

## Pathogenesis of Murine Gammaherpesvirus Infection in Mice Deficient in CD4 and CD8 T Cells

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**Murine gammaherpesvirus is a natural pathogen of wild mice. The virus infects alveolar cells and spleen cells during the primary infection and establishes a latent/persistent infection in B lymphocytes. Little is known about the immunological response to gammaherpesviruses during a primary infection. To address this issue, we investigated the pathogenesis of murine herpesvirus 68 (MHV-68) infection in mice deficient in CD4 or CD8 T-cell populations. Infection of the lung and spleen were greatly exacerbated in CD8-deficient mice, reflected by elevated virus titers in the lung and an increase in the number of infected splenocytes located around germinal centers. This finding contrasts with clearance of virus from the lung and spleen by day 12 postinfection in CD4-depleted animals. These data clearly indicate a major role for CD8 T cells in recovery from an acute MHV-68 infection. Whereas CD4 T cells fail to influence the course of infection in the lung, they do contribute to lymphoproliferation seen in the spleen (splenomegaly) during the primary infection. The significance of these results are discussed in relation to the immune response to other herpesviruses, in particular Epstein-Barr virus, with which MHV-68 shares similar molecular and biological properties.**

Murine herpesvirus 68 (MHV-68) is a naturally occurring murid herpesvirus originally isolated from bank voles (*Clethrionomys glareolus*) in Czechoslovakia (1). Analysis of the structure of the viral genome and limited sequence data of viral genes revealed MHV-68 to be closely related to two gammaherpesviruses of primates, Epstein-Barr virus (EBV) and herpesvirus saimiri (3, 4). Following an intranasal inoculation of virus into BALB/c mice, a productive infection is established in the lung, localized to alveolar epithelium and mononuclear cells (12). As with other gammaherpesviruses, MHV-68 is associated with lymphocytes and latent virus can be detected in the spleen by a cocultivation assay (12). The lymphocyte subset harboring virus during the persistent infection has been identified as an immunoglobulin-positive B lymphocyte (13).

The host defense mechanisms involved in the recovery from MHV-68 infection are unknown. In other herpesvirus infections, T lymphocytes play a key role in the destruction of virus-infected cells and in recovery from the primary infection. Previous studies on herpes simplex virus (HSV) infection of mice identified an important role for virus-specific cytotoxic T lymphocytes in protecting the nervous system from virus infection, whereas in the skin, CD4 T cells were the major effector cell population, possibly functioning via the recruitment and activation of macrophages (8). In contrast to HSV, protective immunity against murine cytomegalovirus infection in BALB/c mice is mediated by CD8 T cells, recognizing an immediate-early gene product in infected cells (6). CD8 T cells are also important in the control of persistent EBV infection in humans and are probably responsible for clearing the infection at epithelial sites (10). However, the role of the immune response in primary EBV infection is still largely unresolved, in part because of the lack of any suitable animal model. Consequently, studies on MHV-68 infection of mice may shed light on the immuno-

logical mechanisms involved in the control of primary EBV infection.

To investigate the role of T cells in a primary MHV-68 infection of BALB/c mice, we have used monoclonal antibodies to deplete T-cell subsets *in vivo* (2, 8). This approach has already been used successfully to study the role of T cells in several virus infections, including HSV (8) and murine cytomegalovirus (6) infections. In this report, we demonstrate that CD8 T cells are central to recovery from a primary infection, whereas CD4 T cells contribute little to recovery but appear to be involved in splenomegaly, an early pathological feature of this virus infection.

### MATERIALS AND METHODS

**Mice.** Female BALB/c mice were obtained from Bantin and Kingman (Grimston, Aldbrough, Hull, United Kingdom) and were infected when 3 to 4 weeks of age.

**Virus and route of inoculation.** Working stocks of MHV-68 were prepared from clone G2.4, the titer of which was  $3 \times 10^7$  PFU/ml. All virus stocks were grown in BHK-21 (baby hamster kidney) cells maintained in Glasgow modified Eagle's medium supplemented with 10% tryptose phosphate broth and 10% newborn calf serum. Each mouse was inoculated intranasally with a dose of  $4 \times 10^5$  PFU of MHV-68. The volume of inoculum was 40  $\mu$ l per mouse, and the mice were lightly anesthetized with ether before administration. Virus dilutions were made in Glasgow modified Eagle's medium immediately before use and kept on ice during the inoculation procedure.

**Intravenous and intraperitoneal administration of antibody.** Rat immunoglobulin G2b monoclonal antibodies YTS 191.1 (anti-CD4) and YTS 169.4 (anti-CD8) were used for depletion of lymphocyte subsets. Mice were injected intravenously in the tail with 0.1 ml of antibody at 10 mg/ml 2 days before virus inoculation and 2 days after and then intraperitoneally. Details of experimental schedule are presented in Fig. 1.

**Cocultivation assay for infectious centers in the spleen.** Mice

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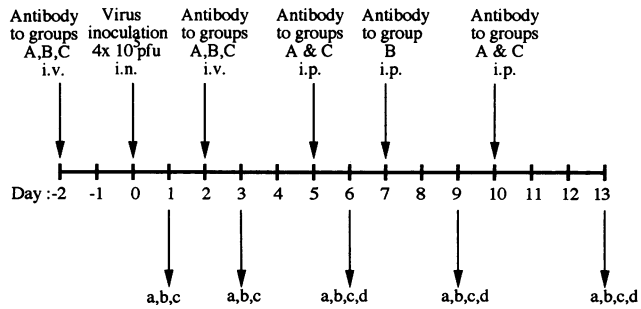


FIG. 1. Experimental protocol for antibody treatment and tissue sampling during MHV-68 infection of BALB/c mice. Groups of mice: A, CD4 depleted; B, CD8 depleted; C, CD4 and CD8 depleted; D, nondepleted. Investigations: a, Spleen infectious center assay; b, lung infectious virus assay; c, spleen cell count; d, flow cytometric analysis. i.v., intravenous administration; i.n., intranasal administration; i.p., intraperitoneal administration.

were killed either by cervical dislocation or by injection of Euthatal B.P. (Vet) anesthesia. Spleens were removed into 2 ml of RPMI 1640 supplemented with 20% fetal calf serum (FCS) and kept individually on ice. A single cell suspension was obtained from each spleen as previously described (13), and the erythrocytes were removed. The leukocytes were counted and resuspended in 4 ml of RPMI-20% FCS; 2 ml was stored at  $-70^{\circ}\text{C}$  for the infectious virus assay, and 1 ml was used for flow cytometric analysis. The rest was diluted (10-fold dilution series) and cocultivated with  $2 \times 10^6$  BHK cells in RPMI-20% FCS to determine the number of infectious centers (12). The monolayers were fixed in 10% formal saline and stained with 1% toluidine blue, and the number of infectious centers was counted.

**Infectious virus assay.** The lungs were removed from the three mice in each group and stored at  $-70^{\circ}\text{C}$  prior to assay. The method was as described by Sunil-Chandra et al. (12). Briefly, the samples were homogenized, and the presence of infectious virus was determined by plaque assay on BHK cells. Dilutions of the lung homogenate or of the spleen cell suspension (see above) were adsorbed with  $2 \times 10^6$  BHK cells and cultured for 4 days. The monolayers were fixed in 10% formal saline and stained with 1% toluidine blue, and plaques were enumerated.

**Flow cytometric analysis.** Spleen cell suspensions from infected mice were adjusted to  $10^7$  cells per ml in Dulbecco's modified Eagle's medium and incubated with 50  $\mu\text{l}$  of monoclonal antibody supernatant to either CD4, CD8, or CD3, or with 50  $\mu\text{l}$  of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 0.1% sodium azide (wash medium) as a negative control, for 30 min. The cells were washed and incubated with 50  $\mu\text{l}$  of STAR 49 anti-rat fluorescein isothiocyanate conjugate (Serotec) diluted 1:100 on ice and in the dark for 30 min. The cells were washed again and fixed in 1% formaldehyde in PBS. The cells were analyzed by flow cytometry (Becton Dickinson FACScan flow cytometer), using the LYSIS II application (Becton Dickinson). The percentage of positive cells was determined by total events obtained from the region of lymphocytes of 5,000 gated cells.

**Histopathological and immunohistochemical studies.** In some experiments, the lungs and spleen were removed and fixed immediately in 10% buffered formal saline. The tissues were paraffin embedded, and 5- $\mu\text{m}$  thin sections were prepared for histopathological studies. Sections were stained

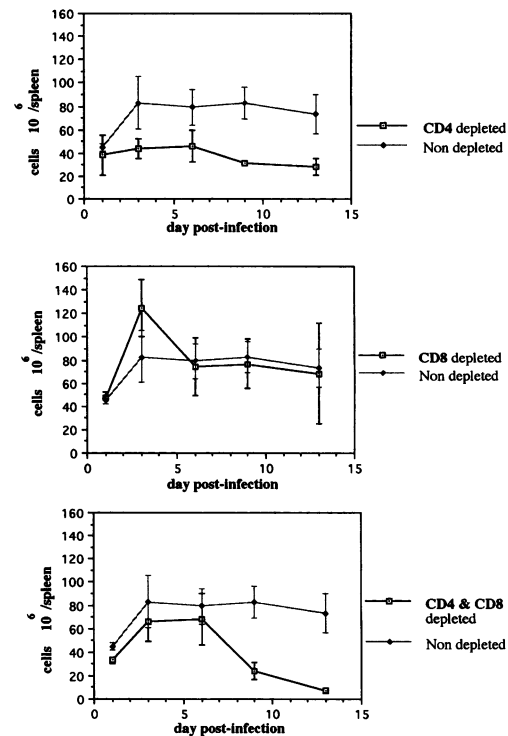


FIG. 2. Spleen cell numbers at different times after infection of depleted and nondepleted mice. Groups are as indicated for each graph. Each T-cell-depleted group is plotted against the nondepleted group. The mean of three mice in each group is shown; horizontal bars represent 1 standard deviation.

with hematoxylin and eosin (H&E). Indirect immunoperoxidase labeling was used to detect virus antigen in sections obtained from the same blocks used for H&E staining. To avoid problems associated with overfixation of tissue specimens and to allow easy penetration of reagents, the sections were treated with 0.1% trypsin (porcine pancreas crude; Sigma Ltd.). Tris-buffered saline was used as a diluent and for rinsing and washing, and the slides were blocked with 5% normal goat serum and 10% BSA in PBS to minimize nonspecific antibody binding. Hyperimmune rabbit serum to MHV-68 was used as the primary antibody (12). To minimize endogenous peroxidase activity, the slides were treated with 0.75% hydrogen peroxide in methanol for 30 min before trypsin treatment and afterwards in 0.3% hydrogen peroxide in Tris-buffered saline for 30 min. Immunoperoxidase labeling using an avidin-biotin detection system was performed with the Vectastain ABC kit (Vector Laboratories, Bredon, Peterborough, United Kingdom).

## RESULTS

**Analysis of spleen T cells in MHV-68-infected mice deficient in CD4 and CD8 T cells.** At various times after infection, mice from different experimental groups were killed, and the spleens were removed to quantitate the number of cells present and to determine the T-cell phenotype by fluorescence-activated cell sorting analysis. In Fig. 2, the spleen cell count for each of the T-cell subset-depleted groups is plotted with the cell counts for the undepleted group. In mice depleted of CD4 or both CD4 and CD8 T cells, spleen cell numbers failed to increase relative to controls and by day 13

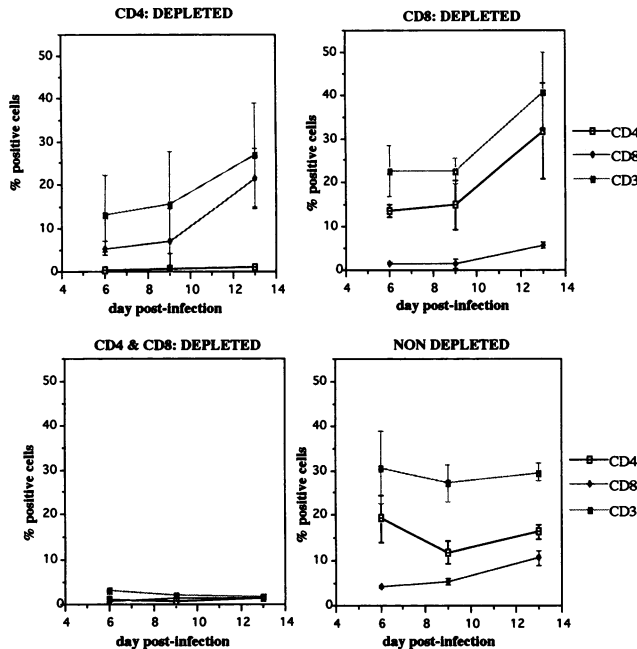


FIG. 3. Flow cytometric analysis of spleen cell suspensions from depleted and nondepleted mice. Groups are as indicated for each graph. The graphs show the percentage positive cells for the markers CD4, CD8, and CD3. The mean of three mice in each group at each time point is shown; horizontal bars represent 1 standard deviation.

had decreased in number. In contrast, in mice depleted of CD8 T cells, the spleen cell numbers increased and in general paralleled the values for the undepleted mice. In the CD8-depleted and the undepleted groups, there was marked evidence of splenomegaly.

Spleen cells taken from mice on days 6, 9, and 13 postinfection were stained for CD3, CD4, and CD8 and analyzed by flow cytometry to check the efficiency of the depletion procedure (Fig. 3). In mice depleted of CD4 cells, the percentage of CD4 cells was negligible, whereas the proportion of CD8 cells increased from 5 to over 20%. Similarly, in the CD8-depleted group, the percentage of CD8 cells was negligible, whereas the proportion of CD4 cells increased from 13 to over 30%. In the group depleted of CD4 and CD8 cells, both populations were absent and there were negligible numbers of CD3<sup>+</sup> cells, suggesting that a compensatory increase in T cells lacking CD4 and CD8 did not occur.

**Effect of T-cell depletion on the growth of virus in the lung and spleen.** The amount of infectious virus in the lungs of T-cell-depleted animals was determined at different times postinfection, using a standard plaque assay (Fig. 4). The nondepleted group had peak virus titers on day 3, with elimination of the virus occurring by day 9. In comparison, the CD4-depleted group showed a more prolonged primary infection, with peak virus titers on day 6 and elimination by day 13. This delayed clearance of virus is also reflected in the delayed clinical recovery of this group compared with the nondepleted group.

Both the CD8-depleted and the CD4-plus-CD8-depleted groups fail to clear the virus from the lung by day 13 postinfection. The high titers seen in these groups correlate with the severity of the clinical signs observed in mice at

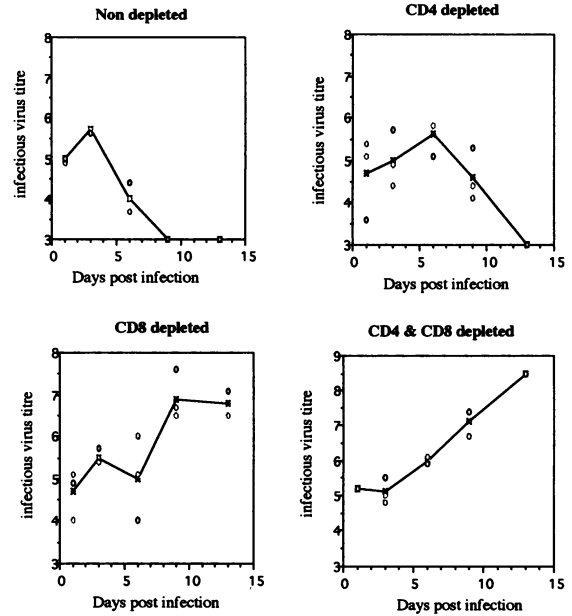


FIG. 4. Infectious virus titers in the lungs of depleted and nondepleted mice. Groups are as indicated for each graph. The mean log<sub>10</sub> PFU per lung for three mice in each group at each time point is shown, with data for individual mice plotted as separate points. The limit of detection of the assay was 3.0 log<sub>10</sub> PFU per lung.

these time points. These data clearly indicate an important role for CD8 T cells in the control of virus in the lung during the primary infection.

Infectious virus was detected in the spleens of CD8-depleted and CD8-plus-CD4-depleted animals at days 9 and 13 (approximately 2 to 3 log<sub>10</sub> PFU). In contrast, little or no virus was detected in spleens of the CD4-depleted and undepleted groups. To improve the sensitivity of the assay, the number of infectious centers was determined in the spleen at different times postinfection (Fig. 5). On day 9, infectious centers were detected in all groups, with the highest number found in CD4-plus-CD8-depleted group. By day 13, the number of infectious centers was lower in the CD4-depleted group than in the other groups, including the nondepleted group; it would appear that CD4 depletion is beneficial for virus clearance from the spleen. The impor-

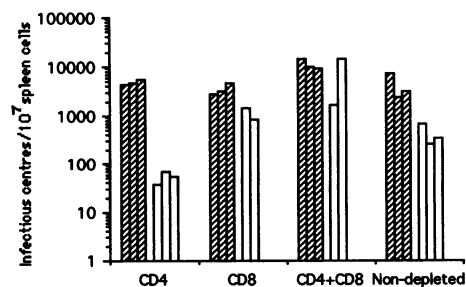
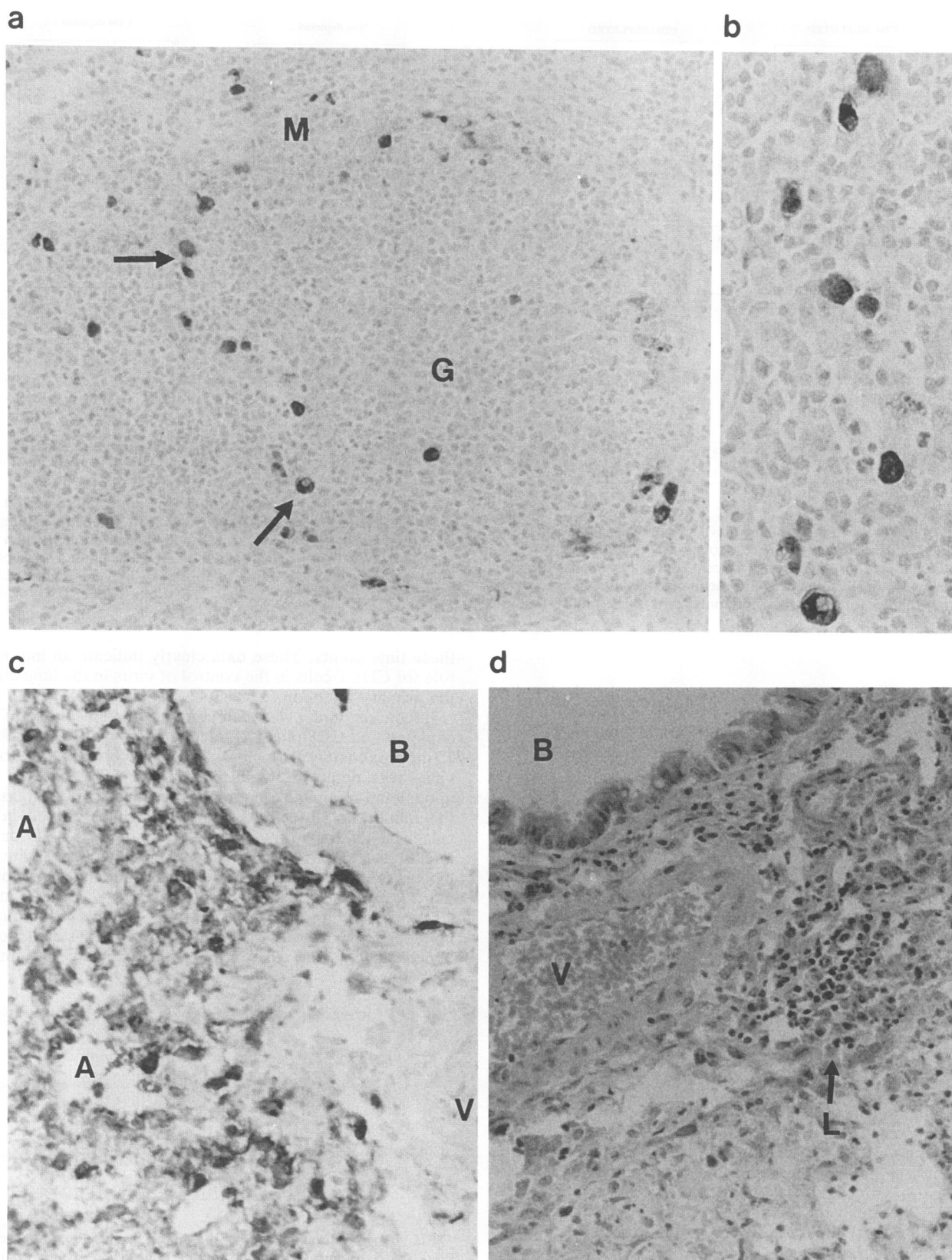


FIG. 5. Infectious centers in the spleens of depleted and nondepleted mice at day 9 (hatched columns) and day 13 (open columns) postinfection. Results for individual animals are shown following depletion of the appropriate T-cell subset as indicated. The number of infectious centers was determined as described in Materials and Methods and expressed as the number per 10<sup>7</sup> spleen cells.



**FIG. 6.** Virus antigen distribution and histopathological changes in CD8-depleted mice at day 12 postinfection. (a) Immunoperoxidase staining for MHV-68 antigen in the spleen (magnification,  $\times 250$ ); arrows indicate the area magnified ( $\times 500$ ) in panel b. (c) Immunoperoxidase staining for MHV-68 antigen in the lung (magnification,  $\times 250$ ). (d) H&E-stained section of the lung (magnification,  $\times 250$ ). M, marginal zone; G, germinal center; B, bronchiole; A, alveolus; V, blood vessel; L, lymphoid cell infiltration.

tance of CD8 cells in control of infectious centers is demonstrated by the larger numbers of infectious centers in both the CD8-depleted and double-depleted groups at day 13 than in the CD4-depleted group.

**Histopathological changes and virus antigen distribution in tissues of T-cell-depleted mice.** At days 6 and 12 postinfection, various tissues were removed and processed for histological examination. Sections were immunostained for MHV-68 antigen and counterstained with H&E as described in Materials and Methods.

At 6 days postinfection, virus was detected in the alveolar epithelium and in mononuclear cells in the lungs of all groups of mice. The intensity and extent of infection was greatest in groups depleted of CD8 and both CD4 and CD8 cells. Other tissues were largely negative at this stage, although an occasional infected cell was detected in the spleen of mice depleted of CD8 or both CD4 and CD8 cells. By day 12, the pattern of staining was markedly different. In mice depleted of CD4 cells, little or no virus antigen was detected in the lung or spleen. Similarly, in the undepleted group, viral antigens were absent from the lung, although an occasional antigen-positive cell was seen in the marginal zone of spleen. However, the lungs of both of these groups showed intense mononuclear cell infiltration in the peribronchiolar, perivascular, and interstitial areas, and there was also evidence of organization and repair (data not shown, but similar to those previously reported by Sunil-Chandra et al. [12]).

In the CD8-depleted mice, the majority of cells in the interstitial area and some cells in the peribronchiolar and perivascular tissues were positive for virus. Lymphoid cell infiltration was seen in the perivascular and peribronchiolar tissues but not in the interstitium. There was also evidence of necrosis, indicating destruction of virus-infected cells. In the spleen, several antigen-positive cells, resembling small lymphocytes or lymphoblasts, were observed in the marginal zones (Fig. 6). In the lungs of CD4-plus-CD8-depleted mice, virus was detected in the perivascular areas and in the alveolar lining cells, where extensive destruction of cells was observed. In the spleen, large blast-like cells and cells of a dendritic morphology were positive for virus. As with the CD4-depleted group, there were no active germinal centers and splenomegaly was not observed.

## DISCUSSION

Much of our knowledge about the immune response to a gammaherpesvirus infection has arisen from studies in humans infected with EBV (7, 10). These studies show the importance of T cells in immune surveillance of latently infected B cells; however, the immune mechanisms associated with the primary infection are largely unknown. To investigate this area, we have used T-cell subset-deficient mice to study a primary infection with MHV-68.

The results clearly demonstrate that CD8 lymphocytes are needed for clearance of infectious virus from the lung and spleen and also for control of the number of infectious centers (presumably B cells) in the spleen. Depletion of CD8 cells results in an overwhelming MHV-68 infection, with uncontrolled virus replication in the lung, and the mice eventually die. In these mice, higher infectious virus titers are detected in the lung and spleen, and these high titers persist until the end of the experiment (day 13). In the spleen, increased numbers of infectious centers are detected in the groups of mice deficient in CD8 T cells. The higher titers and delayed clearance of virus are reflected in the severity of the clinical signs observed in these mice and in

their failure to show clinical recovery during the course of the experiment.

Interestingly, in CD4-depleted mice, virus clearance from the lung was only marginally delayed compared with that in T-cell-intact mice. This finding indicates that in the absence of CD4 T cells, CD8 T cells become activated and function to eliminate the productive infection. However, the elimination of virus is faster in the nondepleted group, which suggests that CD4 cells provide help for CD8 cells to mediate an accelerated virus clearance. In the alphaherpesvirus model used by Nash et al. (8), clearance of HSV from infected skin was markedly delayed in CD4-depleted mice, despite the presence of CD8 T cells. However, in contrast to MHV-68 infection, depletion of CD8<sup>+</sup> lymphocytes had little or no effect on clearance of HSV from the epidermis.

In the spleens of CD8-depleted mice at day 12 postinfection, many viral antigen-positive lymphoid cells are observed, scattered around the marginal zone of the germinal centers. This is consistent with the observation that more infectious centers are detected in the spleens of these mice compared with the other groups. In contrast, in CD4-plus-CD8-depleted mice, clusters of antigen-positive cells of a dendritic morphology are seen in the red pulp. Again, these results highlight the role of CD8 cells in clearance of virus antigen from the spleen and show that CD4 depletion does not have a major effect on virus clearance in this organ.

Although CD4 T cells do not contribute greatly to the recovery from MHV-68 infection, they are involved in the development of splenomegaly and lymphoproliferation seen in response to the virus infection. It can be seen in Fig. 3 that the spleen cell counts are much lower in the CD4-depleted mice than in nondepleted mice and mice depleted of CD8 T cells. This finding is consistent with the splenic enlargement seen in previous experiments (12).

Since there are many similarities between EBV and MHV-68 infections, an understanding of the early immunological events in MHV-68 infection may shed light on the initial host response to EBV. Previous studies on EBV have shown that CD8 T cells act as a major effector mechanism in surveillance against persistently infected B cells; this evidence is based on the recognition of EBV lymphoblastoid cell lines by major histocompatibility complex class I-restricted T cells *in vitro* (10). Furthermore, studies on immunosuppressed transplant recipients (9, 14, 15) and on patients with AIDS (5) shows an increase in productively infected epithelial cells and virus shedding and in lymphoproliferative disorders. This again highlights the importance of cell-mediated immune responses in the control of a persistent infection. In view of the persistence of MHV-68 in mice, it will be interesting to determine whether T cells are important in immunosurveillance of infected cells and suppression of virus-triggered disease. Like EBV infection, MHV-68 infection is associated with lymphoma induction in chronically infected animals (11). The mouse model will provide an opportunity to study immunological control of these tumors.

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