Cytotoxic T lymphocytes specific for herpes simplex virus (HSV) studied using adenovirus vectors expressing HSV glycoproteins

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In previous work, we observed that H-2k-restricted herpes simplex virus (HSV)-specific cytotoxic T lymphocytes (CTLs) were effectively able to lyse transfected target cells expressing HSV glycoprotein C (gC), but not cells expressing gB, gD or gE. To confirm and extend our observations on the specificity of anti-HSV CTLs, recombinant adenovirus (Ad) vectors able to express HSV-1 gB or gC (AdgB2 or AdgC) were constructed. Syngeneic target cells infected with AdgB2 were efficiently lysed by primary H-2b and H-2d, but not by H-2k-restricted HSV-specific CTL. Limiting dilution studies indicated that 4 to 10% of H-2b-restricted HSV-specific CTLs recognize gB. H-2k, H-2b and H-2d-restricted anti-HSV-1 CTLs were unable to lyse AdgC-infected syngeneic target cells. To examine the apparent discrepancy between the previous results involving transfected H-2k cells expressing gC and the present results involving AdgC-infected cells, gC-expressing cell lines used in previous experiments were subcloned and retested in CTL assays. DC2 cells which were lysed by HSV-specific CTLs in the previous experiments remained sensitive to anti-HSV CTLs but two other clones derived from the same transfection were not lysed. Further, L cells transfected with the gC or gD gene coupled to the mouse mammary tumour virus promoter and capable of expressing high levels of the glycoproteins following dexamethasone induction were not lysed by H-2k-restricted anti-HSV CTLs. These results suggest that HSV-specific CTLs do not recognize gC, at least when it is expressed using an Ad vector and in most transfected cell lines, whereas a significant proportion of anti-viral CTLs recognize gB presented in some but not all murine haplotypes.

Introduction

Both helper and cytotoxic T lymphocytes (CTLs) are produced in response to infection with herpes simplex virus (HSV). T cell-mediated immunity and in particular CTLs play an important role in recovery from primary and secondary HSV infections (reviewed in Nash et al., 1985). It has been difficult to determine which viral molecules stimulate these protective T cell responses, primarily because HSV apparently encodes as many as 70 polypeptides, including at least seven membrane glycoproteins (McGeoch et al., 1988). Early studies, partly based on the assumption that cell surface molecules would act as T cell target antigens, suggested that HSV glycoproteins were major CTL targets (Lawman et al., 1980; Carter et al., 1981; Glorioso et al., 1985). Subsequent studies involving transfected cells have partially borne out this conclusion (Blacklaws et al., 1987; Rosenthal et al., 1987).

It is now clear that T lymphocytes recognize internal antigens as well as cell surface molecules (reviewed in Germain, 1988) and thus there is no compelling reason to believe that the HSV structural proteins are preferentially recognized by anti-viral CTLs. Supporting the case for recognition of herpesvirus non-structural polypeptides, an immediate early (IE) non-structural gene product of murine cytomegalovirus (CMV) is recognized by a majority of anti-viral CTLs (Reddehase & Koszinowski, 1984). However, many CMV structural polypeptides are not detected until days after infection (reviewed in Stinski, 1983), whereas HSV structural proteins appear within 2 to 3 h of infection. Recent studies involving drugs that block delayed early and late gene expression suggested that HSV IE gene products act as CTL target antigens (Martin et al., 1988), but it is difficult to exclude the possibility of low level expression of structural polypeptides in drug-treated cells. In this regard it should be noted that others have observed CTL lysis of transfected target cells expressing extremely low levels of viral antigens (Townsend et al., 1984, 1985).
One approach that has been used to define HSV polypeptides recognized by virus-specific CTL has been to construct transfected cell lines expressing individual HSV gene products. Our results using transfected cells suggested that primary H-2 k-restricted anti-HSV CTLs recognized syngeneic target cells expressing low levels of glycoprotein C (gC), but not cells expressing gB, gD or gE (Rosenthal et al., 1987). These conclusions were partly supported by similar observations involving an H-2 k cell line transfected with the HSV-1 gB gene that was lysed, albeit poorly, by restimulated anti-HSV CTLs and a cell line expressing gD that was not lysed (Blacklaws et al., 1987).

An alternative approach to the use of transfected cell lines involves the use of recombinant virus vectors. Recombinant vectors offer some advantages over cell lines in that they can be conveniently used to study CTL responses in mice of different haplotypes and in humans (Yewdell et al., 1985; Zarling et al., 1986; Volkmer et al., 1987; Whitton et al., 1988). Martin et al. (1987) used a recombinant vaccinia virus capable of expressing HSV-1 gD in experiments that demonstrated that syngeneic cells expressing gD were not lysed by HSV-specific CTLs derived from H-2 k, H-2 b and H-2 d mice. McLaughlin-Taylor et al. (1988) immunized BALB/c (H-2 k) mice with a recombinant vaccinia virus expressing HSV-1 gB and observed CTLs capable of lysing syngeneic HSV-infected target cells. From these studies they concluded that gB is a CTL target antigen. However, because the animals were immunized with the vaccinia virus vector expressing gB it is difficult to gauge the relative importance of gB in the immune response to HSV. In the present study the specificity of anti-HSV CTLs was examined using CTLs from HSV-infected mice of different haplotypes and target cells infected with recombinant adenovirus (Ad) vectors or transfected cells expressing HSV glycoproteins.

Methods

Viruses and cells. Wild-type Ad5, AdE3− (previously denoted dE3) (Haj-Ahmad & Graham, 1986), AdgB2 (Johnson et al., 1988) and AdgC were grown in HeLa or KB cells and titrated on 293 cells as described (Graham et al., 1978). The 293 cells (Graham et al., 1977) were maintained in Joklik's modified medium supplemented with 10% foetal bovine serum (FBS; Gibco). Vero cells were grown in alpha minimum essential medium (α-MEM) supplemented with 5% FBS. Mouse Lta cells (H-2 k), Z4 cells (H-2 k) (Persson et al., 1985), L-cgCl (H-2 k), L-gd1 (H-2 k), PAK (H-2 k), MC57 (H-2 b) and B10.D2 (H-2 b) cells were grown in α-MEM supplemented with 10% FBS. L-gc1 and L-gd1 were induced to express gc or gd by exposing the cultures overnight (15 to 20 h) to 10−6 M-dexamethasone (Sigma). HSV-1 strain F (obtained from P. G. Spear, Northwestern University, Chicago, Ill., U.S.A.) was propagated and titrated on Vero cell monolayers. Recombinant vaccinia viruses vyB11, which expresses HSV-1 gB (McLaughlin-Taylor et al., 1988) and VSC8, which expresses β-galactosidase (Chakrabarti et al., 1985), were obtained from Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, Md., U.S.A.) and were propagated on CVI cells.

Construction of plasmids. Restriction endonucleases and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories or Boehringer-Mannheim and were used according to the manufacturers’ recommendations. PviI linkers were purchased from the Molecular Biology and Biotechnology Institute at McMaster University. All plasmids were constructed by standard protocols (Maniatis et al., 1982).

Transfection of 293 cells and screening of recombinant viruses. Subconfluent monolayers of 293 cells were cotransfected with plasmid pFDGdc and Ad5 DNA which had been digested with EcoRI using the calcium technique (Graham & van der Eb, 1973). After 8 to 10 days plaques were picked and grown in 293 cells and virus DNA was characterized by restriction enzyme digestion and agarose gel electrophoresis to identify recombinant vectors. Candidate plaque isolates were twice plaque-purified and re-analysed before preparation of large-scale virus stocks.

51Cr Methylene labelling of cells, immunoprecipitation and gel electrophoresis. Cells growing in 35 mm or 60 mm dishes were infected with AdgB2, AdgC or AdE3− using 50 p.f.u. per cell, or with HSV-1 using 10 p.f.u. per cell and after 1 h of adsorption for HSV or 2 h for Ad recombinants, the virus inoculum was removed and α-MEM containing 5% FBS was added. Cells infected with HSV-1 were labelled with 51Cr methionine from 6 to 12 h post-infection and those infected with AdgB2 or AdgC were labelled from 24 to 30 h post-infection, as described previously (Johnson et al., 1988). Extracts of the cells were immunoprecipitated using a rabbit polyclonal serum (no. 67) specific for gB (Johnson et al., 1988), or a pool of monoclonal antibodies C1, C2 and C3, specific for GC (Holland et al., 1984), or a rabbit polyclonal serum specific for gD (Eisenberg et al., 1982) and Protein A-Sepharose as described (Johnson et al., 1988). Samples were electrophoresed in 8.5% N,N′-diallyltartardiamide-cross-linked polyacrylamide gels; the gels were gelled and exposed to Kodak XAR film as described (Johnson et al., 1988).

Generation of anti-HSV CTLs. CTLs capable of specifically lysing syngeneic HSV-1-infected target cells were generated by the protocol of Pfizenmaier et al. (1977) with modifications (Rosenthal et al., 1987). Briefly, 6 to 9 week old CBA/J (H-2 b), BALB/c (H-2 d) mice (Jackson Laboratories) were immunized in the hind footpads with 5×10 5 p.f.u. of HSV-1 strain F per footpad. Five days later draining lymph nodes were excised and a lymphocyte suspension was prepared by gently pressing lymph nodes through a stainless steel mesh. Viable cells were counted by trypan blue dye exclusion and suspended at 4×10 6 per ml in RPMI 1640 medium (Gibco) containing 10% heat-inactivated FBS, 2×10−6 M-2-mercaptoethanol, 20 mM-HEPES buffer, L-glutamine and penicillin-streptomycin. Lymphocytes (4×10 4) were added to 60 mm2 tissue culture dishes and incubated for 3 to 4 days at 37°C in 5% CO 2.

51 Chromium release assay. Subconfluent monolayers of target cells growing in 75 cm2 flasks or 100 mm dishes were either left uninfected or infected with Ad5, AdE3−, AdgB2 or AdgC at 50 p.f.u./cell or HSV-1 strain F and vaccinia viruses VSC8 and vyB11 at 10 p.f.u./cell. After 2 h of adsorption for Ad viruses or 1 h for HSV and vaccinia viruses, the cells were incubated in medium for various periods at 37°C; generally HSV and vaccinia virus infections proceeded for 6 h, whereas Ad infections were carried out for 24 h. Cells were then removed from dishes using phosphate-buffered saline containing EDTA (200 mg/ml), washed and labelled with Na2 31 CrO 4 (New England Nuclear) for 90 min, washed and counted. 31 Cr-labelled target cells were mixed with non-adherent effector lymphocytes at various effector-target ratios in
duplicate or triplicate using 96-well flat-bottomed microtitre plates (Nunclon) and incubated for 6 h. Percentage specific $^{51}$Cr release was calculated as previously described (Walker et al., 1985).

**Limited dilution analysis.** Responder lymphocytes derived from popliteal lymph nodes of C57BL/6 mice infected with HSV were used to establish microcultures in 96-well U-bottom microtitre dishes with 5 x $10^5$ X-ray-irradiated (2000 rads) syngeneic HSV-infected spleen cells, using replicates of 24 as previously described (Martin et al., 1988). The cells were grown for 6 days in 0.2 ml of RPMI complete medium containing 20% concanavalin A-derived mouse T cell growth factor prepared as described (Von Boehmer et al., 1979). The cytolytic activity was measured using $^{51}$Cr release assays. Microtitre wells considered positive in these assays were two standard deviations above the mean of controls containing no responder cells (Walker et al., 1985). The results of CTL assays were analysed using a computer program, LIMDIL1, provided by David A. Clark (McMaster University), which determines CTL precursor frequencies by the maximum likelihood method (Porter et al., 1973).

**Results**

**Construction of Ad vectors expressing gB and gC**

Previously we described the construction of an Ad recombinant, AdgB2, which induces high-level expression of HSV-1 gB in human and murine cells (Johnson et al., 1988). To investigate further the CTL response to HSV-1-infected cells we constructed a similar vector with HSV-1 sequences encoding gC inserted into the E3 region of Ad5. The gC gene, derived from HSV-1 strain F, in plasmid pCB68 (Rosenthal et al., 1987) was coupled to the SV40 early promoter and the hybrid SV40/gC gene was inserted into the unique XbaI site of plasmid pFGdX1 (Haj-Ahmad & Graham, 1986), which includes the rightward 40% of Ad5 minus a deletion of 1-9 kb within the E3 region (Fig. 1). Plasmid pFGdgC and EcoRI-digested Ad5 DNA were cotransfected into 293 cells. Plaques appeared after 8 to 10 days and viruses were screened for recombinants that included gC sequences. One such recombinant, denoted AdgC, was plaque-purified and used in subsequent experiments.

**Expression of gB and gC in recombinant Ad-infected cells**

Murine cell lines of different haplotypes, Z4 (H-2b), PAK (H-2b) and B10.D2 (H-2b) were infected with AdgB2, AdgC, or AdE3−, an Ad5 mutant lacking E3 sequences, and the levels of expression of gC or gB were examined. Cells infected with Ad vectors were labelled with $^{[35]}$S)methionine 24 h post-infection, although expression of the HSV-1 glycoproteins had not yet reached maximal levels (Johnson et al., 1988) because CTL assays were performed 24 h post-infection to minimize spontaneous release of $^{51}$Cr. AdgB2- and AdgC-infected cells expressed easily detectable amounts of gB and gC, although the levels of expression were often lower than with HSV-1-infected cells (Fig. 2). It should be noted that 10-fold more material immunoprecipitated from Ad vector-infected cell extracts than from HSV-infected cell extracts was loaded on the gels. Also shown in Fig. 2 is the expression of HSV-1 gC in a murine cell line, L-gC1 (Friedman et al., 1989) transfected with a plasmid containing the HSV-1 gC gene coupled to the inducible mouse mammary tumour virus (MMTV) promoter and the hygromycin resistance gene. L-gC1 cells express high levels of HSV-1 gC relative to HSV-infected cells after the MMTV promoter is induced with dexamethasone. A second cell line, L-gD1, was constructed in an identical fashion using the HSV-1 gD gene and also expresses high levels of gD after induction (Fig. 2).

**Susceptibility of AdgB-infected cells to anti-HSV CTL**

L (H-2b) cells, PAK (H-2b) cells or B10.D2 (H-2b) cells were infected with AdgB2 or AdE3−, then used as target cells in CTL assays with unstimulated H-2-compatible lymphocytes from HSV-1-infected CBA/J (H-2b), C57BL/6 (H-2b), or BALB/c (H-2b) mice. H-2b-restricted anti-HSV CTLs were unable to lyse AdgB2-infected L cells, confirming our previous observations involving transfected L cells expressing gB (Table 1). In contrast, H-2b- and H-2b-restricted CTLs efficiently lysed AdgB2-infected cells (Fig. 3, 4 and 5). The results with PAK (H-2b) cells were somewhat surprising in that the AdgB-infected cells were consistently lysed more efficiently than were HSV-infected cells (Fig. 3). In other experiments we used a second H-2b target cell line, MC57, which, after infection with AdgB2, expressed gB at levels similar to that observed with AdgB2-infected PAK cells.

**Table 1. Lysis of AdgB2- and AdgC-infected target cells by HSV-specific CTLs**

<table>
<thead>
<tr>
<th>Target cell*</th>
<th>Effector : target cell ratio</th>
<th>Specific $^{51}$Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lta-uninfected</td>
<td>40:1</td>
<td>11.4 ± 1.5</td>
</tr>
<tr>
<td>Lta-HSV-1</td>
<td>12:1</td>
<td>11.3 ± 0.6</td>
</tr>
<tr>
<td>Lta-AdE3−</td>
<td>40:1</td>
<td>47.3 ± 0.9</td>
</tr>
<tr>
<td>Lta-AdgB</td>
<td>12:1</td>
<td>31.8 ± 0.5</td>
</tr>
<tr>
<td>Lta-AdgC</td>
<td>40:1</td>
<td>13.5 ± 1.1</td>
</tr>
<tr>
<td>Lta-AdgB</td>
<td>12:1</td>
<td>14.6 ± 0.5</td>
</tr>
<tr>
<td>Lta-AdgC</td>
<td>12:1</td>
<td>10.6 ± 2.0</td>
</tr>
<tr>
<td>B10.D2-HSV-1</td>
<td>40:1</td>
<td>17.2 ± 1.4</td>
</tr>
<tr>
<td>B10.D2-HSV-1</td>
<td>12:1</td>
<td>43.3 ± 1.4</td>
</tr>
</tbody>
</table>
| * Target cells were infected with HSV-1 strain F at 10 p.f.u./cell for 6 h. Cells were infected with adenovirus recombinants at 50 p.f.u./cell for 24 h.
| † Assay was performed in triplicate. Spontaneous release was <24%.

* Target cells were infected with HSV-1 strain F at 10 p.f.u./cell for 6 h. Cells were infected with adenovirus recombinants at 50 p.f.u./cell for 24 h.
† Assay was performed in triplicate. Spontaneous release was <24%.
Fig. 1. Construction of AdgC, a recombinant Ad able to express HSV gC. The HSV-1 gC-coding sequences were excised from pCB68 (Rosenthal et al., 1987) using PstI, which cuts approximately 15 nucleotides upstream of the gC translation start site, and BamHI and coupled to the SV40 early promoter in plasmid pSV2XXX (derived from pSV2neo where the SV40 early promoter is delineated by PvuII and HindIII sites) using PstI linkers. The hybrid SV40-gC gene was excised from pSV2gC by partial digestion with XbaI and inserted into the unique XbaI site of pFGdX1, which contains the rightward 40% of Ad5 and a 1.9 kb deletion of E3 sequences (Haj-Ahmad & Graham, 1986), to generate plasmid pFGdgC. Ad5 DNA was digested with EcoRI and cotransfected with pFGdgC into 293 cells; after 8 to 10 days plaques appeared. Viruses were screened for recombinants containing gC sequences by restriction analysis. Restriction sites: BamHI (B), EcoRI (E), PstI (P), PvuII (Pv) and XbaI (X). AP refers to alkaline phosphatase treatment.

(results not shown). HSV-specific CTL efficiently lysed AdgB2-infected MC57 cells (Fig. 4), although lysis of AdgB2-infected MC57 cells never exceeded that observed with HSV-infected cells. The observed differences in lysis of two H-2b target cell lines each infected with AdgB2 highlights another of the potential difficulties in comparing CTL data where different cell lines have been used. MC57 cells infected with a recombinant vaccinia virus, vgB11, expressing HSV-1 gB (McLaugh-lin-Taylor et al., 1988) were also efficiently lysed by HSV-specific CTLs (Fig. 4).

Limiting dilution analysis of HSV-specific CTLs that recognize gB

The frequency of HSV-specific CTLs that recognize cells expressing gB was investigated using limiting dilution cultures of lymphocytes from HSV-infected C57BL/6 (H-
Fig. 2. Expression of gB, gC and gD in mouse cells infected with AdgB2 or AdgC, or in transfected cell lines. Z4 (H-2k), PAK (H-2b), or B10.D2 (H-2d) cells were left uninfected (lanes 1) or were infected with HSV-1 (lanes 2), AdE3+ (lanes 3), AdgB2 (lanes 4) or AdgC (lanes 5) then labelled with [35S]methionine from 3 to 9 h post-infection for HSV-infected cells, or from 24 to 30 h post-infection for Ad-infected cells. L-gC1 and L-gD1 cells were induced with 10⁻⁶ M-dexamethasone for 20 h, then labelled with [35S]methionine for 5 h. Extracts of the cells were mixed with a rabbit polyclonal serum (no. 67) specific for gB (left panel), a pool of monoclonal antibodies specific for gC (centre panel and first lane of right panel), or polyclonal serum specific for gD (second lane of right panel) and subsequently with Protein A-Sepharose. Immunoprecipitated proteins were separated by SDS-gel electrophoresis. One-tenth the amount of immunoprecipitated cell lysate from HSV-infected cells relative to that from Ad recombinant-infected cells was loaded on the gels. The 100K band observed in the L-gD lane is most probably a dimer of gD observed previously (Eisenberg et al., 1982; Gibson & Spear, 1983).

2b) mice. In these experiments we found it necessary to expand CTL cultures in the presence of viral antigen, in this case inactivated HSV-infected cells. The frequencies of HSV-specific CTL precursors varied markedly from experiment to experiment. However, in three representative experiments the frequencies of CTL precursors that recognize AdgB-infected cells represented 9-4%, 4-4% and 7-0% of the total HSV-specific response in each experiment (Table 2). In the third experiment target cells were infected with AdgB2 using 100 p.f.u. per cell instead of 50 p.f.u. per cell and the infection was allowed to proceed for 72 h instead of 24 h so that the level of gB expression was higher. Under these conditions the proportion of HSV-specific precursors able to lyse AdgB-infected cells (7-0%) was not dramatically different from that observed when cells were infected for a shorter time (9-4% and 4-4%).

CTL lysis of cells expressing gC

In previous experiments we observed that L cells transfected with the gC gene coupled to the HSV-1 gD promoter, DC2 cells, were lysed by H-2b-restricted anti-HSV CTLs (Rosenthal et al., 1987). Therefore, we expected that anti-HSV CTLs would recognize and lyse L cells infected with AdgC. However, AdgC-infected Lta cells were not lysed by H-2b-restricted HSV-specific CTLs at levels significantly greater than that observed

Fig. 3. Lysis of AdgB2- and AdgC-infected PAK (H-2b) cells by syngeneic HSV-specific CTLs. Draining lymph nodes from C57BL/6 (H-2b) mice infected with HSV-1 in the footpad were excised 5 days after infection and cells cultured in the absence of viral antigens for 4 days. Target cells consisting of HSV-1-infected PAK (H-2b) cells (O), AdgB2-infected PAK cells (C), PAK cells left uninfected (Δ), PAK cells infected with AdE3- (□) or AdgC (●), or B10.D2 (H-2d) cells infected with HSV (▲) were labelled with 51Cr and mixed with effector lymphocytes for 6 h. Specific 51Cr release was determined and error bars were calculated using one standard deviation.
with cells infected with AdE3- or left uninfected (Table 1). These observations raised important questions concerning the validity of our previous conclusion that gC acts as a target for H-2k-restricted CTLs. In order to address this issue we decided to re-examine the susceptibility of cell transformants used in earlier experiments. In these previous experiments three cell lines, DC1, DC2 and DC3, derived from a single transfection, all expressed similar but low levels of gC (Rosenthal et al., 1987). Detailed CTL experiments were carried out with only one of these, DC2. Unfortunately these cell lines had been stored in a frozen state and less than 1% of the cells survived the freezing and thawing process. Because of this low survival rate we chose to recharacterize clones derived from DC1, DC2 and DC3 cells. All of the cell clones expressed gC at low but detectable levels, comparable in amount to that previously observed (results not shown). A subclone derived from the DC2 cell line, which was lysed by anti-HSV CTL in the previous experiments, was recognized and killed by anti-HSV CTL in the present experiments (Fig. 6). However, the DC1 and DC3 subclones, which had been transfected with the same plasmid and expressed similar levels of gC, were not lysed above background.

The results described above suggested that the DC2 cell line might have been exceptional and not representative of interactions between gC-expressing transformants and HSV-specific CTLs. To investigate further
Table 2. Limiting dilution analysis of H-2b-restricted, HSV-specific CTLs that recognize AdgB-infected cells

<table>
<thead>
<tr>
<th>Experiment†</th>
<th>HSV-specific*</th>
<th>AdgB2-specific</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>frequency 1/n</td>
<td>(range, 95% confidence limits)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5400 (4000 to 7400)</td>
<td>58000 (39000 to 86000)</td>
</tr>
<tr>
<td>2</td>
<td>25000 (19000 to 32000)</td>
<td>580000 (360000 to 920000)</td>
</tr>
<tr>
<td>3</td>
<td>1300 (840 to 2000)</td>
<td>18000 (14000 to 24000)</td>
</tr>
</tbody>
</table>

* Lysis of uninfected or H2-mismatched HSV-infected target cells was not observed with the highest responder cell concentrations.
† Target cells were infected with HSV for 6 h using 10 p.f.u. per cell or AdgB2 for 24 h using 50 p.f.u. per cell, except in experiment 3 where cells were infected for 72 h with AdgB2 using 100 p.f.u. per cell.

Discussion

In a previous report we presented evidence that H-2k-restricted HSV-specific CTLs recognized and lysed transfected target cells expressing gC, but not gB, gD or gE (Rosenthal et al., 1987). Here we have attempted to confirm and extend these observations on the specificity of anti-HSV CTLs using recombinant Ad vectors able to express individual HSV glycoproteins. We utilized a previously described Ad vector, AdgB2, capable of expressing HSV-1 gB (Johnson et al., 1988) and constructed another recombinant Ad vector, AdgC, expressing HSV-1 gC. These vectors can infect a variety of human and murine cells (Johnson et al., 1988) and thus we were able to test CTL responses in mice of a number of different haplotypes. Preliminary results have also suggested that these vectors will be useful in studies of human anti-viral CTLs (C. Posavad & K. L. Rosenthal, unpublished results).

Our results demonstrate that HSV-1 gB expressed by AdgB2 acts as a target antigen for H-2b- and H-2d-restricted anti-HSV CTLs; however, in agreement with our earlier observations involving transfected cells, HSV-specific CTLs did not lyse H-2k target cells infected with AdgB2. Lysis of AdgB2-infected H-2b and H-2d target cells was often as efficient as lysis of HSV-infected target cells, suggesting that a substantial fraction of primary HSV-specific CTLs recognize gB. In the case of H-2b-restricted CTLs approximately 5 to 10% of HSV-specific CTL precursors, cultured under limiting dilution conditions, recognized and lysed AdgB2-infected target cells. This relatively small but significant fraction of HSV-specific CTLs able to recognize a single viral polypeptide probably reflects the large number of viral structural polypeptides expressed after infection by HSV. However, the nature of the assay system may overestimate the fraction of HSV-specific CTLs that recognize gB, especially because CTL precursors were restimulated with u.v.-inactivated, HSV-infected cells.

We recently reported that BALB/c mice inoculated with AdgB2 are protected against lethal challenge with HSV (McDermott et al., 1989). This observation, coupled with the finding that BALB/c mice infected with HSV mount a strong anti-gB CTL response, supports the hypothesis that anti-gB CTLs are protective in vivo. Cell transfer experiments are under way to investigate the cell types involved in protective immunity to HSV.

In contrast to the results with AdgB2, target cells infected with AdgC were not lysed by H-2k-, H-2b-, and H-2d-restricted HSV-specific CTLs. These results, especially those in the H-2k system, were surprising in light of our previous observations with transfected cell expressing gC. Much of the previous work had been carried out
using DC2 cells, which were efficiently lysed by anti-HSV CTLs (Rosenthal et al., 1987). In the present experiments daughter cells derived from DC2 cells remained sensitive to anti-HSV CTLs. In contrast, sister cell clones DC1 and DC3, derived during transfections with the same plasmid and expressing similar quantities of gC, were not lysed. In addition, L-gC1 cells, which express high levels of gC after induction of the transfected gene were not lysed.

At the present time we do not understand the basis for this anomaly and are investigating it further. One interpretation of these observations is that there are clonal differences in the processing or presentation of gC among the cell lines. Preliminary experiments in which vesicular stomatitis virus-specific CTLs were unable to lyse DC2 cells support the hypothesis that lysis of DC2 cells is HSV-specific (L. Witmer & P. Joshua, unpublished results). However, we have been unable to confirm that the response is directed to gC rather than ICP4, thymidine kinase or ICP47, which are all expressed by the parental cell line Z4 (Persson et al., 1985) and presumably also by these cells. These observations serve to highlight potential problems which may be encountered when CTL responses are measured using transfected cell lines. Variability among cell clones, potentially involving antigen processing or presentation pathways, may influence the CTL response to individual viral antigens. This variability may explain our negative findings with H-2k cells expressing gB, gD and gE, but H-2k cells infected with virus vectors expressing gB (this study) and gD (Martin et al., 1987) were also not lysed by HSV-specific CTLs.

The observations involving recombinant virus vectors must also be considered in the context of recent information concerning large DNA viruses commonly used as viral vectors. Evidence is accumulating that such viruses may encode gene products that actively inhibit CTL responses to viral antigens. For example, certain viral polypeptides are not recognized by CTLs when expressed late in the infectious cycle of poxviruses, but are recognized when expressed early, suggesting that vaccinia virus may interfere with T cell antigen processing or presentation (Coupar et al., 1986; Townsend et al., 1988). Further, a 19K Ad glycoprotein encoded in the E3 region can inhibit cell surface expression of MHC gene products (Burgert & Kvist, 1985). In addition, an E3-encoded polypeptide inhibits tumour necrosis factor-induced cytolysis (Goody et al., 1988), a process analogous to CTL-induced cell lysis (Liu et al., 1987). However, it should be noted that Ad E3 sequences encoding these polypeptides have been deleted in AdgB2 and AdgC; nonetheless, it is possible that other Ad polypeptides may inhibit antigen presentation or CTL-induced cell lysis. The observation that AdgB2-infected cells were efficiently lysed at least by H-2b- and H-2d-restricted CTLs suggests that Ad are not inducing generalized defects in antigen presentation, but it is clear that the interpretation of CTL experiments using virus vectors must take into account possible effects of the vector on antigen processing and presentation pathways.

In interpreting studies from different laboratories on HSV-specific CTLs one must also take into account that the specificity and magnitude of the response to individual viral antigens can depend on the strain of mouse used and the conditions used to generate HSV-specific CTLs. In our studies, bulk lymph node cell cultures were incubated for 3 to 4 days without addition of viral antigen, as originally described by Pfenamaier et al. (1977). Using these primary CTLs, which often lyse HSV-infected target cells less efficiently than restimulated CTLs, we were unable to detect lysis of transfected H-2k cells expressing gB or of H-2k cells infected with AdgB2. Blacklaws et al. (1987) observed low but significant levels of HSV-specific CTL lysis of an H-2k cell transformant expressing gB. However, they and others have stimulated CTL activity by culturing spleen cells in the presence of viral antigen (Blacklaws et al., 1987; Martin et al., 1987), which could potentially lead to selective expansion of CTL clones, especially those directed at structural polypeptides. Furthermore, we have observed that cells expressing HSV-1 gD (Fig. 6) or HSV-2 gD (Rosenthal et al., 1987) are not lysed by primary H-2k-restricted anti-HSV CTLs and others have made similar observations involving HSV-1 gD, where CTLs were restimulated using u.v.-inactivated HSV (Blacklaws et al., 1987; Martin et al., 1987). Recent unpublished results demonstrated that HSV-specific CTLs cultured in the presence of infectious HSV were able to lyse transfected L cells expressing gD-1 (R. M. Johnson, D. W. Lanki, F. W. Fitch and P. G. Spear; Abstracts, 13th International Herpesvirus Workshop). One might conclude that in vivo culture conditions affect the ability of HSV-specific CTL to recognize cells expressing gD.

In conclusion, we have identified an HSV membrane glycoprotein, gB, which is recognized by a significant fraction of HSV-specific CTLs. Given that HSV encodes over 65 polypeptides the task of identifying all of the major CTL target antigens using transfected cell or virus vectors will most probably be massive. However, these results should aid studies aimed at understanding the role of HSV-specific CTLs during the infection.

Recently, it was found that cells infected with an Ad vector carrying a mutant gC gene lacking signal recognition sequences were not lysed by HSV-specific CTLs (T. Hanke & D. C. Johnson, unpublished).
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