Herpes Simplex Virus Infection of Human Fibroblasts and Keratinocytes Inhibits Recognition by Cloned CD8$^+$ Cytotoxic T Lymphocytes

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Abstract

CD8$^+$ cytotoxic T lymphocytes (CTL) clones with specificity for herpes simplex virus (HSV) were derived from two donors with genital HSV-2 infection. These CTL clones specifically lysed HSV-infected autologous B lymphoblastoid cells, but not HSV-infected fibroblasts. Exogenous peptide loading sensitized both cell types to lysis by an HSV-specific CTL clone of known specificity. HSV infection rendered fibroblasts refractory to peptide sensitization. HSV infection also rendered fibroblasts and keratinocytes insensitive to lysis by allospecific CD8$^+$ CTL clones. Lysis of B lymphoblastoid cells in this system was only slightly reduced by HSV infection. Reduction of fibroblast allospecific lysis was dose and time dependent and was blocked by acyclovir, indicating the involvement of a late HSV gene product. HSV caused a reduction of fibroblast cell surface HLA class I antigen, at least in part due to reduction of synthesis of heavy chain-β_2 microglobulin heterodimers. These results suggest that HSV-induced blockade of antigen presentation by cutaneous cells to CD8$^+$ CTL may be a mechanism by which HSV limits or evades the immune response of the host. (*J. Clin. Invest.* 1993. 91:961–968.) Key words: HLA A2 • HLA B7 • acyclovir • immune tolerance • radioimmunoprecipitation assay

Introduction

Cell-mediated immunity is important in limiting herpes simplex virus (HSV)[1] infection. One important effector mechanism in limiting cutaneous HSV infections are antigen-specific cytotoxic T lymphocytes (CTLs). In the murine system, adoptive immunotherapy and selective immunodepletion experiments have indicated that CD8$^+$ CTL protect against HSV challenge (1, 2). Human HSV-specific CTL responses detected in bulk culture arise from both CD4$^+$ (3–8) and CD8$^+$ (5, 6, 9) cytolytic effector cells. Recently, by using activated PBMC infected with HSV as in vitro stimulator cells, we reported the isolation of HSV-specific CD8$^+$ CTL clones capable of lysing HSV-infected autologous or HLA class I–matched Epstein-Barr virus–transformed B cell line (LCL) (10). More recently, we have derived an HLA class I–restricted CD8$^+$ CTL clone with specificity for HSV-2 from a cutaneous herpetic lesion (10).

In vivo, HSV primarily infects epidermal keratinocytes and underlying dermal cells (11). As such, we chose to investigate the ability of these clones to lyse HSV-infected dermal fibroblasts. Dermal fibroblasts express HLA class I and can be lysed by virus-specific CTL clones after infection with another herpes virus, cytomegalovirus (12). During the isolation of HSV-specific CD8$^+$ CTL, we noted that HSV-infected fibroblasts did not function as either stimulators of CTL outgrowth in bulk culture (13) or targets for HSV-specific CD8$^+$ CTLs. This paper describes subsequent studies which indicate that while fibroblasts can be sensitized for lysis by an HSV-specific CD8$^+$ CTL clone by incubation with specific peptide, viral infection does not sensitize fibroblasts to lysis by this or other HSV-specific T cell clones. This loss of lysis may be related to the influence of HSV infection on target cell synthesis and cell surface expression of HLA class I.

Methods

Viruses and cell lines. HSV-2 strain 333 (used throughout unless specified) (14, 15), HSV-1 strain E115 (16), and genital wild-type isolates of HSV-2 were passaged on human diploid fibroblast cells at low moi. Cell-associated virus was prepared by sonication of infected human diploid fibroblasts monolayers. After low speed centrifugation, supernatant was stored at −70°C. Virus preparations contained 2.8×10^8 plaque forming units (pfu)/ml (HSV-1), 3×10^8 pfu/ml (HSV-2 333), and 5×10^6 to 3×10^8 pfu/ml (clinical isolates) as measured by plaque assay on Vero cells (17). To inactivate virus, isolates were treated with ultraviolet light for 4 min at 10 cm from a new bulb (GT038; General Electric, Cleveland, OH). This procedure eliminated all detectable infectious virus.

Vero and Madin-Darby canine kidney cells (American Type Culture Collection, Rockville, MD) and human diploid fibroblasts cells (17) were grown in MEM with 10% FCS (Hyclone Laboratories, Inc., Logan, UT). LCL were derived from PBMC (10) and maintained in RPMI 1640 with 25 mM Hepes (Irvine Scientific, Santa Ana, CA) with 10% FCS, 2×10^{-5} M 2-mercaptoethanol (Sigma Immunochemicals, St. Louis, MO), 2 mM L-glutamine, 1 mM pyruvate, 50 μg/ml streptomycin, and 50 U/ml penicillin (Gibco Laboratories, Grand Island, NY) (RPMI-FC).

Fibroblast cell lines from HSV-2 seropositive donors were derived from forearm skin biopsies and cultured in Waymouth's medium (Gibco Laboratories) containing 10–20% FCS, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 1 ng/ml human recombinant basic fibroblast growth factor (Chiron Corp., Emeryville, CA). Cells were used before passage 18.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free calcium concentration; CTL, cytotoxic T lymphocytes; E/T, effector/target; gD_2, glycoprotein D of HSV-2; HSV, herpes simplex virus; LCL, Epstein-Barr virus–transformed B cell line; NK, natural killer; pfu, plaque-forming units.

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Basal keratinocyte cell lines were prepared from neonatal foreskin and cultured in MCDB153 serum-free medium with modifications as previously described using human recombinant epidermal growth factor prepared and generously provided by C. Georges-Nascimento, Chiron Corporation (18). Keratinocytes were used at second through fourth passage (2–4 wk of culture).

The $CD8^+$ T cell clone 3G5 (12, 19, 20) was stimulated every 7–10 d with gamma-irradiated (8,000 rad) HLA A2-positive LCL in RPMI 1640 containing 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, 10% heat-inactivated pooled human serum, and 10–50 U/ml natural human IL-2 (Pharmacia Fine Chemicals, Silver Spring, MD) with fresh medium added every 2–3 d. Cells were used 7–10 d after stimulation. The $CD8^+$ T cell clone C15 was maintained as previously described (20). HSV-specific human $CD8^+$ T cell clones 1H6, 2E7, 2H12, and 3B3 were derived from the peripheral blood of donor 1, maintained as previously described (10) using periodic restimulation with PHA and allogeneic feeder cells, and used 10–14 d after stimulation. 1H6.5 is a subclone of 1H6. HSV-specific T cell $CD8^+$ clone 10H11 was derived from donor 2 from a herpetic buttock lesion (13). This clone was stimulated weekly with PHA, IL-2, and allogeneic irradiated PBMCs, and used 7–8 d after stimulation and 3–4 d after the last addition of IL-2.

Cell lines were tested for mycoplasma infection with the Mylotect Kit (Gibco Laboratories) and were negative at the time they were used for experiments.

Antibodies. mAb W6/32 (21), IgG2a, specific for human HLA class I heavy chain in association with β2-microglobulin, was obtained from Dako Corp., Carpinteria, CA. mAb A1.4 (22), IgG1, specific for HLA class I heavy chain with or without β2-microglobulin, was obtained from Olympus Immunochemicals, Lake Success, NY. mAb 18βB3 (23), IgG2b, specific for a type-common (HSV-1 and HSV-2) determinant of HSV glycoprotein D was obtained from N. Balachandran, University of Kansas Medical Center, Kansas City, KA. mAb MA2.1 (24), IgG1, specific for HLA A2, was obtained from the American Type Culture Collection. mAbs F9B9, IgG1, specific for luteinizing hormone-releasing hormone (Koelle, D. M., unpublished data), and UPC-10 (25), IgG2a, were used as isotype controls. FITC-conjugated mAb to HSV-2 (Syva Co., Palo Alto, CA) was used to confirm infection of fibroblasts and keratinocytes by fluorescence microscopy according to the supplier's instructions.

Cytotoxicity assays. Fibroblasts at 80–100% confluence in six-well cluster plates (9.4 cm^2) were washed once and infected with HSV in serum-free medium for 1 h with constant rocking, washed, and incubated with Waymouth's medium containing 10% FCS and 30–100 μCi $Na_2{}^{51}CrO_4$ for up to 16 h. Cells in parallel replicate wells were counted before infection to calculate the input virus required. LCL targets were prepared as previously described (10) by infection with HSV-2 333 at moi of 0.08–2.5 for 3–18 h. Fibroblast targets were prepared by treatment with 0.05% trypsin (Gibco Laboratories), 2 mM EDTA in PBS. Keratinocyte targets were prepared similarly except that cells were ^{51}Cr-labeled as trypsinized cell suspensions (20). After three washes with RPMI-FC, 5×10^3 targets were combined with the effector cells in a final vol of 200 μl of RPMI-FC. After incubation for 4 h at 37°C in a humidified CO_2 incubator and centrifugation for 2 min at 50 *g*, 100 μl of the supernatant was counted in a counter (Gamma 5500; Beckman Instruments, Inc., Fullerton, CA). Maximal or spontaneous release was measured after addition of 100 μl of 1% NP-40 or RPMI-FC to target cells. Percent specific release (%SR) was calculated by the formula %SR = ([mean experimental cpm − mean spontaneous cpm]/[mean maximal cpm − mean spontaneous cpm]) × 100. Spontaneous release, calculated as ([mean spontaneous cpm − mean machine background cpm]/[mean maximal release − mean machine background cpm]) × 100 was 12% for fibroblast and 17% for LCL targets for a representative subset of the experiments reported and averaged 38% for keratinocytes for the experiments reported. Assays were performed in triplicate.

For experiments involving acyclovir, 150 μM acyclovir (Burroughs Wellcome, Research Triangle Park, NC) was added during infection, wash, and assay periods. For peptide loading, chromium-labeled, washed fibroblasts were incubated with peptide comprising amino acids 301–326 of glycoprotein D of HSV-2 (gD2) (26) at 1 μM for 60 min at 25°C before addition of effector cells. Target cells were plated at 1×10^4 cells/well for this experiment only.

Radioimmunoprecipitation and electrophoresis. Replicate wells (9.4 cm^2) of infected or mock-infected fibroblasts or an equal number (2×10^6) of infected or mock-infected LCL were preincubated for 50 min at 37°C in methionine-free MEM (fibroblasts) or RPMI 1640 (LCL) followed by addition of 184 μCi of ^{35}S-methionine for 70 min at 37°C. Cells were washed three times with PBS, fibroblasts trypsinized as for cytotoxicity assays, and incubated on ice with 30 μl of lysis buffer (10 mM Tris, 0.15 M sodium chloride, 0.02% sodium azide, 0.5% NP-40, 2 mM PMSF) for 30 min. Lysates were centrifuged at 12,500 *g* for 25 min and preabsorbed for 30 min with 25 μl protein A-Sepharose (Sigma Immunochemicals). 20 μl *Staphylococcus aureus* Cowan buffer (PBS, 2 mM methionine, 0.02% sodium azide, 0.5% NP-40) containing 0.6 μg mAb W6/32 was added and after 16 h at 4°C, 30 μl protein A-Sepharose was added for 90 min at 4°C. Samples were washed four times in SAC buffer, immersed in boiling water for 2 min with 2× sample buffer, and electrophoresed through a 10% polyacrylamide gel containing SDS under reducing conditions (27). Gels were treated with 16% salicylate for 30 min and exposed to Kodak XAR film at −70°C.

CA^{2+} analysis. 3G5 cells (10^7/ml in RPMI-FC) were loaded with 5 μg/ml of the acetoxymethyl ester of indo-1 (Molecular Probes, Inc., Eugene, OR) for 45 min at 37°C in darkness, washed, and resuspended at the same concentration in RPMI-FC. Cytoplasmic free (intracellular) calcium concentration ($[Ca^{2+}]_i$) was measured in individual cells by cytofluorimetrically determining their ratio of violet to blue emissions (28). Measurements were performed at 37°C at a rate of 250 cells/s using a Cytofluorograph 50H with a model 2150 computer (Ortho Diagnostic Systems, Inc., Raritan, NJ). When baseline $[Ca^{2+}]_i$ was established for 1 min, flow was interrupted, and an equal vol of fibroblasts at 10^7/ml in RPMI-FC were added, cocentrifuged at 1,500 *g* for 1 min, and analysis continued with gating for lymphocytes. Anti-CD3 mAb G19.4 (28) was added at 25 μg/ml to assess residual reactivity of lymphocytes after mixture with HSV-infected fibroblasts. Computer analysis for $[Ca^{2+}]_i$ vs. time was performed as previously described (29) using Multitime software (Phoenix Flow Systems, San Diego, CA).

Flow cytometry. Replicate samples of cells used for cytotoxicity assays were incubated with the indicated mAbs. After incubation with primary antibody, samples were washed and incubated with phycoerythrin-conjugated goat anti-mouse immunoglobulin (Biomeda, Foster City, CA) and fixed in 2% paraformaldehyde in PBS at 4°C for up to 4 d. A minimum of 10,000 cells were analyzed with an EPICS-C flow cytometer calibrated with beads (Immunocheck, Coulter Electronics, Inc., Hialeah, FL). The percent cells positive for gD2 was calculated using EPICS and Cytologics software (Coulter Electronics Inc., Hialeah, FL).

Informed consent was obtained from all patients. The research protocol was approved by the University of Washington Human Subjects Review Committee.

Results

Susceptibility of HSV-infected target cells to lysis by HSV-specific $CD8^+$ CTL clones. HSV-2–infected autologous but not allogenic LCL were lysed by HSV-specific $CD8^+$ CTL clones derived from donors 1 and 2. Recognition was HLA class 1 restricted and specific for HSV-infected cells (Table I) (10). Both donors are seropositive for HSV-2 and have periodic recurrences of genital herpes. HSV-2–infected autologous fibroblasts were not lysed by any of the HSV-specific $CD8^+$ CTL clones. Since these fibroblasts had been infected for 18 h before incubation with CTL, we tested the possibility that fibroblasts infected with virus for shorter time periods could be recognized by the CTL. As shown in Fig. 1, autologous LCL infected with HSV for as little as 3 h before assay were lysed by clones 2E7,

Table I. Differences in Lysis between HSV-2-infected Autologous LCLs and Fibroblasts by CD8⁺ T Cell Clones

		Donor 1			Donor 2
CLONE		2E7	2H12	3B3	10H11
Cell type	Virus*				
LCL	Mock	1	−1	4	−1
LCL	HSV-2	30	35	43	25
LCL (allo‡)	HSV-2	0.5	−1	19	0.3
Fibroblast	Mock	−6	−0.2	−6	−1
Fibroblast	HSV-2	1	−0.1	−0.2	3

Data are percent specific release at an E/T ratio of 10:1. * Cells were infected for 18 h at an moi of 5 (LCL) or 1 (fibroblasts). ‡ Allogeneic cells are mismatched at HLA A, B, and DR loci.

2H12, and 3B3, but fibroblasts similarly infected for short periods of time were not lysed by these same clones. Inclusion at 150 μM acyclovir during infection and assay did not result in specific lysis. Extending the time of exposure of the fibroblasts to the CTL from 4 h up to 12 h (hours 18–30 after infection) did not lead to specific lysis of infected fibroblasts (data not shown).

Several measures indicated that fibroblasts were productively infected by HSV: HSV titer increased by a factor of 65 over 22 h, HSV-specific cytopathic effect of the fibroblast cultures was observed in > 50% of the cells, HSV-2–specific antigens were demonstrated by immunofluorescence in > 75% of the cells, and Western blots of infected fibroblast lysates probed with serum from HSV-2 seropositive but not HSV seronegative donors showed a full complement of virus-specific bands including glycoprotein B of HSV-2, gD, and MW 65K major transactivating protein of HSV (30) (data not shown).

Omission of trypsin treatment of the infected fibroblasts before assay with the CTL clones 2E7, 2F12, and 3B3 did not render them susceptible to lysis, and conversely, treatment of HSV-infected autologous LCL with 0.25% trypsin solution for 5 min at 37°C before addition of the CTL clones had no effect on their lysis (data not shown).

Sensitization of fibroblasts to CTL lysis by peptide. The HSV-specific CTL clone 1H6 was previously shown to recognize gD2; it lysed autologous LCL infected with recombinant vaccinia virus expressing gD2 (10). The epitope has now been localized to amino acid residues 301–326 of gD2 (Tigges, M. A., S. Leng, D. Koelle, H. Abo, L. Corey, and R. L. Burke, manuscript in preparation). Both autologous uninfected LCL and fibroblasts were sensitized to lysis by the 1H6.5 CTL clone by preincubation with the gD2 peptide 301–326 (Table II).

To determine if the resistance of HSV-infected fibroblasts to lysis by CD8⁺ HSV-specific CTL clones is due to a generalized defect in antigen presentation or a more specific defect in presentation of endogenously synthesized antigen, infected fibroblasts were incubated with peptide prior to assay. Peptide-loaded fibroblasts that were infected for 3 h were lysed by 1H6 cells. However, at 18 h after infection, peptide-loaded fibroblasts were no longer lysed by 1H6 cells.

Effect of HSV infection of various cell types on their lysis by an alloreactive CTL clone. To determine if the failure of HSV-specific CD8⁺ T cell clones to lyse autologous infected fibroblasts was caused by a general impairment of these cells to function as targets for CD8⁺ T cells, or a selective failure to present HSV antigens, the effect of HSV infection on lysis of LCL and fibroblasts by the HLA A2–specific CD8⁺ CTL clone 3G5 was examined (19). Mock-infected HLA A2–bearing fibroblasts and LCL were specifically lysed by clone 3G5 (Tables III and IV). Infection for 18 h with HSV-2 strain 333 or HSV-1 strain 115 at moi of 2–5 greatly reduced lysis of all HLA A2–positive fibroblast lines tested, by a mean of 87% for HSV-2 and 69% for HSV-1 (Table III). This effect was observed in 10 replicate experiments performed with HSV-2 at moi of 1–5. Similar data were obtained when HLA A2–bearing fibroblasts were infected with each of four separate primary clinical isolates of HSV-2 at an moi of 1, and with effector to target ratios of up to 20:1 using either clinical isolates or laboratory strains of HSV-2 at an moi of 1. Rosette formation between CTL and target cells was observed by microscopic examination of wells containing mock-infected fibroblasts but was absent from wells containing HSV-infected fibroblast target cells.

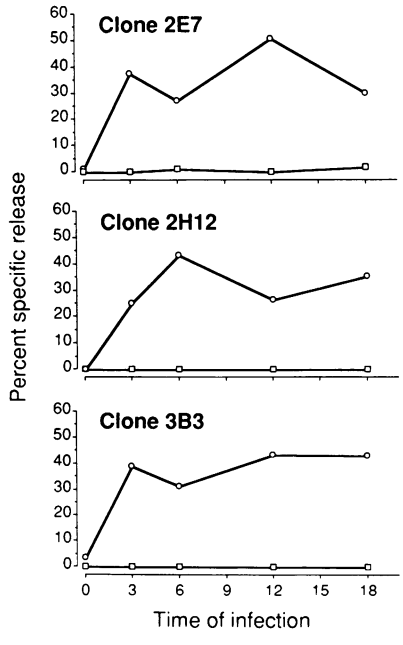

Figure 1. Effect of time of infection with HSV-2, on lysis of autologous LCL (*circles*) and fibroblasts (*squares*) by HSV-specific CD8⁺ T cell clones. Targets were infected at moi of 5 (LCL) or 2.5 (fibroblasts), harvested at the time indicated and then prepared as targets for cytotoxicity assay as described in Methods. Data are percent specific release at an effector/target (E/T) ratio of 10:1.

Table II. Lysis of Autologous Cells by Clone 1HG.5, an HSV-gD2-specific CD8⁺ T Cell Clone

Cell type	Virus*	Time	Peptide‡	Percent specific release
		h		
LCL	Mock	18	None	1
LCL	HSV-2	18	None	44
LCL	Mock	18	301–326	61
Fibroblast	Mock	18	None	−6
Fibroblast	Mock	18	301–326	31
Fibroblast	HSV-2	3	None	−1
Fibroblast	HSV-2	3	301–326	26
Fibroblast	HSV-2	18	None	5
Fibroblast	HSV-2	18	301–326	6

Data are percent specific release at an E:T ratio of 10:1. * Cells were infected for 3 or 18 h at an moi of 5 (LCL) or 1 (fibroblasts). ‡ Peptide comprising amino acid residues 301–326 of gD2.

Table III. Effect of 18-h Infection of Fibroblasts with HSV-1 or HSV-2 on their Lysis by the Allospecific CD8+ CTL Clone 3G5

Donor	HLA A2	Experiment 1* Virus			Experiment 2 Virus		
		Mock	HSV-1	HSV-2	Mock	HSV-1	HSV-2
1	—	2.5‡	0	0	8.0	ND	ND
2	+	37.4	0	3.7	25.9	5.8	4.8
3	+	33.8	9.0	8.3	38.7	ND	3.7
4	+	34.0	2.6	1.1	22.9	6.7	1.2

* Fibroblasts were either mock infected or infected with HSV-1 strain 115 or HSV-2 strain 333 at an moi of 2.5 (experiment 1) or 5 (experiment 2) for 18 h before incubation with 3G5 cells. ‡ Percent specific release at E:T 10:1.

Keratinocytes are the predominant cell type infected with HSV during cutaneous recurrences (11). As with fibroblasts, HSV-2 infection caused cultured keratinocytes to become resistant to lysis by both T cell clone 3G5 and the HLA B7-specific CD8+ CTL clone C15 (20, 31) (Fig. 2).

The effect of HSV-2 infection of LCL on their susceptibility to lysis by 3G5 cells was examined in four experiments. In contrast to the results observed with fibroblasts and keratinocytes, HLA A2-bearing LCL infected for 18 h with 1 to 10 pfu/cell HSV-2 appeared to be lysed equally as well as uninfected LCL (Table IV). At an moi of 30 in three experiments, specific lysis of HLA A2-positive LCL was reduced by only 38%; from a mean of 84% to a mean of 52% at an effector to target ratio of 10:1.

Characteristics of HSV-induced reduction of CD8+ T cell lysis of fibroblasts. The inhibition of fibroblasts lysis by T cell clone 3G5 caused by HSV-2 infection is both time- and dose-dependent (Fig. 3). Inhibition of lysis was detectable at multiplicities of infection as low as 0.03 and was maximal at an moi of 0.3. Inhibition of lysis began by 8 h after infection and was maximal by 16 h after infection. Inhibition of lysis occurred with a similar time course to cell surface expression of gD2 on infected fibroblasts. Viral DNA synthesis is necessary for fibroblasts to express cell surface gD2 detectable by flow cytometry, since cells infected for 18 h with HSV-2 at an moi of 1 in the presence of acyclovir, or after ultraviolet irradiation of the virus, displayed < 2% of cells positive for gD2 by flow cytometry.

To evaluate further the stage of viral replication associated with inhibition of allospecific lysis of fibroblasts, we used acyclovir to block viral DNA replication and the expression of HSV-encoded late gene products. Fibroblasts infected with HSV-2 for 10 h in the presence of acyclovir were lysed by 3G5 cells, indicating the involvement of a late HSV-2 gene in the suppression of allospecific lysis (Fig. 4).

The HSV-mediated block of CTL recognition of fibroblasts could be due to a direct effect on the target cell or to the release of soluble inhibitory substances or large amounts of live virus from infected target cells and subsequent infection or inhibition of the effector cells. Supernatants from 18-h infected fibroblasts had no effect on lysis by the T cell clone 3G5 of mock-infected HLA A2-bearing fibroblasts when added at the beginning of a 4-h cytotoxicity assay. Addition of 10^7 pfu per well of HSV-2 at the beginning of the cytotoxicity assay had no effect on 3G5 lysis of HLA A2-bearing fibroblasts (data not shown). These findings, the similar dose response curves for inhibition of lysis and cell surface gD2 staining and the prevention of blockade of allospecific lysis by acyclovir are consistent with a requirement for target cell infection and late gene expression by HSV-2 for inhibition of allospecific lysis.

Effect of HSV-2 on cell surface expression and synthesis of HLA class I and correlation with susceptibility to allospecific lysis. The effect of HSV-2 infection on the cell surface expression of HLA class 1 in fibroblasts and LCL was examined using flow cytometry. In six experiments with fibroblasts from three donors, infection with HSV-2 333 at an moi of 1 for 18 h re-

Table IV. Effect of HSV-2 Infection on Lysis of LCL by Allospecific CD8+ CTLs

Donor	HLA A2	moi*	Lysis‡
			%
1	—	0	4
2	+	0	71
2	+	1	61
2	+	3	63
2	+	10	57
2	+	30	49

* LCL were either mock infected or infected with HSV-2 strain 333 for 18 h at the indicated moi and used as targets in a cytotoxicity assay with the CD8+ CTL clone 3G5. ‡ Percent specific lysis at E:T 10:1.

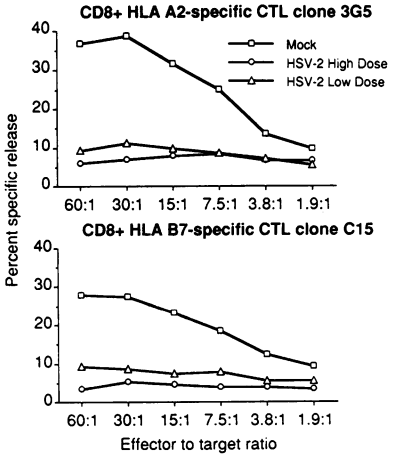

Figure 2. Effect of infection with HSV-2 on lysis of cultured keratinocytes by allospecific CD8+ T cell clones 3G5 (*upper panel*) and C15 (*lower panel*). Keratinocytes were infected for 18 h with HSV-2 at an moi of 5 (high dose) or 1 (low dose) or mock infected. Data are expressed as percent specific release at indicated effector to target ratios.

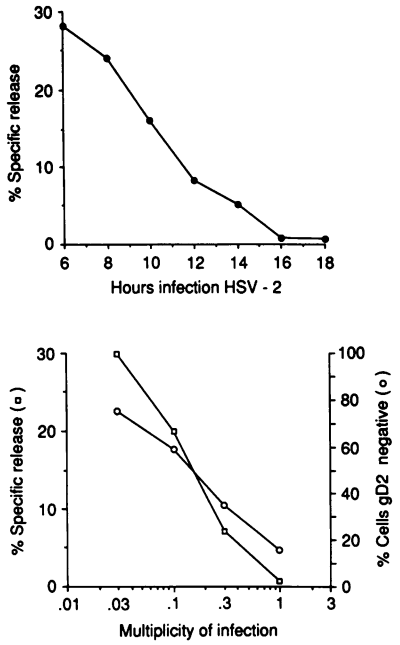

Figure 3. Effect of infection with HSV-2 on lysis of HLA A2-bearing fibroblasts from donor 4 (HLA A2, 28, B27, DR1) by the allospecific $CD8^+$ T cell clone 3G5. Upper panel shows time course of inhibition of target cell lysis after infection at an moi of 1. Bottom panel shows dose-response for inhibition of lysis and cell surface expression of gD2 for 18-h infection. Data are derived from percent specific release at an E:T ratio of 10:1.

duced the intensity of staining with the HLA class I–specific mAb W6/32 (Fig. 5). Similar results were obtained with mAb A1.4 and the HLA A2–specific mAb MA 2.1 (data not shown). The reduction was estimated to be ~ 50–75% by using defined microbead aggregates as standards. In contrast, infection of LCL at an moi of 1 caused no measurable decrease in cell surface expression of HLA class I. A reduction of < 50% was observed 18 h after infection at an moi of 30, the highest virus dose tested.

The reduction of HLA class I on the cell surface of HSV-infected fibroblasts could result from reduced synthesis, interference in intracellular transport or association with β2-microglobulin, or increased turnover of class I molecules. A reduction of synthesis might be expected as a result of the host protein synthesis shut off function of HSV (32). To test these possibilities, infected fibroblasts were pulse labeled for 1 h with [^{35}S]methionine 18 h after infection and immunoprecipitated with mAb W6/32, which binds only heterodimers of HLA class I heavy chain and β2 microglobulin (21). After dissociation of HLA class I heavy chain and β2 microglobulin, fibroblasts and LCL both showed greatly reduced incorporation of newly synthesized HLA class I heavy chain into heterodimers after 18-h infection with HSV-2 (Fig. 6). Inclusion of acyclovir or reduction of the duration of infection to 3 h did not reduce incorporation of newly synthesized HLA class I heavy chain into heterodimers to as great an extent. The reduction of cell surface HLA class I on the surface of 18-h HSV-infected fibroblasts but not LCL may thus be caused by increased turnover of cell surface HLA class I on HSV-infected fibroblasts as compared to LCLs, as well as decreased synthesis of HLA class I.

Effect of HSV-2 infection on fibroblast-triggered increase in CTL intracellular calcium concentration. Fibroblasts were mixed with indo-1 loaded 3G5 cells and $[CA^{2+}]_i$ monitored over time (Fig. 7). HLA A2–bearing fibroblasts triggered a rapid increase in $[Ca^{2+}]_i$ in the lymphocytes, while infection of HLA A2–bearing fibroblasts with HSV-2 reduced their ability to trigger a rapid increase in lymphocyte $[CA^{2+}]_i$. The 3G5 cells incubated with HSV-infected fibroblasts retained their ability to react to anti-CD3 mAb with a rapid rise in $[Ca^{2+}]_i$.

Discussion

In contrast to HSV-infected LCLs, our studies indicate that HSV-infected fibroblasts fail to serve as targets for cloned $CD8^+$ HSV-specific CTL. Specific lysis of HSV-infected autolo-

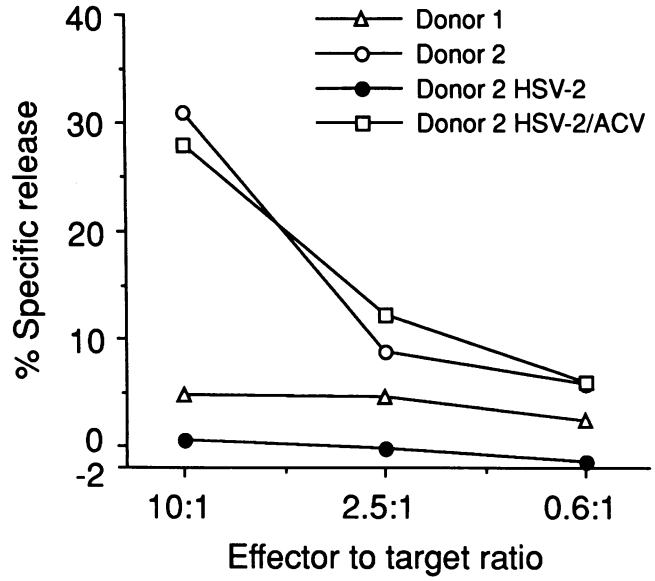

Figure 4. Effect of acyclovir to reverse inhibition of allospecific lysis of HSV-2 infected fibroblasts. Allogeneic fibroblasts were infected with HSV-2 at an moi of 1 for 18 h, with and without 150 μM acyclovir (ACV). Fibroblasts were harvested and used as target cells with the allospecific $CD8^+$ T cell clone 3G5 at E:T ratios as indicated. Donor 1 (HLA A11, B50, 55, DR 4, 6) fibroblasts are included as a negative control.

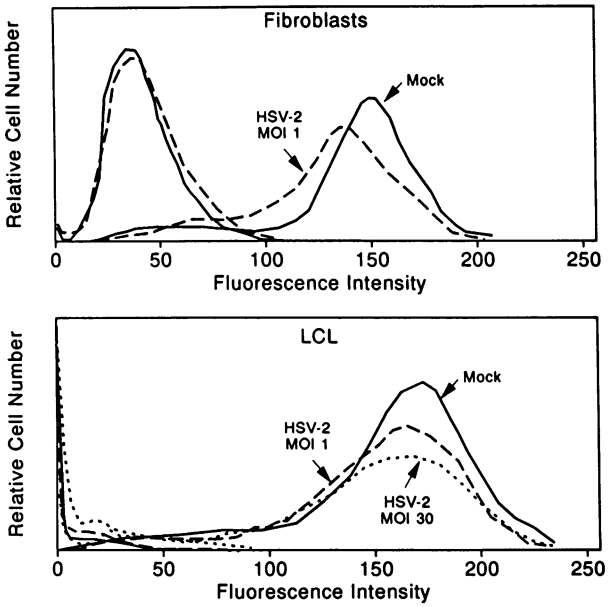

Figure 5. Effect of infection with HSV-2 for 18 h on cell surface expression of HLA class I. Fibroblasts (*top*) were either mock infected (*solid lines*) or HSV-2 infected at an moi of 1 (*dashed lines*) and stained with isotype control mAb (*left two peaks*) or the HLA class I–specific mAb W6/32 (*right two peaks*). LCL (*bottom*) were either mock infected (*solid lines*) or infected for 18 h with HSV-2 at an moi of 1 (*dashed lines*) or 30 (*dotted lines*) and stained as above.

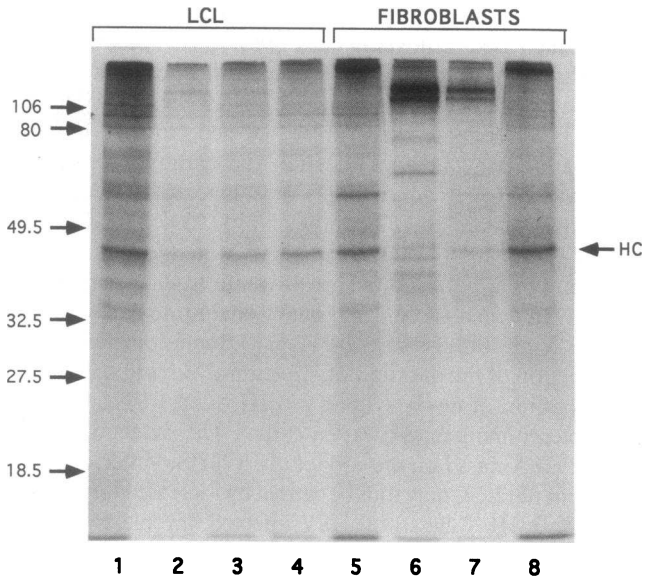

Figure 6. Effect of HSV infection on incorporation of newly synthesized HLA class I heavy chains into heterodimers. LCL (lanes *1–4*) and fibroblasts (lanes *5–8*) were mock infected (lanes *1* and *5*), infected for 18 h with HSV-2 at an moi of 1 without (lanes *2* and *6*) or with (lanes *3* and *7*) 50 μM acyclovir, or infected with HSV-2 for 3 h (lanes *4* and *8*). Cells were metabolically labeled with [^{35}S]methionine and immunoprecipitated with mAb W6/32. Molecular weight markers are shown at right.

gous fibroblasts could not be elicited by varying the experimental conditions, such as time of infection, time of assay, or method of preparation of targets (trypsinization vs. no trypsinization). Peptide sensitization of autologous fibroblasts indicates that this is not related to an inherent inability of fibroblasts to support HSV-specific lysis by CD8$^+$ CTLs, but appears to be related to an effect of HSV infection on antigen presentation by fibroblasts. As fibroblasts and keratinocytes are major targets of the herpes simplex viruses in vivo, this inhibition of specific lysis may be an important mechanism by which HSV helps limit the host's response and prolong viral replication in cutaneous lesions.

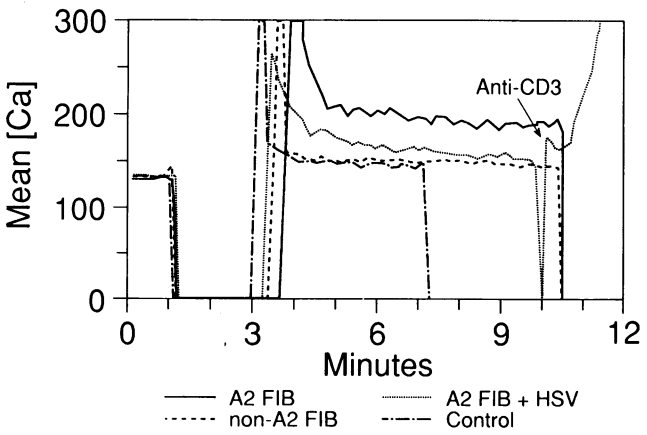

Figure 7. Responses of intracellular free calcium concentration ($[Ca^{2+}]_i$) to addition of fibroblast target cells with and without HSV-2 infection and to anti-CD3 MAb. As controls, indo-1–labeled 3G5 cells were incubated with non-HLA A2–bearing fibroblasts or media. Tracings are off scale during cell mixing and re-establishment of flow.

The inability of HSV-2–infected fibroblasts to present antigen to HSV-specific CD8$^+$ CTL was investigated in two additional experimental systems. HSV-2 infection, in a time-dependent manner, rendered fibroblasts insensitive to loading by an exogenous peptide itself derived from HSV-2. Similarly, in a time-dependent and inoculum-dependent manner, HSV-2 infection of fibroblasts causes them to become insensitive to lysis by the alloreactive CD8$^+$ CTL clone 3G5. In addition, infection of keratinocytes reduced their lysis by 3G5 cells and by a second alloreactive CD8$^+$ CTL clone.

Flow cytometry of HSV-infected fibroblasts but not HSV-infected LCLs demonstrated a significant decrease in cell surface HLA class I heterodimers. The number of MHC–peptide complexes necessary for target cell recognition has been reported to be on the order of 10^2–10^3 (33). The critical threshold level of specific viral peptide–HLA class I complex necessary for recognition of HSV-infected fibroblasts by HSV-specific CD8$^+$ CTL may not be reached in infected fibroblasts. The observation that alloantigen-induced increase in CD8$^+$ CTL intracellular free calcium concentration is reduced by HSV infection of target cells is consistent with the hypothesis that an early step of CTL target recognition is blocked by HSV.

Resistance of HSV-infected fibroblasts to lysis by CD8$^+$ CTL is probably not caused by a generalized resistance to cell killing. HSV-infected fibroblasts can serve as targets for bulk and cloned natural killer (NK) cells (34) and do not differ from control fibroblasts in susceptibility to osmotic shock, NK-like cell-derived granule cytolysin or complement dependent, antibody-mediated lysis (35). The lymphocytic origin and viral transformation of LCL may enable them to serve as more efficient targets for CTL than fibroblasts. For example, EBV transformation of B cells increases the density and number of cell surface molecules that are involved in effector–target interactions, including HLA class I (36), lymphocyte function-associated antigen 3 and intercellular adhesion molecule 1 (37).

Our experiments evaluated three different models of antigen presentation. The first involved the de novo presentation of viral antigens after infection of cells and their recognition by HSV-specific CD8$^+$ CTLs. According to current understanding, viral antigens are synthesized and processed intracellularly. Viral peptides are then loaded into newly synthesized class I molecules in the endoplasmic reticulum associated with β_2 microglobulin and rapidly transported to the cell surface (38). The half life of these peptide-loaded class I molecules on the cell surface is thought to be long and on the order of many hours (38). The second model used was allorecognition of class I molecules by class I–specific CTL. Allorecognition is likely to be caused by CTL engagement of class I molecules loaded with the appropriate allopeptide on the cell surface (39). Thus the allorecognition target preexists on the surface of the cell at the time of HSV infection and could be replenished during the period of viral infection. The third cytolytic model used involved sensitizing target cells to CTL lysis by the in vitro addition of the appropriate peptide epitope. In this case, class I molecules on the cell surface that lack peptide in the binding cleft are loaded by the addition of exogenous peptide. Again, the structure for antigen presentation, the empty class I molecule, is present before virus infection and could be replenished after infection.

Theoretically, all types of antigen presentation could be impacted by a single viral gene function. If after virus infection, HLA class I synthesis or transport to the cell surface were

blocked or turnover were accelerated, the consequence for each of the three models would differ. The de novo presentation of viral antigen derived from intracellular synthesis would be immediately impacted since only newly synthesized class I chains are assembled together with peptide and β_2 microglobulin for transport to the cell surface. Recycling of class I molecules represents a minor pathway (38). In contrast, both allorecognition and recognition of exogenously loaded peptide would be impacted more slowly. The rate of loss of recognition of these two target cells by CTL would be a function of the half life of the appropriate MHC class I molecules on the cell surface, the number of targets present at the time of virus infection, and the number of peptide-loaded class I molecules required to sensitize a target cell to lysis. Our data are consistent with the hypothesis that HSV-2 infection alters HLA class I expression by reducing the synthesis of HLA class I heavy chain. Reduction of cell surface MHC class I by HSV has been noted in murine systems (40, 41) and for several other viruses (42–46). The mechanism has been most completely studied for adenovirus (46). It is, however, also possible that HSV infection interferes with HLA class I heavy chain processing, its association with β_2-microglobulin or peptide, or with the synthesis or function of other elements of antigen processing.

NK cells with activity against HSV-infected fibroblasts have been demonstrated in human PBMC (34) and may have a functional role in limiting primary HSV infection in the neonate (47) and in adults (48). The reduction of cell surface HLA class I on the surface of HSV-infected fibroblasts may enhance their ability to serve as targets for NK cells. Recent experiments indicate that at least some NK cell populations recognize decreased levels of HLA class I on the surface of target cells (49).

The presence of $CD4^+$ CTL activity to HSV-infected LCL has been detected at the bulk and clonal level in human PBMC (50). HLA class II induction on keratinocytes during recurrent HSV infection has been observed in tissue sections (12) and the ability of gamma-interferon–treated keratinocytes to present HSV antigen to $CD4^+$ CTL cells has been demonstrated (51). The present data are consistent with a functional role for $CD4^+$ CTL in control of recurrent HSV infection.

The viral genes mediating the immunosuppressive effects measured in these experiments have not been defined. In a murine system using SV-40–transformed fibroblastoid cells as targets for HSV-specific $CD8^+$ CTL, stronger inhibition of antigen presentation by HSV-2 than HSV-1 was mapped to the 0.82–1.0 region of the HSV-2 genome (40). In our system using alloreactive $CD8^+$ CTL and HSV-infected fibroblast targets, laboratory strains of HSV-2 and HSV-1 gave similar results for two of three fibroblast cell lines. The viral function limiting allospecific lysis of HSV-infected fibroblasts appears to be caused by a late viral gene product. The viral function limiting lysis of HSV-infected fibroblasts by HSV-specific $CD8^+$ CTL may be different, because it is not time dependent and cannot be overcome by inclusion of acyclovir during infection for those CTL clones known to recognize virion components or targets (10). Recent findings (52) suggest that brief contact of $CD8^+$ allospecific CTL with fibroblasts previously infected with HSV for as little as 2 h is capable of inactivating the CTL. HSV may thus be able to inhibit $CD8^+$ CTL recognition of infected cutaneous target cells by more than one mechanism.

Our results with HSV-specific $CD8^+$ T cell clones contrast with some earlier studies. Yasukawa et. al., used autologous fibroblasts infected with HSV-1 strain KOS at high moi overnight as targets for bulk and limiting-dilution–plated (presumably clonal) $CD8^+$ T cells (9). These effectors were initially stimulated with autologous fibroblasts treated with ultraviolet inactivated HSV-1. In another study, autologous fibroblasts infected for 4 h with HSV-1 strain Thea at an moi of 5 were effective as targets for bulk PBMC stimulated with ultraviolet inactivated virus; HLA class I matching between effectors and targets was necessary for a component of the response (5). Potential explanations for these contrasting findings include differences in the actual effector populations used in these studies such as residual NK cell activity, and a lesser effect of HSV-1 than HSV-2 on class I expression.

In summary, autologous dermal fibroblasts infected with HSV-2 do not serve as targets for $CD8^+$ CTL clones that are active against HSV-infected LCL. HSV infection also blocks sensitization to $CD8^+$ CTL lysis by addition of exogenous peptide and prevents lysis of fibroblasts and keratinocytes by cloned allospecific $CD8^+$ CTL. $CD4^+$, HLA class II–restricted HSV-specific CTL clones with cytotoxic activity against HSV-infected LCL also do not lyse autologous HSV-infected fibroblasts (50), most likely due to their lack of cell surface HLA class II molecules. While HSV-specific CTL are detectable in seropositive individuals, the contribution of $CD8^+$ CTL to the antiviral immune response may be limited by the apparent ability of HSV to allow some cell types to escape $CD8^+$ CTL-mediated lysis.

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