent infection, although non-class-switched B cells also carried virus. Importantly, we have also shown that a low level of lytic virus can be detected in the spleen and lung during long-term latency, indicative of persistent viral replication, whereas infectious virus was not consistently detected in circulating cells.

The observation that splenic B cells, macrophages, and dendritic cells maintain long-term MHV-68 latency suggests important similarities with KSHV, which also establishes latency in B cells, monocyte/macrophages, and dendritic cells (30, 31). Another similarity is that MHV-68 is detected in naive B cells (19), and polyclonal naive B cells are also infected with KSHV in Castleman's disease and associated lymphoproliferative disorders (32). It is intriguing that the virus establishes latency in all three types of APCs, but the biological consequences of harboring latency in these multiple cell types have not been established.

An important question is how the virus maintains latency in distinct cellular and anatomical reservoirs. Consistent with the observation that memory B cells are a major reservoir of EBV latency (6, 27), it has been suggested that EBV exploits the biology of the B cell by gaining access to the long-lived memory B cell reservoir (6, 9, 33–35). The major reservoir of MHV-68 latency is also memory B cells (19) and we have recently directly demonstrated that maintenance of MHV-68 latency is dependent on the development of memory B cells.[4] However, it is unclear how γ-herpesviruses maintain latent reservoirs in macrophages and dendritic cells. For example, a recent report showed that murine splenic dendritic cells have a half-life of only 1.5–3 days (36). Potential mechanisms for maintaining latency in macrophages and dendritic cells include the possibilities that the virus may be selectively maintained in specific subsets of long-lived cells or in precursor populations. Alternatively, latency in short-lived macrophages and dendritic cells may be maintained by continual viral reactivation and reinfection. Finally, the viral infection may alter the half-life of the latently infected cells. In support of this latter possibility, recent data indicate that down-regulation of *bcl-2* controls dendritic cell longevity (37). Dendritic cells were shown to down-regulate bcl-2 protein expression upon maturation in vivo, and expression of a *bcl-2* transgene in dendritic cells increased

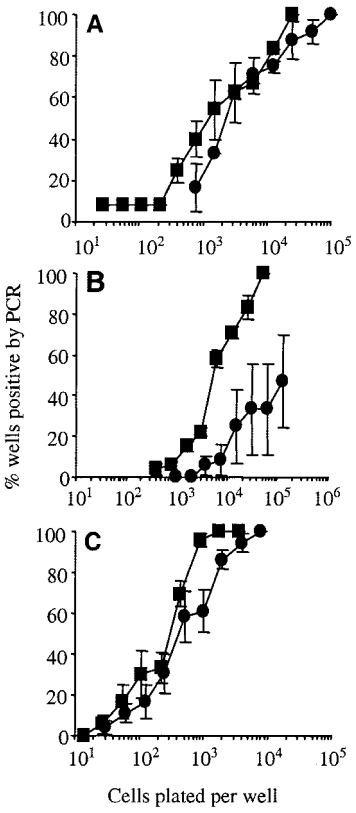

FIGURE 5. MHV-68 establishes and maintains long-term latency in circulating B cells. Blood cells were sorted at 14 days (■) and 3 mo (●) after MHV-68 infection as total B cells (*A*), naive B cells (*B*), and isotype-switched B cells (*C*). The frequency of genome-positive cells was determined by LDA-PCR assay. Shown are the percentages of PCR that scored positive as a function of the number of cells analyzed. Data represent the average of three independent experiments; error bars represent SD.

[4] I.-J. Kim, E. Flaño, D. L. Woodland, F. E. Lund, T. D. Randall, and M. A. Blackman. Maintenance of long-term γ-herpesvirus B cell latency is dependent on CD40 mediated development of memory B cells. *Submitted for publication.*

Table III. *MHV-68 frequencies within blood cell subsets during the establishment and maintenance phases of latent infection*

Cells	Reciprocal Frequency of Genome-Positive Cells[a] (SD)	% of Total Blood[b]
14 days postinfection		
B cells[c]	4,108 (3,347)	50
Naive B cells[d]	11,211 (2,832)	46.8
Isotype-switched B cells[e]	399 (185)	0.5
3 mo postinfection		
B cells	9,286 (9,287)	50
Naive B cells	65,488 (86,226)	45.3
Isotype-switched B cells	1,084 (538)	0.5

[a] Frequencies with 95% confidence limits were determined by linear regression analysis of LDA-PCR data. Data are the mean of three independent experiments, each analyzing pooled blood from 10 mice.

[b] Percentage of each subset of total blood cells was determined by FACS analysis.

[c] B cells were sorted as $CD19^+CD5^-$; purity was 99.44 ± 0.72%.

[d] Naive B cells were sorted as $CD19^+IgM/D^+(IgG_1IgG_{2ab}IgG_3IgA)^-CD5^-$; purity was 99.05 ± 1.71%.

[e] Isotype-switched B cells were sorted as $CD19^+(IgG_1IgG_{2ab}IgG_3IgA)^+IgM/D^-CD5^-$; purity was 95.62 ± 3.13%.

both the frequency and absolute numbers of these cells in transgenic mice. Interestingly, MHV-68 expresses a *bcl-2* homologue, *M11*, that inhibits Fas and TNF-mediated apoptosis (38, 39). It is tempting to speculate that *M11* expression in infected dendritic cells could prolong their half-life. KSHV could use analogous mechanisms as it encodes at least four distinct genes that might serve as apoptosis inhibitors: *K2* (viral (v) IL-6), *K9* (vIRF-1), *ORF16* (vBcl-2), and *K13* (vFLIP) (reviewed in Ref. 40).

The cellular reservoirs in which latency is maintained differ in the three anatomical sites examined. Lytic MHV-68 infection of the lung is cleared by days 11–14 (reviewed in Refs. 10, 11, and 14). As infectious virus is eliminated, latency is established in the spleen and lung in macrophages, dendritic cells, and B cells and, in the case of the lung, null cells. Three months after infection, B cells were the only major reservoir of MHV-68 in the lung, with only a minimal contribution of non-B cells. This contrasts with the spleen and blood. In the spleen, latency was harbored long-term in macrophages, dendritic cells, and B cells, whereas in the blood, latency was mainly found in B cells at both early and late times after infection. Preferential latency in the peripheral blood B cells is probably a consequence of the fact that dendritic cells and macrophages are not usually found at high frequencies in the circulation. However, why the cellular distribution of MHV-68 is different in spleen and lung deserves further analysis.

Our analysis of latency reservoirs in the lung disagrees with that of Stewart et al. (26), in which lung epithelial cells were described as a major reservoir of MHV-68 late during infection using in situ hybridization to detect viral sequences. Our analysis detected virus in only a low frequency of null cells, which had been depleted of B and T cells, macrophages, and dendritic cells, and therefore consisted mainly of endothelial cells, epithelial cells, and fibroblasts.

Importantly, the persistence of a γ-herpesvirus infection in the lung for the life of the host may have important consequences during heterologous infections of the respiratory tract. One possibility is that heterologous infection would trigger viral reactivation, allowing production of infectious virus, which could serve to renew latency reservoirs or transmit the virus to a new host. For example, for EBV it has been suggested that memory B cells transiting the mucosal epithelium may be triggered by Ag or bystander activation to terminal differentiation into plasma cells and associated release of infectious virus (35). This possibility is supported by the observation that cells that replicate EBV during infectious

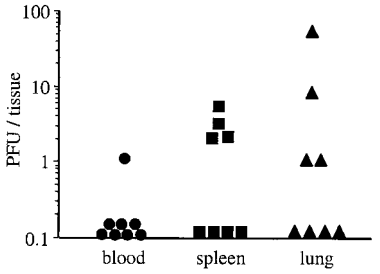

FIGURE 6. MHV-68 maintains persistent replication during long-term latent infection. Infectious viral titers in blood, spleen, and lung cells at 3 mo after infection were determined by a highly sensitive plaque assay as described in *Materials and Methods*. PFU values represent individual mice.

mononucleosis and in the tonsils of healthy carriers resemble plasma cells (6, 41).

The observation that isotype-switched B cells are the major reservoir of latent virus in circulating blood cells is consistent with our previous analysis of MHV-68 latency in splenic B cells (19). In addition, isotype-switched B cells are also a major long-term reservoir of EBV latency (6, 7, 27). An important difference between MHV-68 latency among B cells in blood, spleen, and lung, however, is that the latency frequency remains relatively stable (2-fold differences) in circulating B cells, in contrast to the 10-fold reduction in latently infected B cells in the lung and spleen between 14 days and 3 mo.

The current studies show that MHV-68 reactivates to produce infectious virus in the spleen and lung during long-term infection of immunocompetent mice. Latency and reactivation to form lytic virus are thought to be key events of γ-herpesviruses persistent infection (42). Infectious virus could not previously be recovered from immunocompetent mice by conventional plaque or cytopathic assays during long-term latent infection (15, 17), except in the case of immunosuppressed mice where lytic infection recrudesces (15, 23, 43, 44). In this study, we used a more rigorous cell isolation procedure to increase the yield per organ. We also used a mechanical cell disruption technique that is supposed to have a less severe impact on virus viability. We also analyzed the entire cell population from each organ sampled. This approach showed persistent MHV-68 replication in the spleen and lung with frequencies as low as 2–10 PFU/organ during long-term latent infection, although infectious virus could only be detected in half of the mice analyzed. Persistent lytic virus has also been described for other γ-herpesviruses in the oropharynx, genital tract, tonsils, and spleen during asymptomatic infection (1, 3–8). Continuous viral reactivation of MHV-68 might explain the sustained activation and cycling phenotype of $CD4^+$ and $CD8^+$ T cells specific for viral lytic epitopes during long-term latent infection (45, 46). This phenotype varies from that of memory T cells specific for respiratory viruses that are readily cleared (47). Continuous MHV-68 reactivation may also provide a source of lytic virus for reinfection of cells, which may contribute to the maintenance of latent reservoirs. Importantly, the presence of lytic virus indicates that the immune system is not able to completely control reactivation and persistent production of infectious viral particles during long-term MHV-68 latency.

Together, our results show that MHV-68 latency is established and maintained in a variety of anatomical locations and cell subsets and that long-term latency is accompanied by a low level of persistent virus replication. These findings suggest that γ_2-herpesvirus latency exploits multiple survival strategies. Although B cells are a major reservoir of latency, infection of dendritic cells and macrophages contributes to diversify the cellular reservoirs and

may help to guarantee an efficient maintenance of long-term latency. Long-term latent infection of dendritic cells and macrophages and differences in viral persistence between different anatomical locations are likely to play important roles in the evasion of the host immune control by γ_2-herpesviruses.

Acknowledgments

We thank Simon Monard for assistance with FACS sorting.

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