JOURNAL OF VIROLOGY, Mar. 1992, p. 1622–1634
0022-538X/92/031622-13$02.00/0

Vol. 66, No. 3

Human CD8$^+$ Herpes Simplex Virus-Specific Cytotoxic T-Lymphocyte Clones Recognize Diverse Virion Protein Antigens

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Received 3 September 1991/Accepted 9 December 1991

The role of the HLA class I- restricted, CD8$^+$, herpes simplex virus (HSV)-specific cytotoxic T lymphocytes (CTL) in the control of human HSV infections is controversial because previous reports suggest that a substantial portion of the antigen-specific lytic response is mediated by CD4$^+$ cells. To address this question directly, we isolated HSV-specific CD8$^+$ CTL clones from a patient with recurrent genital herpes. These CTL were cloned by coculturing responder peripheral blood mononuclear cells (PBMC) with phytohemagglutinin-stimulated PBMC that had been infected with live HSV-2 and then irradiated prior to the addition of responder cells. After 1 week, CTL were cloned by limiting dilution using phytohemagglutinin stimulation and allogeneic feeder PBMC. Seven clones were isolated; all seven clones were CD8$^+$ CD4$^-$ CD3$^+$ DR^{bright}, six lysed only HSV-2-infected targets, and one lysed both HSV-1- and HSV-2-infected targets. Antigen presentation was restricted by two to three different HLA class I loci. To determine the antigens recognized by these HSV-specific CTL, target cells were infected with HSV in the presence of acyclovir, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, or cycloheximide in a series of drug block/release protocols to limit the repertoire of viral gene expression to select transcriptional classes. Five of the clones exhibited a different pattern of cytotoxicity, suggesting that each recognized a distinct HSV antigen. One of the clones appears to be directed against an immediate-early antigen; six of the clones recognize virion proteins. Five of these clones recognized internal virion proteins that could be introduced into target cells by HSV infection in the absence of virus gene expression. Antigen specificity was further tested by using vaccinia virus vectors that express glycoproteins gD2 and gB2 or the tegument protein VP16. One clone lysed vaccinia virus/gD2-infected target cells; the remaining clones did not recognize any of these gene products. The diversity of the CD8$^+$ response from a single individual indicated that several different antigens are recognized when presented in the context of a variety of class I HLA alleles, a pattern that markedly differs from that described for another human herpesvirus, cytomegalovirus.

While several lines of evidence suggest that the cell-mediated cytolytic component of the immune response may be crucial in controlling herpes simplex virus (HSV) infections in humans, the exact role of these cells is poorly understood. In mice, CD8$^+$ cytotoxic T lymphocytes (CTL) have been shown to facilitate clearance of infected cells, as demonstrated by immunodepletion experiments (25, 42). CD8$^+$ CTL have also been shown to be responsible for reduction of virus at the site of infection by using adoptive transfer of this subset of lymphocytes from immune donors (6, 48). In humans, however, the existence of a substantial CD8$^+$ HSV-specific response has been surprisingly difficult to demonstrate. Previous workers have used UV-inactivated virus to stimulate HSV-specific memory CTL in vitro. In short-term bulk cultures, the majority of the HLA-restricted HSV-specific lytic activity was shown to be mediated by the CD4$^+$ T-cell subset (47, 54). Using a similar in vitro stimulation protocol with UV-inactivated virus or soluble glycoproteins, Zarling and coworkers isolated bifunctional HSV-specific CTL clones that were cytolytic, secreted interleukin-2 (IL-2), and were, with one exception, CD4$^+$ (63–65). Recently, Yasukawa et al. (62) reported that CD8$^+$, HSV-specific CTL can be demonstrated in HSV-1-seropositive individuals when peripheral blood mononuclear cells (PBMC) are stimulated with cell-associated viral antigens. This study emphasizes the critical role of the in vitro stimulation conditions for the activation of antigen-specific, HLA-restricted CTL responses.

By using HSV-infected antigen-presenting cells (APCs) for in vitro stimulation, we have detected HSV-specific CD8$^+$ CTL in an individual with frequently recurring type 2 herpes and succeeded in isolating seven CD8$^+$, HSV-specific CTL clones from a second individual with recurrent genital herpes. One of the clones and a significant level of activity by CTL in bulk cultures is directed against glycoprotein gD2 as expressed by a recombinant vaccinia virus vector in infected target cells. The other clones exhibit a diversity of antigen recognition, including gene products of the α, β, and possibly γ transcription classes. It is also of interest that most of the CD8$^+$ CTL clones were HSV-2 specific. This diversity of responses from a single individual appears to differ from that described for other herpesviruses such as cytomegalovirus (CMV). However, as with human CMV, many of the target proteins for CD8$^+$ HSV-specific CTL may be internal virion proteins which can be presented in the context of HLA class I in the absence of endogenous protein synthesis, thus rendering an infected cell susceptible to lysis very early in the process of virus infection (46).

MATERIALS AND METHODS

Cell lines and viruses. Virus stocks of HSV-1 strain Patton and HSV-2 strains MS and 333 were prepared from Vero or

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human embryo tonsil cells infected at low multiplicity of infection (MOI), usually 0.01. Cell-associated virus was released from cells by freezing and thawing or by sonication. Low-titer preparations of HSV-2 were concentrated by centrifugation. Vaccinia virus stocks were propagated in BSC-40 cells and purified from cytoplasmic extracts as described elsewhere (31). Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (B-LCL) were obtained by coculture of PBMC with supernatants of B95.8 cells persistently infected with EBV in the presence of 1 μg of cyclosporin A (Sandoz Research Institute, East Hanover, N.J.) per ml (45). The lines were maintained in RPMI-F (RPMI 1640 [J. R. H. Biosciences, Lenexa, Kans.] containing 2 mM glutamine supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum [FBS; HyClone, Logan, Utah]). Human dermal fibroblast lines were initiated from punch biopsies of the skin in the deltoid area. The minced tissue was cultured in Waymouth's medium (GIBCO, Grand Island, N.Y.) supplemented with 20% FBS and 10 ng of recombinant human acidic fibroblast growth factor (Chiron Corp., Emeryville, Calif.) per ml. Once established, the lines were cultured in Waymouth's medium supplemented with 10% FBS and 10 ng of fibroblast growth factor.

Donors. Blood was collected by venipuncture into heparinized blood collection tubes from volunteers with clinical recurrent genital herpes. The donors were seropositive for HSV-2 and seronegative for HSV-1, as determined by Western immunoblot assay (2). Their clinical status was confirmed by isolation of HSV-2 from genital lesions (28). The donors participated in a protocol approved by the Human Subjects Review Committee of the University of Washington.

Antibodies and surface marker analysis. The hybridomas W6/32 and 2.06 were obtained from the American Type Culture Collection, Rockville, Md. Antibodies were prepared from ascites fluid (W6/32) or conditioned culture medium (2.06). The anti-CD8 monoclonal antibody (MAb) Leu 2 used for panning was provided by Chris Walker, Chiron. Goat anti-mouse immunoglobulin was obtained from Protos (South San Francisco, Calif.). Flow cytometry analysis was performed by the Clinical Immunology Laboratory at the University of California, San Francisco.

Construction of recombinant vaccinia viruses. The recombinant viruses vac/gB2-tk$^-$ and vac/gD2-tk$^-$, which contain the gB and gD genes of HSV-2, respectively, under the control of the early/late vaccinia virus promoter p7.5, were obtained from Ian Ramshaw of the John Curtin School of Medical Research, Australian National University, Canberra. The virus vac/VP16-tk$^-$, containing the VP16 gene of HSV-2 also under the control of the vaccinia virus p7.5 promoter, was constructed as described by Sekulovich (47a). To prepare vaccinia virus-infected target cells, B-LCL were infected with the vaccinia virus recombinants at an MOI of 10 18 to 20 h prior to ^{51}Cr loading and CTL assay.

Generation of HSV-specific CTL. (i) Preparation of stimulators. PBMC were purified from heparinized whole blood by centrifugation onto a Ficoll (Pharmacia, Piscataway, N.J.) cushion. The washed PBMC (5×10^6) were seeded into T-25 flasks in 5 ml of RPMI-CF (RPMI 1640 containing 2 mM L-glutamine supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES; pH 7.2], 1% minimal essential medium with nonessential amino acids, 1% minimal essential medium with vitamins, 1 mM sodium pyruvate [GIBCO], 20 μg of asparagine per ml 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, 100 U of streptomycin per ml, and 10% fetal calf serum) and stimulated by incubation with 200 ng of phytohemagglutinin-P (PHA-P; Burroughs-Welcome, Research Triangle Park, N.C.) per ml for 48 h in upright flasks. The cells were then washed once with serum-free RPMI, returned to the flask in 0.5 ml of serum-free RPMI, and infected with HSV-2 strain MS at an MOI of 4. After 1 h, 5 ml of RPMI-F containing serum was added, and the infection was continued for 16 h. The cells were then washed free of virus, resuspended in 5 ml of RPMI-CH (RPMI-CF except that fetal calf serum was replaced with 10% heat-inactivated, pooled human serum), and returned to the flask. Autologous B-LCL stimulators were also infected at an MOI of 4 in serum-free RPMI and then incubated overnight in RPMI-F. Autologous fibroblast stimulator cultures were initiated by seeding T-25 flasks with fibroblasts in Waymouth's medium lacking fibroblast growth factor and cultured until 90% confluent. The monolayers were washed with serum-free medium, infected with HSV-2 at an MOI of 5, and incubated overnight in 5 ml of Waymouth's medium containing 10% FBS. The infected fibroblasts were washed with phosphate-buffered saline (PBS) and treated for 1 h with 1 μg of mitomycin per ml (17). The other infected stimulator cultures were exposed to 7,500 R of γ radiation and placed at 4°C for 1 h. For the PBMC, the nonadherent cells were removed from the flasks and washed with RPMI-CM. The adherent cells remaining in the flasks were also washed, and then the nonadherent irradiated cells were returned to the starting flask in 5 ml of medium. The responder cells were then added to these same flasks for stimulation.

(ii) Preparation of responders. PBMC were purified from fresh whole blood or recovered from cyropreservation, washed, and resuspended in 5 ml of RPMI-CH. Between 1×10^7 and 3×10^7 responders were seeded per flask of stimulator cells, and the flasks were incubated standing upright for 1 week. The cells were then recovered from the stimulation flasks, washed, and assayed or further purified by panning (56, 60). Briefly, the responders were incubated with 1 μg of anti-CD8 MAb per 10^6 cells for 20 min at 4°C. The unbound antibody was removed by washing the cells in PBS containing 1% FBS, and then the cells were added to petri dishes precoated with goat anti-mouse immunoglobulin antibodies and incubated at 4°C for 2 h. Nonadherent cells were removed by washing with PBS, and then CD8$^+$ cells were removed from the plates with a high-pressure stream of buffer. The cells were concentrated by centrifugation and then added to T-25 flasks for bulk culture. Approximately 10% of the stimulated responder cells were recovered as CD8$^+$ cells from the panning plates. The recovered T cells were stimulated with PHA-P and sustained by cocultivation with allogeneic feeder PBMC (37). The allogeneic feeder PBMC were prepared from fresh blood or buffy coats obtained from a blood bank. After Ficoll separation, the cells were exposed to 3,000 R of γ radiation and added to the T-25 flasks at a final concentration of 10^6/ml in RPMI-CH containing 200 ng of PHA-P per ml and purified human IL-2 (32 BRMP units/ml, final concentration; Pharmacia). After 48 h, cells were removed from the T-25 flasks, washed, and returned to the same flask in medium containing IL-2. The cultures were maintained for 10 to 14 days before assay.

Establishment of HSV-specific CTL lines. CD8$^+$ T cells stimulated by infected PHA blasts and isolated by panning were seeded into Terasaki wells at an average of two cells per well in 10 μl of RPMI-CH. Allogeneic feeder cells (10^4) were added in 10 μl of RPMI-CH containing 200 ng of PHA-P per ml and 32 U of IL-2 per ml for each well of the Terasaki plates. The plates were fed at 4- to 5-day intervals

by adding medium containing IL-2 until day 14, when T-cell growth was apparent. Cells were removed from the positive wells and expanded by cultivation in 96-well plates with 10^5 allogeneic feeders per well in medium containing PHA and IL-2. After 48 h, the medium was replaced with medium containing IL-2, and the plates were refed on days 6 and 10. On day 14, the cultures were split into three replicates; two were used for screening assays, and the third was preserved for expansion. The replicates were tested in a ^{51}Cr release assay using uninfected or HSV-2-infected autologous B-LCL. A candidate was scored as positive if the counts per minute released from infected B-LCL was greater than 3 standard deviations above the mean spontaneous release and twofold greater than the value for uninfected B-LCL target cells. Positive cultures were expanded by cultivation in 24-well and then 6-well plates and retested in a ^{51}Cr release assay with graded effector/target cell (E/T) ratios, using autologous and HLA-mismatched targets. The resulting HSV-specific cultures were then cultured in RPMI-F containing 32 U of IL-2 per ml and restimulated with PHA at 2- to 3-week intervals.

Subcloning of $CD8^+$ CTL. The CTL lines were subcloned in U-bottom 96-well plates by seeding 0.3 cells per well in RPMI-CH containing 10^5 irradiated feeder PBMC and 200 ng of PHA-P per ml. After 48 h, the medium was replaced with RPMI-CH containing 48 U of IL-2 per ml, and the plates were refed at 4-day intervals with medium containing 32 U of IL-2 per ml. After 2 weeks, the plates were stimulated a second time with fresh feeders and PHA. T-cell growth was generally apparent 7 to 9 days later. The resulting clones were expanded and tested for HSV-specific lysis. The initial clone is indicated here by the designation 1H6, for example, and the subclone derived from the parent clone is designated 1H6.5

Chromium release assays. Autologous or HLA-mismatched EBV-transformed β-LCL infected with HSV-2 at an MOI of 2 to 4 were used as target cells. After incubation overnight, the cells were washed, concentrated, and resuspended in 0.2 to 0.4 ml of medium containing 50 to 100 μCi of ^{51}Cr for 90 min. The target cells were then washed three times and resuspended at 0.5×10^5 or 1×10^5 cells per ml, and 0.1-ml aliquots were added to the effector cells. For screening of clones, effector cells were removed from their 96-well plates and seeded into two identical replicates in 96-well U-bottom plates. Target cells were either uninfected or infected autologous B-LCL. For assays of bulk cultures or isolated clones, the effector cells were recovered from culture flasks, washed, and seeded into triplicate wells of U-bottom microtiter plates to achieve E/T ratios of 100:1 (bulk cultures) or 10:1 (clones). The effectors were then diluted serially twice in two- or fourfold steps. Control wells containing target cells alone to determine the spontaneous ^{51}Cr counts per minute released or target cells plus 0.5% Nonidet P-40 to determine the total ^{51}Cr counts per minute released were included in all assays. The plates were centrifuged briefly at $800 \times g$ and incubated for 4 h at 37°C in a humidified CO_2 incubator. The assays were completed by removing 100 μl of the supernatants for counting. Percent specific lysis was calculated as [(average cpm released − average spontaneous cpm)/(average total cpm released − average spontaneous cpm)] × 100.

Preparation of drug-treated targets. To prepare target B-LCL treated with IL-2 and acyclovir, the cells were infected with HSV-2 in serum-free RPMI for 1 h, and then RPMI-F containing 32 U of IL-2 per ml or 150 μM acyclovir (Burroughs-Wellcome) was added to the cultures. The cells were cultured with the added drugs present continuously during infection, ^{51}Cr loading, and the CTL assay. To prepare targets treated with the RNA synthesis inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Calbiochem, San Diego, Calif.), B-LCL were treated with 100 μM DRB for 1 h and then the cells were infected at an MOI of 10 in serum-free RPMI containing DRB. After 1 h, RPMI-F containing DRB was added, and the cells were cultured for 0.5 to 2 h before being washed, concentrated, and loaded with ^{51}Cr for 2 h. The inhibitor was present during the loading, washes, and assay. The DRB treatment inhibited transcription by greater than 95%, as measured by incorporation of [^{3}H]uridine into acid-precipitable material. The preparation of targets treated by sequential cycloheximide block and release as described by Del Val et al. (12) is outlined in Fig. 3. Briefly, the B-LCL were pretreated for 1 h with cycloheximide (Sigma, St. Louis, Mo.) at a concentration of 25 μg/ml, infected in serum-free RPMI, and cultured for 4 h after dilution in the presence of cycloheximide. The cells were then washed twice in medium free of drugs or medium containing 5 μg of actinomycin D (ActD; Calbiochem) per ml and then loaded with ^{51}Cr for 2 h in 0.2 ml of medium. The cultures were washed three times in drug-free or ActD-containing medium and then added to the effector cells without additional drugs or in medium containing 20 μg of ActD per ml. ActD was included during loading at a concentration of 10 μg/ml, during subsequent washes at 5 μg/ml, and during culture with the CTL at 10 μg/ml. The cells were kept in the dark as much as possible by wrapping tubes and assay plates with aluminum foil during incubations.

Preparation of RNA and Northern (RNA) blot analysis. RNA was isolated from 3×10^7 infected B-LCL, which were washed once with PBS containing 1% FBS and immediately frozen in a dry ice-ethanol bath prior to storage at −70°C. Total RNA was prepared from the culture samples by a modification of the method of Han and Rutter (18) as described previously (9). To prepare the Northern blots, the RNA was denatured by incubation with 1.1 M formaldehyde–50% formamide in R buffer (5 mM EDTA, 5 mM sodium acetate, 20 mM morpholinepropanesulfonic acid [MOPS], pH 7) for 5 min at 65°C. The RNA was electrophoresed through a 1% agarose gel formed in 1.1 M formaldehyde plus R buffer. The gel was stained for 30 min with 1 μg of ethidium bromide per ml, photographed, and destained for 60 min in R buffer, followed by blot transfer to GeneScreen Plus (Dupont Co., Boston, Mass.) as directed by the manufacturer. The filters were prehybridized for 4 h at 42°C in 60% formamide–10% dextran sulfate–1% sodium dodecyl sulfate (SDS)–1 M NaCl. ^{32}P-labeled probes (specific activity, $>10^9$ cpm/μg) were prepared by using a random-priming kit from Amersham (Chicago, Ill.). For hybridization, ^{32}P-labeled probe (10^6 cpm/ml), total yeast RNA (50 μg/ml; Calbiochem), denatured salmon sperm DNA (10 μg/ml), and poly(A) (1 μg/ml; Pharmacia) were added to the prehybridization mixture and hybridized for 16 h at 42°C. For detection of ICP0, the HSV-2 3.9-kb *Bam*HI fragment pG-Bam5 of pGR90 was used as a probe (35). A 1.2-kb *Bam*HI fragment derived from a plasmid containing the ICP4 gene in a 2.2-kb *Hin*dIII-*Pst*I fragment was used for detection of ICP4 mRNA (49). The VP16 probe was derived from a plasmid carrying the HSV-2 VP16 gene (47a) as a 1.2-kb *Bgl*I fragment. The probe for gD2 consisted of a 845-bp *Sma*I fragment obtained from a plasmid containing the HSV-2 DNA *Hin*dIII L restriction fragment that encodes residues 27 to 313 of the mature gD2 glycoprotein (57). Following

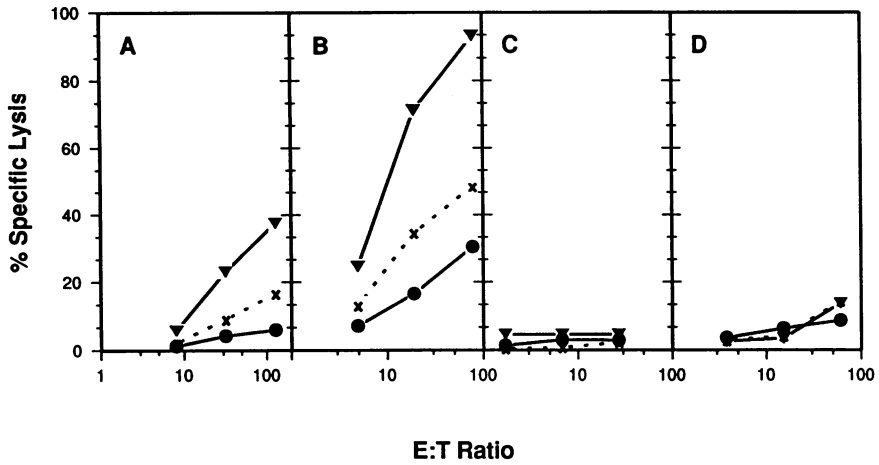

FIG. 1. Comparison of stimulation conditions for HSV-2-specific CTL. PBMC were purified from fresh whole blood (A and C) or recovered from cryopreservation (B and D). Stimulator cells consisted of HSV-2-infected autologous PHA blasts (A and B), infected autologous EBV-transformed B-LCL (C), or infected autologous fibroblasts (D). The infected lymphoblasts were irradiated, and the fibroblasts treated with Mitomycin before responders were added. After 1 week, responder cells were recovered from the stimulation flasks and washed, and graded numbers were added in 100 μl of medium to the assay plates to achieve the E/T ratios indicated. Autologous (▼) or completely mismatched (×) EBV-transformed B-LCL were infected overnight with HSV-2 at an MOI of 4. Uninfected (●) autologous B-LCL and the infected B-LCL were loaded with ^{51}Cr for 90 min, and 10^4 targets were added in 100 μl per well to the assay plate. After 4 h, 100 μl was removed for counting.

hybridization, the filters were washed in 0.1 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C for 3 h. The dried filters were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with an intensifying screen (Dupont) for 16 h.

RESULTS

Conditions of stimulation influence HSV-specific CTL responses. In preliminary experiments, we tested various stimulation conditions to optimize the amplification of HSV-specific CTL. PBMC from an individual with frequently recurring genital herpes were stimulated in vitro by using three different potential APCs, autologous PBMC stimulated with PHA (PHA blasts) to render them susceptible to HSV infection (17), autologous B-LCL, and autologous fibroblasts. To simplify the timing of subsequent experiments, we also compared the cytolytic effector function of cryopreserved versus freshly prepared PBMC. The stimulator cell populations were infected with HSV for 16 h and then irradiated to attenuate viral infectivity prior to addition of the responder cells. The cells were cultured together without added lymphokines for 1 week at a responder/stimulator ratio of approximately 5:1. The cultures were then assayed for the ability to lyse HSV-infected and uninfected autologous B-LCL as monitored by ^{51}Cr release (Fig. 1). A completely HLA-mismatched allogeneic B-LCL was included as a control target cell to determine the extent of HLA-unrestricted (natural killer) lytic activity generated under these conditions. The infected PHA blasts were potent stimulators of CTL for both freshly prepared (Fig. 1A) and previously frozen (Fig. 1B) responder cells, while neither the B-LCL (Fig. 1C) nor the fibroblasts (Fig. 1D) were efficient APCs under these conditions. The cultures stimulated with the infected PHA blasts also exhibited a significant level of unrestricted lytic activity, whereas the infected B-LCL- or fibroblast-stimulated cultures were uniformly unresponsive. The differences observed in the induction of lytic activity in the responder cell populations were not due to differences in viability, since all four cultures were similar in appearance and the number of responders recovered from each culture and their viabilities as determined by trypan blue exclusion were comparable.

This experiment did not distinguish between lysis by $CD4^+$ or $CD8^+$ CTL. To determine whether HLA-restricted lysis mediated by $CD8^+$ CTL could be detected, PBMC from a second individual with frequently recurrent genital herpes were stimulated for 7 days by cocultivation with infected autologous PHA blasts, and then $CD8^+$ cells were isolated by panning (56, 60). The recovered $CD8^+$ cells were stimulated with PHA and then cultured for 14 days in the presence of irradiated allogeneic feeder cells and human IL-2 (37). Lytic activity was assayed against several types of target cells (Fig. 2). This $CD8^+$ cell population efficiently lysed autologous HSV-infected B-LCL as well as HSV-infected, partially HLA-matched B-LCL but not uninfected targets. These bulk cultures had no measurable natural killer activity, as indicated by the absence of lysis of K-562 cells. To determine whether the cytolytic activity was directed against certain abundant virion proteins, recombinant vaccinia viruses expressing the HSV-2 glycoproteins gD and gB or the tegument-associated transactivator protein VP16 were used to infect autologous B-LCL targets. The bulk $CD8^+$ CTL population efficiently lysed vac/gD2-infected target cells and did not lyse target cells infected with the wild-type parental strain. In contrast, gB- or VP16-specific lysis was not detected despite higher levels of expression of these genes than of the gD2 gene in infected B-LCL, as measured by Western blot assay. Moreover, mouse fibroblasts infected with this vac/gB2 vector are recognized by HSV-immune mouse splenocytes (data not shown).

Cloning of HSV-specific CTL. To begin to determine the repertoire of HSV antigens recognized by the $CD8^+$ CTL, clones were isolated from a patient who was HSV-2 solely seropositive and who had culture-proven recurrent genital HSV-2 disease. To obtain these clones, PBMC were stimulated by cocultivation with HSV-infected, PHA-activated lymphoblasts for 1 week. $CD8^+$ cells were isolated by

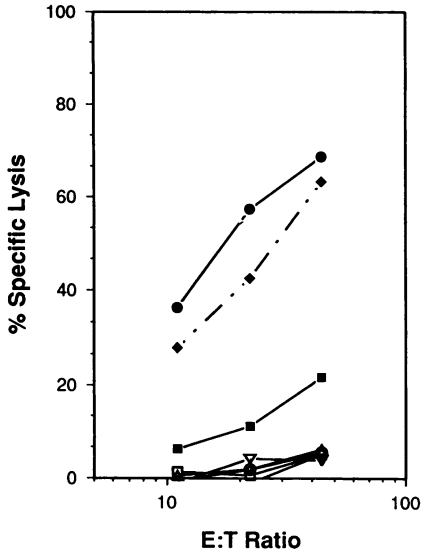

FIG. 2. Lysis by CD8$^+$ CTL expanded in bulk cultures. PBMC were stimulated with HSV-infected PHA blasts for 7 days, and then CD8$^+$ cells were isolated by panning. The recovered cells were cultured in RPMI-H containing 200 ng of PHA-P per ml, 32 U of IL-2 per ml, and 10^7 irradiated allogeneic PBMC for 48 h; then the cells were washed and returned to the flask in RPMI-H containing 32 U of IL-2 per ml for 12 days. For preparation of targets, autologous (●) and partially HLA-matched (◆) B-LCL were infected at an MOI of 4 with HSV-2 or at an MOI of 10 with wild-type vaccinia virus strain WR (□) or recombinant viruses expressing HSV-2 glycoprotein gD2 (■), gB2 (△), or VP16 (▽). After a 16-h infection, the B-LCL, uninfected autologous B-LCL (○), and the natural killer target line K-562 (◇) were washed and loaded with ^{51}Cr, and 10^4 cells in 100 μl of medium were added to the assay plates. After 4 h, 100 μl was removed for counting. The allogeneic B-LCL shared HLA B7, Cw7, and DR2 with the CTL.

panning and were seeded into the wells of Terasaki plates at an average input of two cells per well. The cultures were supplemented by the addition of allogeneic feeder cells, PHA, and IL-2 (37). After 14 days, 206 of 282 (73%) of the wells contained growing cells. The candidates were screened for HSV-specific cytolytic activity, and seven clones were identified.

Because greater than 66.7% of the wells in the initial cloning step generated viable cultures, the isolates could not be considered truly clonal. Nevertheless, the HSV antigen specificity of the isolated lines is probably conferred by a single CTL clone in the potentially oligoclonal populations, since the frequency of HSV-specific CTL among the candidate lines was 0.035. To avoid awkwardness, the HSV-specific candidates will be referred to as clones. Four of the seven clones were subcloned by limiting dilution and have been characterized. The subclones retain the specificity of their parent clones, so it is likely that the results of the analysis of the clones are characteristic of the CTL subclones isolated from that population. One of the clones lost specificity for HSV and was not successfully subcloned. The remaining two clones have been characterized to a more limited extent.

Characterization of HSV-specific CTL. The clones were analyzed in detail to determine virus type specificity, surface phenotype, and HLA restriction. As shown in Table 1, six of the seven HSV-2-specific clones did not recognize target cells infected with HSV-1. The surface phenotype of the

TABLE 1. Type specificity and HLA class restriction of seven HSV-2-specific CTL clones

Clone	% Specific lysis of virus-infected targets[a]		% Inhibition by W6/32[b]
	HSV-2	HSV-1	
1H6	23	4	30 ± 9
3B3	59	48	60 ± 9
2H12	30	3	75 ± 13
2E7	21	0[c]	88 ± 1
2C1	34	0	100
2F11	38[d]	2	ND[e]
2D12	51[d]	1	ND

[a] Autologous B-LCL were infected with HSV-2 strain MS or HSV-1 strain Patton at an MOI of 4 and cultured for 16 h prior to CTL assay. The E/T ratio was 5:1.

[b] HSV-2-infected B-LCL were loaded with ^{51}Cr and then resuspended in medium containing a 1/50 or 1/80 dilution of W6/32 ascites fluid and incubated for 20 min before addition of CTL. Inhibition was calculated by comparison with lysis of target cells treated with the same dilution of an ascites fluid containing an isotype-matched control antibody. The E/T ratio was 5:1. Values are means and standard errors of two to five experiments.

[c] A value of 0 was assigned when the counts per minute released in the test wells were fewer than in the spontaneous release wells.

[d] E/T ratio of 10:1.

[e] ND, not done.

seven CTL clones was CD8$^+$ CD4$^-$ CD3$^+$ HLA DR^{bright}, as characterized by flow cytometry analysis (data not shown). The class of the HLA restriction element recognized by the clones and their subclones was tested by using MAb W6/32, specific for a monomorphic class I determinant, to block lysis (3). The antibody was added to infected, ^{51}Cr-loaded B-LCL targets for 20 min before addition of effector cells (Table 1). The lytic capacity of all clones tested was inhibited by preincubation of target cells with MAb W6/32, although the 1H6.5 subclone was more resistant to blocking than were the other clones. In a similar experiment using MAb 2.06, which recognizes monomorphic determinants on HLA DR (11), lysis was not inhibited (data not shown).

The class I HLA element restricting recognition of HSV epitopes for five clones was determined by using a panel of B-LCL that matched the donor HLA type at one or more loci as target cells. The pattern of recognition of partially HLA-matched target cells and the deduced restriction element for each of the five HSV-specific CTL clones are shown in Table 2. Three of the five clones recognize their cognate HSV-2 antigen in the context of HLA B7, and one clone does so in the context of either B7 or Cw7. The fifth clone, 3B3, recognizes an HSV type-common epitope in the context of HLA A32. In addition to recognizing an HSV antigen, the 1H6.5 subclone exhibited alloreactivity to B-LCL bearing the HLA B13 class I molecule.

Identification of antigen specificity with recombinant vaccinia virus vectors. Vaccinia virus recombinants expressing the HSV-2 gB, gD, or VP16 gene were used to determine whether any of the clones recognized one of these virion proteins. As shown in Table 3, none of the clones lysed vaccinia virus-infected targets expressing gB2 or VP16; one clone, 1H6, recognized vac/gD2-infected targets.

Identification of antigen specificity with drug-treated target cells. As HSV encodes at least 70 proteins (33), reagents to identify all of them are not available. To narrow the focus for further characterization of the antigen-specific CTL clones, infected target cells were treated with selective drugs to limit the repertoire of viral gene expression to specific transcriptional subclasses as outlined in Fig. 3. Four broad classes of

TABLE 2. Lysis pattern of HSV-specific CTL clones from patient JC for partially or fully HLA-matched B-LCL

B-LCL line	HLA type[a]	Lysis by CTL clone:					
		1H6.5[b]		3B3	2H12	2E7	2C1
		−HSV	+HSV				
Autologous	A24,32 B7,57 Cw6,w7 DR2,7 DQw1,w3	−	+	+	+	+	+
GS	A2 B7 Cw7 DR2 DQw1	−	+	−	+	+	+
GH	A2,32 B44,27 Cw2,w5 DR4,11 DQw3	−	−	+	−	−	−
RB	A1,2 B44,37 Cw⁻ DR2,X DQw1,w7	ND[c]	−	−	−	ND	ND
SA H14	A3,32 B44,w60 Cw3,w5 DR2,w6,w52 DQw1,wx	ND	−	+	−	−	ND
KAS 115	A2 B13 Cw7 DR7 DQw2	+	+	−	−	−	ND
SA H3.1	A3,x B13,35 Cw4,wx DR6,w11(5),w52 DQw1,w3	+	+	−	−	−	ND
B Sch	A2,3 B7,w62 C⁻ DR5,w6 DQw7(3),1	−	+	−	+	ND	ND
Autologous	Restriction element for CTL	B13	B7	A32	B7	B7	B7 Cw7

[a] HLA matches with the effectors are underlined.
[b] Subclone of 1H6.
[c] ND, not done.

genes are transcribed sequentially during an infection (reviewed in reference 55). The first class of immediate-early (IE) (α) transcripts is expressed in the absence of viral protein synthesis. Early (β) transcripts are transactivated by α proteins and thus require viral protein synthesis for their expression. As β transcription begins, most α transcription ceases and the corresponding α gene products rapidly disappear (50, 58). Early-late transcripts (γ_1), are normally expressed as viral DNA replication commences, but they are also expressed when replication is blocked (55, 58). The glycoproteins gD and gB are members of the γ_1 transcription class (55). The true late (γ_2) class, which encodes many structural virion proteins, is not expressed in the absence of viral DNA synthesis.

To determine whether any of the remaining six clones also recognize virion proteins as does the gD2-specific CTL clone 1H6.5, target cells were treated with acyclovir after infection to block HSV DNA synthesis and thus inhibit γ_2 expression. Parallel cultures of B-LCL were infected with HSV-2 for 1 h, and then acyclovir was added to one of the cultures at a final concentration of 150 μM. The target cells were incubated for an additional 16 to 18 h and then loaded with ^{51}Cr and mixed with the CTL. As shown in Table 4, two of the subclones, 3B3.4 and 2H12.5, lysed acyclovir-treated target cells with efficiency similar to that of untreated target cells. In a second experiment with the 3B3.4 subclone, specific ^{51}Cr release was 64.4% from acyclovir-treated cells, compared with 69.8% from untreated cells, at a 10:1 E/T ratio (data not shown). The lytic capacity of the 2C1 clone, on the other hand, was enhanced substantially when γ_2 gene expression was suppressed by acyclovir addition compared with untreated cells. In contrast, the 1H6.5 subclone and 2E7 clone exhibited reduced lysis of the acyclovir-treated target cells. The remaining two clones, 2F11 and 2D12, also lysed HSV-2 infected cells with comparable efficiency, with and without acyclovir treatment (data not shown). Thus, three distinct patterns of acyclovir sensitivity of the cytolytic activity were observed for the seven clones: decreased (two clones), enhanced (one clone), and unaltered cytolysis (four clones).

TABLE 3. Antigen specificity of seven HSV-2-specific CTL clones

CTL clone	% Specific lysis of vaccinia virus-infected targets[a]			
	vac/WR	vac/gD2	vac/gB2	vac/VP16-2
1H6	8	64	6	5
3B3	14	10	3	7
2H12	3	2	2	1
2E7	1	0[b]	0	0
2C1	1	2	3	0
2F11	3	0	0	2
2D12	1	1	0	2

[a] Autologous B-LCL were infected with wild-type vaccinia virus (WR) or vaccinia virus recombinants at an MOI of 10 for 16 h prior to CTL assay. The E/T ratio was 10:1.
[b] The counts per minute released in the effector-containing wells were fewer than in the spontaneous release control wells.

During the initial characterization experiments, the CTL were maintained in medium containing IL-2 even during the incubation with target cells. Lysis by the 2C1 clone was quite variable under these conditions. Because B cells express IL-2 receptors and can be induced to differentiate by the lymphokine (5, 40, 41, 53), it was possible that the course of an HSV infection might also be affected by IL-2 treatment. Therefore, the B-LCL targets were infected with HSV-2, IL-2 was added to 32 U/ml (final concentration), and the cells were incubated overnight in parallel with untreated or acyclovir-treated cells (Table 4). Lysis by four of five CTL clones tested was not significantly affected by this treatment, but lysis by 2C1 was abolished.

As an alternate approach to limiting presentation to virion proteins, cells were exposed to DRB prior to infection to prevent any viral RNA synthesis. Under these conditions, only virion proteins released into the cytoplasm after fusion of the envelope with the plasma membrane would be processed and presented. DRB was chosen instead of ActD because it causes less damage to the cells while preventing

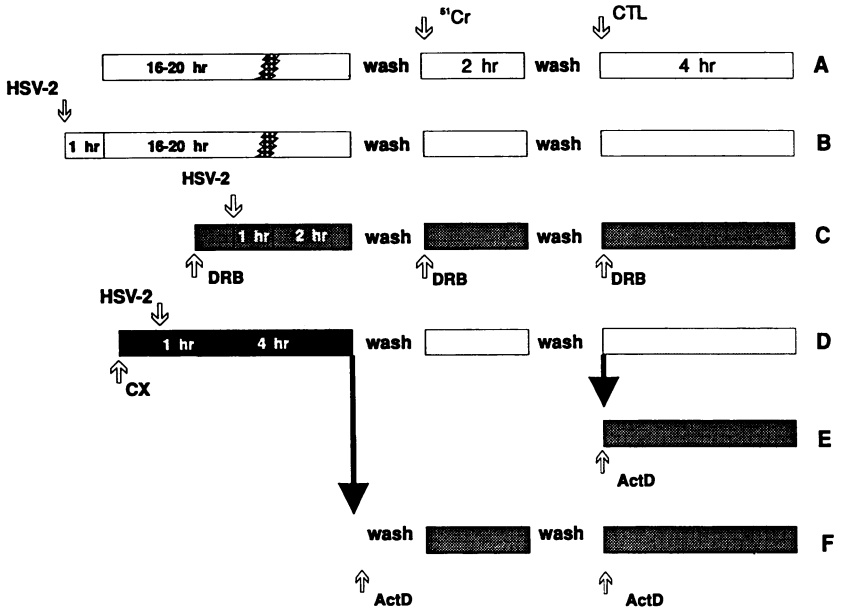

FIG. 3. Schematic for the preparation of drug-blocked/released targets. The inhibitors cycloheximide (CX) and DRB were added to B-LCL 1 h prior to infection with HSV-2. Infections were initiated by incubation of cells and virus in 0.5 ml of serum-free medium for 1 h prior to dilution with medium containing serum and drugs. Drugs were included during washes where appropriate. (A) Uninfected targets; (B) HSV-2 infected targets untreated or treated with 150 μM acyclovir or 32 U of IL-2 per ml; (C) DRB-treated targets; (D) cycloheximide-treated and infected targets released from drug block; (E) cycloheximide-treated targets released from drug block for 2 h and then blocked with ActD; (F) cycloheximide-treated targets shifted to an ActD block.

RNA synthesis as effectively (20). Target B-LCL were pretreated with DRB for 1 h, infected in the presence of DRB, and incubated for 1.5 to 3 h prior to loading with ^{51}Cr and exposure to the CTL. A parallel culture of untreated cells was infected and incubated for the same time periods were as the DRB-treated target cells. A third culture was infected and incubated in the absence of drugs for 16 to 18 h. As summarized in Table 5, three clones, 3B3.4, 2H12.5, and 2F11, were able to lyse DRB-treated cells as efficiently as did target cells infected for the same amount of time in the absence of drug. Another clone, 2D12, lysed DRB-blocked cells with half the efficiency of untreated target cells. In contrast, the 2C1 clone lysed untreated target cells very poorly. However, cells treated with DRB were lysed efficiently. The 2E7 clone and 1H6.5 subclone did not lyse target cells treated with DRB. These same clones also lysed cells infected for less than 6 h with substantially lower efficiency than did targets that had been infected overnight.

The target antigen specificity of the 3B3 and 2H12 clones was further examined by comparing the lysis susceptibilities of target cells infected with HSV-2 and exposed to one of three drug treatment protocols: (i) infection with HSV-2 in the presence of the protein synthesis inhibitor cycloheximide followed by release from drug arrest, (ii) treatment with cycloheximide followed by drug removal and addition of ActD, or (iii) treatment with cycloheximide followed by drug removal and a 2-h delay prior to the addition of ActD as diagramed in Fig. 3D to F. For the first treatment protocol, the cycloheximide was removed 5 h after infection; the cells were loaded with ^{51}Cr and then mixed with effector cells for a 4-h incubation in the absence of drugs (Fig. 3D). Under these conditions, the time course of viral gene expression

TABLE 4. Effect of acyclovir and IL-2 treatment of HSV-infected target cells on lysis by CTL clones

Treatment	E/T ratio	% Specific lysis					% Spontaneous release
		1H6.5	2B3.4	2H12.5	2E7	2C1	
Uninfected	10:1	0[a]	3	8	0	7	11
	2.5:1	0	0	8	0	5	11
None[b]	10:1	55	66	35	37	15	9
	2.5:1	40	29	40	32	12	9
Acyclovir[c]	10:1	12	82	40	5	44	10
	2.5:1	12	58	33	6	42	10
IL-2[c]	10:1	45	54	23	34	1	10
	2.5:1	46	21	15	37	1	10

[a] A value of 0 was assigned when the mean counts per minute released were fewer than in the spontaneous release control wells.
[b] The target B-LCL were infected with HSV-2 at an MOI of 2, incubated overnight (18 h), and then loaded with ^{51}Cr for 90 min prior to assay.
[c] Target cells were treated with 150 μM acyclovir or 32 U of IL-2 per ml from 1 h after addition of virus. The cells were maintained with acyclovir or IL-2 throughout ^{51}Cr loading and incubation with the effector cells.

TABLE 5. Effect of DRB treatment of HSV-infected target cells on lysis by CTL clones

Treatment	E/T ratio	% Specific lysis							% Spontaneous release
		1H6.5	3B3.4	2H12.5	2E7	2C1	2F11	2D12	
Uninfected	10:1	1	0	10	2	2	0[a]	0	12
	2.5:1	0	0	8	0	0	0	0	12
None	10:1	71	82	62	28	2	37	53	13
18 h[b]	2.5:1	21	55	48	18	0	25	42	13
3–5 h[c]	10:1	14	42	29	11	ND[d]	18	30	13
	2.5:1	10	48	28	10	ND	13	29	13
DRB[e]	10:1	4	48	31	0	31	14	15	24
	2.5:1	2	45	26	2	6	5	11	24

[a] A value of 0 was assigned when the counts per minute released in the test wells were fewer than in the spontaneous release wells.
[b] Target cells were infected with an MOI of 5 and then incubated overnight (18 h) prior to ^{51}Cr loading and assay.
[c] Target cells were infected with an MOI of 10 and incubated in parallel with the DRB-treated cells but in the absence of drug.
[d] ND, not done.
[e] Target cells were treated with 100 μM DRB for 30 min before infection with HSV-2 at an MOI of 10. DRB was maintained continuously for the 1.5- to 3-h incubation period after virus addition and during the subsequent loading of the cells with ^{51}Cr, washing, and addition of effector cells for the cytolytic assay.

was delayed by 5 hours such that α, β, and γ_1 proteins were expressed during ^{51}Cr loading and exposure to the CTL. The effects of the drug treatment protocols on the pattern of HSV gene transcription were confirmed by Northern blot analysis as described below. The second set of target cells was treated with cycloheximide for 5 h, followed by the immediate addition and continuous presence of ActD during the washes, ^{51}Cr labeling, and lysis (Fig. 3F). Under these conditions, α gene transcripts accumulate during the cycloheximide block, and these are expressed rapidly after removal of the drug. Because further transcription was blocked, only α antigens were expressed in these cells. The third set of cells was treated with cycloheximide for 5 h, followed by drug removal and ^{51}Cr labeling for 2 h in the absence of drugs and then the addition of ActD and effector cells simultaneously (Fig. 3E). Under these conditions, predominantly β gene products should have been present, since only α transcripts accumulate during the cycloheximide block and are translated immediately after removal of the drug. These proteins activate β gene expression with the concomitant decline of α proteins, as has been described by Del Val et al. (12) for mouse CMV under a similar regimen. As can be seen in Table 6, clone 2H12 lyses virus infected targets treated with cycloheximide followed by drug removal or immediate replacement with ActD but not cells treated with cycloheximide followed by delayed addition of ActD. In contrast, the 3B3 clone lyses all three drug-treated target cells with equal efficiency and at approximately the same level as do target cells infected with virus in the absence of drug treatment. From these results and those of DRB treatment, it would appear that the 2H12 clone may recognize an α antigen that is also packaged in the virion and whose synthesis declines upon the onset of β gene expression. The products of α genes such as ICP4 or ICP0 (61) are likely candidates. The 3B3 clone may recognize a virion protein of the β transcriptional class introduced as a result of viral infection or an α gene product whose synthesis does not decline as rapidly during the period of β gene expression.

It was not possible to apply the same drug block/release strategy to further analyze the other three clones. In the case of the 1H6.5 and 2E7 clones, cognate antigens are expressed at levels too low to sufficiently sensitize the target cells in a short-term infection to distinguish effects of the application and removal of the cycloheximide block. Treatment of the B-LCL with cycloheximide for longer time periods or with higher concentrations of drug rendered them resistant to lysis by the 1H6.5, 2E7, and 3B3.4 clones in addition to causing unacceptably high levels of spontaneously released ^{51}Cr. The 2C1 clone was not analyzed further because of loss of antigen specificity.

Expression of HSV RNA in drug-treated B-LCL target cells. To ensure that the drug treatments were effective in limiting HSV gene expression as predicted, RNA was purified from HSV-infected, drug-treated cells and assayed in Northern blots. IE α gene RNA was detected by using DNA probes that were generated from restriction fragments containing the ICP0 and ICP4 gene sequences. γ_1 transcripts were detected with a gD probe, and γ_2 transcripts were assayed with a VP16 probe. DRB treatment prevented expression of ICP0 or ICP4 α transcripts or the more abundant gD2 γ_1 transcripts (Fig. 4a, lane 4), while all three were readily detected in untreated B-LCL infected for 6 h (lane 2) or in

TABLE 6. Lysis of cells expressing different transcriptional classes of HSV-2 by two CTL clones

Target	Treatment[a]	Transcription class expressed	% Specific lysis[b]		% Spontaneous release
			3B3 (E/T, 8:1)	2H12 (E/T, 13:1)	
Uninfected	CX followed directly by ActD	None	7	8	35
HSV-2 infected	None	α, β, γ_1, γ_2	39	15	21
	CX followed by 6 h in the absence of CX	α, β, γ_1	49	24	17
	CX followed directly to ActD	α	52	23	29
	CX followed by 2-h delay and then ActD	β, (α)	35	2	22

[a] B-LCL targets were treated with cycloheximide (CX) for 5 h before ^{51}Cr loading as outlined in the legend to Fig. 3 and detailed in Materials and Methods.
[b] The CTL were added to U-bottom microtiter plates in 100 μl to achieve the E/T ratios indicated; 10^4 target cells were added, and lysis was allowed to proceed for 4 h.

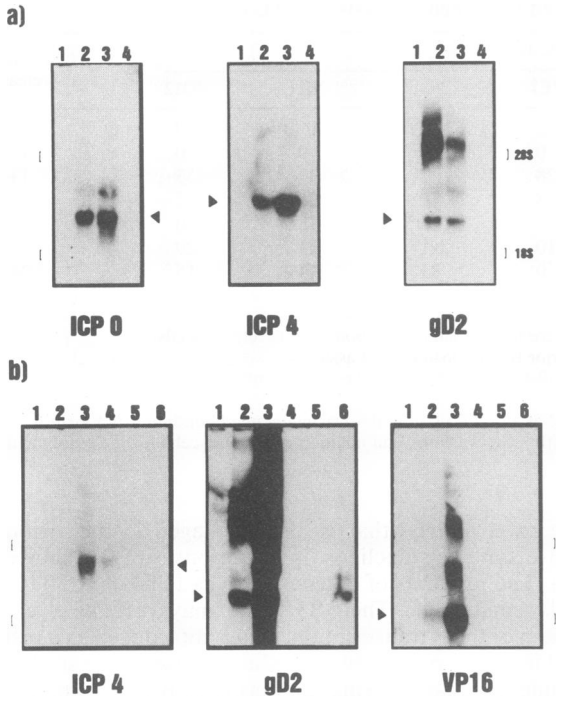

FIG. 4. HSV-2 RNAs in drug-treated B-LCL. RNA was purified from 3×10^7 B-LCL, and 10 μg of RNA was added per lane of 1.1 M formaldehyde–1% agarose gels. The positions of the 28S and 18S rRNAs were determined by ethidium bromide staining prior to blotting and are indicated by brackets. Replicate blots were prepared and hybridized with probes to the genes indicated below the lanes. The lanes in panel a contained RNA from uninfected cells (lane 1), cells infected for 6 h in the absence of drugs (lane 2), cells infected for 5 h in the presence of cycloheximide and then 2 h in the absence of drugs (lane 3), or cells infected for 3 h in the presence of DRB (lane 4). The lanes in panel b contained RNA from uninfected cells (lane 1), cells infected for 6 h (lane 2) or 24 h (lane 3) in the absence of drugs, cells infected for 5 h in the presence of cycloheximide (lane 4), cells infected for 5 h in the presence of cycloheximide and then treated with DRB for 2 h (lane 5), or cells treated for 23 of 24 h with acyclovir (lane 6).

cells treated with cycloheximide for 5 h and then released from the block for an additional 2 h before the cells were harvested for RNA isolation (lane 3). Overexposed Northern blots did not yield signals above background in the lanes containing RNA from DRB-treated cells (data not shown).

Cells maintained in cycloheximide for 5 h before isolation of RNA contained ICP4 transcripts but were devoid of gD2 transcripts (Fig. 4b, lane 4). Treatment of infected cells with 150 μM acyclovir for 23 h blocked VP16 expression (lane 6). The inhibitor also significantly reduced gD2 RNA levels compared with untreated cells (lanes 6 and 3, respectively), characteristic of the γ_1 transcription class. This depression of gD2 expression was reflected as a reduction in sensitivity to lysis by 1H6.5 CTL in acyclovir-treated cells (Table 4). Treating infected cells with cycloheximide and then shifting the cells to DRB for 3 h led to a loss of the ICP4 RNA present at the end of the cycloheximide treatment (lanes 4 and 5). No gD2 or VP16 transcripts were detected in the cells treated with cycloheximide or cycloheximide followed by DRB (lanes 4 and 5). As expected, this treatment limited HSV transcription to the α gene class.

DISCUSSION

We have investigated the nature of the HSV-specific $CD8^+$ CTL response in humans with culture-proven and serologically proven recurrent HSV-2 infection. Although HSV-specific $CD4^+$ CTL have been repeatedly observed and such clones have been isolated (64, 65), the isolation of HLA class I-restricted, HSV-specific $CD8^+$ CTL clones from virus-infected humans has proven considerably more difficult. Other investigators have detected HSV-1 specific, HLA class I-restricted CTL in bulk assays, but the activity represented a minor fraction of the cytolytic activity in the culture (54). A likely explanation for previous failures to clone $CD8^+$ CTL from humans could be that the APCs in the in vitro stimulation phase processed the soluble viral antigens or the noninfectious UV-inactivated virus through the HLA class II pathway in a manner similar to that described for influenza virus antigens (8, 38). In the experiments reported here, $CD8^+$ HSV-specific CTL clones were isolated by stimulating memory CTL in vitro with HSV-infected PBMC rather than using soluble glycoprotein antigens or UV-inactivated virus. In addition, $CD8^+$ T cells were then isolated by panning prior to expansion and cloning. Thus, it is clear that the cellular immune response to HSV infection, like the response to other viruses, includes a broad class I-restricted CTL response.

Five of the seven HSV-specific clones clearly recognize different viral protein antigens. Because two clones, 2F11 and 2D12, were not characterized as extensively, it is not possible to assert that they recognize an HSV antigen distinct from that recognized by either the 3B3 or 2H12 clone. Additional vaccinia virus recombinants expressing specific HSV-2 α and β gene products will be needed to differentiate these clones. The minimum criteria that enable the clones to be distinguished from one another are shown in Table 7. These sorting elements include the type specificity of HSV recognition, recognition of a specific antigen (gD2), and recognition of infected target cells treated continuously with acyclovir, DRB, or IL-2 or treated with cycloheximide followed by drug removal and delayed addition of ActD. All of these sorting elements discriminate between different viral proteins with the sole exception of type specificity. This sorting element could distinguish between different proteins or different epitopes on the same polypeptide chain. On the basis of the pattern of recognition of drug-blocked target cells, a probable target antigen class has been assigned for each clone.

The 1H6.5 subclone recognizes a virion protein, gD2, an abundant glycoprotein inserted in the viral envelope. However, DRB-blocked target cells were not lysed by this gD2-specific clone. Thus, the gD2 epitope was presented on the target cells only as a result of de novo synthesis. Acyclovir treatment reduced gD2 mRNA expression in the target cells. In concordance with the reduced gD2 expression, the ability of the clone to lyse acyclovir-treated target cells was diminished (Fig. 4b, lane 6; Table 4). A similar effect was noted in cells infected for 3 to 5 h compared with target cells infected for longer time periods (Fig. 4b, lanes 2 and 3; Table 5). These data and those of Zarling et al. (64, 65) indicate that both $CD4^+$ and $CD8^+$ CTL specific for gD2 are present in HSV-2 infected patients. Thus, these data suggest that gD2 may be an important target for control of HSV infections.

It is interesting that in this limited sample, five of seven clones recognize exogenous virion proteins introduced into the target cell and presented to the immune system via the

TABLE 7. Assignment of probable CTL target antigens or transcription classes

Clone	Sorting element[a]						Probable target
	HSV-2 Specific	gD2	ACV	IL-2	DRB	Cx/no drug/ ActD[b]	
1H6.5	+	+	↓	+	−	ND[c]	Membrane virion protein, γ_1, gD2
3B3.4	−	−	+	+	+	+	Internal virion protein α, β
2H12.5	+	−	+	+	+	−	Internal virion protein α
2E7.7	+	−	↓	+	−	ND	Nonvirion protein β, γ_1
2C1	+	−	↑	−	↑	ND	Internal virion protein minor β, γ_1
2F11	+	−	+	ND	+	ND	Internal virion protein α, β
2D12	+	−	+	ND	+	ND	Internal virion protein α, β

[a] Condition that distinguishes one clone from the others. Symbols indicate enhancement of lysis (↑), no effect (+), reduction of lysis (↓), resistance to lysis (−).
[b] Infection in the presence of cycloheximide (CX) followed by 2 h without drugs followed by ActD.
[c] ND, not done.

class I pathway in the absence of viral protein synthesis. In most instances, exogenously acquired antigens are processed through endocytic vesicles and presented to the immune system in the context of a class II HLA restriction element, although it is possible to introduce proteins directly into the cytoplasm for class I presentation by osmotic shock (36). By fusion with the plasma membrane (15, 22, 51), HSV virions bypass the endocytic pathway of cellular entry, thus providing a ready source of viral proteins for processing through a pathway that normally handles proteins produced by de novo synthesis. Recently, Riddell et al. (46) have shown for HCMV, the majority of the CMV-specific CTL from seven seropositive individuals also recognize virion protein(s) introduced into the target cell in the absence of any viral gene expression. The class I presentation of these antigens was brefeldin A sensitive, demonstrating that intracellular processing of the antigens was required. Moreover, like the case with HSV gD glycoprotein, the CMV gB homolog to the HSV gB glycoprotein was not recognized in the absence of viral gene expression. This result suggests that the topology of the viral proteins is preserved upon virus-host cell membrane fusion and that viral glycoproteins are not efficiently injected into the cytoplasm. The parallels of these results in CMV and ours in HSV are striking. That the CTL response in this individual appears to be biased to a seemingly minor source of HSV antigens may reflect an adaptation to one way that the virus uses to evade the immune system; an extremely efficient host shutoff function (14, 21, 27). The effects of this shutoff function are reflected in the inability of primary fibroblast lines infected with live virus to serve as APCs in vitro (Fig. 1) and our failure, despite repeated attempts, to use these same primary lines infected with live virus as target cells for three of the CTL clones (26).

For the case of murine CMV, Reddehase and Koszinowski have shown that a single antigen is surprisingly immunodominant; 40 to 50% of all memory CTL are directed against a single protein, the major IE transactivator (IE1, pp89) (44). Similarly, in two CMV-immune humans, it was suggested that the 72-kDa major IE protein is a significant target antigen, since 46% of the CTL lysed target cells infected with a vaccinia virus recombinant expressing the CMV major IE protein, whereas less than 6% lysed vac/CMVgB-infected cells (7). In contrast to these previous results, the diversity of our clones suggest that there is no such extreme immunodominance for the case of HSV. However, in agreement with the results of Riddell et al. (46) for CMV, internal virion proteins appear to be preferentially recognized.

In the mouse, in which HSV-specific CTL are easier to demonstrate, many researchers have focused on the potential of particular glycoproteins to elicit CTL. These efforts have yielded conflicting results. Three groups have detected gB-specific CTL in some but not all haplotypes (4, 34, 59), and Hanke et al. (19) have recently localized a CTL epitope in gB to residues 489 to 515 for the *H-2*^{b} haplotype. In contrast, Martin et al. reported that gC but not gB or gD serves as a class I-restricted target antigen (31). Although several researchers have been unable to detect gD-specific CTL (4, 31, 59), recently Johnson et al. (23) reported the isolation of a gD-specific CTL clone from an *H-2*^{k} mouse. These contradicting results may arise from methodological differences or, more likely, from the possibility the glycoprotein-specific memory CTL are rare. In support of this view, Martin et al. (29, 30, 32) suggested that α gene products may represent as much as 30% of the target antigens and that virion components serve as minor target antigens. In the *H-2*^{k} haplotype, the α gene recognition may be entirely directed to ICP4; ICP0 was not recognized (59). The differences between the results in the mouse and our studies in the human, even with a small sample, i.e., the demonstration of gD-specific CTL in two individuals, the lack of a gB-specific response, and the predominant recognition of internal virion proteins, underscore the necessity to continue to do such studies to more fully characterize the human immune response to HSV.

It is notable that six of the seven CTL clones are HSV-2 type specific. The overall DNA sequence homology of HSV-1 and HSV-2 as measured by the kinetics of solution hybridization is 50% (24). For specific essential genes such as those encoding gB, gD, and VP16, the identity increases up to 86% (47a, 52, 57). Thus, many HSV-specific antibodies are cross-reactive (1, 39). The results presented here suggest that the type specificity of the cellular immune response may be greater than that of the antibody response. Whether this pattern will be maintained in a larger panel of clones from this and other individuals remains to be determined. In mice, the HSV-specific CTL response is also predominantly type specific (16, 43), although type-common recognition has been reported (10, 13). These observations on the type selectivity of the immune response are important for the design of vaccines to prevent or limit HSV disease.

Efficient in vitro stimulation was achieved only with the use of HSV-infected lymphoblasts as stimulator cells. Autologous B-LCL or fibroblasts did not function as APCs. The major difference between the PHA blast cultures and the B-LCL or fibroblast line cultures was the presence of accessory cells, including adherent cells, during the blast transformation, virus infection, and stimulation phases of the cultures. Thus, the failure of both the B-LCL and fibroblasts to stimulate effector cells may be their inability to provide accessory functions such as lymphokine secretion in addition to presenting viral antigens. In addition, the failure of HSV-infected fibroblasts to serve as stimulators may be due to the effect of the efficient HSV host shutoff mechanism on antigen presentation in these cells (14, 21, 27). Consistent with this hypothesis, we have observed that HSV-infected primary fibroblast lines also do not serve as target cells for these CTL clones (26).

In summary, we have, using a novel in vitro stimulation system, been able to demonstrate HSV-specific $CD8^+$ CTL activity in two individuals. In the one individual in whom extensive characterization of $CD8^+$ CTL clones was performed, we found extensive antigenic diversity to HSV-2-specific proteins. While we were able to demonstrate cytolytic activity specific to gD2, we were not able to detect CTL specific to gB2, a more abundant HSV-2 glycoprotein. Most of the cloned CTL were specific to exogenous virion proteins that were processed and presented prior to de novo virion protein synthesis. Quantitative characterization of CTL responses in humans to evaluate their importance in controlling HSV infections in both immunized and naturally infected patients can now be undertaken.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI20381 and AI29681 and by Chiron Corp. D.K. is a research fellow of the American Social Health Association.

We thank John Hansen and Brenda Nesperos, Department of Medicine, University of Washington, for HLA typing several of the B-LCL lines and for providing others used to identify HLA restriction. We also thank Sergio Abrignoni, Ciba-Geigy, and Susan Masecicz, MLC Laboratory, Fred Hutchinson Cancer Research Center, for additional B-LCL lines. We acknowledge the assistance of Jeffrey Neidleman in preparing the manuscript. Finally, we are especially grateful to Chris Walker and Phil Greenberg for many helpful discussions.

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