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Epitope Specificity of H-2K^b-Restricted, HSV-1-, and HSV-2-Cross-Reactive Cytotoxic T Lymphocyte Clones

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HSV-1-specific and HSV-1/HSV-2-cross-reactive H-2K^b-restricted cytotoxic T lymphocyte (CTL) clones were derived from a pool of splenic memory CTL (CTLm) obtained from HSV-1-infected C57BL/6 mice. Two of the HSV-1/HSV-2-cross-reactive CTL clones recognized HSV gB since H-2^b cells infected with a recombinant adenovirus vector expressing HSV glycoprotein B (gB) provided a target for these CTL clones. The CTL recognition epitope was precisely defined as HSV-1 gB residues 498–505 using synthetic peptides and conforms to a predicted H-2K^b-binding motif. Immunization of C57BL/6 mice with the free synthetic peptide corresponding to this predicted minimal epitope (HSV-1 gB498–505) resulted in the generation of HSV-gB epitope-specific CD8$^+$ CTL in the popliteal lymph nodes. The peptide-induced CTL recognize and lyse HSV-1 infected H-2^b cells or cells pulsed with the synthetic peptide, gB498–505. The availability of CTL clones directed to this predicted minimal HSV CTL epitope should be helpful in understanding processing of HSV glycoprotein B and presentation of this CTL recognition epitope.

INTRODUCTION

The nature of the immune response to HSV infection is complex and involves elements of both humoral and cell-mediated immunity. To date, HSV infections have been shown to be modulated by immunizing experimental hosts with the virus or with viral glycoproteins, expressed constitutively in cells, introduced in viral vectors carrying the glycoprotein genes, or administered alone (reviewed in Mester and Rouse, 1991). Immune mechanisms that mediate such resistance to HSV infection include virus neutralizing antibodies, antibody-dependent cellular cytotoxicity (ADCC), macrophages, NK cells, CD4$^+$ helper T lymphocytes, and CD8$^+$ cytotoxic T lymphocytes (CTL) (Mester and Rouse, 1991). Although the neutralizing antibodies should be effective in inactivating extracellular virus, the ability of HSV to remain intracellularly and to infect contiguous cells without entering the extracellular space (Roizman and Sears, 1990) suggests that T lymphocyte-mediated immunity plays a decisive role in limiting the spread of infectious virus by destroying virus-infected cells.

T lymphocytes control the progression of a variety of viral infections (reviewed in Doherty *et al.*, 1992) including HSV (Bonneau and Jennings, 1989, 1990; Mester and Rouse, 1991) and cytomegalovirus (Del Val *et al.*, 1991; Koszinowski *et al.*, 1992; Riddell *et al.*, 1992). These T cells, through their surface receptors, recognize viral antigens on the surface of infected cells in association with MHC class I or class II antigens (Zinkernagel and Doherty, 1979; Townsend and Bodmer, 1989; Braciale and Braciale, 1991; Yewdell and Bennink, 1992). Adoptive transfer studies using experimental mouse models have demonstrated that CTL, if present at the time of HSV infection, are able to protect mice from primary, acute HSV infection and limit the establishment of latent infection in the associated ganglia regardless of the initial site of infection (Bonneau and Jennings, 1989, 1990). However, identification of the epitopes recognized by these T lymphocytes, especially CD8$^+$ CTL, has been difficult, in part, due to a large number of proteins encoded by the HSV genome (McGeoch *et al.*, 1985, 1988; Roizman and Sears, 1990). Early evidence had suggested that HSV-encoded glycoproteins can provide the target antigens for HSV CTL (Lawman *et al.*, 1980; Carter *et al.*, 1981). More recently, both HSV structural and nonstructural proteins have also been shown to provide a target for HSV-specific CTL (McLaughlin-Taylor *et al.*, 1988; Martin *et al.*, 1988; Johnson *et al.*, 1990; Martin *et al.*, 1990; Banks *et al.*, 1991; Hanke *et al.*, 1991).

A key consideration in understanding the role of CD8$^+$ CTL in controlling viral infections is the identification of epitopes recognized by the T cell receptor and an understanding of the T cell repertoire that is induced following infection with a particular virus. The CTL recognition epitopes that are able to associate with a particular MHC haplotype can be predicted on the basis of

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the presence of dominant anchor residues as deduced by sequencing the peptides that associate with the MHC class I antigens (Van-Bleek and Nathenson, 1990; Falk *et al.*, 1991; Rotzschke and Falk, 1991). Because of their ability to bind to MHC class I molecules, it is hypothesized that these epitopes may be able to provide a target for virus-specific CTL and to elicit the generation of CTL *in vivo*.

The T lymphocyte response to at least one HSV encoded glycoprotein, gB, has been demonstrated to play a role in the overall immune response to HSV infection in H-2^b, and H-2^d mice (McLaughlin-Taylor *et al.*, 1988; Witmer *et al.*, 1990; Hanke *et al.*, 1991). In the H-2^b mice, this gB-specific CTL response was shown to constitute approximately 5–10% of all the HSV-specific CTL precursors. Recent studies (Hanke *et al.*, 1991) utilizing adenovirus vectors expressing gB with various deletions, and overlapping synthetic peptides, have localized an H-2K^b-restricted epitope in HSV-1 gB to an 11 amino acid (residues 497–507) segment and an H-2D^d-restricted epitope to within residues 233–379 of gB.

In an attempt to understand the role of a dominant H-2K^b-restricted HSV gB epitope in the induction of a CTL response, we have generated CTL clones from a polyclonal population of HSV-specific memory CTL (CTLm) generated in B6 mice immunized with HSV-1. This CTL population has been previously shown to be active in clearing virus from the site of primary infection and to limit the establishment of latent infection *in vivo* upon adoptive transfer into B6 mice (Bonneau and Jennings, 1989, 1990). Two CTL clones lysed both HSV-1- and HSV-2-infected H-2^b cells and specifically recognize the minimum and optimal gB CTL epitope (residues 498–505) in association with H-2K^b class I antigen. The HSV-1 specific CTL clones did not recognize an epitope within gB. We have further demonstrated that immunization of B6 mice with this predicted minimal epitope peptide is able to elicit the generation of CD8$^+$ epitope-specific CTL.

MATERIALS AND METHODS

Cells and viruses

The B6/WT-3 cell line, an SV40-transformed mouse embryo fibroblast cell line derived from C57BL/6 (H-2^b) mice (Pretell *et al.*, 1979), was maintained in Dulbecco's modified Eagle's medium supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS), 20 m*M* HEPES buffer, 0.075% (wt/vol) $NaHCO_3$, 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. HSV-1 (strain Patton) and HSV-2 (strain 186) stocks were propagated in Vero cells, a continuous line of monkey kidney cells, by infection at a multiplicity of infection (m.o.i.) of 0.01, and virus titers were determined by plaque assay on Vero cells. The adenovirus vector (AdgB2) recombinant (Johnson *et al.*, 1988), which expresses HSV-1 glycoprotein B in infected cells, was propagated in the human embryonic lung cell line 293 (Graham and Smiley, 1977; Harrison *et al.*, 1977). Ad dl327, an E3$^-$ deletion mutant, was kindly provided by Dr. David J. Spector, The Pennsylvania State University College of Medicine. All virus stocks were stored at −70°.

Mice

Male C57BL/6 (B6) mice were obtained from Jackson Laboratories at 5–8 weeks of age and were housed four mice per cage in standard metal cages. All mice were maintained on a 12-hr light/dark cycle and were allowed to acclimate to such conditions for a 2-week period prior to any experimental manipulations. Food and water were provided *ad libitum*.

Synthetic peptides

Synthetic peptides corresponding to the HSV-1 gB residues 498–505 (gB498–505; SSIEFARL) and 497–507 (gB497–507; TSSIEFARLQF) were synthesized at the Macromolecular Core Facility of the M. S. Hershey Medical Center by 9-fluorenylmethyl-oxycarbonyl chemistry using an automated peptide synthesizer. The purity and amino acid composition of each peptide were determined by high-performance liquid chromatography tracing (Waters 600E) and the Pico Tag Amino Acid Analysis System (Water [a division of Milli-Gen]). Peptide stock solutions were made by solubilizing the lyophilized peptides in DMSO (no more than 5% of total volume) and adjusted to the proper concentration with RPMI supplemented with 10% heat-inactivated FBS.

Generation of HSV-specific polyclonal CTL and CTL clones

The generation of a splenic-derived, H-2K^b-restricted polyclonal CTL population, which recognized HSV-1 infected cells has been described previously (Bonneau and Jennings, 1990). CTL clones were established from this polyclonal population by limiting dilution. Briefly, into each well of a 96-well U-bottomed plate (Costar) was placed, on average, one polyclonal CTL, 1×10^4 stimulator cells (mitomycin C-treated, HSV-1 infected B6/WT-3 cells; Bonneau and Jennings, 1990), 1×10^5 γ-irradiated (2000 rad) splenic lymphocytes obtained from nonimmunized C57BL/6 mice, 10% (v/v) Polyclone (Collaborative Biomedical, Bedford, MA), and methyl-α-D-mannopyranoside (0.05 *M*) in a total volume of 200 μl of supplemented Iscoves's-modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 0.0225% $NaHCO_3$, 25 m*M* HEPES buffer, 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 50 μm 2-mercaptoethanol. Cells from

those wells that exhibited growth within 14 days (33% of total wells) were expanded into 24-, and later, 12-well plates in the presence of stimulator cells, Polyclone, and methyl-α-D-mannopyranoside as described above. The continued *in vitro* propagation of these cell lines was maintained as for the polyclonal CTL described above.

Generation of HSV-specific CTL by immunization of mice with HSV

HSV-specific CTL were generated by the procedures based on protocols previously described by Pfizenmaier *et al.* (Pfizenmaier *et al.*, 1977a,b) and Carter *et al.* (Carter *et al.*, 1981). Briefly, mice were infected in the rear footpads by an intraplantar injection of 1×10^6 PFU HSV-1 Patton in a volume of 50 μl. Five days after immunization, the popliteal lymph nodes were removed and a single cell suspension was prepared. Cells (2×10^7) were cultured at 37° in 5% CO_2 for 3 days in supplemented IMDM.

Generation of HSV gB-specific CTL by immunization of B6 mice with HSV gB synthetic peptide

Mice were injected in each rear footpad (intraplantar) with 200 μg gB498–505 peptide in a volume of 25 μl. The peptide was first solubilized in DMSO (no more than 5% of total injected volume), diluted in PBS, then emulsified in TiterMax (CytRx Corporation; Norcross, GA). Popliteal lymph nodes were removed at 8 days postimmunization and lymphocytes were cultured in supplemented IMDM either in the absence or presence of stimulator cells as described in the text.

In vitro depletion of T lymphocytes

Following the 3-day *in vitro* incubation period, lymph node cells were depleted of either $CD4^+$ or $CD8^+$ subpopulations as described previously (Flyer *et al.*, 1982). Briefly, lymphocytes were treated with either the mAb RL172.4 (Ceredig *et al.*, 1985) (rat IgM), specific for murine CD4, or HO-2.2 (Raulet *et al.*, 1980) (mouse IgM), specific for murine CD8 (Lyt 2.2), for 60 min at 4°. MAb RL172.4 was kindly provided by Stephen Jennings, The Louisiana State University School of Medicine at Shreveport. Cells were pelleted by centrifugation followed by resuspension and incubated (37°/45 min) in Low-Tox M rabbit C′ (Cedarlane, Accurate Scientific Products, Westbury, NY). Cells were subjected to a second round of antibody and C′ together for 45 min at 37°, washed twice, and used in the ^{51}Cr release assay.

Fluorescent flow cytometric (FFC) analysis

The efficacy of *in vitro* depletion of the $CD4^+$ and $CD8^+$ populations was accessed by FFC analysis. Briefly, following *in vitro* treatment with antibody and complement, cells were washed and resuspended in PBS containing 2% FCS and 0.1% NaN_3 (FACS buffer) at a concentration of 5×10^6 per ml. A 100 μl volume was pelleted and resuspended in 100 μl of FITC-conjugated anti-Ly-2 (CD8a) or PE-conjugated anti-L3T4 (CD4) (Pharmingen, San Diego, CA). Cells were incubated for 60 min at 4°, washed twice in FACS buffer, and resuspended in FACS buffer and fixed in 1% (w/v) paraformaldehyde. Cells were analyzed using an Epics V flow cytometer/sorter (Coulter Electronics, Inc., Hialeah, FL) at an excitation wavelength of 488 nm.

Cytotoxicity assay

The standard cytotoxicity assay using ^{51}Cr-labeled target cells has been described previously (Carter *et al.*, 1981). Briefly, potential target cells were infected with the respective virus (HSV-1 strain Patton or HSV-2 strain 186) at an m.o.i. of 10 for 1 hr at which time the virus inoculum was removed. The infection was allowed to proceed for a period of time as described in the text. Target cells were labeled with 150 μCi of ^{51}Cr for 1 hr at 37°. To prepare peptide-pulsed target cells, uninfected, labeled cells (5×10^5–1×10^6) were incubated with the appropriate concentration of the respective peptide for 30 min at 37°. The labeled target cells were washed three times and resuspended at a concentration of 1×10^5 cells per ml in medium and 100 μl of suspension was dispensed into 96-well V-bottom tissue culture plates (Costar). To each well was added the appropriate amount of CTL (lymph node-derived polyclonal CTL or CTL clones) in 100 μl to give the desired effector-to-target cell ratio. The plates were centrifuged at 45 *g* for 3 min, incubated at 37° for 4–5 hr, then centrifuged at 180 *g* for 5 min. One hundred microliters of supernatant were removed from each well and the amount of radioactivity released into the supernatant was determined in a Beckman gamma counter. Percent specific lysis was calculated as $[(E - S)/(M - S)] \times 100$, where E is the counts per minute released from the target cells incubated in the presence of effector cells, S is the counts per minute released from target cells in medium alone, and M is the maximum counts per minute released from target cells in the presence of 5% sodium dodecyl sulfate.

RESULTS

Isolation of HSV-1-, HSV-2-cross-reactive, and H-2K^b-restricted CTL clones

The generation of a population of long-term, polyclonal, HSV-specific, H-2K^b-restricted CTL from B6 mice immunized with HSV-1 has been described previously (Bonneau and Jennings, 1989, 1990). The lytic activity of these polyclonal CTL was assessed against B6/WT-

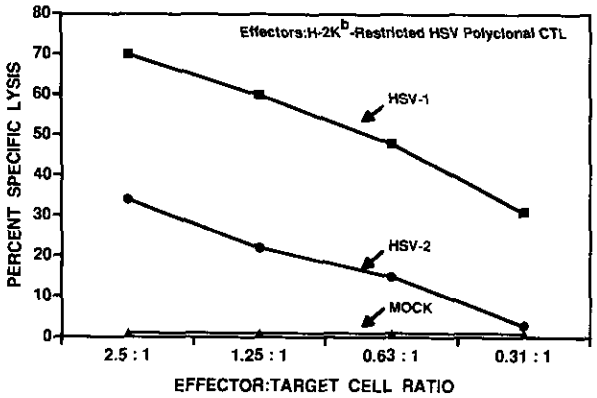

FIG. 1. Recognition of HSV-1- and HSV-2-infected B6/WT-3 (H-2^b) cells by HSV-1 induced H-2K^b-restricted polyclonal CTL. B6/WT-3 (H-2^b) cells were infected with either HSV-1 or HSV-2 at an m.o.i. of 10 and harvested 14 hr postinfection. The infected and uninfected B6/WT-3 cells were labeled with 150 μCi of ^{51}Cr and used as target cells at the indicated effector-to-target cell ratios in a 5-hr ^{51}Cr release assay.

3 syngeneic cells infected with either HSV-1 (strain Patton) or HSV-2 (strain 186). As shown in Fig. 1, these CTL recognized epitopes expressed on both HSV-1- and HSV-2-infected target cells. Lysis of uninfected target cells was at background level.

CTL clones were established from this polyclonal CTL line by limiting dilution as described under Materials and Methods and the lytic activity of the CTL clones was assessed against both HSV-1-infected and HSV-2-infected B6/WT-3 cells (Fig. 2). At least two distinct groups of CTL clones were isolated; one of which exhibited cross-reactive lytic activity against both HSV-1- and HSV-2-infected B6/WT-3 cells (clones 2D5 and 2C7) and another which demonstrated HSV-1 type specificity (clones 1D11, 1G5, 2D1, and 2G5).

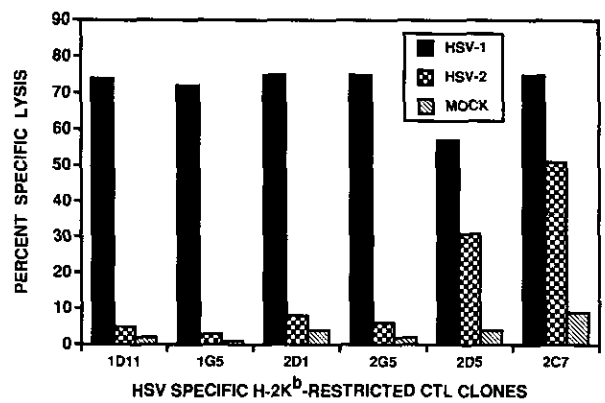

FIG. 2. HSV type-specific recognition exhibited by HSV-specific, H-2K^b-restricted CTL clones. CTL clones were established from the H-2K^b-restricted polyclonal CTL population by limiting dilution as outlined under Materials and Methods. Representative clones were tested for lytic activity against both HSV-1- and HSV-2-infected B6/WT-3 cells as described in the legend for Fig. 1 in a 5-hr ^{51}Cr release assay at an effector-to-target cell ratio of 2:1.

TABLE 1

SPECIFICITY OF HSV-1 AND HSV-2 CROSS-REACTIVE H-2K^b-RESTRICTED CTL CLONES FOR HSV GLYCOPROTEIN gB

	Percent specific lysis from target cells (B6/WT-3) infected with				
CTL clone	HSV-1	HSV-2	AdgB2	Ad dl327	Mock
HSV 1D11	59.1	3.8	8.4	0.0	2.8
HSV 1G5	56.5	6.2	12.4	0.9	3.8
HSV 2D1	53.0	5.8	8.4	3.6	2.8
HSV 2G5	52.0	5.3	8.6	1.4	3.8
HSV 2D5	38.9	47.7	66.9	4.9	8.4
HSV 2C7	49.1	69.5	82.9	14.3	14.4

Note. B6/WT-3 cells were infected with HSV-1 and HSV-2 at an m.o.i. of 10 and harvested 12 hr postinfection. Another group of B6/WT-3 cells were infected with either AdgB2 or Addl327 at an m.o.i. of 20 and harvested 18 hr postinfection. The infected cells along with uninfected B6/WT-3 cells were labeled with 150 μCi of ^{51}Cr for 1 hr and assayed for susceptibility to lysis by the HSV CTL clones at a 2:1 effector to target cell ratio in a standard ^{51}Cr release assay.

Mapping of the epitope recognized by HSV-1/HSV-2-cross-reactive CTL clones

Studies have demonstrated that HSV-encoded glycoprotein B (gB) functions as a recognition site for HSV-specific CTL from B6 mice (Witmer *et al.*, 1990). To determine if any of the representative CTL clones described above recognize an epitope encoded for within gB, a recombinant adenovirus containing the coding region for HSV-1 gB (AdgB2) (Johnson *et al.*, 1988) was used to express gB in B6/WT-3 cells. The results presented in Table 1 show that the HSV-1 and HSV-2 cross-reactive CTL clones 2D5 and 2C7 recognized and lysed B6/WT-3 cells infected with AdgB2, suggesting that these CTL recognize one or more epitopes in HSV gB. In contrast, the HSV-1-specific CTL clones did not lyse the AdgB2-infected cells, suggesting that the epitope(s) recognized by this group of CTL clones were not located within HSV gB. No significant lytic activity was observed against either uninfected cells or those cells infected with the E3$^-$ deletion mutant Ad dl327.

Recent studies have localized an HSV-specific, H-2K^b-restricted CTL epitope within gB to amino acids 497–507 using overlapping synthetic peptides (Hanke *et al.*, 1991). To determine if the gB-specific CTL clones recognized this H-2K^b-restricted CTL recognition site within HSV gB, B6/WT-3 cells were pulsed with a synthetic peptide corresponding to this sequence and used as target cells for recognition by CTL clones 2D5 and 2C7. The results presented in Fig. 3 show that both HSV-1/HSV-2-cross-reactive CTL clones recognized target cells pulsed with the gB497–507 peptide. A representative HSV-1-specific CTL

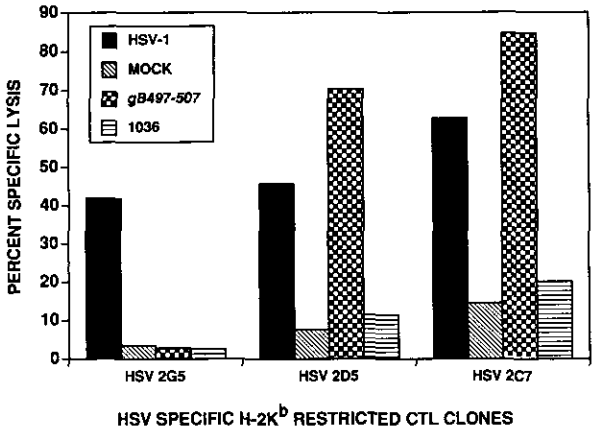

FIG. 3. Recognition of HSV-1 gB synthetic peptide gB497–507 by the HSV-1 and HSV-2 cross-reactive CTL clones. B6/WT-3 cells were labeled with 150 μCi of ^{51}Cr and were incubated with a 1 μM concentration of either the gB497–507 peptide or the unrelated peptide 1036 (LT 195–209) for 30 min at 37°. The peptide-coated cells were washed twice prior to use in a 5-hr ^{51}Cr release assay. HSV-1-infected and uninfected B6/WT-3 cells were included as positive and negative controls, respectively.

clone, (2G5), did not lyse the gB497–507 peptide-pulsed cells. The B6/WT-3 cells pulsed with an unrelated peptide, 1036 (LT 195–209), corresponding to SV40 T antigen residues 195–209 (Tevethia *et al.*, 1990), were not recognized by either of the two CTL clones. These results suggest that amino acid residues 497–507 of HSV-1 gB contain an epitope which is recognized by the CTL clones 2D5 and 2C7. The results also indicate that this H-2K^b-restricted CTL epitope is common between HSV-1 and HSV-2 which is consistent with sequence analysis where this region has been shown to be identical in HSV-1 and HSV-2 (Bzik *et al.*, 1984, 1986).

Peptides which bind to the H-2K^b molecule are octomers which usually contain phenylalanine or tyrosine at position 5 and a leucine at position 8 (Falk *et al.*, 1991). In an attempt to define the minimal epitope necessary for CTL recognition, an octameric peptide (gB498–505; SSIEFARL) corresponding to this predicted H-2K^b binding motif was synthesized and tested for its ability to serve as a determinant for CTL recognition by CTL clone 2D5. B6/WT-3 cells were pulsed with various concentrations of either gB497–507 or gB498–505 and tested for their ability to be recognized and lysed by the gB-specific CTL clone 2D5. The results presented in Fig. 4A show that the 2D5 CTL clone recognized and lysed cells presenting either of these peptides, suggesting that the minimal epitope necessary for recognition by these CTL probably includes residues gB498–505. Likewise, a population of primary, HSV-specific "bulk culture" CTL derived from the popliteal lymph nodes of mice infected in the footpads with HSV were also able to recognize and lyse cells pulsed with either of the peptides (Fig. 4B). However, in both instances, the molar concentration of gB498–505 peptide necessary to render B6/WT-3 cells susceptible to lysis was 100-fold lower than that of gB497–507. These results suggest that the gB498–505 peptide may have a greater affinity for the H-2K^b molecule than does the larger gB497–507 peptide and may represent the naturally processed peptide.

Induction of HSV gB-specific cytotoxic T lymphocytes by gB synthetic peptide

The identification of an H-K^b-restricted CTL recognition epitope within HSV gB (gB498–505) provided an opportunity to determine if a CTL response could be induced by immunizing B6 mice with the synthetic peptide representing this predicted minimal epitope. B6 mice were inoculated in each rear footpad with 200 μg gB498–505 prepared in a low toxicity adjuvant [Ti-

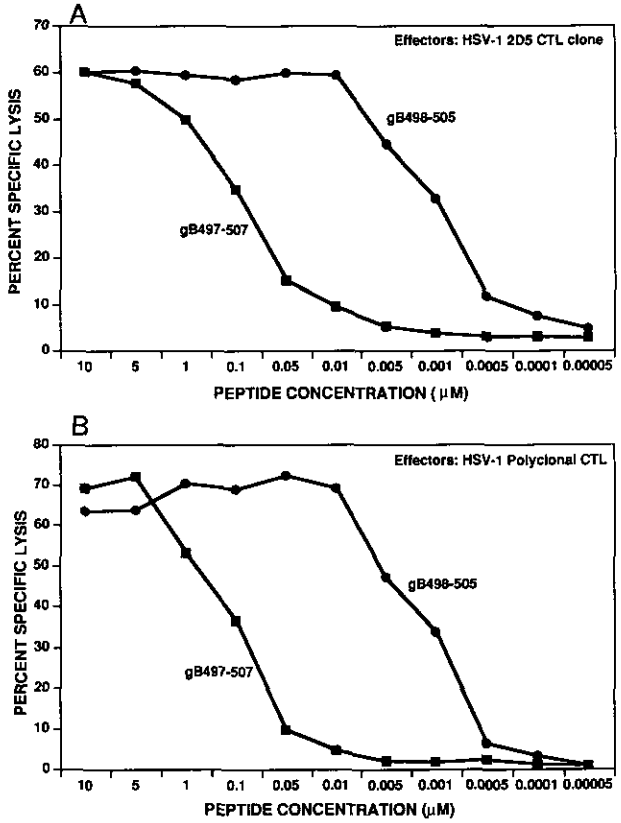

FIG. 4. Preferential recognition of an octomer synthetic peptide gB498–505 by the HSV-1- and HSV-2-cross-reactive CTL clones and by HSV-1 induced primary CTL. B6/WT-3 cells were incubated for 30 min with the indicated concentrations of either an octomer HSV-1 gB peptide 498–505 or the 11 mer HSV gB peptide 497–507 and were used as target cells against HSV gB-specific CTL clone 2D5 at an effector to target cell ratio of 4:1 (A) and against the HSV-1 induced primary CTL at an effector to target cell ratio of 20:1 (B). The primary CTL were generated by immunizing C57BL/6 mice in each rear footpad with 1×10^6 PFU HSV-1 as described under Materials and Methods. Three days later, the popliteal lymph nodes were removed and the lymph node cells cultured *in vitro* for 3 days prior to use in the cytotoxicity assay.

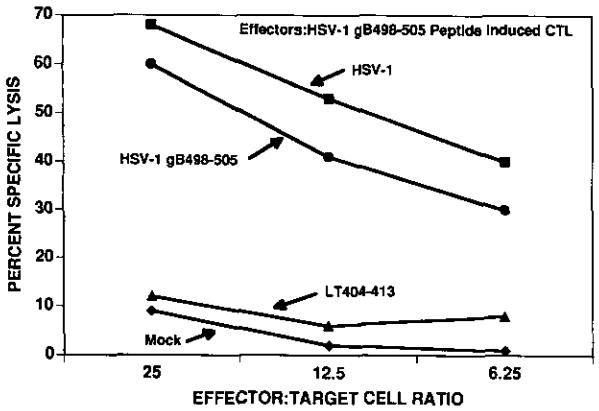

FIG. 5. *In vivo* induction of HSV gB epitope-specific CTL using synthetic peptide gB498–505. C57BL/6 mice were immunized in each rear footpad with 200 μg of HSV gB498–505 peptide emulsified in the adjuvant TiterMax. Eight days later, the popliteal lymph nodes were removed and cultured *in vitro* for 3 days. The lytic activity of the CTL was assessed against HSV-1-infected and uninfected B6/WT-3 cells and against B6/WT-3 cells pulsed with either the gB498–505 peptide or the unrelated LT404–413 peptide at a concentration of 1 μ*M* in the standard cytotoxicity assay.

terMax (TM)]. Eight days later, the popliteal lymph nodes were removed and the cells were cultured *in vitro* for 3 days in the absence of antigenic stimulation. Cells obtained from these cultures exhibited lytic activity against both HSV-1-infected and gB498–505 peptide-pulsed B6/WT-3 cells (Fig. 5). Uninfected cells and cells pulsed with an irrelevant peptide, LT404–413 corresponding to SV40 T antigen residues 404–413 (S. Tevethia, unpublished) were lysed at background levels. Antigenic stimulation during this incubation period was not necessary to induce maturation of these lymph node-derived cells to the lytic phenotype, which is similar to what has been observed with lymph node cells obtained from mice immunized with infectious HSV (Pfizenmaier *et al.*, 1977a,b; Carter *et al.*, 1981; Hanke *et al.*, 1991). Cultures of lymph node cells obtained from mice immunized with only TM failed to develop any cytolytic activity (data not shown). Overall, these results suggest that immunization with the gB498–505 synthetic peptide results in the generation of CTL that are able to progress *in vivo* to the antigen-independent stage of differentiation and are lytic to cells expressing the gB498–505 peptide.

Phenotype of HSV gB498–505 peptide-induced CTL

B6 mice were challenged in each rear footpad with 200 μg gB498–505 in TiterMax and cells exhibiting HSV gB epitope-specific cytolytic activity were generated as described above. In addition, CTL were also generated in response to HSV-1 infection in another group of B6 mice. Prior to assay for cytolytic activity, $CD4^+$ or $CD8^+$ cells were depleted *in vitro* using anti-CD4 and anti-CD8 antibodies and complement as described under Materials and Methods. Depletion of these T cell subsets was confirmed by fluorescent flow cytometry (data not shown). As shown in Fig. 6, depletion of $CD8^+$ cells from the gB498–505 peptide-induced CTL population eliminated lysis of both HSV-infected and gB498–505 peptide-pulsed target cells. In contrast, depletion of $CD4^+$ cells did not reduce the lytic activity of this population. Likewise, depletion of $CD8^+$ cells in cultures prepared using lymph node cells obtained from HSV-infected mice abrogated HSV-specific lytic activity as shown previously (Jennings *et al.*, 1991). Lytic activity against uninfected target cells or target cells pulsed with an unrelated peptide (LT404–413) was at background levels thus confirming the specificity of the cytolytic activity (data not shown). These results show that the lymphocyte subpopulation that is responsible for this gB498–505 peptide-induced lytic activity is mediated by $CD8^+$ CTL.

DISCUSSION

The results presented in this study demonstrate that two H-$2K^b$-restricted, HSV-1/HSV-2 cross-reactive CTL clones derived from B6 mice immunized with HSV-1 recognize an epitope within the HSV gB molecule. These CTL clones, 2D5 and 2C7, recognize HSV-1 gB residues 498–505 (gB498–505). Furthermore, a single immunization with a free synthetic peptide representing this HSV gB epitope resulted in the generation of $CD8^+$ CTL with specificity for this epitope expressed on both virus-infected and peptide-pulsed syngeneic target cells. The H-$2K^b$ restriction of the cross-reacting gB specific CTL clones was demonstrated by testing the ability of the representative CTL clone HSV 2D5 to lyse recombinant cell lines expressing either K^b or D^b class I antigens. The results (not shown) indicated that the K5RSV cells (K^bD^b) after infection with HSV-1 or pulsed with the synthetic peptide 498–505, were lysed by the 2D5 CTL clone whereas KHTGSV cells (K^dD^b) were not lysed.

We have been interested in identifying CTL recognition determinants on proteins encoded by the HSV genome. The approach that we have taken has been to establish a panel of CTL clones which specifically recognize HSV determinants and use these CTL clones to localize epitopes using viral mutants or synthetic peptides corresponding to the predicted MHC binding motifs that have been described for the MHC class I H-$2K^b$, and H-$2D^b$ antigens (Van-Bleek and Nathenson, 1990; Falk *et al.*, 1991; Rotzschke and Falk, 1991). To accomplish this goal, we have isolated HSV-1-specific and HSV-1/HSV-2-cross-reactive H-$2K^b$-restricted CTL clones from a polyclonal CTL population obtained from spleens of C57BL/6 mice immunized with HSV-1 (Bonneau and Jennings, 1990). From this population was established two groups of CTL clones based on

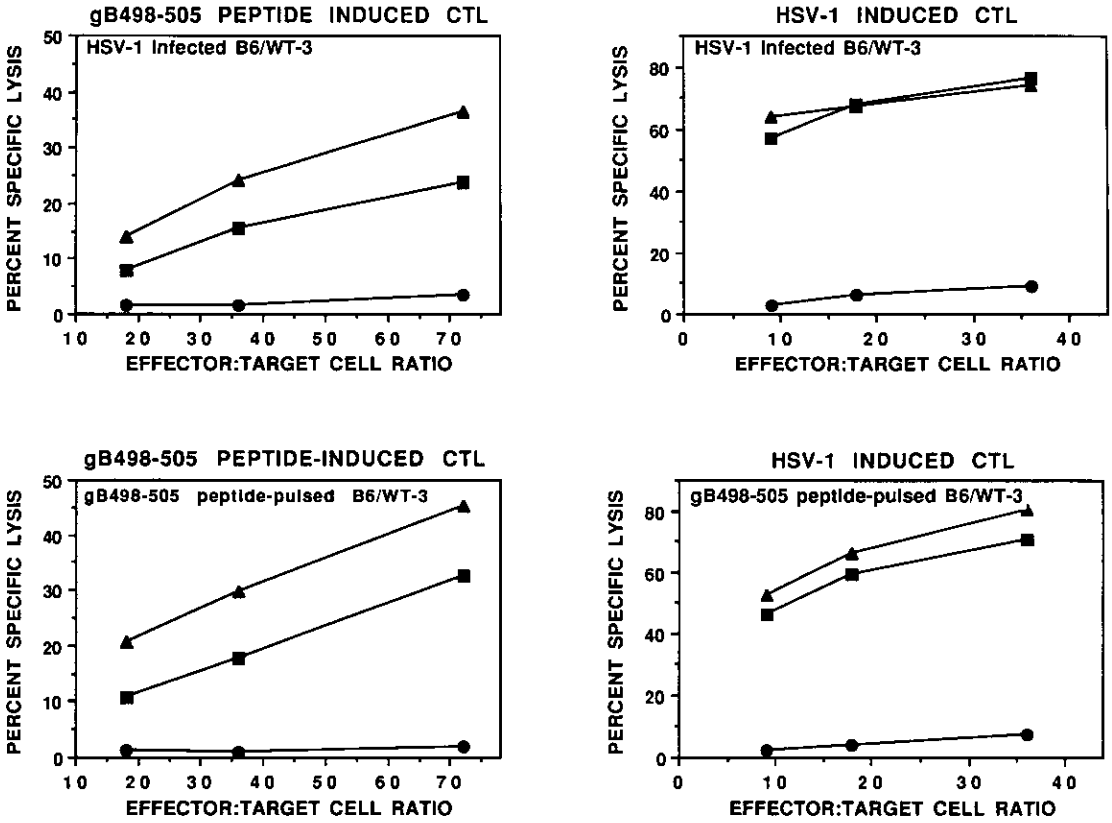

FIG. 6. Phenotype of HSV gB498–505 peptide-induced CTL. CTL generated in C57BL/6 mice against HSV gB peptide 498–505 and against HSV-1 were reacted with either complement alone (■), anti-CD8 antibody and complement (●), or with anti-CD4 and complement (▲) and tested for cytolytic activity against HSV-1-infected or HSV-1 gB peptide 498–505-pulsed B6/WT-3 cells.

their HSV type-specificity. In order to map the specificity of the HSV-1/HSV-2-cross-reactive CTL clones 2D5 and 2C7, we took advantage of the previous studies showing that the H-2K^b-restricted CTL generated in bulk culture in response to primary HSV infection recognized HSV gB as a target antigen (Witmer *et al.*, 1990). The epitope was mapped within gB residues 497–507 (Hanke *et al.*, 1991). By the use of a recombinant adenovirus vector which expresses HSV-1 gB (AdgB2), the CTL clones 2D5 and 2C7 were shown to recognize a determinant within HSV gB. Further, using synthetic peptides, the specificity of these two CTL clones was narrowed to gB residues 498–505.

The observation that the gB498–505 peptide was recognized with a 100-fold greater efficiency than the gB497–507 peptide by both the gB-specific CTL clones and bulk culture CTL obtained from primary HSV infection indicates that the octomer gB498–505 may represent the natural epitope for these CTL. Support for this hypothesis is provided by previous studies in which peptides that bind to the H-2K^b molecules have been identified as octomers which usually contain phenylalanine (F) or lysine (Y) at position 5 and leucine at position 8 (Falk *et al.*, 1991). These residues have been identified as dominant anchor positions in the binding of a peptide to the H-2K^b molecule. The HSV-1 gB498–505 (SSIEFARL) peptide meets these minimum requirements for a H-2K^b-binding peptide.

Since this H-2K^b restricted CTL recognition site which maps in gB appears to be immunodominant as demonstrated by these and previous studies (Hanke *et al.*, 1991), we attempted to induce an anti-HSV CTL response *in vivo* by inoculating mice with the synthetic peptide (498–505). The use of free synthetic peptides representing immunogenic epitopes of virally-encoded gene products is an attractive mechanism for induction of specific antiviral immunity *in vivo*. Of particular interest in the defense against HSV infections is the generation of cell-mediated immunity, specifically the development of HSV-specific CTL and their role in controlling the establishment of primary and latent infection. In other studies, a synthetic peptide representing a T cell epitope of LCMV nucleoprotein has been shown to induce *in vivo* a specific CTL response to LCMV (Aichele *et al.*, 1990). Furthermore, immunization with synthetic peptides have also been shown to confer protection against both LCMV infection (Schultz *et al.*, 1991) and Sendai virus infection (Kast *et al.*, 1991) in experimental systems. In studies presented here, HSV gB peptide 498–505 also is able to induce an HSV epitope-specific CTL response in the popliteal lymph nodes following a single footpad challenge. The necessity for a 3-day *in*

vitro incubation period of lymph node cells from immunized mice in order to allow for maturation of these CTL to the lytic phenotype is similar to what has been shown to be necessary for the development of lytic HSV-specific primary CTL generated in C57BL/6 mice after HSV infection (Pfizenmaier *et al.*, 1977a; Carter *et al.*, 1981). Furthermore, the ability of these peptide-induced CTL to acquire lytic activity in the absence of any *in vitro* antigenic stimulation indicates that these CTL had progressed to an antigen independent stage of differentiation *in vivo* in which additional stimulation is not necessary to achieve full lytic activity (Rouse and Wagner, 1984; Reddehase *et al.*, 1984). These results suggest that a peptide corresponding to an immunodominant HSV CTL epitope may be used to elicit a protective CTL response.

Overall, the results of this study and others (Witmer *et al.*, 1990; Hanke *et al.*, 1991) suggest that the H-2K^b-restricted CTL recognition epitope on HSV gB (gB498–505) is an immunodominant epitope since it is efficiently recognized by both bulk culture CTL generated in response to primary HSV infection in B6 mice as well as a panel of CTL clones derived from a population of splenic HSV-specific memory CTL. Consequently, such an epitope may play a significant role in inducing an *in vivo* immune response capable of providing protection against subsequent HSV challenge in H-2^b mice. The role of HSV gB in the overall immune response to HSV infection has been demonstrated. HSV gB is a major component of virus infected cell membranes and virion envelopes (reviewed in Spear, 1984) and is essential for virus entry into cells (Bzik *et al.*, 1984; Cai *et al.*, 1988). In addition, gB is recognized by CD4$^+$ human CTL clones (Zarling *et al.*, 1986; Torseth *et al.*, 1987) and provides epitopes recognized by HSV-specific CTL in H-2^b and H-2^d mice (Witmer *et al.*, 1990). Studies are in progress to determine the most efficient delivery of this CTL recognition epitope *in vivo* for the induction of an effective antiviral immune response that confers protection against subsequent HSV infection.

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