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Cells Expressing Herpes Simplex Virus Glycoprotein gC but Not gB, gD, or gE Are Recognized by Murine Virus-Specific Cytotoxic T Lymphocytes

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To determine which viral molecule(s) is recognized by herpes simplex virus (HSV)-specific cytotoxic T lymphocytes (CTL), target cells were constructed which express individual HSV glycoproteins. A mouse L cell line, Z4/6, which constitutively expressed high levels of HSV type 2 (HSV-2) gD (gD-2) was isolated and characterized previously (D. C. Johnson and J. R. Smiley, J. Virol. 54:682–689, 1985). Despite the expression of gD on the surface of Z4/6 cells, these cells were not killed by anti-HSV-2 CTL generated following intravaginal infection of syngeneic mice. In contrast, parental Z4 or Z4/6 cells infected with HSV-2 were lysed. Furthermore, unlabeled Z4/6 cells were unable to block the lysis of HSV-2-infected labeled target cells. Cells which express HSV-1 gB (gB-1) were isolated by transfecting L cells with the recombinant plasmid pSV2gBneo, which contains the HSV-1 gB structural sequences and the neomycin resistance gene coupled to the simian virus 40 early promoter and selecting G418-resistant cell lines. One such cell line, Lta/gB15, expressed gB which was detected by immunoprecipitation and at the cell surface by immunofluorescence. Additionally, cells expressing HSV-1 gC (gC-1) or gE (gE-1) were isolated by transfecting Z4 cells, which are L cells expressing ICP4 and ICP47, with either the recombinant plasmid pGE15neo, which contains the gE structural sequences and the neomycin resistance gene, or pDC17, which contains the gC structural gene coupled to the gD-1 promoter. A number of G418-resistant cell lines were isolated which expressed gC-1 or gE-1 at the cell surface. Anti-HSV-1 CTL generated following footpad infection of syngeneic mice were unable to lyse target cells expressing gB-1 or gE-1. In contrast, target cells expressing very low levels of gC-1 were killed as well as HSV-1-infected target cells. Furthermore, infection of gC-1-transformed target cells with wild-type HSV-1 or a strain of HSV-1 that does not express gC did not result in a marked increase in susceptibility to lysis. These results suggest that murine class I major histocompatibility complex-restricted anti-HSV CTL recognize gC-1 but do not recognize gB, gD, or gE as these molecules are expressed in transfected syngeneic target cells. The results are discussed in terms of recent evidence concerning the specificity of antiviral CTL.

Infection with a variety of viruses leads to the induction of cytotoxic T lymphocytes (CTL), which only recognize target cells that express both self-determinants encoded by the major histocompatibility complex (MHC) and foreign antigens of the eliciting virus. This phenomenon of dual specificity of CTL is known as MHC restriction (47). Several studies have demonstrated that the specific lysis of herpes simplex virus (HSV)-infected target cells by anti-HSV CTL also requires H-2 compatibility (21, 29, 34) and the expression of viral glycoproteins (5, 20). The involvement of HSV glycoproteins as the major viral antigenic determinants seen by CTL was suggested by studies which demonstrated that target cells infected with temperature-sensitive mutants of HSV which expressed reduced amounts of cell surface viral glycoproteins at the nonpermissive temperature and target cells treated with known inhibitors of glycosylation showed reduced susceptibility to HSV-specific CTL (5, 20). Furthermore, as has been shown for a number of viruses (48), infection with one serotype of HSV results in the generation of cross-reactive CTL capable of lysing syngeneic target cells infected with a distinct serotype of HSV (4, 11). This suggested that both type-specific and type-common viral determinants were recognized by HSV-specific CTL.

HSV is a complex virus that encodes at least six cell surface glycoproteins, designated gB, gC, gD, gE, gG, and gH (2, 3, 12, 31, 36). Thus, it has been difficult to determine which viral molecule(s) is recognized by anti-HSV CTL. Eberle et al. (11) used a gC-deficient strain of HSV type 1 (HSV-1) in studies which suggested that gC-1 was recognized by anti-HSV CTL as a type-specific antigen. Recently, these results have been confirmed and extended to anti-HSV CTL at the clonal level (13). More recently, Zarling et al. (45) demonstrated that human HSV-specific memory CTL clones could be generated following stimulation with purified cloned glycoproteins gB and gD of HSV-1. Furthermore, human CTL clones were able to lyse autologous targets infected with a recombinant vaccinia-gD-1 virus, demonstrating that gD-1 can serve as a target antigen for human anti-HSV memory CTL (46).

To approach the question of which HSV glycoproteins serve as targets for class I MHC-restricted anti-HSV CTL, we established murine cell lines which express individual glycoproteins by transfecting cells with the glycoprotein genes in conjunction with the neomycin resistance gene and isolating stably transformed cell lines. Our results demonstrate that syngeneic target cells expressing HSV-1 gC were

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lysed by virus-specific CTL, but targets expressing HSV-1 gB or gE or HSV-2 gD were not lysed by virus-specific CTL.

MATERIALS AND METHODS

Cells and viruses. Vero cells and Lta ($H\text{-}2^k$) cells were grown in alpha minimal essential medium (GIBCO Laboratories, Burlington, Ontario) supplemented with 10% fetal bovine serum (FBS) (GIBCO), penicillin-streptomycin, 0.3% glutamine, 0.075% $NaHCO_3$, and 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (complete minimal essential medium). Z4 cells (28) were grown in complete minimal essential medium supplemented with hypoxanthine, thymidine, and methotrexate (HAT) (37). Z4/6 cells (18), Lta/gB15, and E4/13 were grown in complete minimal essential medium containing HAT and G418 (100 μg/ml). HSV-1 strain KOS, HSV-1 strain F, HSV-1 strain MP (16), and HSV-2 strain 333 were propagated at low multiplicity and assayed by plaque production on Vero cell monolayers.

Construction of recombinant plasmids pSV2gBneo, pDC17, and pGE15neo. Restriction enzymes, T4 DNA polymerase, the Klenow fragment of *Escherichia coli* DNA polymerase, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Burlington, Ontario. The HSV-1 gB structural sequences from an *Xho*I site located approximately 38 base pairs (bp) downstream of the mRNA start site to a *Bam*HI site located approximately 475 bp downstream of the polyadenylation signal were excised from pVF30, which contains the *Bam*HI G fragment (map units 0.345 to 0.395) of HSV-1 (KOS) inserted into pUC19, and isolated from an agarose gel by electroelution. The *Xho*I site had been previously blunted with the Klenow fragment of DNA polymerase I before *Bam*HI digestion of pVF30. The purified fragment was ligated to the 3.5-kilobase (kb) *Hin*dIII-*Bam*HI fragment (the *Hin*dIII fragment was blunted with the Klenow fragment of *E. coli* polymerase) of pSV2gpt (23), and plasmid pSV2gB was isolated. A 3.5-kb *Nde*I-*Eco*RI fragment containing the bacterial aminoglycosyl-3'-phosphotransferase gene from transposon Tn*5* coupled to the simian virus 40 (SV40) early promoter from the plasmid pSV2neo (35) was similarly purified, so that the *Nde*I site was blunted with the Klenow fragment of *E. coli* polymerase, and inserted into the *Bam*HI and *Eco*RI sites of pSV2gB to generate plasmid pSV2gBneo.

The HSV-1 gE structural sequences extending from a *Bal*I site at residue 7677 of the U_S region of HSV-1 (KOS) to a *Kpn*I site at residue 12293 of the U_S region (22) was excised from pSG25 (kindly provided by M. Levine, University of Michigan) and inserted into the *Kpn*I and *Hin*cII sites of pUC19, yielding plasmid pGE15. pGE15 was cut with *Pst*I, and the 3' extension was removed with T4 DNA polymerase and subsequently cut with *Eco*RI. The 4.6-kb fragment containing the gE gene was ligated to a 4.6-kb fragment derived from pSV2neo (35) by cutting with *Bam*HI, blunting the ends with T4 DNA polymerase, and cutting with *Eco*RI, and plasmid pGE15neo was isolated.

The HSV-1 gC structural sequences extending from a *Pst*I site 650 bp upstream of the translation initiation site to a *Bam*HI site 320 bp downstream of the translation termination site were inserted into pUC9 to generate plasmid pDJ19. pDJ19 was cut with *Pst*I, treated with exonuclease Bal 31, and ligated to a kanamycin cassette derived from pSKS101. A number of plasmids with various deletions extending from the original *Pst*I site toward the gC translation initiation site were isolated, and one, pCB68, contained a deletion terminating at a *Pst*I site (derived from the kanamycin cassette) 10 bp upstream of the translation initiation site. The HSV-1 gD promoter sequences were fused to gC structural sequences by excising a *Sma*I-*Nco*I fragment containing gD promoter sequences from plasmid pSS17, which contains the *Bam*HI J fragment of HSV-1 cloned into pUC9, and ligating the fragment into the *Pst*I site of pCB68 by using *Pst*I linkers, generating plasmid pDC17.

Isolation of cells expressing HSV-1 gB, gC, or gE. Cells expressing HSV-1 gB were isolated by transfecting subconfluent 60-mm dishes containing Lta cells with 5 μg of pSV2gBneo per dish by the $CaPO_4$ precipitation technique (14), except that 4 h after the precipitate was added the cells were shocked with 15% glycerol for 45 s. Cells expressing HSV-1 gE were isolated by transfecting subconfluent monolayers of Z4 cells (28) in 60-mm dishes with 5 μg of pGE15neo per dish as described above. Cells expressing HSV-1 gC were isolated by transfecting Z4 cells with 5 μg of pDC17 and 0.5 μg of pSV2neo per dish. In all cases the cells in individual dishes were trypsinized 24 h after transfection into five 60-mm dishes containing medium with 400 μg of G418 per ml. The medium was changed every 3 to 4 days, and after 17 to 20 days individual colonies were trypsinized with cloning cylinders and expanded in medium containing G418 (400 μg/ml). The cell lines were screened by labeling monolayers with [^{35}S]methionine and immunoprecipitating gB, gC, or gE.

[^{35}S]methionine labeling of cells, immunoprecipitation, and gel electrophoresis. Monolayers of cells growing in 25-cm^2 plastic flasks were washed twice with medium 199 containing no methionine (199-met) and then incubated for 5 h with 1 ml of 199-met containing 25 to 100 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.). Cell extracts were made by using NP40-DOC extraction buffer (50 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1% Nonidet P-40 [NP40], 0.5% sodium deoxycholate [DOC], 1 mg of bovine serum albumin [BSA; Sigma Chemical Co., St. Louis, Mo.] per ml, 0.5 mM phenylmethylsulfonyl fluoride [Sigma], and 0.1 mg of aprotinin [Sigma] per ml). Extracts were sonicated, centrifuged at 100,000 × *g* for 1 h, and then immunoprecipitated with monoclonal antibody I-59 (27), which is specific for gB, or a mixture of monoclonal antibodies C1, C2, and C3 (17), which is specific for gC, by mixing 0.2 to 0.5 ml of the lysate with 2 to 5 μl of ascites fluid for 1 h at 4°C and adding protein A-Sepharose beads (Pharmacia Chemicals, Dorval, Quebec) for 1 h at 4°C. Immunoprecipitation of HSV-1 gE was carried out in an identical fashion except that 0.1% sodium dodecyl sulfate (SDS) and 50 mM NaCl were added to the extracts and they were heated to 55°C for 5 min and then cooled, and 2 μl of monoclonal antibody II-481 ascites fluid was added. The beads were washed twice in NP40-DOC extraction buffer and once in RIPA buffer (50 mM Tris hydrochloride [pH 7.2], 150 mM NaCl, 0.1% SDS, 1% DOC, 1% Triton X-100). The precipitated proteins were eluted from the beads by boiling in 2× sample buffer (100 mM Tris hydrochloride [pH 6.8], 4% SDS, 4% β-mercaptoethanol, 20% glycerol, and bromophenol blue). Samples were electrophoresed in 8.5% *N,N'*-diallyltartardiamide (DATD) cross-linked polyacrylamide gels as described by Heine et al. (15). Gels were infused with 2,5-diphenyloxazole, dried, and placed in contact with Kodak XAR film.

Immunofluorescence. Monolayers of Lta or Lta/gB15 cells infected with HSV-1 strain F (6 h after infection) growing on glass cover slips in 35-mm dishes were washed twice with phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.5 mM

KH_2PO_4, 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4), containing BSA (10 mg/ml) and then incubated with 1 ml of PBS containing 10 μl of I-59 (27) anti-gB ascites fluid at 4°C for 1 h. The cells were washed three times with PBS containing BSA, incubated with affinity-purified fluoresceinated rabbit anti-mouse immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.) at 4°C for 1 h, washed three times with PBS containing BSA, and fixed with 1.5% paraformaldehyde. The cover slips were mounted in 50% (vol/vol) glycerol–100 mM Tris hydrochloride, pH 8.0, and examined with a Zeiss fluorescence microscope.

Cell surface iodination. Monolayers of Z4 cells, Z4 cells infected for 7 h with HSV-1 (strain F), or E4/13 cells were labeled with $Na^{125}I$ by the procedure of Sefton et al. (33). Cells were subsequently washed once with PBS containing 1 mM NaI and twice with PBS and extracted with NP40-DOC extraction buffer, and the extracts were immunoprecipitated with anti-gE monoclonal antibody II-481 or anti-gD monoclonal antibody II-436.

Generation of CTL. CTL capable of specifically killing syngeneic HSV-1-infected cells were generated by the protocol of Pfizenmaier et al. (29). Briefly, 5- to 8-week-old CBA/J (*H-2*^{k}) mice (Jackson Laboratories, Bar Harbor, Maine) were immunized in the hind footpads with 10^5 PFU of HSV-1 (KOS) per footpad. Five days later, draining lymph nodes were excised, and a lymphocyte suspension was prepared by gently pressing the lymph nodes through stainless steel mesh. Viable cells were counted by trypan blue dye exclusion and suspended at 4×10^6/ml in RPMI 1640 medium (GIBCO) containing 10% heat-inactivated FBS (HyClone Laboratories, Logan, Utah), 2×10^{-5} M 2-mercaptoethanol, 20 mM HEPES buffer, glutamine, and penicillin-streptomycin; 2×10^7 lymphocytes were added to 60-mm^2 tissue culture dishes and incubated for 3 days at 37°C in 5% CO_2.

To duplicate the natural route of infection, anti-HSV-2 CTL were generated following intravaginal infection. Five- to 8-week-old female CBA/J mice were inoculated intravaginally with 10 μl of HSV-2 (strain 333) containing approximately 10^7 PFU. No fluid leakage from the vaginal orifice was observed. Six days later, draining lymph nodes were excised and lymphocyte suspensions were prepared. Lymphocytes were cultured in vitro for an additional 72 h as described above.

^{51}Cr release assay. Semiconfluent monolayers of target cells growing in 75-cm^2 culture flasks were either left uninfected or infected with HSV-1 (KOS) or HSV-2 (333) at 10 PFU/cell. After 1 h of virus adsorption, the cells were incubated in medium for an additional 4 to 5 h at 37°C. Flasks were then washed, and the cells were removed by gently scraping them into PBS without Ca^{2+} or Mg^{2+} to minimize damage to surface glycoproteins. The targets were then labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) for 90 min, washed, and counted. ^{51}Cr-labeled target cells were mixed with nonadherent effector lymphocytes at various effector-target cell ratios in 96-well flat-bottomed microtiter plates (Nunclon, Roskilde, Denmark) and incubated for 4 h. Percent specific ^{51}Cr release was calculated as described previously (42).

Cold target competition cytotoxicity assay. The cold target competition assays were performed essentially as above, except ^{51}Cr-labeled target cells were mixed with various ratios of unlabeled infected or uninfected competitor cells prior to the addition of lymphocyte effector cells. An effector-to-labeled target cell ratio of 100:1 was used in all cold target competition assays.

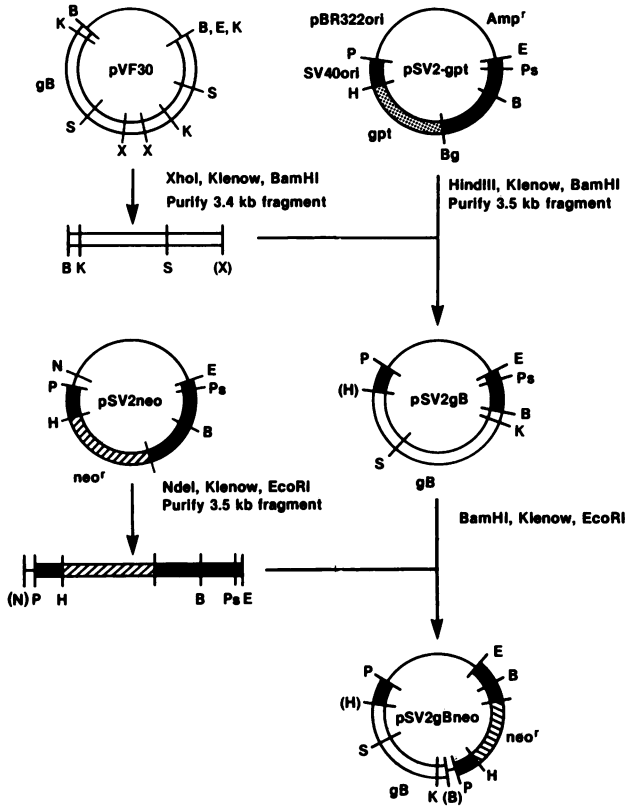

FIG. 1. Construction of recombinant plasmid pSV2gBneo. Plasmid pVF30 contains the *Bam*HI G fragment (map units 0.345 to 0.395) derived from HSV-1 (KOS) inserted into pUC19. A 3.4-kb *Xho*I-*Bam*HI fragment (the *Xho*I end was filled with the Klenow fragment of polymerase I) containing the gB structural sequences (open box) was purified from pVF30 and ligated to a 3.5-kb *Hin*dIII-*Bam*HI fragment derived from pSV2gpt (23) which contains the SV40 early promoter and polyadenylation site (solid boxes) to generate the plasmid pSV2gB. A 3.5-kb *Nde*I-*Bam*HI fragment (the *Nde*I end was filled with Klenow) containing the SV40 early promoter and the neomycin resistance gene (hatched box) was purified from pSV2neo (35) and ligated into the *Bam*HI and *Eco*RI sites of pSV2gB to generate plasmid pSV2gBneo. Abbreviations: K, *Kpn*I; B, *Bam*HI; E, *Eco*RI; S, *Sal*I; X, *Xho*I; P, *Pvu*II; H, *Hin*dIII; Bg, *Bgl*II; Ps, *Pst*I; N, *Nde*I. Parentheses indicate that the restriction site has been lost.

RESULTS

Construction of cell lines expressing HSV-2 gD and HSV-1 gB, gC, and gE. A mouse cell line, Z4/6, expressing HSV-2 gD has been described previously (18). Z4/6 cells synthesize higher levels of gD than infected parental Z4 cells, and the level of cell surface expression of gD in Z4/6 cells, as detected by iodination, was also higher than in infected Z4 cells (18). A high degree of variation in the level of expression of gD on the surface of individual Z4/6 cells was observed by surface immunofluorescence; however, all of the cells expressed detectable gD on their surfaces.

Cells expressing HSV-1 gB were isolated by transfecting Lta cells with the recombinant plasmid pSV2gBneo (Fig. 1), which contains the HSV-1 gB gene coupled to the SV40 early promoter and the Tn*5* neomycin resistance (Neo^r) gene coupled to the SV40 early promoter. Only a small fraction (less than 10%) of the G418-resistant cell lines isolated in three separate transfection experiments by using

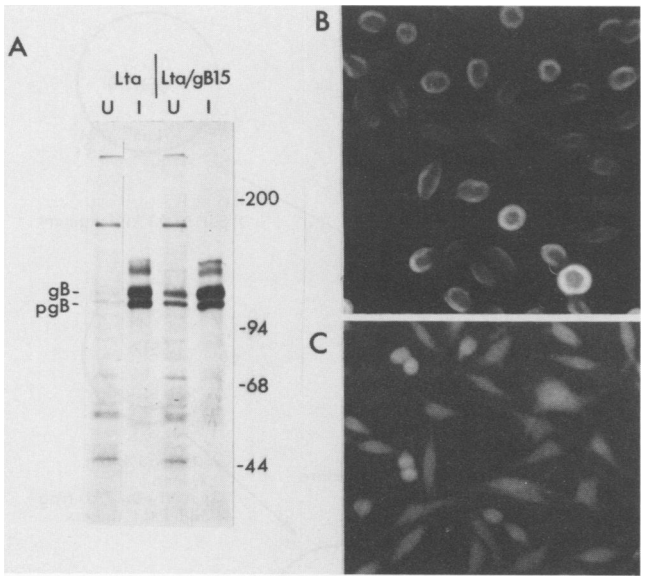

FIG. 2. Expression of gB in Lta/gB15 cells. (A) Lta and Lta/gB15 cells grown in 25-cm^2 flasks were either not infected (U) or infected with 5 PFU of HSV-1 per cell (I). Cells were labeled with [^{35}S]methionine for 5 h from 3 to 8 h after infection. HSV-1 gB was immunoprecipitated with I-59 anti-gB monoclonal antibody, and the precipitated protein was electrophoretically separated on 8.5% polyacrylamide gels cross-linked with DATD. The immature or precursor form of gB is indicated by pgB. The numbers indicate the position of marker polypetides (in kilodaltons). (B) Lta cells infected for 8 h with HSV-1 (5 PFU/cell) and (C) Lta/gB15 cells were stained with monoclonal antibody I-59 followed by fluoresceinated rabbit anti-mouse IgG at 4°C and then fixed with paraformaldehyde as described in Materials and Methods.

pSV2gBneo expressed detectable amounts of gB (results not shown). A G418-resistant cell transformant, Lta/gB15, able to express gB-1 was selected, and the level of expression of gB was compared with that in parental Lta cells infected with HSV-1 by immunoprecipitating gB with the monoclonal antibody I-59, specific for HSV-1 gB (27). Larger amounts of gB were immunoprecipitated from infected Lta and Lta/gB15 cells than uninfected Lta/gB15 cells (Fig. 2A). It was also found that the gB polypeptide turned over more quickly in the transformed Lta/gB15 cells than in infected cells (results not shown), as was the case with gD in Z4/6 cells (18).

The extent to which gB was expressed at the surface of transfected Lta/gB15 was determined by immunofluorescence. Unfixed cells were treated with monoclonal anti-gB antibody I-59 at 4°C, followed by fluoresceinated rabbit anti-mouse IgG. Although the level of gB expression, as judged by the degree of fluorescence, was greater on infected Lta cells (Fig. 2B), Lta/gB15 cells clearly demonstrated surface expression of gB-1 (Fig. 2C). In contrast to the patchy surface fluorescence of infected Lta cells, the surface fluorescence of Lta/gB15 cells was more uniform.

Cells expressing HSV-1 gE were isolated by transfecting Z4 cells (28), which express the viral immediate-early proteins ICP4 and ICP47, with pGE15neo, which includes the gE structural sequences and the neomycin resistance gene coupled to the SV40 early promoter (Fig. 3). Approximately 60% of the cell lines derived from transfections with pGE15neo expressed detectable amounts of gE-1, although the level of expression in these lines varied markedly.

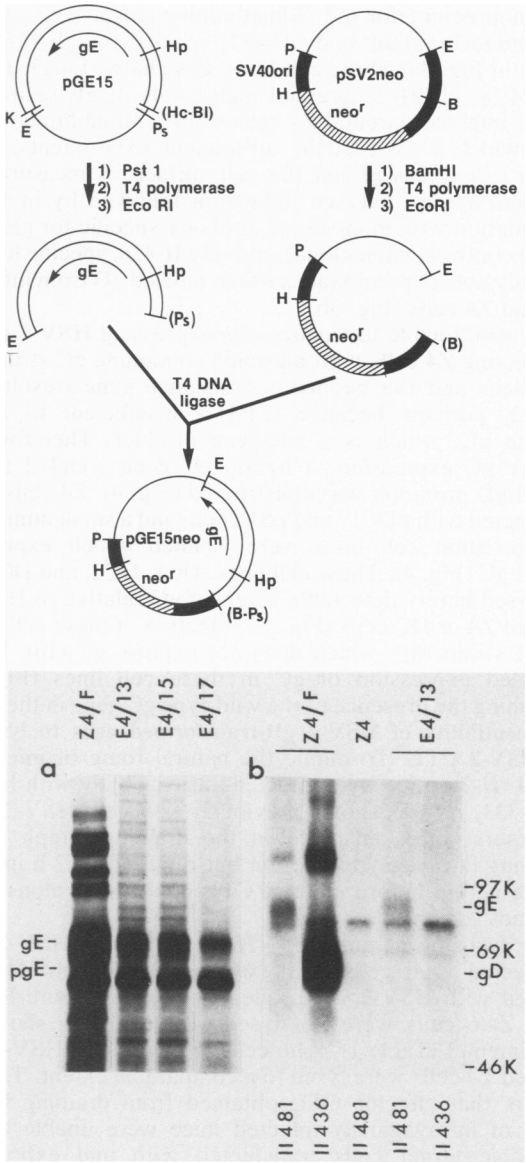

FIG. 3. Construction of recombinant plasmid pGE15neo and expression of HSV-1 gE in cells transformed with pGE15neo. (Top) Construction of pGE15neo containing the HSV-1 gE gene and the Neo^r gene. A *Bal*I-*Kpn*I fragment containing the gE structural sequences was inserted into pUC19 at the *Kpn*I and *Hin*cII sites, yielding pGE15. The gE structural sequences were excised by using *Pst*I (blunted with T4 polymerase) and *Eco*RI and inserted into the *Eco*RI and *Bam*HI (blunted with Klenow) sites of pSV2neo, yielding pGE15neo. (a and b) Expression of gE in E4/13, E4/17, and E4/18 cells. (a) Parental Z4 cells infected with HSV-1 strain F (Z4/F), E4/13, E4/17, and E4/18 cells were labeled for 5 h with [^{35}S]methionine, from 3 to 8 h after infection in the case of Z4/F. Detergent extracts of the cells were immunoprecipitated with monoclonal antibody II-481 for gE (27) and analyzed by polyacrylamide gel electrophoresis. (b) Z4 cells infected with HSV-1 (Z4/F), uninfected Z4 cells, and E4/13 cells were labeled by lactoperoxidase-catalyzed iodination, 7 h after infection in the case of Z4/F, and detergent extracts of the cells were immunoprecipitated with anti-gE monoclonal antibody II-481 or anti-gD monoclonal antibody II-436 (27). The immature form of gE, pgE, and glycoproteins gE and gD are indicated. Sizes are indicated in kilodaltons. Abbreviations: Hp, *Hpa*I; (Hc-Bl), junction of *Hin*cII and *Bal*I, sites lost; (B-P$_S$), junction of *Bam*HI and *Pst*I, sites lost; for other abbreviations, see the legend to Fig. 1.

Immunoprecipitation of [^{35}S]methionine-labeled cell extracts with monoclonal antibody II-481, specific for gE (27), are shown in Fig. 3a. Three cell lines, designated E4/13, E4/17, and E4/18, clearly expressed high levels of gE relative to HSV-1-infected parental Z4 cells (Z4/F). In addition, E4/13 cells, which were used in subsequent experiments, were shown to express gE on the cell surface as measured by lactoperoxidase-catalyzed iodination followed by immunoprecipitation with monoclonal antibody specific for gE (Fig. 3b). In contrast, monoclonal antibody II-436, specific for gD, was only able to precipitate surface-labeled gD from infected parental Z4 cells (Fig. 3b).

We were unable to isolate cells expressing HSV-1 gC by transfecting Z4 cells with plasmids containing gC structural sequences and the neomycin resistance gene (results not shown), perhaps because ICP4 is insufficient to *trans*-activate gC, which is a late-gene product. Therefore, to achieve gC expression, a hybrid gC gene coupled to the HSV-1 gD promoter was constructed (Fig. 4). Z4 cells were transfected with pDC17 and pSV2neo, and a small number of G418-resistant cell lines were isolated which expressed HSV-1 gC (Fig. 4). These cell lines, DC1, DC2, and DC3, all expressed barely detectable levels of gC relative to HSV-1-infected Z4 or DC cells (Fig. 4). Infection of these cells with HSV-1 strain MP, which does not express gC (16), led to increased expression of gC in these cell lines (Fig. 4), confirming the presence of the wild-type gC gene in the cells.

Susceptibility of HSV-2 gD-transformed cells to lysis by anti-HSV-2 CTL. To mimic the natural route of infection, CBA/J (*H-2^k*) mice were infected intravaginally with HSV-2 strain 333. As was shown previously (29), anti-HSV-2 CTL precursors were sensitized in the draining lymph nodes following intravaginal infection but required a 72-h in vitro culture period before effector CTL could be demonstrated (data not shown).

The lysis of parental Z4 (*H-2^k*) cells and HSV-2 gD-transfected Z4/6 cells was examined. Although Z4 cells infected with HSV-2 were killed by syngeneic anti-HSV-2 CTL, Z4/6 cells were not lysed (Table 1). As shown in experiment 3 (Table 1), Z4/6 cells infected with HSV-2 and infected Z4 cells were lysed to a comparable extent. Thus, it appears that effector CTL obtained from draining lymph nodes of intravaginally infected mice were unable to kill syngeneic target cells transfected with and expressing HSV-2 gD and that the presence of gD-2 does not affect the expression of appropriate anti-HSV CTL target structures generated after infection.

To verify that anti-HSV-2 CTL do not recognize HSV-2 gD, the ability of unlabeled Z4/6 cells to block the lysis of labeled HSV-2-infected Z4 cells was examined. Although adding excess unlabeled HSV-2-infected Z4 cells markedly inhibited the lysis of labeled infected Z4 cells, unlabeled Z4/6 cells were not able to inhibit lysis any more than uninfected parental competitor cells (Table 2). These results further suggest that gD-2 expressed on the surface of transfected cells is not recognized by anti-HSV-2 CTL.

Susceptibility of transformed target cells to anti-HSV CTL lysis. Anti-HSV-1 CTL were generated from draining lymph nodes after footpad infection of syngeneic mice. The ability of these lymphocytes to lyse Lta/gB15 cells (which express gB-1), DC2 cells (which express gC-1), or E4/13 cells (which express gE-1) was examined. Despite the ability of anti-HSV-1 CTL to kill HSV-1-infected Lta cells, Lta/gB15 cells were not lysed (Fig. 5). Similarly, although HSV-1-infected Lta or E4/13 cells were lysed to a comparable extent, uninfected E4/13 cells, which express gE, were not killed by syngeneic

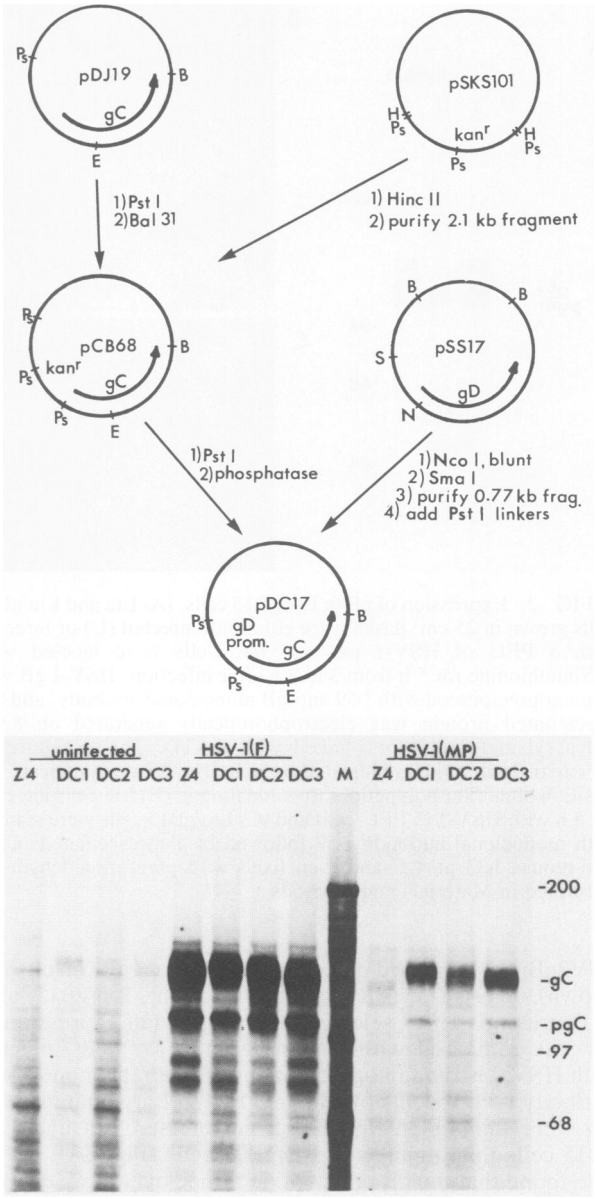

FIG. 4. Construction of plasmid pDC17, containing the HSV-1 gC gene coupled to the HSV-1 gD promoter, and expression of gC in cells transfected with the plasmid. (Top) Procedures used to construct pDC17. Plasmid pDJ19, which contains a *Pst*I-*Bam*HI fragment of HSV-1 (F) DNA cloned into pUC9, was cut with *Pst*I and digested with Bal 31 exonuclease to remove sequences upstream of the gC structural gene. A 2.1-kb fragment derived from pSKS101 and containing the kanamycin resistance gene was ligated to Bal 31-treated pDC19, which was used to transform bacterial cells to kanamycin resistance. One colony contained pCB68, which has a *Pst*I site (derived from the kanamycin resistance gene) approximately 10 bp upstream of the translation initiation site for gC. Plasmid pCB68 was cut with *Pst*I, treated with alkaline phosphatase, and ligated to a fragment containing the gD promoter, derived from pSS17, which has been ligated to *Pst*I linkers, producing plasmid pDC17. (Bottom) Expression of HSV-1 gC in cell lines derived by transfecting Z4 cells with pDC17. Cells were left uninfected or were infected with HSV-1 strain F or MP, which does not express gC, and then labeled with [^{35}S]methionine for 7 h (from 3 to 10 h postinfection). Extracts of the cells were immunoprecipitated with anti-gC monoclonal antibodies as described in Materials and Methods. Sizes are indicated in kilodaltons. See the legends to Fig. 1 and 3 for abbreviations.

TABLE 1. Lysis of HSV-2 gD-expressing Z4/6 target cells by CTL generated after intravaginal infection of syngeneic mice

Expt no.	Target cells	HSV-2 strain 333	Effector-target cell ratio	% Specific ^{51}Cr release[a]
1	Z4	−	40:1	6
			12:1	2
			4:1	1
	Z4	+	40:1	42
			12:1	25
			4:1	12
	Z4/6	−	40:1	2
			12:1	2
			4:1	1
2	Z4	−	40:1	4
			12:1	4
			4:1	2
	Z4	+	40:1	54
			12:1	30
			4:1	17
	Z4/6	−	40:1	3
			12:1	6
			4:1	6
3	Z4	−	40:1	1
			12:1	1
			4:1	1
	Z4	+	40:1	24
			12:1	15
			4:1	5
	Z4/6	−	40:1	2
			12:1	2
			4:1	1
	Z4/6	+	40:1	19
			12:1	10
			4:1	3

[a] Assay time, 4 h; spontaneous release from each target was <25%.

HSV-1-specific CTL (Fig. 6). Thus, cells transfected with and expressing HSV-1 gB or gE were not susceptible to lysis by anti-HSV-1 CTL.

In contrast, uninfected DC2 cells, which express very low levels of gC-1, were lysed as well as HSV-1-infected Z4 target cells (Fig. 7). Thus, gC-1 was recognized by class I MHC-restricted HSV-1-specific CTL. Interestingly, in agreement with the results of Eberle et al. (11), we found that Z4 cells infected with HSV-1 strain MP, which does not express gC, were effectively killed by anti-HSV CTL (Fig. 7). Infection of DC2 with either wild-type HSV-1 (KOS) or gC-deficient strain MP enhanced expression of gC (Fig. 4) yet did not markedly increase the susceptibility of these cells to lysis (Fig. 7).

TABLE 2. Cold target competition of HSV-2-specific CTL lysis by HSV-2 gD-expressing cells

Cold competitor	HSV-2 strain 333	Unlabeled-labeled target cell ratio	% Specific inhibition of lysis[a]
None	−		0
Z4	−	8:1	12
		4:1	23
	+	8:1	89
		4:1	78
Z4/6	−	8:1	23
		4:1	0

[a] Effector-labeled target cell ratio of 100:1; assay time, 4 h.

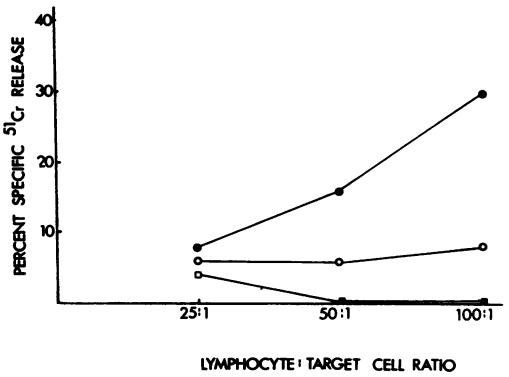

FIG. 5. Resistance of HSV-1 gB-expressing target cells to lysis by anti-HSV-1 CTL. HSV-specific CTL generated following footpad infection of syngeneic mice specifically lysed HSV-1 (KOS)-infected Lta cells (●) but did not lyse uninfected Lta cells (○) or Lta/gB15 cells (□).

Expression of H-2 antigens on transfected target cells. To demonstrate that the resistance of HSV-1 gB- and HSV-2 gD-transfected target cells was not due to altered H2 antigen expression, the relative level of H2 antigen expression was examined. Anti-H-2^k CTL were generated in a 5-day mixed lymphocyte culture and used as effector cells to determine the lytic susceptibility of the various infected and transfected targets used in these studies. Both infected and uninfected transfected target cells were lysed, as were infected and uninfected parental cells (Table 3). Thus, the inability of anti-HSV CTL to kill targets that express gB-1 or gD-2 cannot be attributed to the lack of decreased expression of H-2 antigens.

DISCUSSION

Cell lines expressing individual HSV glycoproteins were used to determine which viral molecule(s) specifies recognition by anti-HSV CTL. Our results demonstrate that cells transfected with the gC structural gene and expressing very low levels of HSV-1 gC were lysed as effectively as virus-infected target cells. In contrast, target cells expressing HSV-1 gB or gE or HSV-2 gD were not killed by syngeneic

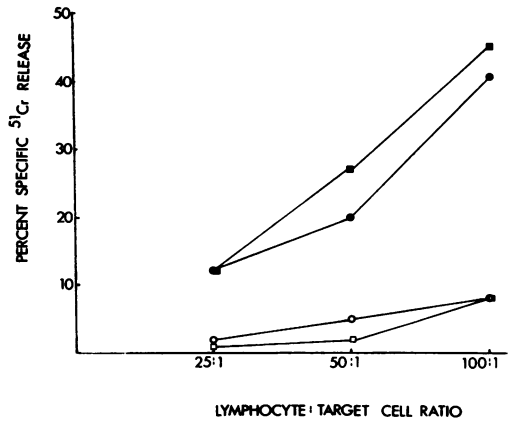

FIG. 6. Resistance of HSV-1 gE-expressing target cells to lysis by anti-HSV-1 CTL. HSV-specific CTL generated following footpad infection of syngeneic mice specifically lysed HSV-1 (KOS)-infected Z4 (●) or E4/13 (■) cells but did not lyse uninfected Z4 (○) or E4/13 (□) cells.

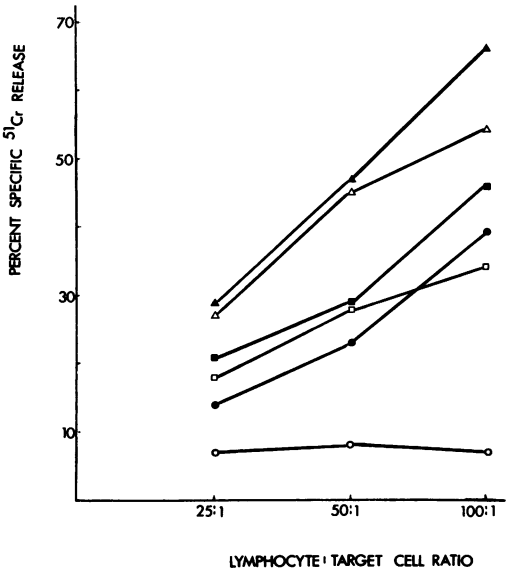

FIG. 7. Susceptibility of HSV-1 gC-expressing target cells to lysis by HSV-specific CTL. Anti-HSV CTL generated following footpad infection of syngeneic mice specifically lysed HSV-1 (KOS)-infected Z4 cells (●), uninfected DC2 cells (□), and DC2 cells infected with HSV-1 strain KOS (■) to a comparable extent. Similarly, DC2 cells infected with HSV-1 strain MP (▲) were lysed as well as Z4 cells infected with HSV-1 strain MP (△). In contrast, uninfected Z4 cells (○) were not lysed by HSV-specific CTL.

TABLE 3. Expression of H-2 antigens on various transfected and infected target cells as detected by alloantigen-specific CTL

Expt no.	Target cells	Infecting virus	Effector-target cell ratio	% Specific ^{51}Cr release[a]
1	Z4	None	40:1	51
			12:1	29
			4:1	15
		HSV-2 333	40:1	27
			12:1	16
			4:1	6
	Z4/6	None	40:1	59
			12:1	37
			4:1	23
		HSV-2 333	40:1	36
			12:1	27
			4:1	12
2	Lta	None	30:1	74
			15:1	61
			5:1	37
		HSV-2 333	30:1	57
			15:1	36
			5:1	20
	Lta/gB15	None	30:1	63
			15:1	40
			5:1	21
	Z4/6	None	30:1	54
			15:1	47
			5:1	25

[a] Assay time, 6 h; spontaneous release from each target was <25%.

CTL, and infection of these cells did not enhance lysis above the level seen in infected cells. We conclude from these results that class I MHC-restricted CTL generated from the draining lymph nodes of infected syngeneic mice recognize gC-1 but do not recognize HSV-1 gB or gE or HSV-2 gD as expressed on transfected mouse L cells.

Our results confirm and extend those of earlier studies (11, 13) concerning cell-mediated immunity to HSV, in which it was found that HSV-1 gC is recognized by anti-HSV CTL. Eberle et al. (11) used the gC-deficient HSV-1 strain MP in cold target inhibition assays and showed that MP-infected cells were less efficient in competing with restimulated anti-HSV CTL than were wild-type HSV-infected cells. More recently, Glorioso et al. (13) used gC-deficient variants of HSV-1 to show that targets lacking gC-1 were not killed by anti-HSV-1 memory CTL or CTL clones. In addition, most monoclonal-antibody-resistant (*mar*) mutations in gC-1, with one exception, did not affect CTL recognition of virus-infected cells.

We found, as did Eberle et al. (11), that target cells infected with HSV-1 strain MP, which does not express gC (16), were lysed by HSV-specific CTL. These results suggest that antigens other than gC-1 were recognized by HSV-specific CTL. However, Glorioso et al. (13) demonstrated that, although antigens other than gC-1 may be recognized, a majority of anti-HSV memory CTL were specific for gC-1. In light of our data, it is possible that a fragment of HSV-1 gC capable of serving as a CTL target is produced in strain MP-infected cells. Indeed, the inability of MP to produce gC is due to a frameshift mutation in the gC-1 coding sequence, which has been localized to a position 290 bp downstream of the initiator codon (10). Although no full-sized gC polypeptide has been detected in MP-infected cells, it is possible that a polypeptide bearing N-terminal amino acid sequences of gC is synthesized in MP-infected cells and can be recognized by anti-HSV CTL. Supporting this view, Draper et al. (10) detected two polypeptides produced by in vitro translation of MP mRNA which were precipitated by anti-gC antibodies. Furthermore, Townsend et al. (38) recently demonstrated that signal sequence-deleted hemagglutinin (HA) was recognized by anti-influenza virus CTL. Since signal-deleted HA was not expressed at the cell surface and was rapidly degraded in the cytosol, these results suggest that class I-restricted CTL recognized degraded forms of HA.

HSV gB, gC, gD, and gE are major structural antigens of the virus and are easily detected on the surface of infected cells. Each of these proteins is capable of inducing neutralizing antibodies (1, 6, 7, 9, 24, 26, 27, 30, 32). Therefore, it is somewhat surprising that of these glycoproteins, only gC-1 was recognized by murine anti-HSV CTL. Immunization of mice with purified HSV-1 gD (6, 19) or vaccinia virus recombinants that contain gD-1 (7, 25) resulted in the generation of neutralizing antibodies and protection against subsequent lethal challenge. Furthermore, synthetic peptides of HSV gD were shown to activate human T cells in vitro (8). Recent work by Zarling et al. (45) demonstrated that human HSV-specific memory CTL clones could be generated from the peripheral blood of seropositive individuals following culture with purified cloned gB-1 and gD-1. More recently, these investigators used recombinant vaccinia viruses that expressed gD-1 to show that human CTL clones generated with inactivated HSV-1 or purified cloned gD-1 lysed autologous cells expressing gD-1 (46). Furthermore, 98% of these clones were $T3^+$ $T4^+$ and appeared to be human leukocyte antigen class II restricted (45, 46), demonstrating that gD-1 serves as a target antigen for human anti-HSV-1 class II-restricted CTL.

The apparent discrepancy between the results reported by Zarling et al. (45, 46) and those reported here could simply be due to a species difference or a difference in the vectors used to introduce and achieve glycoprotein gene expression.

Alternatively, distinct viral molecules may serve as targets dependent on the MHC-restricting element. In support of this, Townsend et al. (39) have shown that CTL from different inbred strains of mice recognize distinct fragments of influenza A virus nucleoprotein (NP). Thus, although gD-1 may serve as the major viral target for class II-restricted human CTL, gC-1 was recognized by the murine class I-restricted CTL which were investigated here. Lastly, the discrepancy could be due to the fact that Zarling et al. (45, 46) examined clones of memory CTL that were restimulated in vitro with purified cloned gB-1 or gD-1 or recombinant vaccinia viruses. Thus, although these glycoproteins can serve as targets, we have no idea how representative gB-1- and gD-1-specific CTL clones are in a population of human anti-HSV CTL. In contrast, we have examined primary CTL derived from draining lymph nodes of infected mice. Clearly, the identity of the viral protein(s) which is recognized by the majority of HSV-specific CTL must await precise limiting dilution analysis with targets expressing specific viral proteins and will likely be dependent on the species and MHC-restricting element examined.

The inability of gB-1-, gE-1-, or gD-2-expressing target cells to be lysed by HSV-specific CTL can be interpreted in a number of ways. First, the level of cell surface expression of the HSV glycoproteins in the transformed cell lines may be insufficient to allow cytotoxic T cells to recognize and lyse the targets. Although the level of synthesis of these polypeptides in the transformed cells was much higher than in infected L cells, with the exception of gB synthesis in Lta/gB15 cells, the steady-state level of glycoproteins in the transformed cells was lower, possibly due to a rapid turnover rate. Additionally, the cell surface expression of gD-2, gB-1, and gE-1 was variable in most of the transformed cells. Although little is known about the amount of cell surface expression of a given polypeptide required for CTL recognition and lysis, recent studies by Townsend et al. (40) demonstrate that high levels of lysis of L cells transfected with the influenza virus NP gene occurred despite low levels of NP expression and the absence of detectable NP on the cell surface. Our results demonstrate that gC-expressing target cells were efficiently lysed despite lower levels of expression than in HSV-1-infected targets. Furthermore, cells which expressed high levels of HSV-1 gB following infection with a recombinant adenovirus vector which carries the HSV-1 gB structural sequences were not lysed by anti-HSV CTL (D. C. Johnson and K. L. Rosenthal, unpublished results).

Second, the distribution or topography of glycoprotein molecules at the surface of transformed cells may be different from that on infected cells. We observed a more uniform distribution of glycoprotein molecules on the surface of cells expressing gB (Fig. 3) and gE (results not shown) than on infected L cells. An altered distribution of viral cell surface molecules may lead to an inability to associate properly with H-2 molecules.

Third, the conformation of cell surface molecules may differ on cells expressing individual glycoproteins and infected cells due to an absence of other viral proteins that would normally associate with surface viral glycoprotein molecules. Epitopes recognized by anti-HSV CTL may be formed by the interaction of two or more glycoprotein molecules that interact to form a particular structure on the virion and infected-cell surfaces. Alternatively, the conformation of individual glycoproteins may be markedly affected by interaction with internal viral polypeptides.

Last, the inability of primary anti-HSV CTL to lyse syngeneic L cells transformed with HSV-1 gB or gE or HSV-2 gD may reflect a true inability of these molecules to serve as major targets for HSV-specific CTL.

Previous studies of anti-HSV cell-mediated immunity suggested that HSV glycoproteins function as the targets for CTL lysis (5, 20). We have conducted a systematic study of the role of individual glycoproteins as CTL targets. Although cell surface glycoproteins seem like obvious candidates for the target of CTL recognition, recent work by Townsend et al. (39–41) in the influenza virus system has demonstrated interesting and unexpected results. Notably, they used recombinant influenza A viruses to map recognition by CTL to the NP gene (41). They then went on to show that although L cells expressing influenza virus HA following DNA-mediated gene transfer became specific targets for a subpopulation of HA-specific CTL, the major population of cross-reactive CTL recognized L cells expressing NP (40). Similar results were obtained by Yewdell et al. (43) with recombinant vaccinia viruses as a means of expressing influenza virus NP. More recently, Yewdell et al. (44) used recombinant vaccinia viruses to demonstrate that although glycoprotein G of vesicular stomatitis virus (VSV) was recognized by VSV-specific CTL, the nucleocapsid (N) protein of VSV served as the major target antigen for cross-reactive anti-VSV CTL. Since neither influenza virus NP or VSV N is glycosylated and neither is considered a cell surface viral protein, a number of interesting questions arise about which viral proteins and in what form viral proteins are recognized by CTL. It is hoped that further studies on the viral target antigenic specificity of CTL by DNA-mediated gene transfer will contribute to our understanding of this vital issue.

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