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

David M. Koelle, Hongbo B. Chen, Marc A. Gavin, Anna Wald, William W. Kwok, and Lawrence Corey

HSV-2 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; UL41, rapidly degrades host cell mRNA, contributing to decreased synthesis of new HLA class I. As a functional consequence of these activities, HSV-2-infected human fibroblasts and keratinocytes are poorly recognized by HSV- or allospecific CD8 CTL clones (10). Using knockout viruses, contributions of both genes can be discerned. They can be overcome, and CTL lysis restored, if fibroblasts are pretreated with IFN-γ (15). IFN-γ up-regulates proteasome, TAP, and HLA genes involved in class I Ag processing and presentation. In vivo, IFN-γ levels are very high in recurrent HSV-2 lesions (16).

3
HSV encodes ~85 proteins (1). Little is known concerning the targets of HSV-specific CD8 T cell responses. The repertoire is shaped by immune evasion genes. Clones recognizing type-common 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 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–appropriate persons.

Materials and Methods

Cell lines and viruses

EBV-transformed lymphocyte cell lines (EBV-LCL) were derived from PBMC and maintained in LCL medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 1% penicillin-streptomycin, 2 × 10^{-5} M 2-ME, 1 mM pyruvate) as described (10). Autologous EBV-LCL 1847, 3100, and 5491 and EBV-LCL 5085 were initiated in house, and HLA B*45-bearing EBV-LCL, HM0983-022A-3216 was provided by Dr. L. Musey. TAP-deficient cell lines 721.174 (20), T2 (21), and T2/B7.63 (T2 transfected with HLA B*0702) were maintained in LCL medium. Cell lines T2/B7.63, made by Dr. Peter Cresswell and provided by Dr. Charles Lutz, was maintained in 600 μg/ml G418. Primate kidney epithelial COS-7 cells (22) were grown in MEM with 10% FCS, 2 mM L-glutamine, and 1% penicillin-streptomycin. Fibroblasts were grown from skin biopsies as described (10). Single donor neonatal foreskin keratinocytes (Cascade Biologicals, Portland, OR) were HLA typed, and HLA A*0201 cells were expanded per the manufacturer’s recommendations with Epilife medium containing growth supplement (HKGS), penicillin-streptomycin-ampicillin, trypsin, and trypsin inhibitor supplied by the Cascade Biologicals.

Donors for lesion and PBMC studies had their HSV-1 and HSV-2 serostatus determined by type-specific serology (23). All subjects gave informed consent. Lesion HSV-specific T cells were obtained from three subjects from HSV-reactive cultures subsequent to HSV reactivation with IM (11, 12). Lesions were 4 days old at the time of specimen collection. For subjects 1874 and 5491, lesion lymphocytes were expanded in bulk with PHA and IL-2 in the presence of acyclovir (11, 12) and CD8+ cells were positively selected with CD8 immunomagnetic beads (Mini-macs; Miltenyi Biotec, Auburn, CA) and cloned (11). Two input numbers (3 and 1 cell/well) were used, and clones selected for workup were from plates with <37% of wells positive for IFN-γ. Tissue was digested with collagenase IV-S (C1889; Sigma, St. Louis, MO) in PBS, and the resultant cell suspension was immediately cloned (24) in serial 10-fold dilutions. Clones selected for study were from plates with <37% of wells positive for growth. To expand clones after positive screening, 5 × 10^5 cells were mixed in 25 ml T cell medium (TCM) (11) with 2.5 × 10^10 irradiated (3300 rad) allogeneic PBMC. 5 × 10^5 irradiated allogeneic (8000 rad) EBV-LCL, and 30 ng/ml anti-CD3 mAb OKT3 (Ortho, Raritan, NJ) (25). At 24 h and approximately every 3 days, human recombinant IL-2 (50 U/ml; Chiron, Emeryville, CA) was added with fresh medium, and cells were split as needed. The nomenclature for clones lists subject number, year of specimen, and clone number.

CTL were restimulated from PBMC by incubating 4 × 10^6 PBMC (Ficoll–Hypaque density gradient centrifugation) with 100 μM peptide synthesized by F-moc chemistry in ~100 μl TCM for 1 h (1% DMSO was present). Cells were diluted to 20^6/ml and cultured in 1.88-cm^2 wells in the presence of 20 ng/ml recombinant human (rh) IL-7 (R&D systems), rhIL-2 (Chiron) was included at 20 U/ml starting on day 3. Cultures were fed with 0.5 volume medium containing the same cytokines every 2–3 days with further addition of growth factors every 12–14 days. For IFN-γ responses, a modified protocol was used. PBMC (4 × 10^5) were stimulated with 1 μM peptide in 1.88-cm^2 wells with rhIL-2 (10 U/ml) added on day 3. Cells were restimulated on day 7 with 2 × 10^4 autologous PBMC, 1 μM peptide, and IL-2 and fed with 0.5 volume medium containing IL-2 every 2–3 days until assay on days 14–16. For some bulk PBMC-derived cultures, CD8 cells were positively selected, cloned at 1 cell/well, and expanded as described above.

HSV-1 strain E1115 (26) and HSV-2 strains 333 (27) and HG52 (28) were raised and titered in Vero cells (29). The ICPR deletion mutant of HSV-2, hr259, was grown on complement E4 cells (30). Recombinant vaccinia expressing ICPO of HSV-2 (31) (kindly provided by Dr. B. Rousse) and wild-type vaccinia NY were raised and titered in BSC-40 cells.

Expression cloning

The strategy of Boon et al. (32) was adapted to genomic HSV-2 DNA. Cytoplasmic and supernatant virus purified from HSV-2 strain HG52-infected Vero cells (33) were combined. DNA was digested with Sau3AI, reextracted, and partially filled in with DNA polymerase Klenow fragment (F-Moc chemistry) (New England BioLabs, Beverly, MA). Plasmids pcDNA3.1(+)/His A, B, and C (Invitrogen, Carlsbad, CA) were digested with XhoI and partially filled with Klenow fragment, dATP, and dGTP. After ligation of purified (organic extraction, ethanol precipitation) insert mixture (~100 ng) and individual vectors (~1 μg), DNA was precipitated, washed, and electroporated into Escherichia coli strain DH10B (Life Technologies, Gaithersburg, MD). Each library contained several thousand primary transformants. The majority of each library was immediately amplified in bulk. Among 20 random clones, all contained single HSV-2 Sau3AI fragments. Sequencing (Taq Dye-Deoxy F5; Perkin-Elmer ABI, Foster City, CA) was performed per the manufacturer’s instructions.

To make library DNA for transfection, 96-well plates (140504; Beckman, Fullerton, CA) were inoculated either with libraries at ~15 colonies/well or with selected individual clones. After overnight incubation at 37°C at 300 rpm agitation, DNA was prepared with 96-well filters (Millipore, Bedford, MA) per the manufacturer. Some bacteria in each well were saved. Average yield was 10 μg of DNA per well. IFN-γ secretion as the primary readout of T cell activation.

Cell lines and viruses

EBV-LCL in 96-well flat-bottom plates were transfected the next day (day 2) with 50 ng LA heavy chain cDNA and Fugene-6 (Boehringer Mannheim-Roche, Indianapolis, IN), using the manufacturer’s protocol. On day 3, cells were infected with HSV-2 strain 333 at an estimated MOI of 10. On day 4, 0.7–1.0 × 10^5 cloned CD8 T cells in 150 μl FCS-medium were added. Supernatants were saved on day five for IFN-γ ELISA (below).

To make libraries, COS-7 were cotransfected with 50 ng HLA cDNA and 100 ng library DNA (pool or single colony). Two days later, 1 × 10^5 cloned T cells/well (with rhIL-2 at 5 U/ml for some assays) were added, and supernatants were saved as above. DNA was prepared as above from 96 individual colonies derived from positive pools, and the process was repeated to identify individual active plasmids which were then sequenced to identify antigenic regions.

To make HLA B*4501 cDNA, total RNA (5 μg) from subject 1 (HLA A1, A*0201, B7, B*4501) EBV-LCL, purified using guanidinium-acid phenol (34), was incubated in 15 μl water with 0.5 μg oligo(dT)_12–18 (Pharmacia, Piscataway, NJ) at 70°C for 10 min and chilled. The reaction mixture (5 μl) containing 1 μl Moloney murine leukemia virus reverse transcriptase, 2.5 mM MgCl₂, 5 mM DTT, 2.5 μl 10× PCR buffer II (500 mM KCl, 100 mM Tris, pH 9.0), and 500 μM each dNTP was incubated at 42°C for 1 h and boiled for 5 min. For PCR, a 50-μl reaction containing 1× pfu buffer and 1 U pfu DNA polymerase (Inviron), 5 μl 2.5 mM each dNTP, 1 μl cDNA, and 50 pmol each primer (AAGGTCACCGACCACCCGAA and GGTCTAGAAGTTCGACACT) was heated to 94°C for 1 min, cycled 30× with 94°C for 30 s, 50°C for 30 s, and 72°C for 3 min; cycled 30× with 60°C annealing; and extended for 10 min at 72°C. Amplifier purified by organic extraction/alcohol precipitation was digested with appropriate enzymes and ligated into pcDNA3.0 (Invitrogen). The
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 (TGCTCTAGAGCTCGATCCCTGCGCGTCGG; Xbol site underlined) was from the 3′-end of the HSV-2 DNA in the ICP0 genomic clone. Moloney murine leukemia virus reverse transcriptase (Life Technologies) was used per the manufacturer. To examine splicing, PCR used pfu cDNA polymerase, the above 3′-primer, and 5′-primer TAA GTGATCTGAAACCGGGGCCGGCGGCGGACG (KpnI site). To isolate exon 1 (28) of ICP0, PCR used the same 5′-primer and 3′-primer TGGCTCTAGATCTGGTGTGTCG (BamHI) and Xbol sites underlined. Full-length UL46 of HSV-2 was cloned by PCR into pCDNA3.1/His-C using 5′-primer CGAGGATCCGTCTCCGCCATGCAACGCCG and 3′-primer CGAGAATTCCTGGCGCGGTGGCGGGC (BamHI and Xbol sites underlined). Expression of aa 1–590 of UL47 was made by PCR, using the above 5′-primer, an appropriate 3′-primer, and pCDNA3.1/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 NolI 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 pCDNA3.1/His series, and UL49 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 3Cr 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 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 5 μg/ml for 30 min before infection. Actinomycin D was maintained throughout the 90-min infection, wash, and assay periods. For fibroblast targets, cells were plated in 9.6-cm2 plates and infected at 70–90% confluence, usually the next day, with the exception of IFN-γ experiments, in which some wells were treated with 500 U/ml IFN-γ for 90 min at 37°C in 2 ml volumes. In experiments with 5′ end-labeled [32P]RNA, the reaction conditions were reaction mixture (above) and full-length HSV-2 US8, US2, US4, US6, and US7, each cloned into pCDNA3.1/His series, and UL49 cloned into pEGFP-C1 (Clontech, Palo Alto, CA) as described and validated (35, 36).

**HLA-peak tetramers**

A tetramer reagent 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-FITC (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 DMSO (Sigma) 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*0401 (One Lambda, Canoga Park, CA) or PE-labeled mAb 1288 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 18B8B3 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 CD3+, CD8+, TCR αβ+, CD4−, and CD16/56− and recognized an HSV-2 type-specific epitope. For each clone, most HLA-A- and B-mismatched target cells were not lysed, regardless of viral infection. HLA-restricting alleles were preliminarily 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 proteins, we checked their cytolytic activity against EBV-LCL infected in the presence of actinomycin D. Clones 1874.1999.22, 5101.1999.23, and 5491.2000.48 lysed these target cells (Table I). Lysis by clone 1874.1999.51 was significantly inhibited by blockade of transfection. Each of the clones was able to lyse target cells infected with the mutant virus hr259, which lacks the ICP4 transactivator 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).

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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 Sau3AI 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 Sau3AI 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 Sau3AI 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 5′-vector-insert junction in C:2:C10:B9 revealed out-of-frame translation of the initial UL47 DNA. The insert is expected to contain the UL46 promoter (40). The epitope, therefore, could be encoded by UL46. In addition to 445 bp of 5′-untranslated sequence, all of UL47 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 Sau3AI fragment from nucleotides 1858–3022 (28). Nucleotide 2007 listed as T in the published sequence was A (not shown). 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, 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.dcrtr.nih.gov/molbio/hla_bind/). Peptide UL47 (HSV-2) 289–298 had a 50% effective concentration (EC50) in the 1–10 nM range in cytology 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).

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

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Specificity

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* 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).

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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>
</tr>
</thead>
<tbody>
<tr>
<td>5101.1999.23</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UL47 aa 1–696 (full length)</td>
<td>A*0201</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>1874.1991.22</td>
<td>None</td>
<td>A*0201</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UL47 aa 1–696</td>
<td>A*0201</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>
<td>A*0201</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UL47 aa 1–696</td>
<td>A*0201</td>
<td>2984</td>
</tr>
<tr>
<td></td>
<td>UL47 aa 1–535</td>
<td>A*0201</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UL47 aa 1–590</td>
<td>A*0201</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>1874.1997.51</td>
<td>Genomic, nucleotides 1858–3022</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>B*4501</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Genomic, nucleotides 1858–3022</td>
<td>B*4501</td>
<td>&gt;600</td>
</tr>
<tr>
<td></td>
<td>ICP0 exon 1 cDNA aa 1–25</td>
<td>B*4501</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>ICP0 exon 1 (start of exon 2) cDNA aa 1–105</td>
<td>B*4501</td>
<td>&gt;600</td>
</tr>
</tbody>
</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.
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-2-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.

TAP dependence of Ag processing for recognition by HSV-2 tegument protein epitopes by CD8 CTL

For each of the three CD8 clones studied, lysis of TAP-deficient cells after HSV-2 infection was greatly reduced in comparison to wild-type EBV-LCL (Table IV). Greater than 90% of each of the TAP-deficient cell lines, as well as control wild-type LCL, were permissive for viral infection and protein synthesis as evaluated by flow cytometry using mAb specific for envelope glycoprotein gD. Peptide loading was able to sensitize the TAP-deficient cells, confirming HLA class I expression.

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-1HSV-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...
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-<b>b</b>-microglobulin-peptide complexes by inhibition of TAP. This effect occurs quickly during viral infection (67).

FIGURE 5. CTL activity and tetramer binding by cultured PBMC of A*0201-bearing persons restimulated for 12–14 days with UL47 peptides. Top. Data are percent specific 51Cr release at E: T 20:1. Donor 1659 is HSV-1 and HSV-2 seronegative; the other donors are HSV-2 seropositive. Cells were stimulated with the indicated peptide as discussed in Materials and Methods. Target cells were EBV-LCL either with or without HLA A*0201 and were incubated with 1 µM peptide for 90 min or infected for 18 h with HSV-2 at MOI 10. Bottom. Detection of tetramer-binding cells among PBMC restimulated with HSV-2 UL47 peptide 551–559. The percentages listed are the proportion of CD8-staining cells that also stain with tetramer, using the thresholds shown. A, CD8 clone 1874.1991.22, specific for UL47 551–559, stained with anti-CD8-FITC only. All other panels are double stained with anti-CD8-FITC and a PE-labeled tetrameric complex of HLA A*0201 and peptide 551–559 (tetramer A2/551); B, clone 1874.1999.22 which is specific for peptide UL47 551–559 in the context of A*0201 (see text); C, negative control clone 5491.2000.48; D–J, bulk PBMC cultures restimulated with peptide UL47 551–559 as discussed in Materials and Methods. D, Subject 1659, HSV-uninfected; E–J, the six HSV-2-infected A*0201 subjects in the same order as in the top: 7282, 1874, 9107, 10081, 9383, and 5101, respectively. FL, Fluorescence.

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 51Cr 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 m551–559 for clone 1874.1991.22; UL47 551–559 for clone 5101.1999.23; 1p UL4702-restricted. Lysis of the A0201-bearing wild-type EBV-LCL twice.

Activity with two virion tegument proteins and one immediate early protein might outpace the down-regulation of HLA class I. It is rational to predict that processing of both vhs-encoded tegument protein VP13/14, which is present among lesion-infiltrating HSV-2-specific clones (41). Because responses to UL49 are also present in the cornea in herpes stromal keratitis in humans (35), a disease that may be driven by pathogenic 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 presentation 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 T cells, 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 IPC0 genomic clone started well upstream of the ATG start, extending to bp 2-infection, was able to sensitize target cells to lysis. 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 sensitization 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 fibroblasts by noninfectious particles. After 18 h of infection, the fibroblasts 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-infected cells. In contrast, 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.

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 Controls</td>
<td>1874</td>
<td>2.5</td>
<td>54.8</td>
</tr>
<tr>
<td>CD8 clone 5491.2000.48 Controls</td>
<td>1874</td>
<td>0.8</td>
<td>52.5</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.

**addition, vhs destabilizes host mRNA and reduces synthesis of new HLA class I. It is rational to predict that processing of both virion input proteins and immediate early proteins might outpace the down-regulation of HLA class I. A similar model has been proposed for human CMV, which also potently down-regulates.HLA class I. It is rational to predict that processing of both vhs-encoded tegument protein VP13/14, which is present among lesion-infiltrating HSV-2-specific clones (41). Because responses to UL49 are also present in the cornea in herpes stromal keratitis in humans (35), a disease that may be driven by pathogenic 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 processing pathways.

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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/Ago 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 CDS 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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We thank Al Farrand for essential advice concerning ELISA. Specimens were collected by the staff of the Virology Research Clinic. Sigrid N. Reymond, Christopher McClurcan, Matthew Wavra, Randal Cavallos, and Lonnie Yeung provided valuable technical assistance. HLA-typed EBV-LCL and T2 cells were kindly provided by Drs. J. Hansen, G. Nepom, and L. Musey. Dr. T. Spies provided cell line 721.174. Dr. C. E. Rouse provided vaccinia expressing ICP0 of HSV-2. Dr. A. Davison provided cell line T2/B7.63, originally made by Dr. P. Cresswell. Dr. S. Barcy provided valuable molecular biologic advice.

References