Concomitant Helper Response Rescues Otherwise Low Avidity CD8+ Memory CTLs to Become Efficient Effectors In Vivo

Udayasankar Kumaraguru, Susmit Suvas, Partha S. Biswas, Ahmet Kursat Azkur and Barry T. Rouse

*J Immunol* 2004; 172:3719-3724; doi: 10.4049/jimmunol.172.6.3719
http://www.jimmunol.org/content/172/6/3719

**References**
This article cites 27 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/172/6/3719.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Concomitant Helper Response Rescues Otherwise Low Avidity CD8+ Memory CTLs to Become Efficient Effectors In Vivo

Udayasankar Kumaraguru,2 Susmit Suvas, Partha S. Biswas, Ahmet Kursat Azkur, and Barry T. Rouse2

This report seeks a means of maximizing memory CD8 T cell responses to peptide immunization. Delivery of the CD8 peptide epitope by stress protein, heat shock protein (hsp70), results in excellent immunogenicity at the acute phase but memory responses were poor both in terms of the number of responding cells as well as their functional avidity. We demonstrate for the first time that hsp70 can also be used as a vehicle to achieve CD4 T cell responses to loaded peptide epitopes and that coimmunization with hsp70 loaded with both CD8 and CD4 peptide epitopes may increase memory up to 3-fold. Furthermore, CD8+ T cell memory responses were of higher avidity measured both by in vitro cytotoxicity assays and a new methodology that measures the avidity of CTL activity in vivo in mice. Our results emphasize that peptide immunization remains a viable approach to induce long-term CD8+ T cell function, providing steps are taken to assure appropriate stimulation of Th cell responses. The Journal of Immunology, 2004, 172: 3719–3724.

Defense against many virus infections requires an effective CD8 T cell response which, even for complex pathogens, may be directed against only a few peptide epitopes (1). Such peptides represent potential vaccine candidates, and include therapeutic use to boost CD8+ T cell responses upon a background of ineffective existing immunity. Such a procedure could be valuable to enhance resistance against agents such as HSV where the frequent recurrences suffered by some patients are likely associated with defective immunity (2). However, used alone peptides induce minimal CD8+ T cell responses, a shortfall that may be overcome by coupling them to various carrier molecules and coadministering appropriate adjuvants. One approach that results in immunogenicity is to link peptides to stress proteins such as heat shock protein (hsp)70 or gp96 (3–8), or administer molecular patterns such as CpG-containing oligodeoxynucleotides (9, 10). Using an 8-mer peptide, SSIEFARL, of the HSV protein gp B, both hsp70-SSIEFARL and CpG-SSIEFARL were shown to induce potent CD8+ T cell responses in C57BL/6 mice (6, 9). Unfortunately, such responses, although robust and immunoprotective in the acute phase, rapidly declined leaving a poor memory response (6). Thus, few memory cells were still present after 6 wk, and these expressed low avidity, killing peptide-sensitized targets better than those infected with virus. Low avidity CD8+ T cell responses have also been noted in other studies that have used peptide immunization (11, 12).

Immunologists have long recognized that optimal CD8+ T cell responses often require the concomitant participation of CD4+ helper cells during immune induction (13). This issue has recently been a focus topic with several groups reporting impaired CD8+ memory if immunization is performed in the absence of helper cell function (14–18). In some reports, the effects of helper cells could be substituted by using anti-CD40 Ab to costimulate CD8+ T cells or providing the cytokine products of CD4 cells such as IL-2 (19, 20). Conceivably, the durability and functional efficacy of the CD8+ T cell response to peptide immunization could also be enhanced by additionally providing CD4+ Th cell stimulation. We have analyzed this situation using the hsp70-peptide system that in previous studies was shown to induce poor memory (6). We show for the first time that the hsp70-peptide system can also be used to induce Th cell and Ab responses. More importantly, the coadministration of hsp70 linked to a CD8 peptide along with the hsp70 linked to helper peptides, resulted in 3-fold improvement in CD8+ T cell memory with cells also showing high avidity cytotoxic activity. The relevance of our observations to future vaccine formulations is briefly discussed.

Materials and Methods

Mice

Four- to 5-wk-old BALB/c (H-2b) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). In conducting the research described in this work, we adhered to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Peptides

HSV-ICP27 (aa 448–456) peptide DYATLGGVV, HSV-gD (aa 4–23, ALVDASLMADPNRFRGKDLP) and chicken OVA class II (aa 323–339, ISQAVHAAHAEINEAGR) were synthesized and supplied by Research Genetics (Huntsville, AL).

Viruses

HSV-1 KOS was grown on Vero cell monolayers (CCL81; American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at −80°C until used.

1 This work was supported by National Institutes of Health Grants AI14981 and AI46462.
2 Address correspondence and reprint requests to Dr. Udayasankar Kumaraguru or Dr. Barry T. Rouse, M409 Department of Microbiology, University of Tennessee, Knoxville, TN 37996. E-mail addresses: udayk@utk.edu or btr@utk.edu
3 Abbreviations used in this paper: hsp, heat shock protein; ICS, intracellular cytokine staining.
Proteins
We purchased rhp70 from StressGen Biotechnologies (ESP-755; Victoria, BC, Canada). The protein was ~90% pure and was determined by SDS-PAGE analysis. The endotoxin concentration was <50 endotoxin U/ml as determined by Limulus amebocyte lysate assay.

Cell lines
Vero (African green monkey kidney cell line) MC38, (C57BL/6, colon carcinoma, H-2b), EMT6 (BALB/c mammary adenocarcinoma cells, H-2d) were used. All cell lines were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 100 U of penicillin G per milliliter, 100 μg of streptomycin sulfate per milliliter, and 2 mM l-glutamine.

Hsp70 and peptide binding
The ICP27 or gD peptides were adsorbed onto the hsp70 by the procedure described by Ciupiti et al. (3). In brief, the peptides were incubated with rhsp70 in binding buffer (PBS with 2 mM MgCl₂) at 37°C for 60 min. Then, 0.5 mM ADP (Sigma-Aldrich, St. Louis, MO) was added, and the incubation was continued for another 60 min at the same temperature. As a control, carrier peptides were complexed to BSA by glutaraldehyde conjugation.

Immunizations
BALB/c mice were immunized with 1) 2.5 μg of ICP27/gD peptide and 2.5 μg of rhsp70 or BSA, 2) 2.5 μg of peptide mixed with 2.5 μg of rhsp70 or BSA, or 3) with binding buffer only. The immunizations were done i.p. on days 0 and 21. The i.p. route was chosen after experimenting with i.m. and footpad injections. Mice belonging to the virus control group were injected i.p. with 1 × 10⁶ UV-inactivated HSV-KOS.

HSV and peptide-specific lymphoproliferation
Splenocytes from experimental mice were restimulated in vitro for assessing proliferative ability as described earlier (6). In brief, responders were stimulated with either peptide-pulsed or virus-infected APCs for 3 days. The last 18 h was conducted in the presence of [3H]thymidine. After incubation, plates were harvested and read using the Inotech cell harvester and reader (Inotech, Biosystems International, Lansing, MI). Proliferative responses tested in quadruplicate wells were expressed as mean cpm + SD.

ELISA for gD-specific Abs
The gD-specific Ab in the samples were determined by standard ELISA. ELISA plates were coated with gD peptide and then incubated overnight at 4°C. Serially diluted samples were analyzed and Ab concentrations were calculated with an automated ELISA reader (Spectra MAX340; Molecular Devices, Sunnyvale, CA).

CTL assays
The CTL assay was performed as described earlier (21). Briefly, effector cells generated after in vitro expansion (with peptide or HSV) were analyzed for their ability to kill MHC-matched Ag-presenting targets. The targets included ⁵¹Cr-pulsed, MHC-matched, and HSV-infected (EMT6-HSV), MHC-matched and DYATLGVV-pulsed (EMT6-DYATLGVV), MHC-mismatched, HSV-infected, and DYATLGVV-pulsed (MC38-HSV and MC38-DYATLGVV), and MHC-matched uninfected or unpulsed (EMT6) targets. The chromium release results were compiled and are expressed as lytic units as described elsewhere (21).

Intracellular IFN-γ staining (intracellular cytokine staining (ICS) assay)
To enumerate the number of IFN-γ-producing cells, ICS was performed as previously described (6). In brief, 10⁵ freshly explanted splenocytes were cultured in flat-bottom 96-well plates. Cells were either left untreated, stimulated with relevant or irrelevant peptide (1 μg/ml), or treated with PMA (10 ng/ml) and ionomycin (500 ng/ml), and then incubated for 6 h at 37°C in 5% CO₂. Brefeldin A was added for the duration of the culture period to facilitate intracellular cytokine accumulation. After this period, cell surface staining was performed, followed by ICS by using the Cytofix/Cytoperm kit (BD PharMingen, San Diego, CA) in accordance with the manufacturer’s recommendations. For ICS, the Abs used were anti-IFN-γ (clone XMG1.2). All Abs were purchased from BD PharMingen, and data were analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Tetramer staining and flow cytometry
MHC class I (H-2b⁺) tetramers to measure DYATLGVV-specific T cells were provided by the National Tetramer Facility (Athens, GA). A total of 10⁵ T cells obtained from these mice was stained with a mixture of FITC-labeled anti-CD8 (Caltag Laboratories, Burlingame, CA) and PE-labeled tetramers for 45 min at 4°C. The controls included isotype control, stained cells, and unstained cells. They were then analyzed by using a FACSscan machine and CellQuest software (BD Biosciences). The percentage values shown are the double-positive cells (CD8⁺ and DYATLGVV-specific TCR).

ELISPOT assay
ELISPOT was used for quantification of cytokine-producing cells. ELISPOT plates (Millipore, Molsheim, France) were previously coated with IFN-γ anti-mouse Ab. The nylon wool-purified immune T cells (responder cells) were mixed with syngeneic splenocytes (stimulator cells) pulsed with relevant or irrelevant peptide. Coincubation of the responder and stimulator cells was continued for 72 h at 37°C. The ELISPOT plates were washed three times with PBS and three times with PBST, then biotinylated IFN-γ Ab was added to the plates for 1 h at 37°C. The spots were developed using nitroblue tetrazolium (Sigma-Aldrich) and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich) as a substrate following incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h and counted 24 h later under a stereomicroscope.

In vivo assay for CTL avidity measure
Splenocytes from naive mice were stained with PKH26 (PKH26-GL; Sigma-Aldrich) or 1.5, 0.15, or 0.03 μM CFSE (Molecular Probes, Eugene, OR). The CFSE-labeled cells alone were then coated with different concentrations of ICP27 peptide (1, 0.1, or 0.01 μM) while the PKH26 stained cells were used as peptide unpulsed control. They were then transferred i.v. (5 × 10⁶ cells of each population) into the indicated groups of mice. Five hours later, the lymphocytes were isolated from the spleen as previously described. Target cells were distinguished from recipient cells based on PKH26 and CFSE staining. Dot plot was used to calculate the number of recovered PKH26-labeled peptide-unpulsed targets. Histogram plots were used to demonstrate the difference in separation pattern based on intensity of CFSE staining. The recovery and percent killing of the various CFSE-labeled peptide-pulsed targets were calculated as follows: 100 — [(percent of peptide pulsed in immunized/percentage of unpulsed in immunized)/(percentage of peptide pulsed in unimmunized/percentage of unpulsed in unimmunized)] × 100.

Statistical analysis
The data were analyzed by dependent-sample t test by using SPSS for Windows, release 10.1.3 (SPSS, Chicago, IL), and the Student t test.

Results
Responses to hsp70 loaded with CD8- and CD4-recognized peptides
Previous studies had demonstrated that the protein ICP27 contains a CD8⁻-recognized peptide epitope (22) and that the gD peptide 4–23 stimulates CD4⁺ T cells in BALB/c mice (23). These peptides, along with the CD4⁺ recognized OVA peptide 323–339, were loaded onto hsp70 protein and used in various combinations to immunize groups of mice (days 0 and 21). Control groups were given UV-HSV. Responses, focusing on the CD8⁻ reactions to ICP27 448–456, were measured on day 28 (acute phase) and day 60 (memory phase). As is evident in Table I, the acute phase response to hsp70-DYATLGVV alone was approximately equal to those coimmunized with helper peptide or immunized with UV-inactivated virus. The differences were not statistically significant between groups at the acute phase. Additional experiments also showed that mice immunized with hsp70-gD peptide induced CD4⁺ T cell responses, as measured by peptide- and HSV-induced in vitro proliferation assay (Fig. 1), as well as induced peptide-specific Ab responses (Fig. 2). Mice also responded positively by enhanced CD4⁺ T cell responses to hsp70 OVA peptide immunization as measured by peptide-induced ELISPOT and intracellular IFN-γ staining (Fig. 3). Whereas the acute phase CD8⁺ T cell
responses to hsp70-ICP27 peptide showed no influence of helper cell coimmunization, a notable effect was evident in the memory cell responses (Table I). At this stage (day 60), the response of animals coimmunized with the CD8 and helper cell peptides or UV-HSV was significantly higher than those immunized with hsp70-ICP27 alone (p < 0.0001). In fact, the response induced by coimmunization gave a memory response equivalent to that induced by UV-HSV.

Thus coimmunization with hsp70-gD resulted in a memory CD8+ T cell response to ICP27 that was almost 3-fold higher than that to hsp-ICP27 alone. However, coimmunization with the heterologous hsp70-OVA peptide, which was shown to be immunogenic, resulted in minimal and statistically insignificant improvement of memory CD8+ responses (p < 0.242).

**Differences in avidity of CD8+ T cells generated in the presence or absence of help**

In addition to measuring CD8+ T cell responses quantitatively to the different peptide immunizations, CTL responses were also measured in the memory phase against both peptide- and virus-infected target cells. The results, shown in Table II, demonstrate that coimmunization with the CD8+ and CD4+ peptide epitopes resulted in CTL response to ICP27 that showed higher avidity than those to the ICP27 peptide alone. Accordingly, animals immunized with HSV showed CTL responses (recorded as lytic units) against viral targets that were around 31% of peptide-specific responses. The avidity of CD8+ T cells generated concomitantly in the presence of gD CD4-specific helper was an effective means of inducing high avidity CTL.

In **vivo CTL challenge with targets pulsed with different molar peptide concentration delineates the difference between helped and unhelped CD8+ T cells**

The difference in the quality of CTL activity observed in the above described in vitro assay was further investigated using a modification of the recently described in vivo CTL assay. The differences in the avidity at the acute and memory phase were measured by assessing the ability to lyse syngeneic targets pulsed with varying concentrations of peptide ranging from 1 to 0.01 μM. Accordingly, mice belonging to different groups were challenged in vivo by injecting i.v. equal amounts of peptide-pulsed (1, 0.1, or 0.01 μM concentration of ICP27 peptide) targets. They were labeled with different concentrations of CFSE (1.5, 0.15, and 0.03 μM) so as to

![FIGURE 1. Hsp70 + ICP27 peptide or hsp70 + gD peptide immunization induces peptide-specific CD8+ or CD4+ T cell responses. BALB/c mice were immunized with rhp70-DYATLGVGV alone or in combination with rhp70-gD peptide or rhp70-OVA class II peptide i.p. on days 0 and 21. Control for immunization included peptide alone, HSV-1 KOS (106 PFU before UV inactivation), or buffer alone. One week later, the splenocytes were harvested, and nylon wool-nonadherent T cells were assessed for in vitro proliferative response. The cells were harvested and read with an Inotech automatic cell harvester and reader; the results were expressed as cpm.](http://www.jimmunol.org/)

### Table 1. Frequency of ICP27 peptide-specific CD8+ T cells

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Frequency Per 10^6 Cells</th>
<th>Frequency Per 10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp70 + ICP27 pep (A)</td>
<td>0</td>
<td>1/100000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1/141</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1/336</td>
</tr>
<tr>
<td>Hsp70 + ICP27 pep + gD pep (B)</td>
<td>0</td>
<td>1/100000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1/158</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1/184</td>
</tr>
<tr>
<td>Hsp70 + ICP27 pep + OVA pep (C)</td>
<td>0</td>
<td>1/100000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1/172</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1/297</td>
</tr>
<tr>
<td>UV-HSV (D)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1/149</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1/206</td>
</tr>
</tbody>
</table>

*The mice were immunized as indicated and sacrificed on days 0, 28, and 60. They were then analyzed for tetramer-positive CD8+ T cells and peptide-induced IFN-γ production, as described in Materials and Methods. The tetramer and ICS values are percentage of double-positive cells (CD8+Tet or CD8+IFN-γ, respectively). They were calculated back to total CD8s in each spleen, and the average was used to arrive at the frequency. The experiments were done with 10 individual mice in each group at a given time point. The data represent the average result. Individual values were used to compute the statistics by using SPSS software to analyze the significance by the dependent-sample t test. The differences were not statistically significant between all groups.*
FIGURE 2. Hsp70 + gD peptide immunization induces gD peptide-specific IgG. Serum was obtained from groups of BALB/c mice immunized with hsp70-DYATLGVGV alone or in combination with hsp70-gD peptide or hsp70-OVA class II peptide i.p. on days 0 and 21. Control for immunization included peptide alone, HSV-1 KOS (10^6 PFU before UV inactivation), or buffer alone. It was analyzed for the presence of gD-specific Ab by ELISA. Ab concentrations were calculated with an automated ELISA reader. The IgG concentration shown is an average of values obtained from five individual mice. There was significant production of Abs in hsp70-gD-immunized mice compared with buffer alone (p < 0.05). However, it did not compare very well to virus control, and the response was far better in UV-HSV immunized groups (p < 0.05).

enable effective separation during acquisition for flow cytometric analyses. In addition, as mentioned in Materials and Methods, peptide-unpulsed targets labeled with PKH26 were used. As shown in Fig. 4, all three targets were recovered (in comparison to peptide-unpulsed PKH labeled targets) in negative control mice that received PBS, indicating the absence of peptide-specific CTLs and hence, no killing. However, in the positive control mice (UV-HSV infected), 1 and 0.1 μM peptide-pulsed targets were completely lysed and could not be recovered. Furthermore, ~5% of 0.01 μM targets were also lysed.

Mice belonging to test groups (CD8 epitope, CD8 and CD4 gD epitope, or CD8 and CD4 OVA epitope) were also challenged in vivo as described above. The mice that received gD-specific help along with CD8 peptide immunization showed lysis of both 1 and 0.1 μM targets and were similar to virus-infected controls. However, the CD8+ CTLs generated in the absence of help or in the presence of OVA-specific CD4 help were of low avidity as demonstrated by their inability to lyse even 1 μM concentration of peptide-pulsed targets. The difference in ability to lyse low concentration peptide-pulsed targets between the helped and unhelped was distinct and also statistically significant (p < 0.005). In contrast, the results from similar assays performed at the acute phase showed minimal or no changes between the test groups (CD4 help and none). In all instances, lysis of targets sensitized with the lowest concentration of peptides was ~80%. The results from these studies indicate that effective high avidity memory CD8+ T cells can be generated with hsp70-peptide immunization and maintained, provided concomitant appropriate help is made available during priming.

Discussion
This report seeks a means of maximizing memory CD8+ T cell responses to peptide immunization. Confirming previous reports, we show that loading CD8+ peptide epitope onto the stress protein hsp70 results in excellent immunogenicity (6). However, this was true only at the acute phase. Memory responses were poor both in terms of the number of responding cells as well as their functional avidity. We demonstrate, for the first time, that hsp70 can also be used as a vehicle to achieve CD4+ T cell response to loaded peptide epitopes and that coimmunization with hsp70 loaded with both CD8+ and CD4+ peptide epitopes may increase memory up to 3-fold. Furthermore, CD8+ T cell memory responses were of higher avidity measured both by in vitro cytotoxicity assays and a new methodology that measures the avidity of CTL activity in vivo. Our results emphasize that peptide immunization remains a viable approach to induce long-term CD8+ T cell function, providing steps are taken to assure appropriate stimulation of Th cell responses.

In the search for new vaccines to replace ineffective or toxigenic older formulations, peptides representing the target epitope of T and B cells represent attractive candidates (24). However, peptides are usually nonimmunogenic or induce tolerance unless given along with potent adjuvants (25). As this report shows, peptide
immunogenic systems may succeed at inducing acute responses but memory and functional efficacy may be suboptimal. With regard to sustainable CD8+ T cell responses, several recent reports have demonstrated the necessity for appropriate Th cell activation during CD8+ T cell induction if durable memory is to be established. Thus the Bevan and Shen groups (15, 16) showed defective CD8+ memory if Ag priming occurred in animals genetically unable to concomitantly mount CD4+ T cell responses. Others, using specific Ab to deplete T cells, came to similar conclusions (14, 16). Our studies, using a different approach, further substantiate the need for help to obtain appropriate memory. Moreover, our studies also demonstrate that satisfactory T cell help is also required if a memory population is to be induced that expresses optimal functional activity.

A few other studies have shown that hsp70 is a useful vehicle to obtain CD8 responses against loaded peptide epitopes (3–5, 7). However, the issue of memory was not usually studied. As we have shown, the hsp70 peptide system may induce responses comparable to those induced by virus immunization, but responses are far inferior when measured in the memory phase (6). Previous studies had not used the hsp70 carrier system to induce CD4+ T cell or B cell responses. However, using the well-studied peptide 4–23 of HSV gD that is known to possess two epitope recognized by B cells and at least two by CD4+ T cells (23, 26, 27), both CD4 and Ab responses were induced. More of interest, coimmunization with hsp70-gD and hsp70-ICP27 peptide resulted in ~3-fold increase in potency of the CD8 T cell memory response. No effect of the helper peptide coimmunization was noted in the acute phase. Curiously, all helper peptide-epitope coimmunizations did not achieve similar effects. Thus, hsp-OVA (aa 323–339), while inducing a specific response measured by ELISPOT, had only a marginal, and not significant, influence on the memory response to the hsp70-ICP27 peptide. The reasons for this are not clear but likely relate to the quantity or quality of responses to gD 4–23 compared with OVA. This issue is under further investigation.

Likely of more significance than the qualitative effects of helper peptide coimmunization is the marked influence on T cell avidity. Thus, using a concept first elaborated by Zinkernagel and colleagues (28), we observed that T cells induced by some

Table II. Comparison of avidity of CD8+ T cells generated in the presence or absence of help

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Phase</th>
<th>Peptide targets</th>
<th>HSV targets</th>
<th>HSV/Pept%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8</td>
<td>Acute</td>
<td>63</td>
<td>54</td>
<td>86</td>
</tr>
<tr>
<td>CD8 + CD4 (gD)</td>
<td>Acute</td>
<td>74</td>
<td>67</td>
<td>90</td>
</tr>
<tr>
<td>CD8 + CD4 (OVA)</td>
<td>Acute</td>
<td>68</td>
<td>59</td>
<td>87</td>
</tr>
<tr>
<td>UV-HSV</td>
<td>Acute</td>
<td>52</td>
<td>49</td>
<td>94</td>
</tr>
</tbody>
</table>

“ The mice were sacrificed on day 7 (acute) and day 60 (memory) after the second immunization and analyzed for CTL activity by 51Cr release assay as described in Materials and Methods. The CTL data are expressed as lytic units and show results accrued from UV-treated HSV, CD8 epitope alone, CD8 epitope plus cognate CD4 epitope, and CD8 epitope plus noncognate CD4 help. The targets are DYATLGVGV-pulsed and HSV-infected targets (EMT6). The experiments were done with 10 individual mice in each group at a given time point. The data represent the average result. Individual values were used to compute the statistics by using SPSS software to analyze the significance by the dependent-sample test.

HSV/Pept% = (HSV targets/peptide targets) × 100. Values of p are indicated by numbers in parentheses as follows: 1) p < 0.001 (t = 13.93); 2) p < 0.001 (t = 3.99); 3) p < 0.001 (t = 0.74); and 4) p < 0.032 (t = 1.37).

FIGURE 4. In vivo CTL challenge with targets pulsed with different molar peptide concentration. Targets were prepared with splenocytes from naive mice. They were stained with PKH26 or 1.5, 0.15, or 0.03 μM CFSE. The CFSE-labeled cells were coated with different concentrations of ICP27 peptide (1.01, 0.01 μM), while the PKH26-stained cells were used as peptide-unpulsed control. Equal volume of all four targets was transferred i.v. into test mice (three per group). Five hours later lymphocytes were isolated from spleen as previously described. Target cells were distinguished from recipient cells based on PKH26 and CFSE staining. Dot plot was used to calculate the number of recovered PKH26-labeled peptide-unpulsed targets. Histogram plots were used to demonstrate the difference in separation pattern based on intensity of CFSE staining. The recovery and percent killing of the various CFSE-labeled peptide-pulsed targets were calculated as follows: 100 − [(percentage of peptide pulsed in immunized/ percentage of peptide pulsed in unimmunized)/(percentage of peptide pulsed in unimmunized/ percentage of peptide pulsed in unimmunized)] × 100). This assay was performed at day 28 to represent acute stage and 60 days later for assessing the memory response. The figure shows a representation of data obtained from (both acute and memory) one mouse in each group, but the numbers above the histogram are averages of percent killing observed in three mice.

immunization procedures yield low avidity CTL able to readily lyse peptide-sensitized targets but not those infected by virus. Our results, using coimmunization with the 4–23 peptide, show that this procedure resulted in high avidity T cells in contrast to the memory population tested from those immunized with the CD8 peptide alone. This phenomenon was also observed in vivo using a novel approach to record avidity. This was done by comparing the in vivo lyses of targets sensitized with different concentrations of peptide. We judged high avidity CD8 to be those that could lyse targets sensitized with low as well as with high concentrations of peptides.

Unfortunately, we could not test the functional significance of the more numerous high avidity anti-HSV CD8+ T cells in vivo by
viral challenge. In fact, we have observed that mice coimmunized with ICP27 peptide + gD 4–23 are markedly more resistant to HSV challenge than are ICP27 peptide-immunized mice. However, the recipients of gD 4–23 also generate CD4+ and Ab response to HSV, which as shown previously also protect against challenge (29). The issue of assessing the in vivo significance of high avidity CTL requires evaluation in circumstances which avoid such complications.

In conclusion, our results add further evidence that appropriate helper cell stimulation is necessary during the induction of CD8+ T cells if sufficient functional memory is to be induced. We have also developed an immunization procedure that achieves successful memory response against peptide Ags which may prove useful for future vaccine formulations.

Acknowledgments

Tetramers were provided by the National Institute of Allergy and Infectious Diseases, MHC Tetramer Core Facility, National Institutes of Health (Bethesda, MD) and Yerkes Regional Primate Research Center (Atlanta, GA).

References