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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-$2D^b$- and H-$2K^b$-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-$2D^b$-restricted epitope. In this report, the preferential recognition of the H-$2D^b$-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-$2K^b$- and H-$2D^b$-restricted epitopes were not significantly different. Thus, the results suggest that *in vitro* stimulation of splenocytes specific for the H-$2K^b$-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-$2K^b$-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-2K^b-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-2^b) 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-2D^b-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-2K^b-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-2D^b, but not the H-2K^b-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-2K^b-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-2K^b-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-2K^b-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-2D^b- and H-2K^b-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.

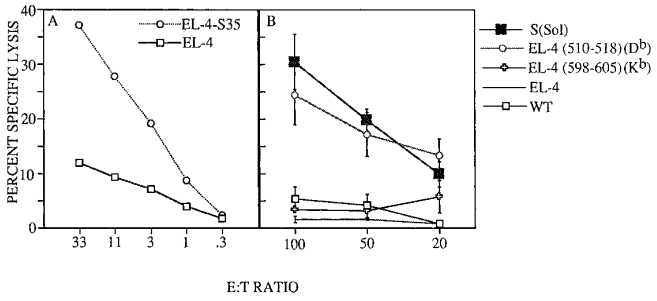

FIG. 1. Recognition of CTL epitopes at amino acids 510–518 (H-2D^b) and 598–605 (H-2K^b) by spleen-derived CD8$^+$ cytotoxic T lymphocytes. (A) An anti-S CD8$^+$ T cell line was developed and tested for cytotoxic activity against EL-4-S35 in chromium release assays. To develop this CD8$^+$ T cell line specific for the S protein, B6 mice were infected intraperitoneally (i.p.) with a sublethal dose of MHV-JHM (2.5×10^5 PFU). Eight days later, splenocytes were harvested as described previously (*35*). $35–50 \times 10^6$ splenocytes were incubated for 5 days with 4×10^6 irradiated (20,000 rad) EL-4-S35 transfectants in 20 ml RPMI media supplemented with 10% FCS, 2 m*M* glutamine, 5×10^{-5} *M* β-mercaptoethanol, and antibiotics (complete RPMI). Subsequently, viable cells were harvested, washed, and restimulated every 7 to 9 days in 24-well flat-bottom tissue culture plates. For this purpose, $1.25–2.5 \times 10^5$ CD8$^+$ T cells were mixed with 1.5×10^5 irradiated EL-4-S35 cells and $3–5 \times 10^6$ irradiated (3000 rad) syngeneic spleen cells in 2 ml complete RPMI supplemented with 5% rat concanavalin A supernatant containing 50 m*M* methyl-α-D-mannopyranoside. (B) Spleen cells were harvested 8 days after i.p. inoculation with MHV-JHM. After 5 days *in vitro* stimulation with EL-4-S35 in complete RPMI, cells were harvested and analyzed in ^{51}Cr release assays using the indicated peptides (1 μM final concentration) and E:T ratios. Each point represents an average of at least three mice and error bars show the standard error for each point. The same results were obtained if 5% rat concanavalin A supernatant containing 50 m*M* methyl-α-D-mannopyranoside was added to the media to provide exogenous growth factors (data not shown). S(Sol) and WT were MC57 cells infected with recombinant VV expressing a C-terminal truncated form of the S protein (26) and wild-type VV, respectively. EL-4 cells without any peptide added served as negative control (EL-4). The average spontaneous release for EL-4 targets corresponded to $<$13% and for MC57 cells $<$25%. Percentage of specific release was calculated as described previously (*26*).

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-2K^b-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-2D^b-restricted epitope, cells responding to the two epitopes should be present in the spleen at different concentra-

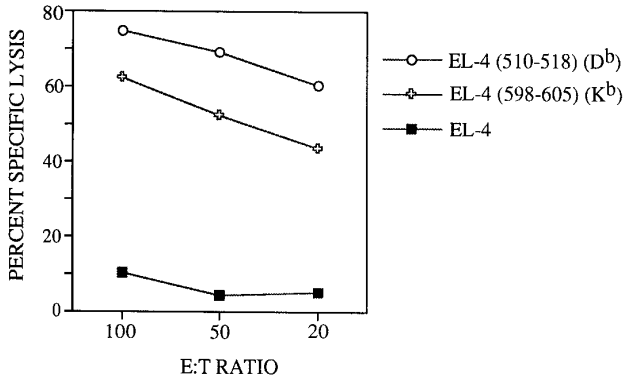

FIG. 2. Recognition of CTL epitopes after stimulation *in vitro* with exogenous peptides. Six week-old B6 mice were inoculated intraperitoneally with MHV-JHM. Splenocytes were harvested at 8 days p.i., stimulated *in vitro* with irradiated, syngeneic splenocytes coated with peptides corresponding to the H-2K^b- and H-2D^b-restricted epitopes (final concentration: 5 μM) and analyzed as the effector population in ^{51}Cr 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.

tions. On the other hand, if inefficient expansion occurring *in vitro* is the sole explanation for the different results presented above, precursors to the two epitopes should be present at similar concentrations in the spleen.

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 in the spleen at this time (*29*), although only memory cells may be measured in this secondary CTL assay (*30*). In these assays, threefold serial dilutions of responder spleen cells from individual mice were plated in a volume of 200 μl in the wells of 96-well round bottom tissue culture plates (Costar, Cambridge, MA) in complete RPMI media supplemented with 5% rat concanavalin A supernatant containing 50 m*M* methyl-α-D-mannopyranoside. In addition to immune or naive responder splenocytes, individual wells received 5×10^5 irradiated (3000 rad) syngeneic spleen cells which had been coated with peptide (10 μM) corresponding to either the H-2K^b-restricted (amino acids 598–605) or the H-2D^b-restricted (amino acids 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 8–10×10^3 ^{51}Cr-labeled EL-4 target cells coated with either the H-2K^b-restricted or the H-2D^b-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^b- (average 1/1291, range 1/927–1/1542 spleen cells) and H-2D^b-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^b- and H-2D^b-restricted epitopes, respectively). Therefore, *in vivo* priming was equally effective for the two S-specific CD8$^+$ T cell epitopes after peripheral inoculation with virus.

Previous reports have indicated that the surface expression of H-2K^b is down-regulated in cells acutely or persistently infected with MHV-JHM (*31, 32*). To determine if surface expression of H-2K^b was down regulated in EL-4-S35 cells, the surface levels of H-2D^b and H-2K^b 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^b molecule was not the explanation for the differences in recognition between the H-2D^b- and H-2K^b-restricted epitopes.

Three independent studies showed that spleen-derived CD8$^+$ cytotoxic T cells from intraperitoneally infected B6 mice recognize the H-2D^b- but not the H-2K^b-restricted epitope in secondary CTL assays. First, Berg-

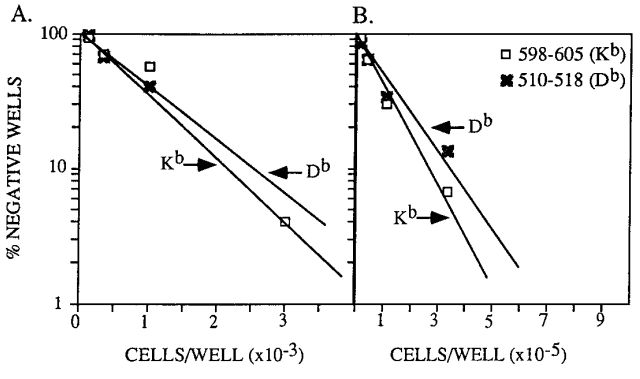

FIG. 3. CTL precursor frequency assessed by limiting dilution analysis of spleen cells from MHV-JHM-infected and naive B6 mice. Limiting dilution analyses using infected and naive mice were performed on the same day to minimize variability. Splenocytes from B6 mice inoculated intraperitoneally with virus (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^b-restricted-aa 598-605 and H-2D^b-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, ^{51}Cr-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.

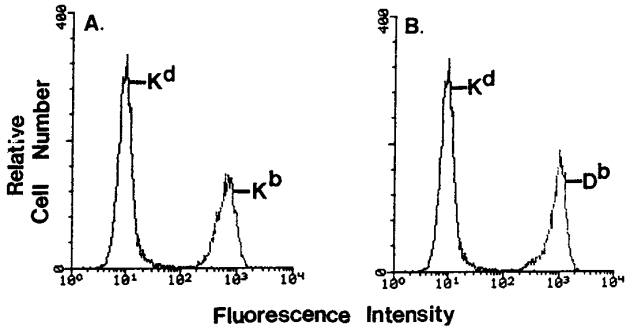

FIG. 4. FACS analysis of H-2K^b and H-2D^b expression by the EL-4-S35 cell clone. (A) EL-4-S35 cells were stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-H-2K^b antibody (Y3, ATCC HB 176) (K^b). (B) EL-4-S35 cells were stained sequentially with unconjugated mouse anti-H-2D^b antibody (B22-249) and FITC-conjugated goat anti-mouse antibody (D^b). Background fluorescence was determined by staining cells with FITC-conjugated anti-H-2K^d antibody (SF1-1.1.1, ATCC HB 159). Stained cells were analyzed on a FACScan (Becton–Dickinson, Mountain View, CA) interfaced to a Hewlett–Packard computer. Forward angle light scatter and side scatter were used to exclude dead cells from analysis. Data were collected on 5,000–10,000 cells.

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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