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CD8 CTL from Genital Herpes Simplex Lesions: Recognition of Viral Tegument and Immediate Early Proteins and Lysis of Infected Cutaneous Cells

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AIDS recognizes by HSV-specific CD8 CTL, are rational candidate vaccine compounds. The Journal of Immunology, 2001, 166: 4049–4058.

AIDS causes chronic infections. CD8 CTL may play several protective roles, and stimulation of a CD8 response is a rational element of vaccine design for this pathogen. The viral Ags recognized by CD8 T cells are largely unknown. It has been hypothesized that HSV inhibition of TAP may favor recognition of virion input proteins or viral immediate early proteins. We tested this prediction using HSV-specific CD8 CTL, clones obtained from genital HSV-2 lesions. Drug and replication block experiments were consistent with specificity for the above-named classes of viral proteins. Fine specificity was determined by expression cloning using molecular libraries of viral DNA, and peptide epitopes recognized at nanomolar concentrations were identified. Three of four clones recognized the viral tegument proteins encoded by genes UL47 and UL49. These proteins are transferred into the cytoplasm on virus entry. Processing of the tegument Ag-derived epitopes was TAP dependent. The tegument-specific CTL were able to lyse HLA class I-appropriate fibroblasts after short times of infection. Lysis of keratinocytes required longer infection and pretreatment with IFN-γ. Another clone recognized an immediate early protein, ICP0. Lymphocytes specific for these lesion-defined epitopes could be reactivated from the PBMC of additional subjects. These data are consistent with an influence of HSV immune evasion genes upon the selection of proteins recognized by CD8 CTL in lesions. Tegument proteins, identified for the first time as Ags recognized by HSV-specific CD8 CTL, are rational candidate vaccine compounds.

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3 Abbreviations used in this paper: ICP47, infected cell protein number 47 (other gene numbers are similar); US12, unique short region of the HSV genome, gene 12 (other gene numbers are similar); vhs, virion host shutoff; UL47, unique long region of the HSV genome, gene 47 (other gene numbers are similar); EBV-LCL, EBV-transformed lymphocyte cell line; MOI, multiplicity of infection, the number of PFUs of virus added per cell; TCM, T cell medium; th, recombinant human; EC50, 50% effective concentration.
HSV encodes ~85 proteins (1). Little is known concerning the targets of HSV-specific CD8 CTL or how the CD8 repertoire is shaped by immune evasion genes. Clones recognizing type-specific epitopes in HSV envelope glycoproteins B and D (15, 17) have been derived from PBMC using secondary in vitro restimulation. Other clones were active against targets infected in the presence of transcriptional inhibitors (17), consistent with recognition of viral protein(s) loaded into APC upon virion binding and entry. The molecular identity of these targets was not determined. Recently, Mikloska et al. (18) found a high prevalence of bulk CD8 CTL responses specific for immediate early proteins ICP27 and ICP4, using IFN-γ-treated keratinocytes as stimulator and readout cells. The kinetics of HSV-induced down-regulation of HLA class I may predict that either viral proteins injected directly into the cytoplasm, as is the case for CMV protein pp65, or viral immediate early proteins, might “outpace” immune evasion. Alternatively, it is possible that TAP-independent Ag processing might sidestep TAP inhibition.

We used genetic approaches to test these predictions. T cell clones were recovered from recurrent genital HSV-2 lesions, without secondary in vitro restimulation with Ag. We have tried to minimize bias that might be introduced by such restimulation, as recently reported for CMV pp65 (19), and to study cells that have physiologically localized to the site of infection. Expression cloning was used to assign antigenic specificity. This paper describes the first identification of three HSV-2 proteins as targets of the local HSV-2-specific CD8 T cell response. We further investigate the ability of these CTL to recognize skin-derived cells, their TAP dependence, and their reactivation from the PBMC of HLA-appe-
sequence was identical with GenBank 61710. HLA A*0201 and B*0702 cDNAs previously similarly cloned by RT-PCR, and shown by sequencing to be identical with wild-type sequences, were obtained from Dr. Stanley Riddell.

To study the cDNA species derived from the positive genomic clone containing portions of ICP0 (Results), COS-7 cells (100 mm2) were transfected with the ICP0 genomic clone, and total RNA was prepared after 48 h. The primer used for cDNA synthesis (TGGTCTGATAAGCTGATCCCTGGCGGCGGCCGC (Xhol site underlined)) was cloned into pGEM-T easyvector (Promega) at the Xhol site. Sequencing was performed by serological and in vivo methods at the Fuji Photo Film (Tokyo, Japan). cDNAs obtained were polyadenylated and transfected into CHO cells for expression cloning.

Full-length UL47 of HSV-2 was cloned by PCR into pCNA3.1/His-C using 5′-primer CTAGGATCCCCTCCGGCCACCATGTCC and 3′-primer CTAGCTAGACTCATTTTAGGGCTGGCAGG (BamHI and Xhol sites underlined). Full-length UL46 of HSV-2 was constructed by cloning into pCNA3.1/His-C using 5′-primer CGAAGATCTCGTCTGCGCATTGCAACGGCGC and 3′-primer CGCCGTCATTATTTATGGCCTGTGGTGC (BamHI and Xhol sites underlined). Similarly, a construct expressing aa 1–590 of UL47 was made by PCR, using the above 5′-primer, an appropriate 3′-primer, and pCNA3.1/His-C. Expression of aa 1–535 and 536–696 of UL47 was driven by constructs derived from full-length UL47 using a naturally occurring Nofl site at aa 535. In-frame vector-HSV-2 fusion at the 5′-end of the HSV-2 DNA was confirmed by sequencing in each case.

We investigated some CD8 CTL clones in the COS-7 system using a panel of cloned HSV-2 genes. Cells were cotransfected with HLA class I heavy chain cDNA (50 ng/well) and HSV-2 constructs (25 ng/well) and assayed for stimulation of IFN-γ. Our gene panel included UL46 and UL47 (above) and full-length HSV-2 UL19, UL21, UL50, and US3, each cloned into pCNA3.1/His series, and UL40 cloned into pEGFP-C1 (Clontech, Palo Alto, CA) as described and validated (35, 36).

Cytotoxicity assays
Cytotoxicity assays were performed as previously described (10) using 4-h 51Cr release. Target EBV-LCL were typically infected for 18 h with HSV or vaccinia strains at multiplicity of infection (MOI) 10, and the usual E:T ratio was 20:1, or loaded with peptide for 90 min at 37°C in 200 μl volumes. In some assays, purified mAb W6/32 (37) was included at 10 μg/ml. To inhibit viral RNA expression, EBV-LCL were preincubated with actinomycin D (Sigma) at 1 μg/ml for 30 min before infection. Actinomycin D was maintained throughout the 90-min incubation, in the presence of 10 μg/ml capture mAb IgG (Sigma) as previously described (10). All targets were used in CTL assays at 2 × 104/well, and 2.5% Ipegal (Sigma, St. Louis, MO) was used to measure total release. Assays were performed in triplicate and spontaneous release was usually <25%.

HLA peptide tetramers
A tetrameric receptor containing HLA A*0201 heavy chains, β2-microglobulin, and peptide UL47 551–559 was synthesized by the Tetramer Facility of the National Institute of Allergy and Infectious Diseases (Bethesda, MD). Tetramers were biotinylated and labeled with streptavidin-PE.

Flow cytometry
Lymphocytes were washed and incubated with anti-CD4-FITC/anti-CD8-PE (Sigma), anti-CD3-FITC/anti-CD16PE and CD56-PE, or anti-TCR αβ-FITC or anti-TCR γδ-PE (Becton Dickinson, San Jose, CA), or a mixture of control FITC- and PE-labeled mAb (Sigma) on ice for 30 min. For tetramer staining, cells were centrifuged, resuspended in 100 μl TCM, and incubated with 1 μl tetramer for 1 h at room temperature. After this, 1 μl DAPI (4′,6-diamidino-2-phenylindole) was added, and cells were incubated for 30 min on ice. To measure HLA transfection, trypsinized COS-7 cells were stained with 1 μg FITC-labeled mAb B12 reactive with HLA B*0702 (Chemicon, Temecula, CA), or supernatant of MA2.1 cells (38) reactive with HLA A*0201, followed by FITC-labeled goat anti-mouse IgG (Sigma). Washed cells fixed with 1% paraformaldehyde in PBS were analyzed with a FACScan cytometer (Becton Dickinson) and WinMDI version 2.8 shareware (http://facs.scripps.edu). Cells in the appropriate gates on forward vs side scatter were analyzed. To measure the infection of wild-type and mutant LCL with HSV-2, uninfected or infected (18 h, MOI 10) cells were stained with mAb 18B8 specific for envelope glycoprotein D or control mouse IgG1, followed by FITC-conjugated goat anti-mouse IgG (Sigma) as previously described (10).

HLA typing

Subjects were typed serologically and by DNA methods at the Puget Sound Blood Center (Seattle, WA).

ELISA
IFN-γ was measured with reagents from Endogen. Costar No. 3369 plates (Corning, NY) were coated with 100 μl 0.25 μg/ml capture mAb (M700A-E) diluted in 0.1 M sodium carbonate (pH 9.4) overnight at 4°C, and blocked with 1% BSA in 0.2 M NaCl, 3 mM KCl, 0.05 M Tris, pH 9 (TBS) for 1 h. Subsequent incubations were each 100 μl, preceded by three to five washes with PBS/0.2% Tween 20, and performed with rotation at room temperature. Samples and standards diluted in TBS with 0.1% BSA, 0.05% Tween 20, and 4 μg/ml Ig-Inhibiting Reagent No. 6LD1068 (Bioreclamation, East Meadow, NY; sample buffer) were added for 2 h. Biotinylated detection mAb (M701B) diluted to 100 ng/ml in sample buffer was added for 1 h. Avidin-D HRP (A-2004) diluted to 100 ng/ml in TBS with 1% BSA, 0.05% Tween 20 was added for 1 h. TMB substrate was added for 10 min, reactions were stopped with 1 M phosphoric acid, and results were read on a plate reader at 450 and 650 nm. The lower limit of detection was 10 pg/ml.

Results

Cloning of HSV-2 type-specific CD8 CTL from genital HSV-2 lesions and assignment of HLA-restricting alleles
CD8 CTL clones specific for HSV-2 were obtained from herpetic lesions, without secondary in vitro restimulation with Ag, as previously described (12). Clones with HSV-specific cytotoxic activity in screening assays, and CD8 but not CD4 expression, were expanded for further study. For subjects 1874 and 5491, multiple T cell clones with the same apparent pattern of HSV-2 type specificity and HLA restriction were derived. Representative clones, based on HLA restriction analysis and HSV-2 type specificity (39), were chosen for detailed study (Table I). Clone 5101.1999.23 was obtained by collagenase digestion of a lesion biopsy and was the single CD8 CTL clone obtained in screening 60 clones. Each clone was assigned as HLA A*0201, B*0702, or B*4501 by HLA typing the source subjects and using partially matched EBV-LCL as APC (Table I). A transfection/infection assay (Fig. 1) confirmed the CTL results (Table I) and also established the suitability of HLA-transfected COS-7 for expression cloning.

Requirement for viral protein expression for lysis of HSV-2-infected target cells
To evaluate whether the T cell clones recognized virion input protein peers, we checked their cytolytic activity against EBV-LCL infected in the presence of actinomycin D. Clones 1874.1991.22, 5101.1999.23, and 5491.2000.48 lysed these target cells (Table I). Lysis by clone 1874.1991.51 was significantly inhibited by blockade of transcription. Each of the clones was able to lyse target cells infected with the mutant virus hr259, which lacks the ICP4 trans-activator protein, and is only able to newly express the other immediate early proteins, ICP0, ICP27, ICP22, ICP47, and the small unit of ribonucleotide reductase (30).
Recognition of tegument HSV-2 Ags by CD8 T cells

For expression cloning, HSV-2 genomic DNA fragments were cotransfected together with HLA class I cDNA into COS-7 cells, and IFN-γ secretion again used as the readout for T cell activation. The genomic HSV-2 Sau3A1 libraries, in each reading frame, were screened to oversample the HSV-2 genome ~6-fold. Each of the first three CD8 T cell clones studied responded to cells transfected with plasmid DNA prepared from individual bacterial colonies, which were sequenced to preliminarily identify T cell Ags (Table I).

CD8 clone 5101.1999.23 recognized COS-7 cells cotransfected with HLA A*0201 and a HSV-2 Sau3A1 fragment from bp 102,943–102,876 (28) (Table I). The predicted fusion protein contains HSV-2 UL47 aa 278–298. Reactivity with UL47 was confirmed by cotransfection of A*0201 and full-length HSV-2 UL47 (Table II).

The CD8 T cell clone 1874.1991.22 recognized COS-7 cells cotransfected with HLA A*0201 and a HSV-2 Sau3A1 fragment from bp 102,875–101,383 (Table I). This fragment was predicted to contain the DNA encoding UL47 aa 300–696, intervening DNA, and then aa 1–71 of UL46. Analysis of the Sau3A1 fragment from nucleotides 1858–3022 (Table II) was similarly analyzed. Peptide 551–559 was active at 1 nM (Fig. 3). Potential HLA B*0702-binding peptides in UL47 (HSV-2) 289–298 had a 50% effective concentration (EC50) in the 1–10 nM range in cytolysis assays (Fig. 3). The predicted epitope lay within exon 1 or exon 2, PCR was repeated with specific primers. The exon 1-partial exon 2 cDNA, but not exon 1 cDNA, was synthesized from total cellular RNA followed by PCR de novo to amplify the spliced transcript. The size of the PCR product was 300 bp, consistent with the splicing out of intron 1.

The specificity of CD8 clone 5491.2000.48 was determined with a panel of partial- and full-length HSV-2 genes. The HSV-2 genes studied were previously shown to be recognized by CD4 T cell clones (35, 36, 41). Only HSV-2 UL49, when cotransfected with HLA B*0702, stimulated IFN-γ release by clone 5491.2000.48 (Fig. 2).

HSV-2 gene UL47 encodes protein VP13/14, whereas UL49 encodes VP22 (1); both tegument proteins are loaded into the cytoplasm on virion binding and entry (42). The small genomic HSV-2 fragment of UL47 recognized by clone 5101.1999.23 was scanned for peptides fitting the A*0201 binding motif (http://134.2.96.221/ and http://bimas.dctr.nih.gov/molbio/hla_bind/). Peptide UL47 (HSV-2) 289–298 had a 50% effective concentration (EC50) in the 1–10 nM range in cytolysis assays (Fig. 3). UL47 535–590 (Table II) was similarly analyzed. Peptide 551–559 was active at 1 nM (Fig. 3). Potential HLA B*0702-binding peptides in UL49 of HSV-2 were synthesized, and two (aa 47–55 and 14–22) were active at 1 μM (data not shown). Titration (Fig. 3) showed that UL49 49–55 was highly active, with an EC50 of <10 nM, whereas UL49 14–22 had activity only at 1 μM (not shown). The antigenic peptides in UL47 and UL49 contain significant amino acid sequence differences from the corresponding predicted HSV-1 peptides (28, 43), explaining type-specific recognition of HSV-2 (Table I).

Recognition of immediate early HSV-2 protein ICP0 by CD8 T cells

For clone 1874.1997.51, positive reactions to plasmid pools were present in each library. The active plasmids in each library contained a genomic Sau3A1 fragment from nucleotides 1858–3022 (28). Nucleotide 2007 listed as T in the published sequence was read as C. In addition to 445 bp of 5'-untranslated sequence, all of predicted exon 1, intron 1, and the first 234 bp of predicted exon 2 of ICP0 were present, preliminarily identifying the Ag as ICP0. Because alternative splicing of HSV-1 ICP0 has been documented at both the DNA and protein levels (44, 45), we first identified the Ag-encoding mRNA species in COS-7 cells to determine how the ICP0 genomic clone was spliced in our system. COS-7 cells were transfected with genomic clone C:1:H3:B8 (Table I), and cDNA was synthesized from total cellular RNA followed by PCR designed to amplify the spliced transcript. The size of the PCR product (~300 bp) was consistent with the splicing out of intron 1. Sequencing showed a slight difference from the reported (28) splice point for mature HSV-2 ICP0 mRNA. Three base pairs encoding aa Q26 (28) were missing. We have retained Q26 for peptide numbering (below). To determine whether the antigenic peptide lay within exon 1 or exon 2, PCR was repeated with specific primers. The exon 1-partial exon 2 cDNA, but not exon 1 cDNA,

Table I. CTL activity and HLA restriction of CD8 clones, and initial results of expression cloning

<table>
<thead>
<tr>
<th></th>
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<td></td>
</tr>
<tr>
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<td>B*0702</td>
<td>B*4501</td>
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</tr>
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<td>0</td>
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<td>Predicted HSV-2 ORF(s)</td>
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<td></td>
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<td>UL49 1–300</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ICP0 1–105</td>
<td></td>
<td></td>
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</tr>
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</table>

* Data are percent specific release in 51Cr release assays at E:T 20:1. For actinomycin D experiments, target cells infected with wild-type HSV-2 were assayed in the presence of 5 μg/ml actinomycin D from 0.5 h before infection through the assay. To assess HLA restriction, allogeneic EBV-LCL were either mismatched at HLA-A and -B or matched with the index subject at only the indicated HLA class I allele. The positive HSV-2 genomic clones are listed by indicating the positive pCDNA3.1/His A, B, or C library: positive library plate: positive library well: positive final well. For 5491.2000.48, full-length UL49 of HSV-2 in pEGFP-C1 (Clontech) was positive (see text). The nucleotide numbers and predicted amino acid numbers within the antigenic HSV-2 DNA fragments are given as reported for the HSV-2 strain HG52 genomic sequence (28).
was stimulatory for T cell clone 1874.1997.51 (Table II), localizing the epitope to aa 26–105 in exon 2. Reactivity was confirmed in CTL assays using a recombinant vaccinia virus expressing ICP0. At E:T 20:1, lysis of vaccinia ICP0 (31)-infected target cells was 52.1% compared with 2.3% for vaccinia wild type. Having determined the RNA splicing pattern, we proceeded to find the peptide epitope.

Two reported HLA B45-restricted epitopes (46–48), AEEAA-GIGIL and GAETFYVDGA, share with the B44 supertype (49) a preference for negatively charged and hydrophobic amino acid side chains at the P2 and P9 anchor positions. ICP0 (HSV-2) 92–105, containing this motif, was active at 1 μM (not shown). Truncation yielded ICP0 (HSV-2) 92–101, with an EC50 in the 1 nM range (Fig. 3).

Recognition of skin-derived fibroblasts and keratinocytes by CD8 CTL clones

Within lesions, HSV-2 is mainly present in keratinocytes (16). We investigated how MOI (amount of virus), time of infection, and pretreatment with IFN-γ influenced lysis of dermal fibroblasts and keratinocytes. For fibroblasts (Fig. 4), in the absence of IFN-γ pretreatment, infection for 2 h led to detectable lysis, which increased with increasing MOI. Lysis was undetectable (<5% specific release at E:T of 20:1) after overnight infection with MOI 1, 5, or 25. With IFN-γ pretreatment, lysis was generally increased, but 2-h infection was still superior. HLA-mismatched target cells were not lysed, even after peptide loading (data not shown).

Keratinocytes showed some similarities and differences from fibroblast as target cells (Fig. 4). IFN-γ pretreatment generally increased recognition, without leading to lysis of control cells. In contrast to fibroblasts, 18-h infection was generally required. Weak cytolysis of cells infected for 2 h was noted only for IFN-γ-pretreated targets. Chromium release again correlated directly with the amount of infectious virus added, because no specific lysis was noted at MOI 1 or 5 (data not shown).

PBMC responses to HSV-2 T cell epitopes

The A*0201-restricted responses to UL47 were studied in six HLA A*0201-bearing, HSV-2 infected persons (Fig. 5). No response was seen in a HSV-uninfected control subject. One HSV-2-infected subject, 9383, who has infrequently recurring genital HSV-2, had strong cytolytic responses, with effector populations

Table II. Confirmation and localization of epitopes recognized by CD8+ clones

<table>
<thead>
<tr>
<th>T Cell Clone</th>
<th>HSV-2 ORF and Predicted Amino Acids</th>
<th>HLA cDNA</th>
<th>IFN-γ (pg/ml)</th>
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<tr>
<td>5101.1999.23</td>
<td>None</td>
<td>None</td>
<td>0</td>
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<tr>
<td></td>
<td>UL47 aa 1–696 (full length)</td>
<td>A*0201</td>
<td>&gt;3000</td>
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<tr>
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<td>None</td>
<td>A*0201</td>
<td>0</td>
</tr>
<tr>
<td>1874.1991.22</td>
<td>UL47 aa 1–696</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>A*0201</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UL46 aa 1–722 (full length)</td>
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<td>UL47 aa 1–696</td>
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<td>A*0201</td>
<td>0</td>
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<td>UL47 aa 1–590</td>
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<tr>
<td>1874.1997.51</td>
<td>Genomic, nucleotides 1858–3022</td>
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<td>B*4501</td>
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<tr>
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<td>ICP0 exon 2 cDNA aa 1–105</td>
<td>B*4501</td>
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</table>

*The indicated CD8 CTL clones were reactive with HSV-2 genomic clones indicated in Table I. COS-7 cells were transfected with HLA cDNA and HSV-2 DNA or cDNA as shown. T cell activation was detected by IFN-γ secretion, reported as the mean of duplicate wells. Two CTL clones are shown to react with UL47. The epitope recognized by clone 1874.1991.22 is localized to aa 536–590 of UL47, and the epitope recognized by clone 1874.1997.51 is localized to aa 26–105 of ICP0. Values are mean of duplicate IFN-γ secretion into the medium as measured by ELISA. ORF, Open reading frame.
killing both HSV-2-infected A*0201-bearing EBV-LCL and peptide-loaded targets. Subject 1874, from whom the 551–559-specific clone was derived, also had low but detectable PBMC CTL responses to peptide 551–559. In contrast, subject 5101, from whom the 289–298-specific clone was derived, did not have a detectable PBMC CTL response after peptide restimulation. Confirmation of CTL activity was obtained by deriving CD8 clones from peptide-stimulated PBMC from donor 9383. For both UL47 289–298 and 551–559, clones were obtained which lysed HLA A*0201-bearing EBV-LCL loaded with the stimulating peptide or infected with HSV-2 (data not shown).

A tetrameric form of HLA A*0201, loaded with UL47 peptide 551–559 and labeled with PE, was used to study these effector populations (Fig. 5). The tetramer bound specifically to the index T cell clone 1874.1991.22. Flow cytometric assays showed the highest level of CD8$^+$ and tetramer$^+$ cells for subject 9383. The index subject, 1874, also had tetramer$^+$ CD8 cells detected. However, other subjects who did not have detectable CTL, such as 7282 and 5101, appeared to have enriched numbers of tetramer-binding cells compared with the HSV-1 seronegative control subject.

Three HSV-2-infected, HLA-compatible (B*4501) persons were available for study of the epitope in ICP0. Two subjects had strong CTL responses (Table III). Lysis of infected targets was inhibited by anti-class I mAb, and not observed if the target cells did not express B*4501.

**Discussion**

HSV-2 causes considerable morbidity and mortality, especially in neonates (50). Because of the chronic nature of the infection, the limitations of antiviral therapy, and the frequency with which transmission is caused by asymptomatic shedding of the virus, vaccination is likely to be required to reduce new HSV-2 infections (51). To date, most vaccines have been ineffective in phase III clinical trials (52). The recent report that vaccination with a specific adjuvant and an envelope glycoprotein induced partial protection in HSV-1/HSV-2-seronegative women (53) highlights both the potential efficacy of vaccination and the need for improved formulations and markers of effective immunity.

The possible functional importance of HSV-specific CD8 CTL in humans has been addressed in several recent studies (4, 12, 13, 16, 54). Murine studies also illustrate protective roles for CD8 CTL (55–57). Because the HSV-2-encoded protein ICP47 is a relatively inefficient inhibitor of murine TAP (14), we chose the human system for our Ag discovery work.

Earlier work has shown that the majority of human PBMC- and lesion-derived HSV-2-reactive CD8 CTL clones are type specific for HSV-2 (12, 15, 17, 58). This is also consistent with a possible functional role for CD8 responses in host defense, because prior HSV-1 infection provides poor protection against subsequent HSV-2 infection (59), whereas both neutralizing Ab and CD4 responses have a strong type-common component (60, 61). We emphasized study of HSV-2 type-specific clones. Clones recovered

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Lysis by lesion-derived CD8 clones of autologous LCL loaded with HSV-2 peptides at the indicated concentrations. Data are percent specific $^{[3]}$Cr release at E:T 20:1. $\bullet$, Lysis by clone 5101.1999.23 of targets loaded with UL47 551–559; $\circ$, clone 1874.1991.22 and UL47 289–298; $\bigtriangledown$, clone 1874.1997.51 and ICP0 92–101; $\blacktriangle$, clone 5491.2000.48 and UL49 49–57. Lysis of mock-loaded targets was <5% specific release for each clone.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Cytolytic activity of CD8 CTL clones against cutaneous cells. **Top,** UL49-specific clone 5491.2000.48, HLA B*0702-expressing fibroblasts from subject SJ, and peptide UL49 aa 49–47. **Middle,** UL47-specific clone 1874.1991.22, HLA A*0201-bearing fibroblasts from subject 1874, and peptide UL47 aa 551–559. **Bottom,** HLA A*0201-bearing keratinocytes were used as target cells. **Left,** UL47-specific clone 5101.1999.23 and peptide UL47 aa 289–298; **right,** UL47-specific clone 1874.1991.22 and peptide UL47 aa 551–559. HLA A*0201-bearing keratinocytes were mock treated or treated with 500 U/ml IFN-γ and then infected or treated with peptide. In each case, HLA-mismatched target fibroblasts had <5% specific release at E:T 2:1, 6:1, and 20:1.
directly from the site of infection, derived without secondary in vitro restimulation with Ag (62), were used to study physiological responses at the site of disease.

Little is known about the specificity of human HSV-2-specific CD8 CTL. The two published epitopes are type-common peptides within glycoproteins B and D (10, 15). At the nonclonal level, experiments using restimulation of PBMC, drug blocks, and vaccinia recombinants show that HSV-1 ICP4, ICP27, ICP0, all immediate early proteins, HSV-1 early protein ICP6, and possibly other true early proteins may be targets of human CTL (18, 63, 64). HSV-1 early protein thymidine kinase (tk) is recognized by CD8 clones from PBMC of subjects treated with tk-transfected autologous cells, but this is likely a primary immune response (65). A PBMC-derived CD8 T cell clone specific for a melanoma-associated protein (Melan A/MART-1) also reacted with a peptide from HSV-1 glycoprotein C (66).

It has been hypothesized that the selection of Ags recognized by HSV-specific CD8 CTL is influenced by immune evasion genes within HSV (7, 9). ICP47 blocks assembly of mature HLA-b2-microglobulin-peptide complexes by inhibition of TAP. This effect occurs quickly during viral infection (67). In

Table III. Recognition of HSV-2 ICP0 by peptide-stimulated PBMC from HSV-2-infected, HLA-appropriate persons

<table>
<thead>
<tr>
<th>Effector</th>
<th>B=4501 Target EBV-LCL</th>
<th>Non-B=4501 Target EBV-LCL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mock</td>
<td>Peptide</td>
</tr>
<tr>
<td>1874 PBMC</td>
<td>1</td>
<td>45.3</td>
</tr>
<tr>
<td>8915 PBMC</td>
<td>0</td>
<td>54.9</td>
</tr>
<tr>
<td>10061 PBMC</td>
<td>1.3</td>
<td>-0.6</td>
</tr>
<tr>
<td>1874.1997.51</td>
<td>0</td>
<td>65.3</td>
</tr>
</tbody>
</table>

* Data are percent specific release in 4-h $^{51}$Cr release assays at E:T 20:1. Each subject was HSV-2 infected and HLA B=4501 positive. PBMC were stimulated for two cycles with peptide ICP0 92–101; see Materials and Methods. Target B=4501-bearing (subject 1874), or non-B=4501-bearing (subject 5085) EBV-LCL were sensitized with 1 μg/ml ICP0 92–101 for 90 min or treated with anti-class I mAb at 10 μg/ml. As a positive control, CD8 clone 1874.1997.51 was included.
As an additional control, T2 cells, which do not express B0702, were not lysed after p0201 EBV-LCL 5491, was detected after peptide loading (clones are HLA A UL47 activity with two virion tegument proteins and one immediate genes on the selection of CD8 Ags, given that we uncovered re-

pace the down-regulation of HLA class I. A similar model has virion input proteins and immediate early proteins might out-

new HLA class I. It is rational to predict that processing of both vhs

encodes tegument protein VP13/14, which is present in large amounts in virions (71). The physiological function(s) of this protein as a vaccine. Overall, UL49 is the only known HSV-2 protein. As manipulation of virus or host is not possible with the natural host species for HSV-2, formal proof of an effect of immune evasion genes on the CD8 CTL repertoire will be difficult to obtain. If additional epitopes can be accrued with this or other approaches, they can be examined to determine whether a pattern consistent with antigenicity of virion input and immediate early proteins is present.

TAP-independent processing has been reported in other viral systems (76–78). Thus far, in our examination of three discrete epitopes in tegument proteins, we did not find evidence for TAP-independent Ag processing of HSV epitopes. The CD8 response seems to “evade the evasion,” at least in the cases examined to date, while continuing to rely on TAP for Ag processing. The TAP dependence of responses to immunodominant Ags, if these can be identified as such, will also be of interest.

Most studies of clonal CD8 responses have used EBV-LCL as target cells. These cells are relatively resistant to HSV-mediated class I down-regulation (10). For dermal fibroblasts, we found that a short time of infection (2 h) was adequate for target cell sensi-
tization for lysis by tegument protein-specific CTL. Because the UL47 and UL49 tegument proteins are synthesized with “late” kinetics (1), typically starting after 6 h or more of viral infection, these data are also consistent with recognition of preformed Ag in fibroblasts. Lysis was MOI dependent. Because HSV preparations typically contain a large number of defective particles (1), it is likely that tegument proteins were also being delivered into fibro-

blasts by noninfectious particles. After 18 h of infection, the fi-

broblasts were not lysed, regardless of MOI, similar to previous results with CD8 CTL clones of unknown fine specificity (10). IFN-γ pretreatment was able to partially restore lysis of 18-h-in-

fected cells. In contrast to fibroblasts, recognition of keratinocytes after 18 h of infection was superior to recognition after 2 h of infection. The reason for the difference between fibroblasts and keratinocytes is unknown. IFN-γ pretreatment was able to restore some lysis of 2-h-infected cells, and further improved recognition of 18-h-infected cells. In future studies, we hope to compare the recognition of IFN-γ-treated keratinocytes by both CD4 and CD8 CTL (79–81), given that both are present in lesions.

Tegument proteins have not previously been described as targets of the HSV-specific CD8 T cell response. CD4 responses to HSV-1 UL47 have been detected in HSV-mediated acute retinal necrosis (82). CD4 responses to UL49 are commonly detected among lesion-infiltrating HSV-2-specific clones (41). Because re-

sponses to UL49 are also present in the cornea in herpes stromal keratitis in humans (35), a disease that may be driven by patho-

genic Th1-like T cells (82), caution is clearly warranted in using this protein as a vaccine. Overall, UL49 is the only known HSV-2 protein recognized by both CD4 and CD8 T cell clones recovered from herpetic lesions. A unique intercellular transport pathway allows highly efficient uptake of soluble UL49 protein into a variety of epithelial cell types (83–85) which could also intersect Ag pro-

cessing pathways.

A few technical aspects of the methods warrant brief comment. Our library approach worked each of the first three consecutive times it was applied to CD8 CTL clones, so we do not think that technical factors greatly biased the viral kinetic or structural classes of the CTL epitopes detected. A library created, as was ours, with a single restriction endonuclease will contain “holes.” The initial positive ICP0 genomic clone started well upstream of the ATG start, extending to bp −445. As noted above, the ATG start of ICP0 was out of frame with the vector-derived peptide expressed by the positive genomic clone. The ICP0 promoter appears to be functioning without additional viral factors such as the major trans activator VP16 as previously reported for HSV-1 (86).

### Table IV. TAP dependence of processing of HSV-2 tegument for presentation to CD8 T cells

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Mock</th>
<th>Peptide</th>
<th>HSV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 clone 1874.1991.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>2.5</td>
<td>54.8</td>
<td>30.7</td>
</tr>
<tr>
<td>5491</td>
<td>2.0</td>
<td>−2.5</td>
<td>−1.2</td>
</tr>
<tr>
<td>TAP (−)</td>
<td>721.174</td>
<td>−3.9</td>
<td>90.0</td>
</tr>
<tr>
<td>T2</td>
<td>0.7</td>
<td>94.7</td>
<td>2.2</td>
</tr>
<tr>
<td>CD8 clone 5101.1999.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1874</td>
<td>0.8</td>
<td>52.5</td>
</tr>
<tr>
<td>5491</td>
<td>1.7</td>
<td>−2.1</td>
<td>−1.2</td>
</tr>
<tr>
<td>TAP (−)</td>
<td>721.174</td>
<td>0</td>
<td>31.7</td>
</tr>
<tr>
<td>T2</td>
<td>−0.7</td>
<td>71.0</td>
<td>0.8</td>
</tr>
<tr>
<td>CD8 clone 5491.2000.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1874</td>
<td>0.8</td>
<td>−2.7</td>
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<tr>
<td>5491</td>
<td>0.2</td>
<td>68.3</td>
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<tr>
<td>TAP (−)</td>
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<td>−0.4</td>
<td>57.8</td>
</tr>
<tr>
<td>T2</td>
<td>0.2</td>
<td>0.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Data are percent specific release in 51Cr release assays at E:T 20:1. The first two clones are HLA A*0201-restricted. Lysis of the A*0201-bearing wild-type EBV-LCL 1874, but not non-A*0201 EBV-LCL 5491, was detected after peptide loading (UL47 551–559 for clone 1874.1991.22; UL47 551–559 for clone 5101.1999.23; 1 μM, 90 min) or HSV-2 infection (MOI 10, 18 h). In contrast, peptide loading, but not HSV-2-infection, was able to sensitize TAP-deficient cell lines. Similar data are shown for the third clone, a HLA B*0702-restricted, UL49-specific CTL clone and peptide UL49 49–57, using the B*0702 autologous EBV-LCL, 5491, the non-B*0702 EBV-LCL, 1874, and the TAP-deficient, HLA B*0702-containing transfectant T2/B7.63. As an additional control, T2 cells, which do not express B*0702, were not lysed after peptide loading.
Not all viral promoters will necessarily be active outside of the context of natural viral infection. This problem can be overcome by fragmenting the HSV-2 DNA with alternative methods before library creation.

We chose the sequenced strain (28) of HSV-2, HGS2, for library creation. HSV-2 strains are relatively invariant due to the high fidelity of the HSV DNA polymerase (1). Possibly, our approaches may fail if strain-specific epitopes are recognized in vivo. The library is a relatively efficient method for epitope/Ag discovery once conditions are optimized. Neither “holes” in the library nor strain-specific epitopes have interfered as of yet.

In summary, reactivity of lesion-infiltrating, HSV-2 type-specific CD8 T cell clones with the tegument proteins encoded by genes UL47 and UL49 (VP13/14 and VP22, respectively), and ICPO, are described for the first time. The data are consistent with a modulatory effect of ICPO and/or vhs on the CD8 response to HSV. TAP function, but not viral gene transcription, is required for recognition by UL47- and UL49-specific clones, consistent with processing of preformed virion input protein. Tegument-specific CD8 clones are able to recognize skin-derived fibroblasts and keratinocytes. Responses were also detectable in the PBMC of additional subjects. Further studies are required to define the prevalence and dominance of these virus-specific responses and the potential role of these Ags in immunologic approaches to reduce HSV-2 infection and disease.

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References


