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# Turnover of T Cells in Murine Gammaherpesvirus 68-Infected Mice

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**Respiratory challenge of C57BL/6 mice with murine gammaherpesvirus 68 induces proliferation of T lymphocytes early after infection, as evidenced by incorporation of the DNA precursor bromodeoxyuridine. Using pulse-chase analysis, splenic and peripheral blood activated T lymphocytes were found to continue dividing for at least a month after the initial virus challenge. The results are in accord with the idea that T cells are stimulated for a substantial time after the acute, lytic phase of virus infection is resolved.**

Intranasal infection of mice with murine gammaherpesvirus 68 (MHV-68) leads to productive infection of the respiratory epithelium which is controlled by $CD8^+$ T lymphocytes within 10 to 13 days of the initial exposure (3, 6) and establishes a persistent, latent infection in B lymphocytes (12, 17, 18). The infected mice show marked splenomegaly within 14 to 18 days of challenge (16), developing a blood picture similar (15) to that associated with Epstein-Barr virus-induced infectious mononucleosis (IM) of humans. Proliferating splenic lymphoid cells have been detected in mice as early as 3 to 5 days after infection and continue to be evident during the onset of IM (5, 15). Similar to the blood picture of Epstein-Barr virus-infected adolescents (9, 10), the IM-like phase is characterized by increased numbers of activated T lymphocytes in the blood of MHV-68-infected mice for at least 2 months after infection (15). Although activated $CD8^+$ T cells are more prominent, the effect is also apparent for the $CD4^+$ set. The general impression is that the massive proliferation of lymphoid cells in the spleen leads to the IM-like syndrome in the peripheral blood.

A central question is whether the protracted presence of activated lymphoid cells in the blood reflects continued proliferation or accumulation. The extent of proliferation was addressed by giving MHV-68-infected mice drinking water containing a DNA precursor, bromodeoxyuridine (BrdU). Following a pulse-labeling period, the various lymphocyte subsets were stained for expression of surface markers and BrdU in accordance with published methods (2, 14). The results confirm the earlier impression, generated by staining for cellular DNA with propidium iodide, that splenic T lymphocytes begin to proliferate early after infection and continue throughout the later IM-like phase of this disease process (5, 15). The blood picture mimics that seen in the spleen, indicating that activated lymphoid cells multiply for several weeks following the initial encounter with MHV-68.

**Characterization of cell proliferation with BrdU.** Female C57BL/6J (B6) mice, purchased from The Jackson Laboratory (Bar Harbor, Maine) were infected with MHV-68 at 6 to 10 weeks of age as previously described (15). Groups of MHV-68-infected B6 mice were given drinking water containing BrdU (Sigma Chemical Co., St. Louis, Mo.) at 0.8 mg/ml, which was made fresh and changed every day. The protocol for

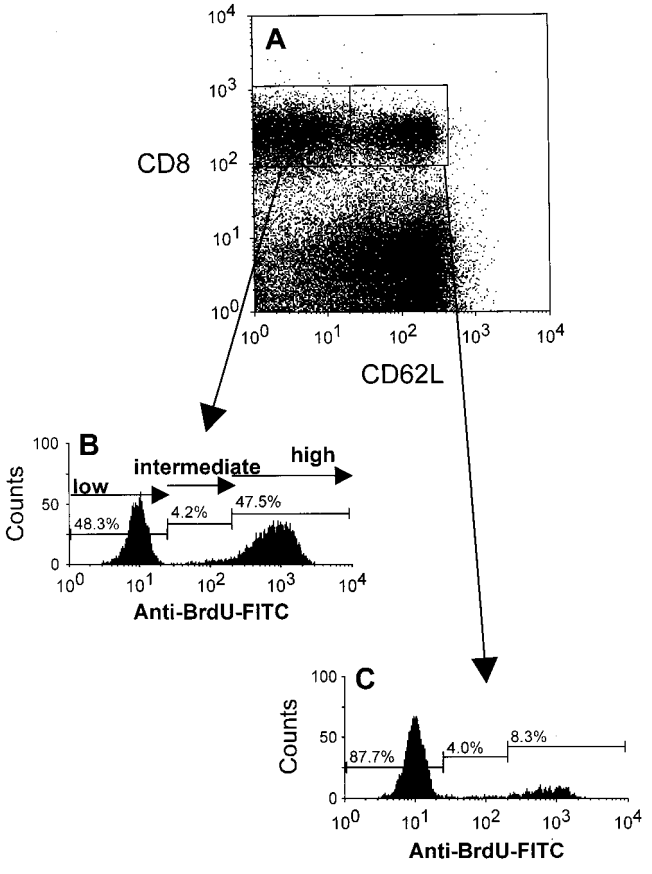


FIG. 1. Representation of the method used to determine BrdU expression in a given population of cells. After a 6-day pulse with BrdU-containing drinking water, single-cell suspensions of spleens from MHV-68-infected mice were stained for flow cytometric analysis using a phycoerythrin-conjugated anti-CD8 antibody (53-6.72; Pharmingen, San Diego, Calif.), a biotin-conjugated anti-CD62L antibody (MEL-14; Pharmingen), streptavidin-conjugated RED670 (Becton Dickinson, San Jose, Calif.), and a fluorescein isothiocyanate-conjugated anti-BrdU antibody (Becton Dickinson). (A) Gates were set around the activated ($CD8^+$ $CD62L^{lo}$) and resting ($CD8^+$ $CD62L^{hi}$) populations based on surface expression, and (B and C) histograms showing BrdU expression were drawn for each population. Based on these histograms, BrdU expression was separated into low, intermediate, and high groups and percentages of all three were determined.

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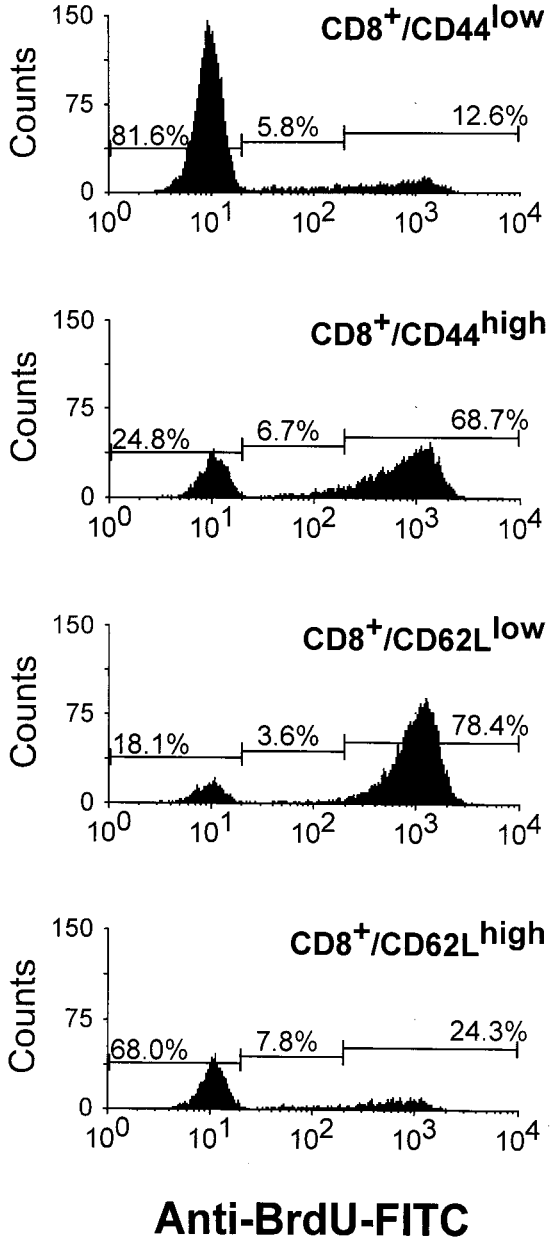


FIG. 2. Either CD44 or CD62L expression can be used to monitor BrdU incorporation in activated-T-cell populations. Mice were infected with MHV-68 and given drinking water containing BrdU for 6 days prior to days 0, 9, 15, 21, and 33 after infection, when spleens were removed and single-cell suspensions were stained. The anti-CD44–biotin monoclonal antibody (IM7) was obtained from Pharmingen. After the 6-day pulse, BrdU was removed from the drinking water of the remaining mice and the analysis was repeated at various times during the chase period. The top two panels show the BrdU staining profiles for $CD8^+$ T cells using CD44 as a measurement of activation. The lower two panels characterize the same population of $CD8^+$ T cells using CD62L for the same purpose. The percentages of cells expressing low, intermediate, and high levels of BrdU are shown in each panel. The lymphocytes were analyzed from pools of three or four mice.

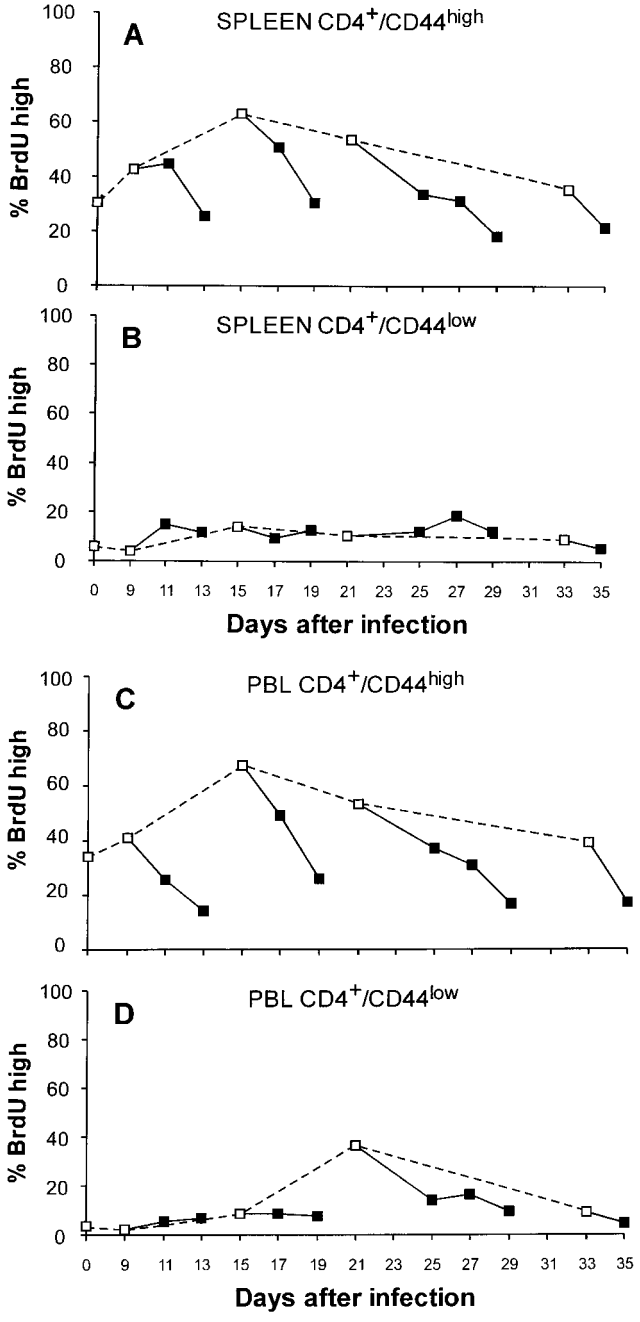


FIG. 3. BrdU incorporation into $CD4^+$ T cells from the spleens and peripheral blood of MHV-68-infected mice. B6 mice were infected with MHV-68 and given BrdU as described in the legend to Fig. 2. Flow cytometric analysis was performed as described in the legend to Fig. 1, with the exception that cells were stained with antibodies to the CD4 (phycoerythrin-conjugated anti-CD4 antibody RM4-5; Pharmingen) and CD44 surface proteins. The percentages of $CD4^+$ $CD44^{hi}$ cells that incorporated high levels of BrdU during a 6-day pulse (open squares) and during subsequent chase periods (closed squares) are depicted for the spleen (A) and peripheral blood (C). Percentages of resting $CD4^+$ T cells, those depicted in the $CD44^{lo}$ population, that express high levels of BrdU are similarly depicted for the spleen (B) and peripheral blood (D). Cells were pooled from three or four mice.

the pulse-labeling studies was that mice were given BrdU for 6 days prior to analysis and then the turnover rate of lymphoid cells was evaluated by analyzing for the disappearance of the BrdU-labeled cells through a chase period after withdrawal of the water containing BrdU. This BrdU feeding protocol (14) is sufficient to label >95% of $CD4^+8^+$ thymocytes (data not shown). At various times after infection, peripheral blood cells and spleens were taken and the cell phenotypes and activation status were determined by flow cytometric methods described elsewhere (15). Lymphocytes were then analyzed for BrdU incorporation in accordance with published methods (14).

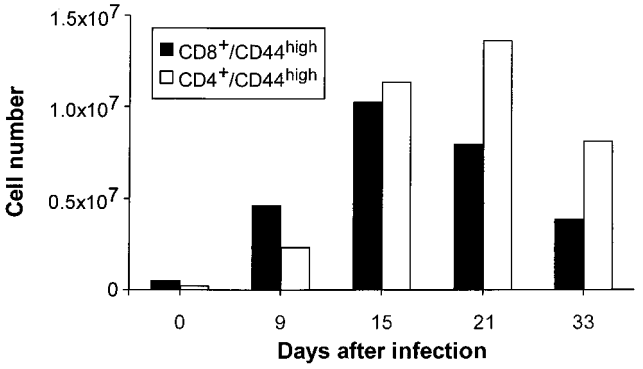


FIG. 4. Total numbers of proliferating activated T cells in the spleens of MHV-68-infected mice. The total numbers of $CD8^+$ $CD44^{hi}$ and $CD4^+$ $CD44^{hi}$ cells incorporating BrdU during a 6-day pulse were determined based on the percentages of cells incorporating BrdU within the aforementioned cell populations and the total spleen cell number (using standard hemacytometer and trypan blue exclusion tests). Each time point represents a pool of three or four mice.

Using cells obtained after a 6-day pulse, gates were set around the population of choice to obtain the percentage of BrdU incorporation (Fig. 1A, $CD8^+$ $CD62L^{lo}$ versus $CD8^+$ $CD62L^{hi}$). A histogram of BrdU expression was then plotted for the delineated set (Fig. 1B versus C), and the BrdU expression profile was characterized as low, intermediate, or high; this convention was used throughout. However, for clarity, only the percentages of cells incorporating high levels of BrdU are shown in the data that follow.

**Correlation of T-cell proliferation with activation status.** High levels of CD44 expression or low levels of CD62L identify activated T lymphocytes (1, 7, 8, 11). Those T lymphocytes with an "activated" phenotype ($CD44^{hi}$ or $CD62L^{lo}$) showed much greater levels of BrdU incorporation (Fig. 2) than those with the characteristics of naive ($CD62L^{hi}$) or resting ($CD44^{lo}$) cells.

Kinetic analysis of both spleens and peripheral blood lymphocytes established that the $CD4^+$ $CD44^{hi}$ (Fig. 3) sets began proliferating as early as day 9 after infection. This is in accord with the observation that virus-specific $CD4^+$ T cells can be detected early in the course of this infectious process by ELISpot analysis of mediastinal lymph nodes (4). These $CD4^+$ T lymphocytes continued to turn over for at least a month after MHV-68 infection. The high levels of proliferation found for activated $CD4^+$ T cells were also reflected in increased total counts for the $CD4^+$ $CD44^{hi}$ cells in the spleen, with maximal numbers being found at days 15 and 21 after infection (Fig. 4).

The level of proliferation appears to be greatest during the IM-like stage of the infection. During this time, there is also marked turnover of $CD4^+$ $CD44^{lo}$ cells in the peripheral blood. It is conceivable that these cells begin proliferating prior to increasing their levels of expression of CD44. If, indeed, at least some of the increase in activated T cells during this time is cytokine driven, it is possible that cytokines might also promote the "bystander" proliferation of antigen nonspecific cells

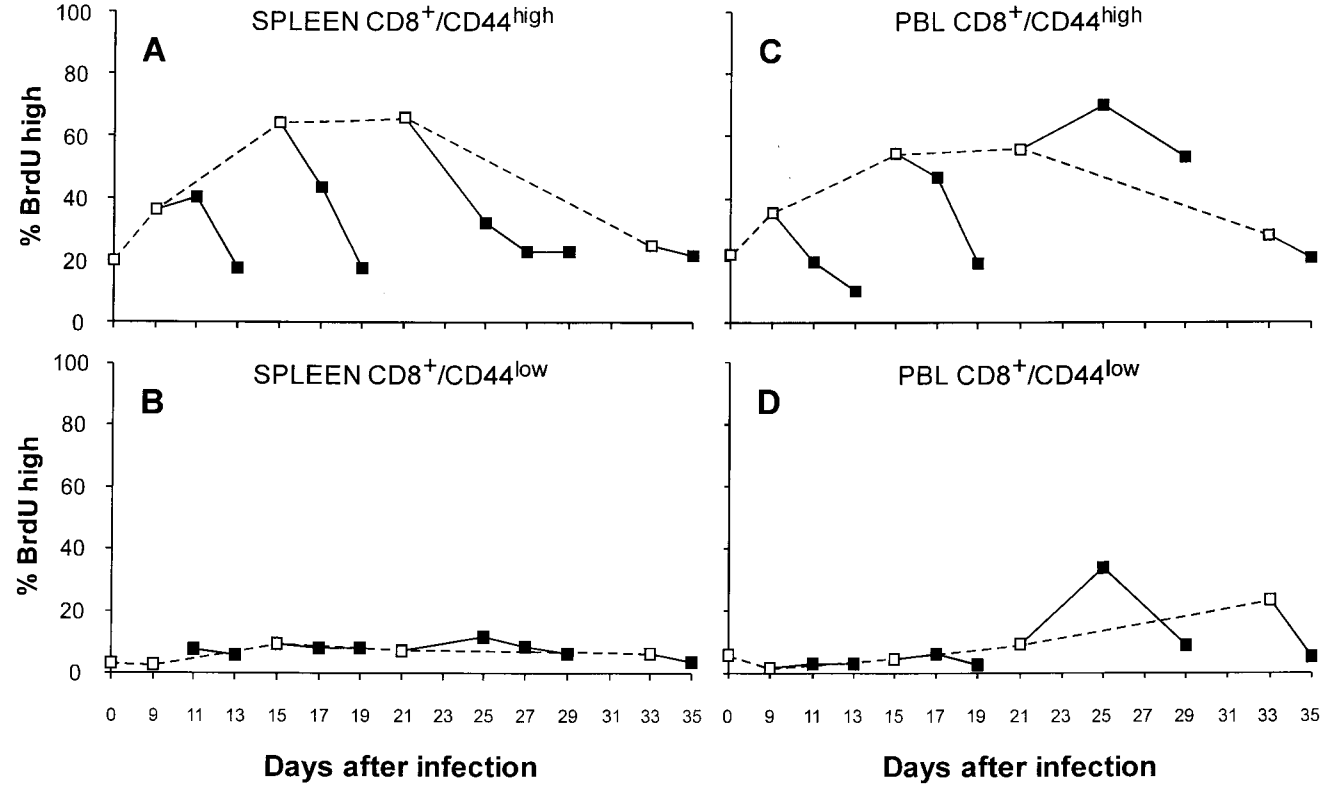


FIG. 5. BrdU incorporation into $CD8^+$ T cells from the spleens and peripheral blood of MHV-68-infected mice. Mice were infected with MHV-68 and given BrdU as described in the legend to Fig. 2. Flow cytometric analysis was performed as described in the legend to Fig. 1, with the exception that cells were stained with antibodies to the CD8 and CD44 surface proteins. The percentages of $CD8^+$ $CD44^{hi}$ cells that incorporated high levels of BrdU during a 6-day pulse (open squares) and during subsequent chase periods (closed squares) are depicted for the spleen (A) and peripheral blood (C). Percentages of resting $CD8^+$ T cells, those depicted in the $CD44^{lo}$ population, that express high levels of BrdU are similarly depicted for the spleen (B) and peripheral blood (D). Cells were pooled from the spleens and peripheral blood of three or four mice for analysis.

without a concurrent switch in the activation phenotype (13). Alternatively, there is such a high degree of turnover in the spleen at this time that trafficking of cells into and out of this and other peripheral lymphoid organs could account for the apparent increase in the level of proliferation of nonactivated $CD4^+$ T lymphocytes in the peripheral blood.

Similar to the activated $CD4^+$ T lymphocytes, proliferation of splenic $CD8^+$ $CD44^{hi}$ cells also began early after mice were infected with MHV-68 and continued for several weeks (Fig. 5). This finding was also reflected in increased total counts for the activated $CD8^+$ T lymphocytes in the spleen (Fig. 4). The proliferative activity in the peripheral blood lymphocytes was generally reflective of that in the spleen, although the result seen during the chase on day 25 could be thought to reflect an increased rate of export of proliferating T cells from the spleen. During the IM-like stage of the infection, there appear to be higher percentages of proliferating $CD8^+$ cells, both $CD44^{lo}$ and $CD44^{hi}$, in the peripheral blood. It is known that there is considerable expansion of $CD8^+$ $V\beta4^+$ cells at this time (15). What is not understood is the cell loss in this population. A high level of cell death, possibly due to the apoptosis of $CD8^+$ T cells with exhausted proliferative potential that did not incorporate BrdU during the previous pulse period, could account for the relative increase in proliferating $CD8^+$ cells on days 25 and 29 compared to day 21.

In conclusion, results from this study indicate that there are significant changes in population dynamics during MHV-68 infection. Activated T lymphocytes undergo enormous proliferation for at least the first month after infection. Evaluation of the levels of cell death due to apoptosis within these populations would add to the overall picture of cell turnover and population dynamics associated with MHV-68 infection.

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