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Mature B Cells Are Required for Acute Splenic Infection, but Not for Establishment of Latency, by Murine Gammaherpesvirus 68

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Murine gammaherpesvirus 68 (γHV-68; also referred to as MHV-68) is a gammaherpesvirus which infects murid rodents. Previous studies showed that CD8 T cells are important for controlling γHV-68 replication during the first 2 weeks of infection and suggested a role for B cells in latent or persistent γHV-68 infection. To further define the importance of B cells and CD8 T cells during acute and chronic γHV-68 infection, we examined splenic infection in mice with null mutations in the transmembrane domain of the μ-heavy-chain constant region (MuMT; B-cell and antibody deficient) or in the β_2-microglobulin gene ($\beta_2^{-/-}$; CD8 deficient). Immunocompetent mice infected intraperitoneally with γHV-68 demonstrated peak splenic titers 9 to 10 days postinfection, cleared infectious virus 15 to 20 days postinfection, and harbored low levels of latent virus at 6 weeks postinfection. $\beta_2^{-/-}$ mice showed peak splenic γHV-68 titers similar to those of normal mice but were unable to clear infectious virus completely from the spleen, demonstrating persistent infectious virus 6 weeks postinfection. These data indicate that CD8 T cells are important for clearing infectious γHV-68 from the spleen. Infected MuMT mice did not demonstrate detectable infectious γHV-68 in the spleen at any time after infection, indicating that mature B lymphocytes are necessary for acute splenic infection by γHV-68. Despite the lack of measurable acute infection, MuMT spleen cells harbored latent virus 6 weeks postinfection at a level about 100-fold higher than that in normal mice. These data demonstrate establishment of latency by a herpesvirus in an organ in the absence of acute viral replication in that organ. In addition, they demonstrate that γHV-68 can establish latency in a cell type other than mature B lymphocytes.

Gammaherpesviruses are characterized biologically by their ability to establish latency in lymphocytes. To help elucidate the pathogenesis of acute and chronic gammaherpesvirus infection, a mouse model of gammaherpesvirus infection has recently been developed. Murine gammaherpesvirus 68 (γHV-68; also referred to as MHV-68) is a natural pathogen of wild murid rodents (3) and is capable of infecting both outbred and inbred mice (2, 8, 24, 27, 31, 32). Viral genome structure and limited sequence analysis indicate that γHV-68 is related to the primate gammaherpesviruses herpesvirus saimiri and Epstein-Barr virus (EBV) (6, 7, 30). γHV-68 infects multiple organs of inbred mice and can establish a latent or persistent infection in the spleen (2, 27, 31, 32). Depletion of CD8 T lymphocytes results in increased γHV-68 titers during the first 2 weeks of infection (8). Previous studies have implicated B lymphocytes as a potential reservoir of persistent and/or latent virus in infected mouse spleens (32). On the basis of sequence homology to gammaherpesviruses and the reported ability to establish latency in B lymphocytes, it has been proposed that γHV-68 may provide a useful small animal model for primate gammaherpesviruses.

In this report, the role of B lymphocytes and CD8 T lymphocytes in acute and chronic infection of the spleen and establishment of viral latency by γHV-68 is examined. We have developed quantitative assays that clearly differentiate between infectious virus and latently infected cells. Using these assays, we show that mature B cells are important for acute splenic infection by γHV-68, since mice lacking mature B lymphocytes (MuMT mice) did not contain detectable viral titers in the spleen during the acute stages of infection. However, MuMT mice did harbor latent virus, demonstrating that mature B lymphocytes are not necessary for establishment of latency by γHV-68. In addition, mice lacking CD8 T cells (β_2-microglobulin-deficient [$\beta_2^{-/-}$] mice) did not completely clear infectious virus from the spleen up to 6 weeks postinfection, demonstrating a critical role for CD8 T cells in clearance of infectious γHV-68.

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MATERIALS AND METHODS

Viruses and tissue culture. γHV-68 was kindly provided by P. Doherty (St. Jude's Hospital, Memphis, Tenn.). γHV-68 was passaged once in baby hamster kidney cells (ATCC CRL 6281). After complete cytopathic effect (CPE) was observed, total cell lysate was harvested and stored at −70°C. Virus stocks used for mouse infections were derived after an additional passage in NIH 3T12 cells (ATCC CCL 164). The titers of the virus stocks were 3×10^6 to 1×10^7 PFU/ml, as measured by plaque assay on NIH 3T12 cells. γHV-68 was handled with biosafety level 2 precautions, as it has been shown to infect human cell lines (3). NIH 3T12 and MEF (mouse embryonic fibroblast) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 2 mM L-glutamine. MEF cells were obtained as previously described (26) by homogenizing BALB/c mouse embryos, plating the homogenate in tissue culture flasks for 1 to 2 weeks, and harvesting adherent cells with trypsin.

Mice and inoculations of mice. Mice were housed and bred at the Washington University School of Medicine at biosafety level 2 in accordance with all federal and university policies or were purchased from the Jackson Laboratory (Bar Harbor, Maine). Sentinel mice screened negative every 2 to 4 months for adventitious mouse pathogens. $\beta_2^{-/-}$ and MuMT mice were initially obtained from D. Roopenian and L. Schultz, respectively, at the Jackson Laboratory. These mice were of a mixed 129Ev/Sv and C57BL/6 background, and thus 129/J or 129Ev/Sv and C57BL/6J mice were used as immunocompetent controls. In some

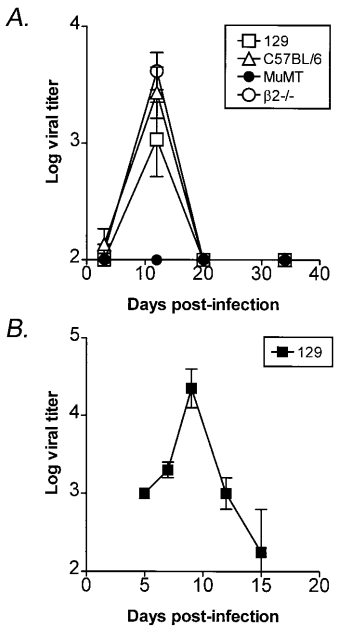

FIG. 1. Time course of γHV-68 titers detected in spleen after i.p. inoculation. The indicated strains of mice were injected i.p. with 10^5 PFU (A) or 10^6 PFU (B) of γHV-68 in complete medium. At the indicated days after infection, spleens were harvested, and a portion of the spleen was homogenized and assayed for viral titer by plaque assay on NIH 3T12 mouse fibroblasts. Shown are the calculated log viral titers detected per spleen. The limit of detection of the plaque assay was 100 PFU per spleen. Data shown are averages of three mice per group. Error bars represent standard errors from the mean, calculated using GraphPad Prizm (Microsoft Corporation).

later experiments, $\beta_2^{-/-}$ and MuMT mice were derived from breeder mice purchased from the Jackson Laboratory after backcrossing to C57BL/6 for 10 generations. No differences were detected between mice on a 129 × C57BL/6 mixed background and mice on a C57BL/6 background, and thus data from mice with null mutations were pooled and compared with 129 and C57BL/6 controls. $\beta_2^{-/-}$ mice were derived by disruption of the β_2-microglobulin gene and have a significant deficiency in the number and function of CD8 T cells (19). MuMT mice were derived by interruption of a transmembrane exon of immunoglobulin M and are deficient in functional B cells (18). We confirmed the expected cell deficiencies in these mice by fluorescence-activated cell sorter analysis (data not shown).

Mice were inoculated intraperitoneally (i.p.) with 10^5 to 10^6 PFU of γHV-68 in 1.0 ml of complete DMEM or were mock infected with 1.0 ml of NIH 3T12 control cell lysate in complete DMEM. Adult mice from 1 to 11 months old were used for all experiments. At various times postinfection, mice were sacrificed by cervical dislocation after metofane anesthesia. A portion of the spleen was fixed in 10% formalin, sectioned, and stained by hematoxylin and eosin. The remainder of the spleen was disrupted in complete DMEM in a tissue homogenizer that preserves cell viability. Spleen homogenates were either frozen at −70°C or assayed immediately for infectious virus by plaque assay. For spleen cell counts and limiting-dilution assays, a portion of the spleen homogenate was passed over a Nitex filter to remove splenic stroma, erythrocytes were destroyed by ammonium chloride lysis, and the remaining splenocytes were washed and resuspended in complete DMEM.

Plaque assay. Plaque assays were performed on NIH 3T12 cells. A total of 2×10^5 to 2.5×10^5 NIH 3T12 cells were plated per well in six-well tissue culture plates. Serial 10-fold dilutions of spleen homogenates from infected animals were plated on the NIH 3T12 monolayers and, after infection for 1 h at 37°C, were overlaid with 1.3% Noble agar in DMEM supplemented with 7.5% fetal calf serum. Six days after infection, monolayers were stained with neutral red, and plaques were counted the next day. The limit of detection of the plaque assay was 100 PFU per spleen.

Limiting-dilution analysis. Serial twofold dilutions of stock γHV-68 virus or of infected mouse splenocytes were plated on monolayers of 1×10^4 to 1.5×10^4 MEF cells per well in 96-well tissue culture plates; 24 to 48 wells were plated per dilution. MEF cells were observed microscopically for viral CPE for up to 4 weeks. To differentiate between latently infected cells and infectious virus in cell samples, serial twofold dilutions of spleen cells were plated before or after mechanical disruption of viable cells. For mechanical disruption, cells were resuspended at a concentration of 1×10^6 to 5×10^6 cells per ml in hypotonic 1/3× DMEM in the presence of 100 μl of 0.5-mm-diameter zirconia-silica beads per ml and shaken twice for 1 min at high speed in a Mini-Beadbeater-8 (Biospec Products, Bartlesville Okla.).

RESULTS

Absence of infectious γHV-68 in spleens of B-cell-deficient mice. To determine the role of B cells and CD8 T cells in acute and chronic γHV-68 infection, MuMT and $\beta_2^{-/-}$ mice were infected. Viral plaque assays of spleens harvested at various times after i.p. inoculation of 10^5 PFU of γHV-68 detected between 10^3 and 10^4 PFU per spleen 12 days postinfection in immunocompetent and $\beta_2^{-/-}$ mice (Fig. 1A). However, no virus was detected by plaque assay at any time in the spleens of MuMT (B-cell and antibody-deficient) mice. To further assess the lack of detectable γHV-68 in the spleens of MuMT mice, we performed a kinetic experiment with a higher dose (10^6 PFU) of γHV-68 and found that day 9 was the peak of acute splenic infection in immunocompetent mice after i.p. inoculation of γHV-68 (Fig. 1B). The peak of infection in the spleen was somewhat later than that previously reported for intranasal or intravenous infection by γHV-68 (31). In five separate experiments, no infectious virus was ever detected in MuMT mouse spleens harvested 9 to 10 days after infection with 10^6 PFU of γHV-68 (Fig. 2). Thus, MuMT mice had an acute titer of less than 100 PFU per spleen of γHV-68, which was the limit of detection of the plaque assay. Approximately 10^4 PFU per spleen was detected by plaque assay in immunocompetent and $\beta_2^{-/-}$ mice. Mock-infected mice tested negative at all times (data not shown). Thus, increasing the virus inoculum and harvesting the spleens on the peak days of acute infection did not lead to detection of acute viral replication from the spleens of MuMT mice.

At the peak of acute infection, splenomegaly was observed in immunocompetent and $\beta_2^{-/-}$ mice but not in MuMT mice. Nine to 10 days after infection with γHV-68, control C57BL/6 and 129 mice demonstrated a 2- to 3-fold increase in both spleen weight and spleen cell number compared with mock-infected controls (Fig. 3). Spleens from MuMT mice did not change in weight or cell number during acute infection. Interestingly, while acute titers of γHV-68 were similar in control and $\beta_2^{-/-}$ mice (Fig. 1 and 2), splenomegaly and increases in total spleen cell numbers persisted in $\beta_2^{-/-}$ mice after virus was cleared, as measured by plaque assay. This persistence of splenomegaly in $\beta_2^{-/-}$ mice is likely explained by persistence of infectious γHV-68 at levels below the sensitivity of plaque assay detection (see below).

Hematoxylin-and-eosin-stained sections of infected mouse spleens revealed expansion of follicles and prominent germinal center activation in immunocompetent and $\beta_2^{-/-}$ mice (data

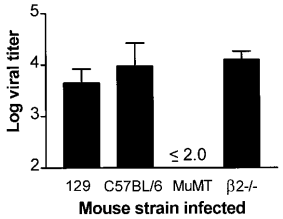

FIG. 2. Lack of acute splenic titers after γHV-68 infection in mice lacking mature B cells. The indicated strains of mice were injected i.p. with 10^6 PFU of γHV-68 in complete medium. Nine to 10 days later, spleens were harvested and analyzed by plaque assay as described for Fig. 1. Shown are the calculated log viral titers detected per spleen. Data shown are averages of results from five separate experiments using 3 to 5 mice per group; 10 to 17 mice were separately analyzed per group. Error bars represent standard errors from the mean.

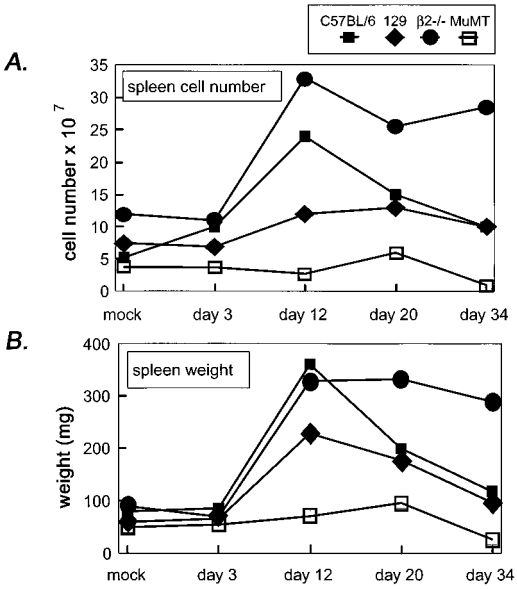

FIG. 3. γHV-68 infection causes splenomegaly in all mice analyzed except MuMT mice. Shown are spleen cell number after ammonium chloride lysis of erythrocytes (A) and spleen weight (B) at the indicated days after i.p. inoculation of 10^5 PFU of γHV-68 or after mock infection with uninfected NIH 3T12 cell lysate. The data represent averages of measurements from three mice.

not shown). No changes were observed in the spleens of mock-infected mice. These changes were maximal at day 10 and were still present at day 22 postinfection. At 29 days postinfection, spleens from immunocompetent mice appeared relatively normal, while spleens from $\beta_2^{-/-}$ mice still showed continued enlargement of the white pulp and prominence of active germinal centers on days 29 and 36 postinfection. This finding correlates with the data in Fig. 3 demonstrating persistent splenomegaly in $\beta_2^{-/-}$ mice and indicates that $\beta_2^{-/-}$ mice cannot normally control γHV-68 infection. In spleens from MuMT mice, neither splenomegaly nor any of these histological changes were observed, supporting the absence of acute splenic infection in mice lacking mature B cells.

Limiting-dilution analysis confirms absence of acute splenic infection in mice lacking mature B cells. The possibility existed that the plaque assay was not sensitive enough to detect infectious γHV-68 in acutely infected spleens from MuMT mice or in spleens from normal or $\beta_2^{-/-}$ mice during chronic infection (Fig. 1 and 2). The limit of detection of the plaque assay was determined to be 100 PFU per spleen. Acute viral titers detected by plaque assay in normal and $\beta_2^{-/-}$ spleens were 10 to 100 times higher than the limit of detection (Fig. 2). Since the number of cells in MuMT spleens during acute infection was about 10-fold lower than the number of cells in control spleens (Fig. 3), it was possible that we did not detect γHV-68 in MuMT spleens because we were plating fewer cells in the plaque assay. Therefore, we developed a more sensitive assay to measure infectious γHV-68, which involved coculturing a known number of spleen cells with MEF cells in a limiting-dilution assay. To determine the sensitivity of the limiting-dilution assay using MEF cells for detecting infectious γHV-68, we cultured serial twofold dilutions of a stock of γHV-68, which had a known titer as determined by plaque assay on NIH 3T12 cells, with MEF cells in 96-well plates. Eight days after infection, wells were scored microscopically for viral CPE (Fig. 4). If limiting-dilution analysis on MEF cells and plaque assay on NIH 3T12 cells were similarly sensitive, the Poisson distribution predicts that 1 PFU per well should result in CPE in 63% of wells. However, limiting-dilution analysis showed that approximately 0.1 PFU scored positive in 63% of the wells. Thus, the limiting-dilution assay on MEF cells is about 10 times more sensitive than the plaque assay on NIH 3T12 cells for detection of infectious γHV-68.

TABLE 1. Mechanical disruption of cells without inactivating infectious γHV-68

Assay	No treatment (%)	% Mechanical disruption[a]
Cell viability (trypan blue)[b]	100	0.75 (0.15)[c]
Virus stability[d] (plaque assay) without added cells	100	88 (34)
Virus stability (plaque assay) with 1-5 × 10^6 cells/ml	100	85 (14)

[a] Cell viability and virus stability were tested after mechanical disruption in 1/3× DMEM containing < 1% FCS and 100 μl of 0.5 mm silica beads per ml.
[b] Cells tested were splenocytes obtained from naive or infected mice and were suspended at 1 × 10^6 to 5 × 10^6 cells/ml.
[c] Numbers obtained were normalized to results with no treatment (incubated in DMEM with 10% FCS). Results for cell viability represent averages of 20 datum points. Numbers in parentheses indicate standard errors from the mean.
[d] Virus tested was a stock of γHV-68 virus diluted to 1,000 PFU per ml either in hypotonic medium alone or in the presence of 1 × 10^6 to 5 × 10^6 naive spleen cells per ml.

Limiting-dilution analyses of splenocytes harvested from mice 9 days postinfection and cocultured with MEF cells confirmed the lack of detectable acute splenic infection in MuMT mice (Fig. 5). With normal mice, 10^2 to 10^3 splenocytes per well released γHV-68 with consequent CPE in a significant percentage of wells. Consistent with the results observed by plaque assay, the level of infectious γHV-68 detected in the $\beta_2^{-/-}$ mice was comparable to that observed in the normal mouse controls. In contrast, little or no infectious γHV-68 was detected in MuMT splenocytes even when 5 × 10^4 splenocytes were added per well. Since the limiting-dilution analysis is based on input cell number, it is clear that MuMT spleens have greatly reduced (at least 100-fold) viral replication during acute γHV-68 infection.

Splenocytes from mice lacking mature B cells harbor latent virus. To analyze establishment of latency in the spleen by γHV-68, limiting dilution analysis was performed with spleen cells from mice infected with 10^6 PFU of γHV-68 6 weeks prior to harvest (Fig. 6). Serial twofold dilutions of splenocytes ranging from 10^5 to 1.6 × 10^3 spleen cells per well were analyzed.

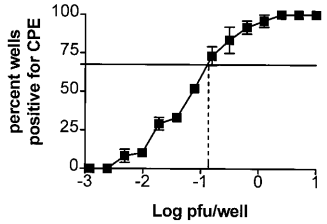

FIG. 4. Limiting-dilution analysis is 10-fold more sensitive than plaque assay for detecting infectious γHV-68. Serial twofold dilutions of a stock of γHV-68 virus, which had a known titer as determined by plaque assay on NIH 3T12 fibroblasts, were plated on a monolayer of MEF cells in 96-well plates. Eight days after infection, wells were scored microscopically for viral CPE. Shown are the percentage of wells which scored positive for viral CPE as a function of log PFU (as determined by plaque assay) plated per well; 24 wells were plated per dilution. Data shown are averages of two experiments. Error bars represent standard errors from the mean. The hatched line indicates the log PFU per well in which 63% of the wells scored positive for viral CPE.

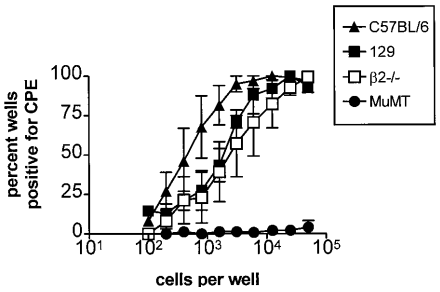

FIG. 5. Limiting-dilution analysis confirms lack of detectable acute splenic infection in mice lacking B cells. Nine days after i.p. infection with 10^6 PFU of γHV-68, spleens from the indicated mice were harvested, homogenized, filtered through a Nitex filter, and treated with ammonium chloride to remove erythrocytes. Serial twofold dilutions of splenocytes ranging from 5×10^4 to 10^2 cells per well were plated on monolayers of MEF cells in 96-well plates, which were scored microscopically for viral CPE 5 to 7 days later. Shown are the percentages of wells which scored positive for viral CPE as a function of the number of cells plated per well; 24 to 48 wells were plated per dilution. Data shown are averages of three experiments. Spleen cells from three to five mice per group were pooled and assayed per experiment. Error bars represent standard errors from the mean.

To distinguish infectious from latent virus, a parallel series of limiting-dilution wells was plated with 10^5 to 1.6×10^3 cell equivalents of spleen cells which had been previously subjected to mechanical disruption. Mechanical disruption killed >99% of cells, as measured by trypan blue exclusion, but had at most a twofold effect on virus stability (Table 1). In the limiting dilution assay, both productively and latently infected cells can release virus in serial dilutions of intact splenocytes. After mechanical disruption, the latent component is destroyed and only preformed infectious virus is detectable. When persistent infectious virus is present, most wells show CPE by 5 to 7 days after plating. In contrast, most wells demonstrating reactivation from latency showed CPE 7 to 21 days after plating. This assay takes advantage of the fact that limiting dilution on MEF cells is more sensitive than plaque assay for detecting infectious γHV-68 and can quantitatively discriminate between latency and productively infected cells.

Six weeks postinfection, immunocompetent 129 and C57BL/6 mice harbored a low level of latently infected cells, as defined by detection of infectious γHV-68 when live splenocytes were assayed, but not after the cells were destroyed (Fig. 6A and B). Since with 10^5 intact splenocytes per well we detected viral CPE in approximately 10% of the wells, a lower estimate of latently infected cells in the spleens of these animals is 1 in 10^6 cells. This is a lower limit estimate since it is possible that not all latent virus will reactivate under these conditions.

$\beta_2^{-/-}$ mice were persistently infected, as defined by the detection of infectious γHV-68 in the absence of live cells (Fig. 6C). Thus, β_2-microglobulin expression, and likely CD8 T lymphocytes, are important for clearing infectious γHV-68 from the spleen. It is notable that we detected infectious virus in $\beta_2^{-/-}$ mice when limiting dilution was used despite lack of detection of virus by plaque assay at late times after infection (Fig. 1). Thus, persistent infection is present in $\beta_2^{-/-}$ mice despite the fact that at least 90% of the virus detected by plaque assay on day 9 has been cleared by 6 weeks postinfection (Fig. 1). This result demonstrates that lack of infectious virus by plaque assay is not sufficient grounds for establishing that an organ or cell population is latently rather than persistently infected.

Surprisingly, MuMT mice harbored latent virus at significantly higher levels than normal mice (Fig. 6D). From the levels of viral reactivation, we estimate that at least 1 in 2×10^4 splenocytes are latently infected in MuMT spleens. These results demonstrate that although mature B lymphocytes are required for acute splenic infection by γHV-68, they are not required for establishment of latency.

DISCUSSION

In this report, we make four important points regarding γHV-68, a murine herpesvirus which has been classified as a gammaherpesvirus on the basis of sequence homology and genome organization (6, 7, 30). First, mature B lymphocytes are critical for the pathogenesis of acute γHV-68 infection. Second, CD8 T cells are important for clearing infectious γHV-68 from the spleen. Third, γHV-68 can efficiently establish latent infection in the spleen even in the absence of detectable acute virus replication in the spleen. Last, γHV-68 can establish latency in animals lacking mature B lymphocytes. These data show that B lymphocytes can play an important role in the acute pathogenesis of gammaherpesvirus infection even when they are not the obligate seat of latency.

Role of B cells in acute γHV-68 pathogenesis. By using MuMT mice, which lack surface immunoglobulin-bearing B cells (18), we showed that B lymphocytes are essential for acute γHV-68 infection of the spleen. We failed to detect infectious γHV-68 by either plaque assay on NIH 3T12 cells (Fig. 1 and 2) or limiting-dilution assay on MEF cells (Fig. 5 and 6) in spleens from MuMT mice. The lack of detectable γHV-68 could not be explained by the lower number of spleen cells in γHV-68-infected MuMT mice (Fig. 5). This observation is consistent with the hypothesis that B lymphocytes are the acutely infected cell type in mouse spleens. An alternate possibility is that B cells or antibodies are necessary for the acute

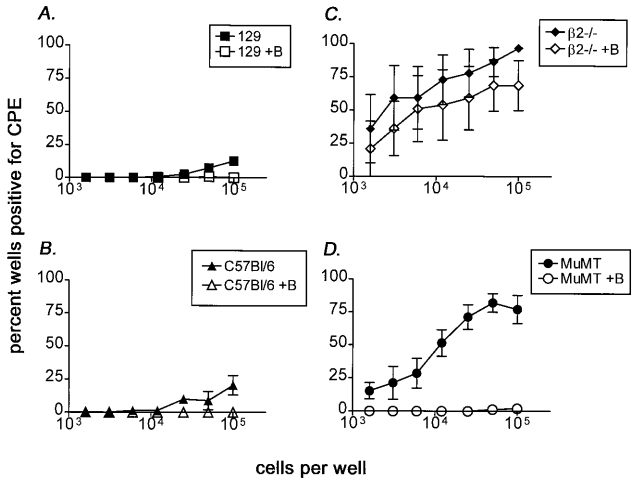

FIG. 6. Splenocytes from B-cell-deficient mice harbor latent γHV-68, while CD8 T-cell-deficient mice are persistently infected with γHV-68. Limiting-dilution analysis was performed with splenocytes from the indicated strains of mice harvested 39 to 46 days after i.p. inoculation of 10^6 PFU of γHV-68. A parallel series of dilutions compared twofold dilutions of splenocytes (closed symbols) with twofold dilutions of mechanically disrupted spleen cells (open symbols; "+ B" denotes samples to which beads were added and cells were mechanically disrupted); 24 to 48 wells were plated per each dilution on a monolayer of MEF cells in 96-well plates, which were scored microscopically for viral CPE. Shown are the percentages of wells which scored positive for viral CPE 2 to 3 weeks after plating as a function of the number of cells plated per well. Data shown are averages of four to five experiments per mouse group. Spleen cells from three to five mice per group were pooled and assayed per experiment. Error bars represent standard errors from the mean.

infection seen in the spleen in an indirect manner. For example, B cells could be necessary for spread of infectious virus to the spleen via the circulation.

Role of B cells in γHV-68 latency. Previous work has suggested that B cells are important for γHV-68 latency. In an infectious center assay, which could theoretically detect both infectious virus and latently infected cells, γHV-68 was detectable in a B-cell-enriched population of mouse spleen cells up to 36 days postinfection (32). However, significant virus was also detected in adherent spleen cells, raising the possibility that B cells are not the only site of γHV-68 infection (32).

Despite the lack of detectable acute infection in the spleens of MuMT mice, latent virus was readily detected in MuMT mouse spleens by limiting-dilution assay 6 weeks postinfection (Fig. 6). Use of limiting-dilution analysis combined with mechanical disruption of cells, an assay system that distinguishes productive from latent infection, demonstrates that MuMT spleens are latently but not productively infected. Since MuMT mice lack mature B cells, γHV-68 is able to establish latency in a cell type other than mature B cells. It is unclear at this time what cell type is latently infected in MuMT spleens or normal mouse spleens. Identification of γHV-68 as a gammaherpesvirus on the basis of sequence homology predicts latency in lymphocytes. Candidates include T cells, natural killer cells, and surface immunoglobulin M-negative pre-B cells.

Reactivatable virus was detected more readily from MuMT than from immunocompetent spleen cells (Fig. 6). This finding implies that there are more latently infected cells in MuMT spleens, suggesting that antibody-producing cells are important for controlling the extent of latent infection. Alternatively, virus could reactivate more efficiently in limiting-dilution analysis in the absence of B cells or antibody. The latter scenario seems unlikely, since mixing equal numbers of splenocytes from latently infected immunocompetent and MuMT mice did not diminish detection of latent virus from MuMT splenocytes by limiting-dilution assay (data not shown).

The contrast between acute and latent infection in spleens of MuMT mice is interesting and instructive. Since γHV-68 establishes latency in the spleens of MuMT mice in the absence of acute infection, acute replication in the spleen is not required for latency. We consider it most likely that γHV-68 establishes acute infection in another organ(s) in MuMT mice, resulting in the establishment of latency in a cell type that then traffics hematogenously to the spleen. This would predict that a circulating cell is latently infected with γHV-68. A similar scenario has been hypothesized for EBV, in which epithelial cells in the oropharynx have been implicated as the site of acute viral infection. Tonsillar B cells may then become latently infected and via hematogenous spread establish latency in the bone marrow. The role of the bone marrow in EBV latency has been shown by the eradication of latent EBV by allogeneic bone marrow transplantation of EBV-negative donor marrow and by the reconstitution of latency by transplantation of EBV-positive marrow (10 to 14). Interestingly, we have found latent γHV-68 in the bone marrow of MuMT and normal mice (unpublished data), suggesting that a similar scenario may apply to γHV-68.

Role of CD8 T cells in γHV-68 pathogenesis. A previous study showed that CD8 T cells control γHV-68 lung titer 1 to 2 weeks after intranasal infection (8). We wished to evaluate the importance of CD8 T cells during chronic infection and viral clearance and thus examined γHV-68 infection in $\beta_2^{-/-}$ mice, which lack efficient surface major histocompatibility complex class I expression as a result of a null mutation in the β_2-microglobulin gene (19), with consequent deficiency in CD8 T cells (5, 15, 19, 21, 23, 25). This model permits evaluation of the role of CD8 T cells during both acute and chronic infection, as it does not rely on transfer of immunogenic anti-CD8 antibody to mice for prolonged periods. The deficiency of CD8 T cells in $\beta_2^{-/-}$ mice, while not absolute (4, 20), is sufficient to increase acute titers of many viruses, including lymphocytic choriomeningitis virus, Sendai virus, and Theiler's virus (5, 9, 16, 21, 23, 29).

The importance of CD8 T lymphocytes in chronic γHV-68 infection was evidenced by (i) the presence of persistent virus in $\beta_2^{-/-}$ mouse spleens 6 weeks postinfection (Fig. 6), (ii) prolonged splenomegaly in $\beta_2^{-/-}$ mice (Fig. 3), and (iii) the more severe and prolonged histological changes observed in $\beta_2^{-/-}$ mouse spleens (data not shown). In contrast to the finding of increased lung titer after anti-CD8 administration (8), we found that $\beta_2^{-/-}$ mice did not exhibit increased spleen titers during acute infection (Fig. 1, 2, and 5). We have no explanation for this difference but note that experiments presented here differed from published studies (8) in the nature of CD8 depletion, route of infection, and organ evaluated. $\beta_2^{-/-}$ mice did not show clinical illness despite persistent infection 6 weeks after infection. This is in contrast to the finding of significant clinical illness in CD8-depleted mice challenged by the intranasal route (8). This difference could be due to compensatory changes in the $\beta_2^{-/-}$ mouse immune system. For example, $\beta_2^{-/-}$ mice have altered antibody and CD4 T-cell responses compared with normal mice (1, 15, 17, 21, 25, 29). However, we feel that the difference in clinical illness between $\beta_2^{-/-}$ and CD8-depleted mice is more likely due to the prominent pneumonia seen after intranasal inoculation (8, 27).

These data demonstrate that the CD8 T lymphocytes are important during chronic γHV-68 infection. In the case of the gammaherpesvirus EBV, CD8 T cells are thought to play a role in immunosurveillance against latently infected cells. Thus, EBV-infected lymphoblastoid cell lines are recognized by major histocompatibility complex class I-restricted T cells in vitro, and cyclosporin A, a known inhibitor of T-cell activation, promotes outgrowth of latently infected lymphoblastoid cells in patients (reviewed in references 22, 28, and 33). One possibility that would explain persistent γHV-68 infection in $\beta_2^{-/-}$ mice would therefore be that CD8 T cells are involved in immunosurveillance against γHV-68-infected cells expressing viral antigens during reactivation. Definition of the genes expressed in latently infected cells and their potential recognition by CD8 T cells is therefore an important goal.

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