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SHORT COMMUNICATION

Differential Antigen Recognition by T Cells from the Spleen and Central Nervous System of Coronavirus-Infected Mice

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CD8+ cytotoxic T lymphocytes (CTLs) isolated from the central nervous system (CNS) of C57Bl/6 mice acutely infected with mouse hepatitis virus, strain JHM (MHV-JHM), and analyzed in a direct ex vivo cytotoxicity assay recognize two epitopes (H-2Db- and H-2Kb-restricted encompassing amino acids 510–518 and 598–605, respectively) within the surface (S) glycoprotein. In contrast, CD8+ T cells isolated from the spleens of mice inoculated intraperitoneally with MHV-JHM and restimulated in vitro only respond to the H-2Db-restricted epitope. In this report, the preferential recognition of the H-2Db-restricted epitope is confirmed using splenocytes stimulated in vitro with either MHV-JHM-infected MC57 cells or with a cell line expressing the S protein and analyzed in secondary CTL assays. To determine whether these results represent a difference in epitope recognition between the spleen and CNS, secondary CTL assays were performed using spleen cells coated with peptides encompassing the CTL epitopes as stimulators. Under these conditions, both epitopes sensitized cells for lysis by spleen-derived CTLs, suggesting that both epitopes were recognized by splenic CD8+ T cells after infection in vivo. Furthermore, limiting dilution analysis indicated that the precursor frequency of splenic CD8+ T cells specific for both the H-2Kb- and H-2Db-restricted epitopes were not significantly different. Thus, the results suggest that in vitro stimulation of splenocytes specific for the H-2Kb-restricted epitope is inefficient after endogenous processing but that this inefficiency can be corrected if peptide is provided exogenously at sufficiently high concentrations. As a consequence, the results also show that cells responsive to both of the previously identified CNS-derived CD8+ T cell epitopes are present in the infected spleen at nearly the same frequency.

MHV-JHM causes acute encephalitis and acute and chronic demyelinating encephalomyelitis in susceptible mice and rats (3–8). The chronic disease results either if mice are infected with an attenuated variant of MHV or if they are protected from the acute encephalitis by a passive infusion of anti-viral antibodies, CD4+ T cells or CD8+ T cells (6, 9–22). This chronic neurological disease has been studied as an animal model of human demyelinating disease, including multiple sclerosis.

Both CD4+ and CD8+ T cells are required for virus clearance (23–25). Target proteins for CD8+ T cell cytotoxicity have been identified in MHV-JHM-infected BALB/c and C57Bl/6 (B6) mice. In BALB/c mice, the majority of the CTL activity is directed against the nucleocapsid (N) protein, whereas in B6 mice, the S glycoprotein is the predominant target (26, 27). Using cells isolated from the CNS of B6 mice with acute encephalitis and analyzed directly ex vivo in cytotoxicity assays, we identified two immunogenic CD8+ T cell epitopes within the S protein (1). In contrast to these results, we could not identify a significant amount of CTL activity against the S protein (26) or S-specific immunogenic peptides S510–518 or S598–605 (unpublished observations) in direct ex vivo CTL assays using cells isolated from the spleens of mice with acute encephalitis. Bergmann et al., however, were able to isolate S protein-specific CD8+ T cell clones from the spleens of B6 mice infected intraperitoneally with a sublethal dose of virus. These clones only recognized one of the two epitopes which we identified in the direct ex vivo CTL assays. The same results were obtained when spleen cells were analyzed in CTL assays after secondary stimulation in vitro with MHV-infected spleen cells (2).

In many cases, antigen-specific cytotoxicity by cells primed in vivo can only be demonstrated after secondary in vitro stimulation with antigen. Conversely, there are no reports identifying epitopes which are observed in primary but not secondary cytotoxic T cell assays. The results described above, therefore, would be most consistent with different epitope recognition between effectors harvested from peripheral lymphoid tissue and those harvested from the site of inflammation, the brain. Alternatively, insufficient presentation of the H-2Kb-restricted epitope in vitro and consequent inefficient expansion of lymphocytes primed in vivo to respond to this epitope could also explain these results.

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To determine if inadequate presentation of the H-2Kb-restricted epitope could be overcome by using a different source of antigen for stimulation, two alternative approaches were tried. First, irradiated (9000 rad), MHV-infected MC57 cells (H-2k) were used as stimulators. MC57 cells in general are not readily infected by MHV-JHM (28), but a line which was partially susceptible to the virus (approximately 10–20% of the cells were positive for viral antigen in an immunofluorescence assay) was developed. When these cells were used to stimulate splenocytes from MHV-infected mice prior to assay for cytotoxicity, only the H-2Dβ-restricted epitope was recognized (data not shown).

In a second approach, we constructed EL-4 cell clones which constitutively expressed the S glycoprotein as described previously (1). Individual G418-resistant clones were selected and monitored for S protein expression in primary CTL assays using CNS-derived lymphocytes from B6 mice with acute encephalitis. One transfectant, EL-4-S35, although not lysed as efficiently as some other transfectants, consistently maintained its stimulatory activity over an extended period of time and was used in the experiments described below. To show the long-term stimulatory capability of EL-4-S35, this clone was used as the source of antigen to stimulate the expansion of an S-specific CD8+ T cell line from the spleens of intraperitoneally infected B6 mice (Harlan – Sprague – Dawley, Indianapolis, IN). As shown in Fig. 1A, this cell line specifically lysed cells expressing S, thus showing that EL-4-S35 expressed sufficient S protein for the stimulation of precursors for analysis in secondary CTL assays. Next, we determined if the use of EL-4-S35 as a stimulator facilitated the recognition of the H-2Kb-restricted epitope by splenocytes from intraperitoneally infected B6 mice. For this purpose, splenocytes were harvested 8 days after intraperitoneal inoculation and restimulated in vitro with EL-4-S35 cells. When analyzed in CTL assays, these cells recognized the H-2Dβ, but not the H-2Kb-restricted epitope (Fig. 1B). The results from these experiments using two different types of cells expressing S protein as stimulators are the same as those obtained by Bergmann et al. using MHV-JHM-infected syngeneic spleen cells. They showed that when S protein was processed endogenously, the H-2Kb-restricted epitope was not recognized by spleen-derived lymphocytes in secondary CTL assays.

The results presented thus far are consistent with inefficient presentation of the H-2Kb-restricted epitope either in vivo during the primary response or later in vitro. Stimulation with exogenous peptide should circumvent difficulties associated with presentation of a suboptimal concentration of the H-2Kb-restricted epitope in vitro after endogenous processing. To determine if this in fact occurred, splenocytes from intraperitoneally infected mice were stimulated in vitro with splenocytes coated with peptides corresponding to the H-2Dβ- and H-2Kb-restricted epitopes. Irradiated splenocytes were coated with both peptides at a concentration of 5 μM each, washed, and added to bulk cultures of spleen cells from MHV-infected mice. As shown in Fig. 2, splenocytes stimulated in vitro with both peptides for 5 days were able to efficiently lyse targets coated with either peptide in secondary cytotoxicity assays.

These results suggested that both epitopes were recognized efficiently in the mouse and that stimulation in vitro was inefficient in the case of the H-2Kb-restricted epitope. It was not possible from these secondary CTL assays using bulk cultures to determine if there were quantitative differences in the number of CD8+ T cells responding in vivo to the two epitopes. Such quantitative differences could contribute to the results shown in Figs. 1 and 2. If in vivo priming after intraperitoneal inoculation with MHV-JHM occurred more efficiently with the H-2Dβ-restricted epitope, cells responding to the two epitopes should be present in the spleen at different concentra-
Epitopes, limiting dilution analyses (LDA) using spleen-infected B6 mice recognize the H-2D<sup>b</sup> but not the H-2K<sup>b</sup>.

Consideration to immune or naive responder splenocytes, individual wells received 50,000 viable cells. Each well was tested for cytolytic activity against 8 × 10<sup>4</sup> – 10<sup>5</sup> cells coated with peptides encompassing the two immunogenic epitopes (H-2K<sup>b</sup>-restricted aa 598-605 and H-2D<sup>b</sup>-restricted aa 510 – 518) epitope. Thirty wells were prepared for each dilution of responder cells. After 7 days of incubation at 37°, each well was tested for cytolytic activity against 8 – 10 × 10<sup>5</sup> Cr-labeled EL-4 target cells coated with either the H-2K<sup>b</sup>-restricted or the H-2D<sup>b</sup>-restricted peptide (1 µM) plated directly into the LDA plates. Control wells received uncoated EL-4 cells. Samples were processed as previously described (26). Individual cultures were considered positive if the percentage of specific release was greater than 3 standard deviations above the mean of cultures that contained stimulators alone without responders.

These assays using peptide-coated splenocytes as stimulators revealed comparable CTL precursor/effector frequencies for the H-2K<sup>b</sup>-restricted (average 1/1291, range 1/927–1/1542 spleen cells) and H-2D<sup>b</sup>-restricted (average 1/987, range 1/380–1/1323 spleen cells) epitopes in these mice. As expected, the CTL precursor/effector frequencies obtained from infected B6 mice were significantly higher than those obtained from naive B6 mice (average 1/171, 872 spleen cells and 1/660,803 spleen cells for the H-2K<sup>b</sup>- and H-2D<sup>b</sup>-restricted epitopes, respectively). Therefore, in vivo priming was equally effective for the two S-specific CD8<sup>+</sup> T cell epitopes after peripheral inoculation with virus.

Previous reports have indicated that the surface expression of H-2K<sup>b</sup> is down-regulated in cells acutely or persistently infected with MHV-JHM (31, 32). To determine if surface expression of H-2K<sup>b</sup> was down regulated in EL-4-S35 cells, the surface levels of H-2D<sup>b</sup> and H-2K<sup>b</sup> molecules were measured using FACS analysis. As shown in Fig. 4, similar amounts of the two H-2 molecules were present on EL-4-S35 cells, suggesting that preferential down-regulation of the H-2K<sup>b</sup> molecule was not the explanation for the differences in recognition between the H-2D<sup>b</sup>- and H-2K<sup>b</sup>-restricted epitopes.

Three independent studies showed that spleen-derived CD8<sup>+</sup> cytotoxic T cells from intraperitoneally infected B6 mice recognize the H-2D<sup>b</sup> but not the H-2K<sup>b</sup>-restricted epitope in secondary CTL assays. First, Berg-derived lymphocytes from B6 mice were incubated in vitro with irradiated, syngeneic spleen cells containing peptide (final concentration: 1 µM). EL-4 cells without any peptide added served as a negative control (EL-4). Average spontaneous release was <12%. Percentage of specific release was calculated as described previously (26). An average of two experiments is shown.

To compare the CTL precursor frequency for the two epitopes, limiting dilution analyses (LDA) using spleen-derived lymphocytes from B6 mice intraperitoneally infected with MHV-JHM 8 days previously and from naive B6 mice were performed (Fig. 3). Both virus-specific memory and effector cells should be present at similar concentrations in the spleen.

![FIG. 2. Recognition of CTL epitopes after stimulation in vitro with exogenous peptides. Six week-old B6 mice were inoculated intraperitoneally with MHV-JHM. Splenocytes were harvested at 8 days p.i., stimulated in vitro with irradiated, syngeneic spleen cells coated with peptides corresponding to the H-2K<sup>b</sup>- and H-2D<sup>b</sup>-restricted epitopes (final concentration: 5 µM) and analyzed as the effector population in ¹¹Cr release assays. Targets were EL-4 cells coated with the indicated peptides (final concentration: 1 µM). EL-4 cells without any peptide added served as a negative control (EL-4). Average spontaneous release was <12%. Percentage of specific release was calculated as described previously (26). An average of two experiments is shown.](image-url)

![FIG. 3. CTL precursor frequency assessed by limiting dilution analysis of spleen cells from MHV-JHM-infected and naive B6 mice. Limiting dilution analyses using infected and naive mice were performed on the same day to minimize variability. Splenocytes from B6 mice inoculated intraperitoneally with virus (A) or naive mice (B) were harvested 8 days p.i. They were then stimulated in vitro with irradiated, syngeneic spleen cells coated with peptides encompassing the two immunogenic epitopes (H-2K<sup>b</sup>-restricted aa 598-605 and H-2D<sup>b</sup>-restricted aa 510 – 518) in a limiting dilution assay as described in the text. Each group comprised 3 – 4 mice for each experiment. Cytotoxic activity was assayed by lysis of peptide-coated, ¹¹⁵Cr-labeled, EL-4 cells. The average spontaneous release was <13%. CTL precursor frequencies were calculated using the weighted mean method (36). A representative experiment of four similar studies is shown for both MHV-JHM-infected and naive B6 mice.](image-url)
mann et al. isolated splenic lymphocytes from MHV-JHM-infected B6 mice and analyzed their antigen specificity in cytotoxic T cell assays after in vitro stimulation with MHV-infected splenocytes. The results indicated that only the H-2D^b-restricted epitope was recognized. Similar results were obtained when a panel of CD8^+ T cell clones were analyzed (2). Second, Heemskerk et al., using mice intraperitoneally infected with MHV-A59, a hepatotropic strain closely related to MHV-JHM, were unable to demonstrate any CD8^+ class I-restricted cytotoxicity to MHV-A59 in secondary cytotoxic T cell assays (33). The S glycoprotein of MHV-A59 is deleted in the region that includes the H-2D^b-restricted epitope although the H-2K^b-restricted epitope is present. MHV-A59 causes lethal hepatitis with prominent cellular infiltration in susceptible strains. It is possible that direct ex vivo analysis of lymphocytes isolated from infected livers would demonstrate the presence of an H-2K^b-restricted cytotoxic response. All of the experiments described by Heemskerk et al. included secondary stimulation in vitro with MHV-infected syngeneic spleen cells, a step which our study indicates inadequately stimulates the H-2K^b-restricted precursors, leading to a skewed result. Third, the data presented in this study show that splenocytes derived from mice intraperitoneally infected with MHV-JHM and stimulated in vitro with MHV-JHM-infected MC57 cells or a cell line expressing the S protein recognized only the H-2D^b-restricted epitope in cytotoxicity assays.

In contrast to these data, use of peptide-coated splenocytes as stimulators in secondary CTL assays or in limiting dilution assays resulted in proliferation of cells able to recognize both the H-2D^b- and H-2K^b-restricted epitopes. The LDA showed that similar CTL precursor frequencies were measured for the two epitopes and suggested that in vivo priming in the spleen was similarly efficient for both epitopes. These data are most consistent with inefficient in vitro stimulation of the H-2K^b-restricted CD8^+ T cells. Antigen is likely to be processed by different antigen presenting cells (APCs) in vivo and in vitro and at different sites in vivo. The microenvironment may allow adequate presentation of the H-2K^b-restricted epitope in vivo but this may not occur in vitro. Although some H-2K^b-restricted epitope is likely expressed on the cell surface of APCs in vitro, this amount may be inadequate to stimulate in vivo primed cells, in part, because sensitization of target cells for half maximal lysis by the H-2K^b-restricted peptide requires 250-fold as much peptide as required for sensitization by the H-2D^b-restricted peptide (1). Use of peptide-coated spleen cells as stimulators probably resulted in a higher concentration of the H-2K^b peptide on the cell surface, providing adequate levels of MHC–peptide complexes to restimulate cells primed previously in vivo.

Many previous experiments have shown that cytotoxic T cell assays performed after secondary stimulation in vitro are far more sensitive than are primary CTL assays (34). In the case of viruses, secondary stimulation is often done using virus-infected APCs. Our results show that, in at least one case, secondary stimulation by infected APCs with the requirement for protein processing prior to peptide presentation on the surface may be inadequate to allow the expansion of CTLs adequately primed in vivo.

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