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Effect of Herpes Simplex Virus Types 1 and 2 on Surface Expression of Class I Major Histocompatibility Complex Antigens on Infected Cells

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Cytotoxic T lymphocytes (CTL) generated in C57BL/6 (*H-2*^{b}) mice in response to infection with the serologically distinct herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) were cross-reactive against target cells infected with either serotype. However, HSV-2-infected cells were shown to be much less susceptible to CTL-mediated lysis, and analysis through the use of HSV-1 × HSV-2 intertypic recombinants mapped the reduced susceptibility to a region contained within 0.82 to 1.00 map units of the HSV-2 genome. The study reported here was undertaken to determine the possible reasons for the reduced susceptibility of HSV-2-infected cells to lysis by CTL. Competition for the specific lysis of labeled HSV-1-infected cells by either HSV-1- or HSV-2-infected, unlabeled inhibitor cells and frequency analysis of the CTL precursor able to recognize HSV-1- and HSV-2-infected cells suggested that the reduced susceptibility of HSV-2-infected cells to lysis could be explained, at least in part, by reduced levels of target cell recognition. A determination of the surface expression of the critical elements involved in target cell recognition by CTL following infection with HSV-1 or HSV-2 revealed that all the major HSV-specific glycoprotein species were expressed. Infection with both HSV-1 and HSV-2 caused a reduction in the expression of the class I *H-2* antigens. However, this reduction was much greater following infection with HSV-2. This suggested that one important factor contributing to reduced lysis of HSV-2-infected cells may be the altered or reduced expression of the class I *H-2* self-antigens.

The recognition of viral antigens, inserted into the host cell membrane as a consequence of infection, by cytotoxic T lymphocytes (CTL) is restricted by the association of these antigens with the class I self-antigens of the major histocompatibility complex. In the mouse, these so-called restricting elements are the gene products encoded within the *K*-, *D*-, and *L*-loci of the *H-2* complex (9, 40). Several studies have confirmed that these basic principles are true in the CTL response to herpes simplex virus (HSV) in mice (5, 6, 14, 18, 22, 26, 33, 34). We focused on the primary CTL response in C57BL/6 (*H-2*^{b}) mice following the local injection of infectious virus in the hind footpads, and it was demonstrated that one or more of the HSV-encoded envelope glycoproteins, designated gB, gC, gD, and gE (23), expressed on the surface of HSV-infected *H-2*^{b} (B6/WT-3) cells serve as the target antigens for HSV-specific CTL (6, 18); that CTL generated in response to HSV type 1 (HSV-1) or HSV type 2 (HSV-2) exhibit cross-reactive recognition of target cells infected with either serotype (5); and that the effector CTL population recognizes HSV-encoded glycoprotein(s) primarily in association with the *H-2K*^{b} self-antigen (14, 18).

Despite the observation that HSV-specific CTL are cross-reactive, it has been shown that HSV-2-infected target cells are consistently less susceptible to lysis than HSV-1-infected cells (5). The reduced susceptibility is not due to the inability of HSV-2 to replicate within B6/WT-3 cells, nor is it due to an inability of the infected cells to express serologically recognizable HSV-encoded glycoproteins on the cell surface (5). The HSV-2-encoded function(s) responsible for the reduced susceptibility maps between 0.82 and 1.00 map units of the HSV-2 genome (4), a region which encompasses the short unique and terminal repeat sequences (21). However, neither the viral product(s) responsible for this phenomenon nor the mechanism by which it is facilitated are known.

The complex, multistaged process by which CTL effect the lysis of target cells has been described previously in great detail (1). The critical initial stage is the formation of CTL-target cell conjugates. The efficiency of this step is dependent on the levels of expression of the appropriate antigenic structures on the target cell surface. In this study, we examined the effect of HSV infection on the expression of the class I *H-2* antigens on the infected cell surface to examine the possibility that the reduced susceptibility of HSV-2-infected cells to lysis by cross-reactive, HSV-specific CTL may be the consequence of a reduced CTL recognition of the target cell. The results show that both HSV-1 and HSV-2 cause a reduction in serologically detectable *H-2* but that the effect of HSV-2 is more pronounced. Furthermore, the ability of HSV-2 to reduce *H-2* expression maps to the same region of the HSV-2 genome associated with reduced susceptibility to lysis. Finally, the reduced *H-2* expression correlates with a reduced susceptibility to lysis by CTL specific for a third-party antigen expressed on the surface of B6/WT-3 cells, namely the simian virus 40 (SV40) tumor-specific transplantation antigen. The significance of these findings in the context of recognition of HSV-infected cells by CTL is discussed.

MATERIALS AND METHODS

Mice. Male C57BL/6 (B6, *H-2*^{b}) mice were obtained from Jackson Laboratories, Bar Harbor, Maine, at 4 to 5 weeks of age and were used routinely between the ages of 6 and 12 weeks.

Cells and cell culture. The clonally derived cell line

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B6/WT-3, an SV40-transformed mouse embryo fibroblast line of B6 origin, has been described previously (28). The cells were maintained in monolayer culture at 37°C in Dulbecco modified Eagle medium supplemented with 5% (vol/vol) heat-inactivated fetal calf serum–0.075% $NaHCO_3$–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer–100 U of penicillin per ml–100 μg of streptomycin sulfate per ml–20 μg of gentamicin per ml–0.03% glutamine.

Viruses. The original stocks of HSV-1 strain KOS and HSV-2 strain 186 were kindly provided by P. A. Schaffer, the Dana-Farber Cancer Institute, Boston, Mass. The original stocks of the HSV-1 × HSV-2 intertypic recombinants R50BG10, RH1G44, and RS1G25 were kindly provided by B. Roizman, the University of Chicago, Chicago, Ill. The origin and genetic characteristics of these intertypic recombinants has been summarized previously (4). In addition, the HSV-1 × HSV-2 intertypic recombinant R50BG13 1A1 (designated 1A1) and a derivative (designated 3-3) were kindly provided by P. G. Spear, the University of Chicago. 1A1 (11) is a TK^+, arabinosylthymidine-sensitive modification of the original stock isolated by Tognan et al. (38). Derivative 3-3 is an insertion mutant of 1A1 which has the HSV-1 gD gene (*Sac*I fragment, 0.906 to 0.924 map units) inserted into the *tk* locus. This mutant expresses gD of both HSV-1 and HSV-2 (11).

Generation of HSV and SV40-specific CTL. Effector cells capable of lysing HSV-infected or SV40-transformed target cells were generated as described previously (6, 26). Briefly, donor mice were immunized by injection of 10^5 PFU of HSV or 10^7 PFU of SV40 virus into each hind footpad. Five or nine days later, respectively, the draining popliteal lymph nodes were excised and collected in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum, 2×10^{-5} M 2-mercaptoethanol, 20 mM HEPES buffer, 0.225% $NaHCO_3$, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 0.03% glutamine. Single-cell suspensions were incubated in tissue culture dishes (60 by 15 mm; Costar, Cambridge, Mass.) at a final concentration of 2×10^7 cells in 6 ml of medium at 37°C and in 5% CO_2 for 3 days. Control cultures of lymphocytes were isolated from the inguinal, superficial and deep axillary, and cervical lymph nodes of nonimmunized mice and established under identical conditions.

Generation of SV40-specific CTL clones. The SV40-specific CTL clones used in this study were 10H5 (*H-2K*^{b}-restricted), 20C7, and B12 (*H-2D*^{b}-restricted). They were generated as described by Campbell et al. (3). Briefly, mice were immunized twice by the intraperitoneal route with the SV40-transformed cell line B6/WT-19. A single-cell suspension of 9-day-old immune spleen cells was cultured in vitro at a concentration of 1.5×10^7 with 3×10^5 gamma-irradiated B6/WT-19 stimulator cells per well in 12-well tissue culture plates (Costar). On day 4 of culture, the lymphocytes were harvested, fractionated on lymphocyte-separating media (Litton Bionetics, Kensington, Md.), and cultured at a concentration of 6×10^4 cells per well in the presence of 3×10^5 stimulator cells and interleukin-2 (Collaborative Research, Inc., Lexington, Mass.) in 12-well plates. The clones were isolated from mass cultures by limiting dilution in the presence of irradiated, syngeneic spleen cells as fillers, stimulator cells, and interleukin-2 into flat-bottomed, 96-well microtiter plates and were expanded.

Assay for lymphocyte-mediated cytolysis. The ability of lymphocytes obtained from mice immunized with HSV to lyse HSV-infected target cells was determined as described previously (6). Briefly, confluent monolayers of target cells were infected by adsorption for 1 h with HSV diluted in Tris-buffered physiological saline at a multiplicity of infection of 2.5. At this time, the inoculum was removed and replaced with Dulbecco modified Eagle medium containing 10% fetal calf serum and labeled with 250 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, Ill.) for 12 to 14 h. Infected cells were removed by treatment with trypsin (0.5 mg/ml, 1 min, 37°C), washed three times, and suspended in RPMI 1640 medium at 2×10^5 cells per ml. Cytolytic activity was determined in a standard 5-h assay by adding 2×10^4 labeled target cells in 100 μl to glass culture tubes (10 by 75 mm) containing 100 μl of effector cells at an effector to target cell ratio of 50:1. Spontaneous release from target cells over the 5-h incubation period ranged from 15 to 25%. Maximal release was determined by the addition of 5% (wt/vol) sodium dodecyl sulfate.

Cold target inhibition assay. The ability of unlabeled cell populations to compete against labeled target cells for the lytic activity of HSV-specific CTL was determined in a cold target inhibition assay. Briefly, lymphocytes were added to glass tubes (10 by 75 mm) at a concentration to give the required effector to labeled target cell ratio. Unlabeled inhibitor cells were added at various concentrations, followed immediately by the addition of a fixed concentration of ^{51}Cr-labeled target cells. The ability of the unlabeled cells to compete was determined by measuring the reduction of specific ^{51}Cr release following a 5-h incubation at 37°C.

Antibody-dependent complement-mediated cytolysis of HSV-infected cells. The expression of HSV-encoded antigens on the cell surface was determined as described previously (5). HSV-specific antibody, used at a dilution of 1:10, was prepared by immunizing rabbits three times with HSV-infected rabbit kidney cells. Rabbit complement (C'; Low Tox M; Cedarlane, London, Ontario, Canada) was used at a final dilution of 1:20.

Determination of CTL precursor frequency by limiting dilution analysis. The frequency of HSV-specific CTL precursors (CTLp) that give rise to lytic progeny was determined as described previously (14, 30, 31). Briefly, graded numbers (usually 2.5×10^2 to 3.2×10^4) of lymphocytes from mice immunized for 5 days were cultured in vitro in flat-bottomed, 96-well microtiter plates (Costar) containing 3×10^5 irradiated (1200 R) syngeneic spleen cells as fillers, 2.0 half-maximal units of interleukin-2 (AMGen Biologicals, Thousand Oaks, Calif.), with HSV-infected, mitomycin-C-treated peritoneal washing cells as stimulators. Control cultures were established as described above, except no responder cells were included. Cultures were incubated for 5 to 7 days at 37°C and in 5% CO_2. Cultures were split three- or fourfold, and the cytolytic activity was determined against 2×10^3 labeled target cells. Individual cultures were considered positive if the level of ^{51}Cr release exceeded the mean value of the control cultures by at least 3 standard deviations. Precursor frequencies were estimated by the minimum chi-square method (36). The estimates were considered valid only if the single-hit hypothesis was fulfilled with a probability of $P > 0.05$.

Monoclonal antibodies. The monoclonal antibodies specific for the *H-2* antigens were EH-144 (*H-2K*^{b} [27]) and H-141-51 (H-2D^b [19]), both kindly provided by T. V. Rajan, the Albert Einstein College of Medicine, New York, N.Y. Monoclonal antibodies specific for the HSV glycoprotein species were I-144 (gB-1 [15]) and II-436 (gD-1 [15]), which were kindly provided by P. G. Spear; HC-1 (gC-1 [25]) and H600 (gE-1 [24]) were provided by L. Pereira, the California

Department of Health, Berkeley, Calif.; 20 D4 (gB-2,1), 17 A2 (gC-2), 17 A3 (gD-2), 17 C2 (gE-2), and 13 C5 (gG-2) were provided by W. Rawls and N. Balachandrin, McMaster University, Hamilton, Ontario, Canada (16).

Preparation of target cells for analysis by flow cytometry. HSV- and mock-infected cells were removed from the flasks by treatment with trypsin (0.5 mg/ml, 1 min, 37°C), washed once in phosphate-buffered saline (pH 7.4) containing 2% fetal calf serum and 0.1% NaN_3, and 10^6 cells were dispensed in glass culture tubes (10 by 75 mm). The cells were pelleted by centrifugation (400 × *g*, 5 min), thoroughly drained, and suspended in 100 μl of the appropriate monoclonal antibody, and incubated at 4°C for 1 h. Controls were suspended in phosphate-buffered saline. The cells were washed once, suspended in 100 μl of a 1:25 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (heavy and light chain specific; Becton Dickinson and Co., Mountain View, Calif.), and incubated at 4°C for 1 h. Samples (400 μl) were analyzed immediately or fixed by the addition of 1 ml of 2% (wt/vol) paraformaldehyde in phosphate-buffered saline (pH 7.4) and stored at 4°C for no longer than 48 h.

Flow cytometry analysis. Samples were analyzed with an Epics V Flow cytometer/sorter (Coulter Electronics, Inc., Hialeah, Fla.) with excitation of fluorescence of 500 mW at 488 nm. Instrument calibration was performed with 10-μm fluorospheres (grade II; Coulter Electronics) and the coefficientsof variation were < 2.5. Ninety-degree light scatter was collected with a 488-nm dichroic mirror and a neutral density filter. Green fluorescence was collected with a 515-nm interference filter, a 560-nm short-pass filter, and a 515-nm long-pass glass filter. For each sample, 10,000 cells were analyzed at 500/s by collecting the logarithm of integrated green fluorescence gated on forward angle light scatter and 90° light scatter. Dead cells, usually less than 5%, were effectively gated out with forward angle light scatter gates or by the exclusion of red fluorescing cells after the addition of propidium iodide. Histograms were analyzed by simple integration and the immuno program provided by Coulter Electronics. Results were presented as fluorescence profile histograms with the number of cells on the *y* axis, and the fluorescence intensity is presented as arbitrary units on a three-decade logarithmic scale on the *x* axis.

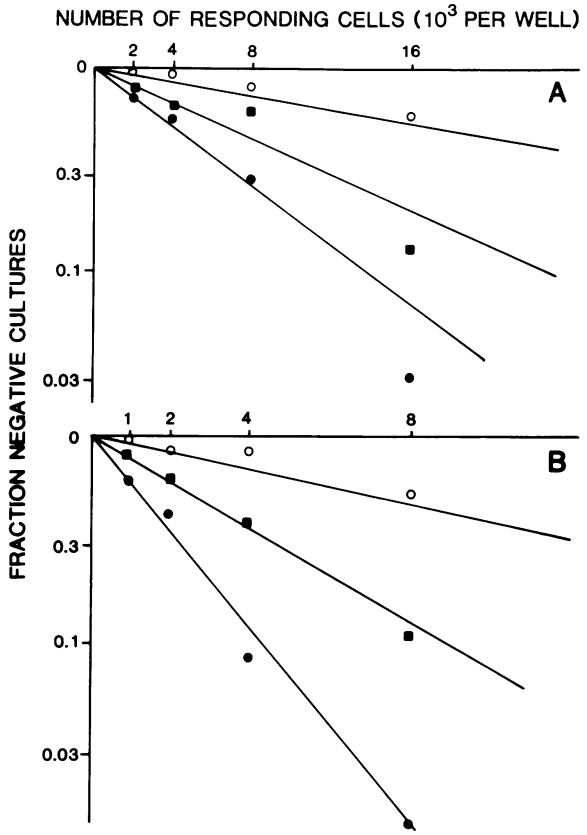

FIG. 1. Frequency of HSV-specific CTLp able to recognize KOS-, 186-, and R50BG10-infected B6/WT-3 cells. CTLp from mice immunized 5 days previously with HSV-1 KOS (A) or HSV-2 186 (B) were expanded under limiting dilution conditions (see the text). The lytic activity of individual cultures was determined by splitting each culture fourfold and assaying against KOS- (●), 186- (○), R50BG10- (□), or mock-infected (data not shown) target cells. HSV-specific CTLp frequency estimates against infected target cells were as follows: panel A, KOS, f = 1/6030, P = 0.55; 186, f = 1/24290, P = 0.10; R50BG10, f = 1/9003, P = 0.15; panel B, KOS, f = 1/1914, P = 0.82; 186, f = 1/10219, P = 0.21; R50BG10, f = 1/3932, P = 0.90. The frequency of positive cultures against mock-infected cells was too low to determine.

TABLE 1. Mapping of the HSV-2-encoded function(s) causing reduced susceptibility to lysis by HSV-specific CTL

B6/WT-3 cells infected by[a]:	Location of HSV-2 DNA in recombinants (map units)[b]	Percent specific ^{51}Cr release in:	
		Anti-HSV-1 CTL[c]	Antibody + C'[d]
HSV-1 KOS		58.7	73.6
HSV-2 186		17.1	78.5
HSV-1 × HSV-2 (R50BG10)	0.82-1.0	24.7	81.9
HSV-1 × HSV-2 (RH1G44)	0.35-0.43	51.8	67.6
HSV-1 × HSV-2 (RS1G25)	0.58-0.72	42.3	44.6
Mock		2.6	0.7

[a] Target cells were infected with the designated virus strain at a multiplicity of infection of 2.5 or mock infected with Tris-buffered saline. The cells were labeled with 250 μCi of ^{51}Cr and harvested 14 h postinfection.

[b] Approximate location.

[c] Lymphocyte cytotoxicity was determined in a 5-h ^{51}Cr release assay at 37°C. CTL were used at an effector-to-target cell ratio of 50:1.

[d] Target cells were treated with hyperimmune rabbit anti-HSV sera (1:10) at 4°C for 1 h followed by rabbit low toxicity C' (1:20) for 1 h at 37°C.

RESULTS

Differential susceptibility of HSV-1- and HSV-2-infected B6/WT-3 cells to lysis by CTL. In Table 1 is summarized the relative susceptibility of B6/WT-3 cells infected with HSV-1 strain KOS or HSV-2 strain 186 to lysis by HSV-specific CTL. First, HSV-2-infected cells exhibited a reduced susceptibility to lysis by CTL compared with cells infected with HSV-1. Second, by using HSV-1 × HSV-2 intertypic recombinants with a precisely characterized genome structure, it was possible to map the HSV-2-encoded function(s) associated with the reduced susceptibility to 0.82 to 1.00 map units of the HSV-2 genome. Cells infected with the recombinant R50BG10, the genome of which contains only these sequences of HSV-2 DNA, exhibited a level of susceptibility comparable to that of HSV-2-infected cells. It should be noted that there was not an absolute reduction to the same levels as those of HSV-2. R50BG10-infected cells exhibited a susceptibility to lysis intermediate to those of KOS- and

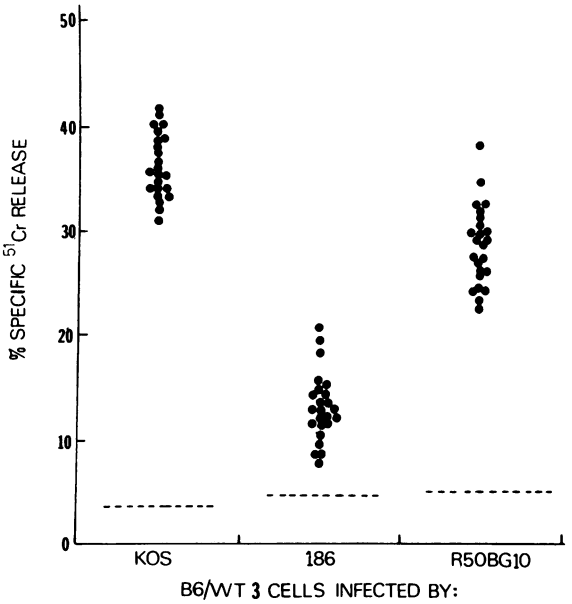

FIG. 2. Lytic activity of individual oligoclonal cultures against KOS-, 186-, and R50BG10-infected B6/WT-3 cells. Each point represents the percent specific ^{51}Cr release obtained from individual cultures at the 32,000 responder cell input level. The broken lines represent the 95% tolerance limits for the determination of positive lytic activity against each labeled target cell.

186-infected cells. Recombinant genomes expressing regions of HSV-2 DNA other than the 0.82- to 1.00-map-unit sequences exhibited a susceptibility to lysis comparable to that of HSV-1-infected cells. These results are in agreement with our previously published findings (4, 5).

Despite the differential susceptibility to lysis by CTL, cells infected with the wild-type and the recombinant strains of HSV were equally susceptible to lysis mediated by polyclonal anti-HSV antibody in the presence of C′ (Table 1 [4]). This indicates that the infected cells are able to process and express viral antigens important to the humoral response to HSV infection and that the reduced susceptibility of HSV-2-infected cells to lysis by CTL is probably not due to an innate resistance of the cells to the mechanisms of immune cytolysis.

The ability of unlabeled HSV-1- and HSV-2-infected cells to inhibit the specific lysis of labeled HSV-1-infected targets was investigated in a cold target inhibition assay. Although the overall levels of inhibition obtained were low, it appeared that HSV-2-infected cells were less effective. This suggests that HSV-2-infected cells may be less efficiently recognized by the CTL population (data not shown).

Frequency of CTL precursors with specificity for HSV-1- and HSV-2-infected cells. There were potentially two possible explanations for the reduced susceptibility of HSV-2-infected cells to lysis by HSV-specific CTL. One possibility, as mentioned previously, may be that HSV-2-infected cells are recognized less efficiently by the cross-reactive, HSV-specific CTL population. An alternative explanation may be that fewer CTLp with the potential of recognizing HSV-2-infected cells are generated in vivo during the primary response to HSV. These possibilities were tested by assessing the frequency and lytic potential of the progeny of limited numbers of HSV-specific CTLp. Lymph node cells obtained from mice immunized 5 days previously were cultured under limiting dilution conditions under which potentially all virus-specific CTLp may expand (30, 31). We determined that the effector cells that lyse HSV-infected cells in this assay express the Thy1$^+$, Lyt2$^+$ phenotype, with a lower but significant level of Lyt1 expression (data not shown). Figure 1 shows the relative frequencies of CTLp generated in response to immunization with HSV-1 KOS. The frequency of cultures with lytic activity against HSV-1 KOS-infected cells (f = 1/6030; P = 0.55) was higher than for either R50BG10-infected (f = 1/9004; P = 0.15) or HSV-2 186-infected (f = 1/24920; P = 0.10) cells. Moreover, this finding was not due to the generation of an HSV-1 KOS-specific response, as similar results (Fig. 1) were observed for CTLp generated in response to HSV-2 186 (KOS-infected, f = 1/1914, P = 0.82; R50BG10-infected, f = 1/3932, P = 0.90; 186-infected, f = 1/10219, P = 0.26), which indicates that cross-reactive HSV-specific CTL were generated. Therefore, the consistent finding of these experiments is that the reduced susceptibility of HSV-2 186-infected cells to lysis by CTL reflects a lower frequency of CTLp with the potential to recognize the HSV-2-infected cells.

The results shown in Fig. 2 illustrate an important point obtained from this assay system. At a responder cell input number at which all lethal dose cultures were positive against KOS-, 186-, and R50BG10-infected cells, the levels of the percentage of specific ^{51}Cr release obtained from 186-infected targets were demonstrably lower than those obtained from the KOS-infected cells. The lysis of the R50BG10-infected cells was intermediate. No individual culture exhibited equivalent levels of lytic activity against all three target cells, which suggests a fundamental difference in the ability of the different target cells to be recognized by the lytic progeny of the HSV-specific CTLp generated in vivo.

Levels of expression of HSV-encoded glycoproteins on the surface of HSV-infected B6/WT-3 cells. If the reduced lysis of HSV-2-infected cells were due to a decreased level of recognition by CTL, this implies a reduction or alteration in the essential antigenic structures expressed on the infected cell surface. Virus-specific CTL recognize antigenic determinants resulting from an association between the viral antigen and the class I *H-2* antigens on the surface of infected cells (9, 40). Therefore, reduced recognition may indicate an inadequate expression of either or both of these antigens. The experiments shown in Table 1 indicate that all wild-type, mutant, and intertypic strains of HSV so far tested are susceptible to lysis by anti-HSV antisera in an antibody-dependent, complement-mediated cytolysis assay. While providing evidence for the presence of the glycoprotein species on the surface of infected cells, no information on the relative expression of the individual glycoproteins could be determined by this method. Therefore, the expression of HSV-specific glycoproteins was assessed by fluorescent flow cytometric analysis with monoclonal antibodies specific for individual glycoprotein species of HSV-1 and HSV-2. The monoclonal antibodies were extensively titrated and used at the dilution corresponding to maximal binding.

The fluorescent profiles of cells infected with HSV-1 KOS, HSV-2 186, or the intertypic recombinants RH1G44 or R50BG10 for 12 h and stained for the surface expression of the individual glycoprotein species are shown in Fig. 3. Negligible binding to the mock-infected cells was observed. It is evident from the fluorescence profiles obtained for KOS- and 186-infected cells that each of the glycoprotein species is expressed on the cell surface. By this analysis, it was found that strain 186-infected cells express approximately a twofold lower level of all the glycoproteins tested

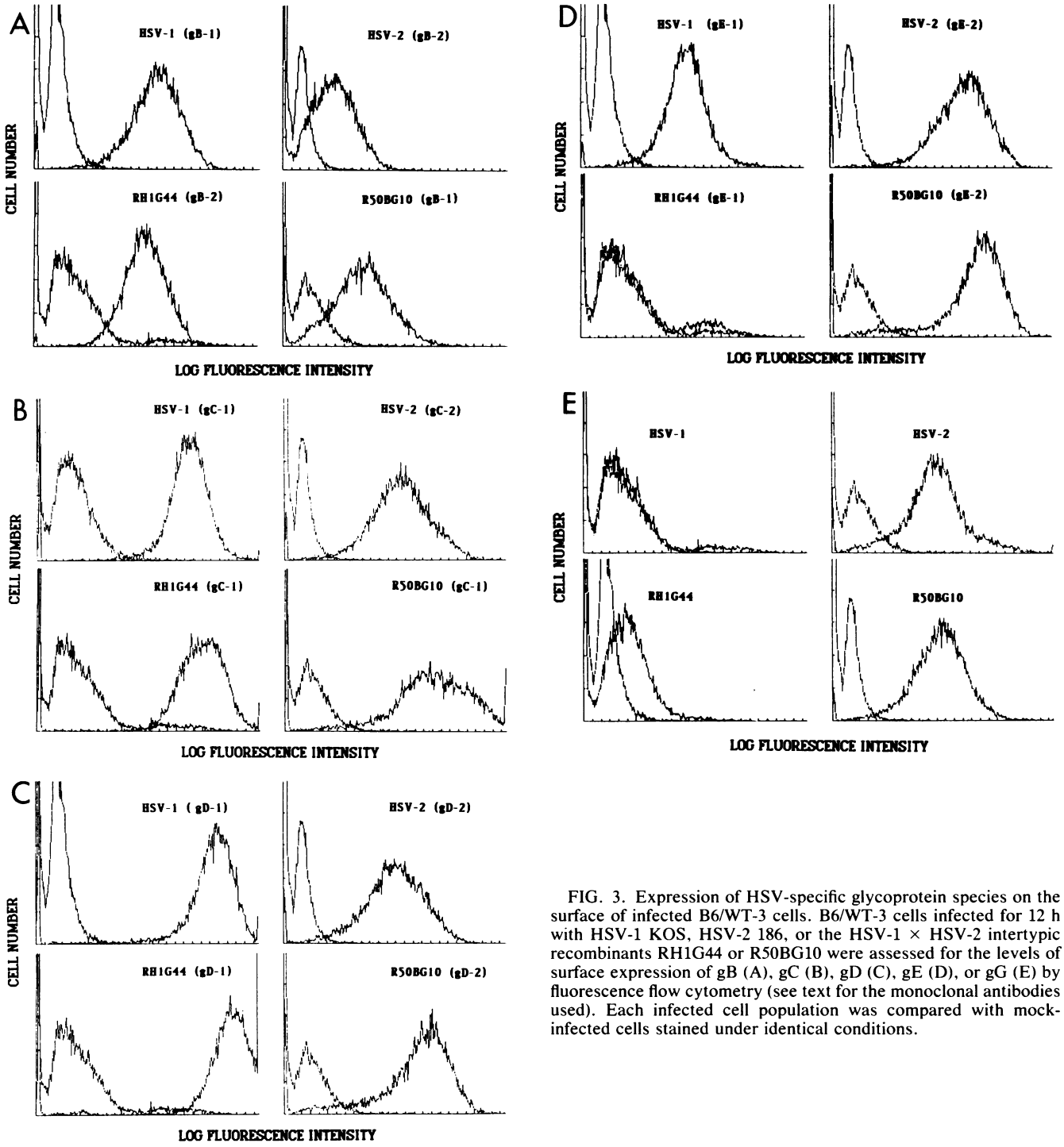

FIG. 3. Expression of HSV-specific glycoprotein species on the surface of infected B6/WT-3 cells. B6/WT-3 cells infected for 12 h with HSV-1 KOS, HSV-2 186, or the HSV-1 × HSV-2 intertypic recombinants RH1G44 or R50BG10 were assessed for the levels of surface expression of gB (A), gC (B), gD (C), gE (D), or gG (E) by fluorescence flow cytometry (see text for the monoclonal antibodies used). Each infected cell population was compared with mock-infected cells stained under identical conditions.

compared with KOS-infected cells. Consistently quantitative differences in the expression of certain glycoproteins, especially gB, were observed, but results of repeated experiments suggest that this is most likely attributable to variations in the affinity or avidity of the individual monoclonal antibodies. With this qualification, the results suggest that the reduced susceptibility of strain 186-infected cells to lysis by HSV-specific CTL may be attributable in part to an inappropriate or inadequate expression of certain glycoprotein species, but this alone could not account for the observation.

The HSV-2-encoded function(s) responsible for the reduced susceptibility to lysis by CTL map to 0.82 to 1.00 map units of the HSV-2 genome. It is possible that the reduced lysis may be directly attributable to the expression of a glycoprotein species encoded in this region of HSV-2 DNA. This could be because a glycoprotein in this region is the major target antigen for CTL, but the HSV-2 glycoprotein

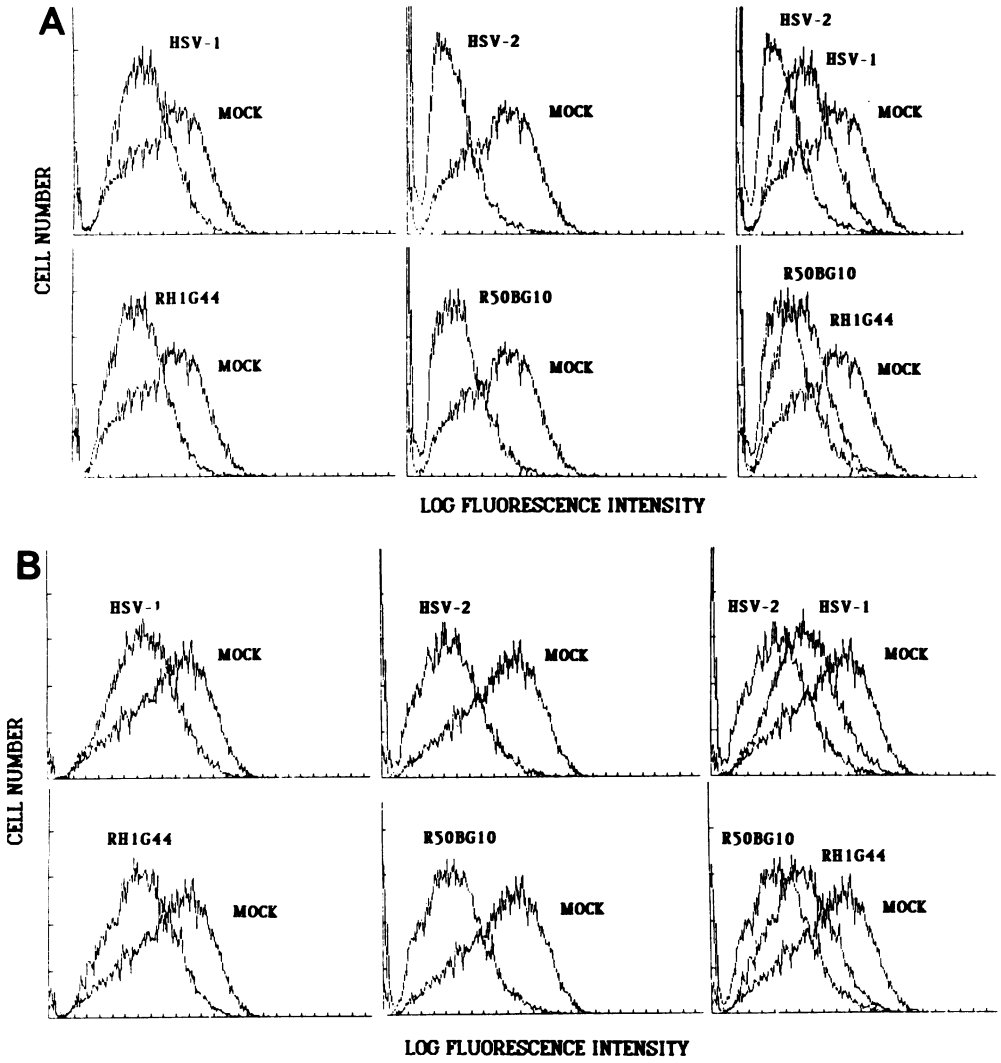

FIG. 4. Expression of the class I *H-2* antigens on the surfce of HSV-infected B6/WT-3 cells. B6/WT-3 cells were infected for 12 h with HSV-1 KOS, HSV-2 186, or the HSV-1 × HSV-2 intertypic recombinants RH1G44 or R50BG10, and assessed for the expression of $H\text{-}2K^b$ (A) or $H\text{-}2D^b$ (B) antigens on the cell surface by fluorescence flow cytometry (see text for the monoclonal antibodies used). Each infected cell population was compared with mock-infected cells stained under identical conditions.

may not serve as well as its HSV-1 counterpart in this regard. As pointed out above, primary HSV-specific CTL are cross-reactive, and it has been shown that of the glycoproteins encoded within this region, gD-2 and gE-2 express mainly cross-reactive determinants (23), while gG is apparently type specific (20). We were interested to determine whether the reduced susceptibility to lysis could be overcome if both gD-1 and gD-2 were expressed on the infected cell surface. B6/WT-3 cells were infected with the intertypic recombinant 3-3, an insertion mutant which coexpresses gD-1 and gD-2 (11). Using a monoclonal antibody that recognizes determinants exclusive to gD-1 and a second that recognizes cross-reactive determinants, we were able to confirm that both gD species are expressed on the surface of infected B6/WT-3 cells (data not shown). As shown in Table 2, 3-3-infected cells were lysed to the same level as cells infected with the parental recombinant R50BG13 and similar to the level observed for 186-infected cells. Therefore, the coexpression of gD-1 and gD-2 did not overcome the reduced susceptibility to lysis, which indicates either that the HSV-2-encoded function(s) responsible was indepenent of gD expression or that the HSV-2 encoded function was dominant.

Expression of *H-2* antigens on the surface of HSV-infected B6/WT-3 cells. If the reduced susceptibility of HSV-2-infected cells to lysis by CTL is the result of reduced target cell recognition, but not totally attributable to the expression of HSV-2 glycoprotein species on the surface of infected cells, an alternative explanation may be that HSV-1 and HSV-2 cause a differential reduction or alteration in the expression of the class I *H-2* self-antigens. This possibility was also investigated by fluorescent flow cytometric analysis of infected cells stained with monoclonal antibodies specific for $H\text{-}2K^b$ or $H\text{-}2D^b$. Mock-infected B6/WT-3 cells expressed high levels of $H\text{-}2K^b$ and $H\text{-}2D^b$ (Fig. 4). Although not formally tested by two-color fluorescence, it was expected that the antigens are coexpressed, because greater than 90% of cells were graded as positive for each antigen.

TABLE 2. Coexpression of gD of HSV-1 and HSV-2 does not overcome HSV-2-induced lowered susceptibility to lysis by HSV-specific CTL

B6/WT-3 cells infected by[a]:	Percent specific ^{51}Cr release in:			
	anti-HSV-1 CTL[b]		Antibody + C'[c]	
	Expt 1	Expt 2	Expt 1	Expt 2
HSV-1 KOS	57.1	66.3	76.5	80.9
HSV-2 186	8.1	26.4	85.0	84.6
HSV-1 × HSV-2 (R50BG13-1A1)[d]	10.7	18.7	30.8	31.2
3-3[d]	15.3	24.6	64.1	61.9
Mock	3.1	6.1	1.7	1.6

[a] Target cells were infected with the designated virus strain at a multiplicity of infection of 2.5 or mock infected with Tris-buffered saline. The cells were labeled with 250 μCi of ^{51}Cr and harvested 14 h postinfection.

[b] Lymphocyte cytotoxicity was determined in a 5-h ^{51}Cr release assay at 37°C. CTL were used at an effector to target cell ratio of 50:1.

[c] Target cells were treated with hyperimmune rabbit anti-HSV sera (1:10) at 4°C for 1 h followed by rabbit low toxicity C' (1:20) for 1 h at 37°C.

[d] R50BG13-1A1 is the HSV-1 × HSV-2 recombinant virus R50BG13 made TK^+ and $araT^s$. 3-3 is an insertion mutant containing the DNA for HSV-1 gD in the *tk* gene of R50BG13-1A1.

However, it was consistently found that *H-2K*^{b} expression was lower than *H-2D*^{b} expression, as indicated by a lower mean peak fluorescence.

Infection with both HSV-1 KOS or HSV-2 186 resulted in a decrease in the expression of serologically detectable *H-2K*^{b} and *H-2D*^{b} (Fig. 4) but was more pronounced following HSV-2 infection. This was evident from the shift to the left and lower mean peak fluorescence of the HSV-2-infected cells, which indicates that a greater number of cells are registering in the lower fluorescence channels. This effect could be attributed to function(s) associated with the 0.82- to 1.00-map-unit region of the HSV-2 genome, as infection with the intertypic recombinant R50BG10 had a similar effect on *H-2* expression as that with HSV-2 186. This was emphasized by the results presented in Table 3, which represents an integration analysis of the fluorescence profiles of infected and uninfected cells, showing the percentage of the total cells that registered within certain arbitrary fluorescence intensity limits. It can be seen that HSV-1 KOS and the recombinants RH1G44 and RS1G25 caused a similar reduction in *H-2* expression, as shown by the increase in the percentage of cells that register in the lower intensity channels. HSV-2 186 and the recombinant R50BG10 had a more pronounced effect. Therefore, the ability of a particular virus to reduce *H-2* expression closely paralleled the susceptibility of cells infected with that virus to lysis by HSV-specific CTL. Furthermore, the increased ability of HSV-2 to reduce *H-2* appeared to map to the 0.82- to 1.00-map-unit region of the HSV-2 genome.

TABLE 4. Lysis of HSV-infected B6/WT-3 cells by SV40-specific CTL

B6/WT-3 cells infected by:	Percent specific ^{51}Cr release in the following CTL and the indicated expt no.[a]:					
	Anti-SV40			Anti-HSV		
	1	2	3	1	2	3
Mock	58.9	49.6	79.0	0.5	2.9	25.6
HSV-1 KOS	27.7	21.7	71.4	33.3	42.1	75.3
HSV-2 186	7.8	19.7	37.8	4.7	15.0	45.5
HSV-1 × HSV-2 (R50BG10)	18.4	20.1	54.6	12.3	22.2	64.0

[a] SV40-specific and HSV-specific CTL were generated in C57BL/6mice and used at an effector to target ratio of 50:1 in a 5-h assay.

Recognition of HSV-infected B6/WT-3 cells by SV40-specific CTL. The results presented above suggest that the reduced susceptibility of HSV-2-infected cells to lysis by HSV-specific CTL is a direct consequence of the reduction in *H-2* expression on the surface of the infected cell. However, it cannot be assumed that a reduction in serologically detectable *H-2* necessarily reflects a decreased ability of these molecules to act as restriction elements for self-restricted CTL. Therefore, we investigated the effect of infection with HSV-1, HSV-2, and the intertypic recombinants on the recognition and lysis of B6/WT-3 cells by *H-2*^{b}-restricted CTL clones specific for an unrelated third-party antigen, the SV40-specific large T antigen present on the surface of B6/WT-3 cells. Large T antigen has been shown to be the most likely candidate for the SV40 tumor-specific transplantation antigen (37). The results obtained (Tables 4 and 5) demonstrate three distinct groups with respect to susceptibility to lysis by SV40-specific CTL. These groups directly reflect the ability of the virus strain to reduce the expression of class I *H-2* antigen expression. Furthermore, infection with HSV did not cause a decrease in the surface expression of SV40 large T antigen on the cell surface (data not shown). Therefore, it appears that the reduction in expression of

TABLE 3. Integration analysis of *H-2* expression fluorescence profiles of HSV-infected and uninfected B6/WT-3 cells

B6/WT-3 infected by[a]:	Percentage of cells positive for the following antigens within the indicated integral boundaries[b]							
	H-2K^b				*H-2D*^{b}			
	10–40	41–70	71–100	101–140	10–40	41–70	71–100	101–140
Mock	21.1	34.7	35.9	8.3	9.4	21.7	35.2	33.7
HSV-1 KOS	17.9	55.5	24.2	2.4	12.2	43.7	35.8	13.0
HSV-1 × HSV-2 (RH1G44)	26.6	54.0	18.1	1.3	9.6	36.8	39.8	13.8
HSV-1 × HSV-2 (RS1G25)	21.8	45.8	25.6	6.8	12.0	27.9	33.8	16.3
HSV-2 186	53.3	40.0	5.9	0.8	25.1	55.4	17.1	2.4
HSV-1 × HSV-2 (R50BG10)	42.8	47.2	9.2	0.8	21.3	49.5	24.6	4.6

[a] Target cells were infected with the designated virus strain at multiplicity of infection of 2.5 for 14 h or mock infected with TBS. The cells were reacted with monoclonal antibodies specific for *H-2K*^{b} or *H-2D*^{b} and labeled with a fluorescein isothiocyanate-conjugated second-step antibody.

[b] Boundaries are defined by fluorescence units.

TABLE 5. Lysis of HSV-infected B6/WT-3 cells by SV40-specific CTL clones

B6/WT-3 cells infected by:	Percent specific ^{51}Cr release by the following SV40-specific CTL clones[a]:			
	Expt 1		Expt 2	
	10H5	20C7	10H5	B12
Mock	54.8	92.3	83.3	87.6
HSV-1 KOS	39.0	78.5	78.8	85.5
HSV-1 × HSV-2 (RH1G44)	39.1	75.5	77.5	84.0
HSV-2 186	14.5	52.8	59.5	72.2
HSV-1 × HSV-2 (R50BG10)	14.0	60.6	62.5	78.2

[a] ^{51}Cr-labeled target cells were reacted with SV40-specific CTL clones at an effector to target ratio of 10:1 in a 5-h assay.

serologically detectable *H-2* does indeed reflect a decreased ability of these molecules to function as restriction elements.

DISCUSSION

Murine virus-specific CTL recognize infected cells via a receptor specific for virus-encoded antigens present in the infected cell membrane only in association with the class I antigens of the *H-2* complex (9, 40). Reduced recognition by CTL may be due to either low or inappropriate expression of a particular viral antigen that serves as the major target antigen or to a reduced or altered expression of *H-2K* or *H-2D* self-antigens or both. However, the first explanation appears to be unlikely because both HSV-1- and HSV-2-infected cells were equally susceptible to lysis by anti-HSV antibody and C′ (Table 1 [4, 5]), and analysis of the expression of individual HSV-1- and HSV-2-specific glycoprotein species by fluorescent flow cytometry confirmed that cells infected with either virus strain expressed a full complement of viral glycoproteins on their surface. It was observed that the detectable levels of gB-2 expression were consistently lower than those of gB-1. This either may be due to a real inability of gB-2 to be expressed at high levels on infected cells or may reflect a lower affinity of the gB-2-specific monoclonal antibody for this glycoprotein species. Nevertheless, it is unlikely that the low expression of gB-2 is responsible for the reduced susceptibility of HSV-2-infected cells to lysis, as the intertypic recombinant RH1G44, which confers the HSV-1 level of susceptibility to infected cells, also expresses relatively low levels of gB-2.

It was found that the expression of both *H-2K* and *H-2D* antigens were reduced after infection with both HSV-1 and HSV-2. However, HSV-2 caused a greater reduction in *H-2* expression than HSV-1, and the HSV-2-encoded function responsible for the greater reduction in expression of *H-2* mapped to 0.82 to 1.00 map units of the HSV-2 genome. This provides strong circumstantial evidence that the reduction in the class I *H-2* antigens may be directly responsible for the HSV-2-associated reduction in susceptibility to lysis by HSV-specific CTL. The possibility that the reduction in *H-2* expression causes the reduced susceptibility to lysis of HSV-2-infected cells was further reinforced in an assay in which an unrelated third-party antigen present on the surface of B6/WT-3 cells was used. It was found that B6/WT-3 cells infected with HSV were less susceptible to lysis by both bulk culture CTL and CTL clones specific for the SV40 tumor-specific transplantation antigen and that the ability of a particular virus strain to reduce target cell lysis was directly related to its ability to alter *H-2* expression. The surface expression of SV40 large T antigen, the likely candidate for SV40 tumor-specific transplantation antigen (37), was unaffected by HSV infection, further emphasizing the importance of *H-2* reduction in reduced susceptibility to lysis.

The reduction in *H-2* expression on host cells as a consequence of viral infection has been reported in a number of investigations (2, 10, 12, 29). There are many ways in which the interaction between an infecting virus and a host cell could lead to the qualitative change or quantitative reduction in the expression of *H-2* antigens. One possibility is that the virus may inhibit host cell protein synthesis, leading to decreased de novo synthesis of *H-2*. It is known that HSV-2 shuts off host cell protein synthesis more rapidly than HSV-1 (8), and it is likely that this property plays some role in *H-2* reduction in infected B6/WT-3 cells. There is reason to suggest that this is not the sole explanation. The HSV-1 × HSV-2 intertypic recombinant R50BG10, with a genome containing HSV-2 DNA from 0.82 to 1.00 map units, causes a decrease in *H-2* expression equivalent to that of HSV-2 186. However, the HSV-2-encoded functions associated with the rapid shutoff of host cell protein synthesis map to 0.52 to 0.59 map units (7, 13), and R50BG10 shuts down the protein synthesis of B6/WT-3 cells much like HSV-1 KOS (S. R. Jennings, P. L. Rice, and S. S. Tevethia, unpublished data).

A second possibility is that the interaction between the viral antigen and *H-2* in the cell membrane that leads to the formation of determinants recognizable by *H-2*-restricted CTL causes a qualitative alteration in the native *H-2* molecule (10). Such an altered *H-2* molecule may no longer express the epitopes recognized by the *H-2*-specific monoclonal antibodies used in this study. We have shown previously that $H\text{-}2^b$-restricted, HSV-specific CTL recognize viral antigen primarily in association with the $H\text{-}2K^b$ gene product, suggesting a preferential association with this self-antigen (14), and it was observed in this study that HSV infection apparently causes a greater reduction in the expression of $H\text{-}2K^b$ than $H\text{-}2D^b$. The difference in the ability of HSV-1 and HSV-2 to reduce *H-2* expression in all likelihood represents one aspect of a series of biological differences between the two serotypes. Other possible explanations include a virus-induced destabilization of the *H-2* molecules within the cell membrane, leading to increased shedding, destabilization of the protein itself, leading to more rapid turnover, a reduction in the transcription of the class I *H-2* mRNA, or a destabilization of the class I *H-2* mRNA, leading to a higher rate of degradation or decreased translation. These possibilities are currently under investigation.

A question that arises from this study is the potential biological significance of a reduction in the class I *H-2* antigens following infection with HSV. It has been shown in the experimental adenovirus model that the ability of adenovirus 12 to affect the expression of the class I molecules was directly related to its oncogenic ability in vivo (32, 35). The oncogenic potential presumably reflects the ability of the transformed cells to evade the immune system (35). Whether such an effect of HSV is occurring in vivo has yet to be established, but the differential effects of HSV-1 and HSV-2 on class I *H-2* expression may explain in part the higher pathogenicity of HSV-2 in mice (17). It should be noted that Yasukawa and Zarling (39), studying human HSV-specific CTL clones, did not observe a reduced susceptibility of HSV-2-infected target cells compared with HSV-1-infected cells. However, the CTL in their experiments were restricted to the recognition of HSV glycoproteins in association with the class II major histocompatibility

complex antigens. A differential effect of HSV-1 and HSV-2 on class I-restricted human HSV-specific CTL has not been determined.

An important further consideration is the critical levels of surface *H-2* expression that will provide an adequate target for CTL recognition. Although we demonstrated that there is a reduction in the expression of class I *H-2* antigens and a concomitant reduction in lysis by CTL, the reduction in *H-2* expression was relative, not absolute. The results suggest that a high level of *H-2* must be expressed for adequate recognition by HSV-specific CTL and that expression below this critical level leads to reduced recognition. This is supported by the finding that cell lines that are able to process and express HSV glycoproteins but that express low levels of *H-2*, compared with those of B6/WT-3, are poor targets for HSV-specific CTL (S. R. Jennings, unpublished data). Another consideration alluded to earlier is the possibility of an innate resistance of HSV-2-infected cells to lysis by CTL. Thus, although the cell is recognized and the lethal hit is delivered, lysis does not occur. Although the equal susceptibility of all HSV-infected cells to lysis by antibody and C′ makes this possibility unlikely, it has not been definitively proven. Our current research is concerned with a more detailed analysis of the events following the interaction of CTL with the HSV-infected target cell.

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