Herpes simplex virus type 1 glycoproteins gB, gC and gD are major targets for CD4 T-lymphocyte cytotoxicity in HLA-DR expressing human epidermal keratinocytes

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T lymphocytes are the main mediators of the protective immune response in recurrent herpes simplex. Early in the development of recurrent lesions, macrophages and CD4 T lymphocytes predominate in the mononuclear infiltrate surrounding infected epidermal cells. Human epidermal keratinocytes allow herpes simplex virus type 1 (HSV-1) replication and human leukocyte antigen (HLA)-DR is strongly expressed in vivo. In vitro, their pre-treatment with IFN-γ induced HLA-DR expression and partially reversed major histocompatibility complex class I down-regulation by the virus. Mononuclear cell cytotoxicity for these cells was mediated predominantly by CD4 and also by CD8 T cells. Late HSV-1 proteins were the major targets for CD4 CTL, while CD8 CTL predominantly targeted early HSV-1 proteins. Here it is shown that both mononuclear and CD4 CTL consistently recognized the major HSV-1 glycoproteins, gB, gC, gD and gH, using IFN-γ-pretreated keratinocytes infected with vaccinia virus–HSV glycoprotein recombinants (VvgB, VvgC, VvgD or VvgH). CD4 cytotoxicity was highest for VvgD-infected keratinocytes, followed by VvgB or VvgC and then VvgH in seven patients. CD4 CTL from two of 13 patients also recognized an epitope in the HSV tegument protein VP16, demonstrated by comparing cytotoxicity for the partial deletion mutants RP3 or RP4 and the parental RP1 HSV strain. In summary, the major HSV glycoproteins gB, gC and gD were consistently the major targets for CD4 CTL in VvgB-, VvgC-, VvgD- and VvgH-infected, IFN-γ-pretreated human epidermal keratinocytes in vitro.

Introduction

The immune control of recurrences of human herpes simplex infection appears to be mainly mediated by T lymphocytes and macrophages. CD4 lymphocytes and macrophages are the predominant infiltrating cells in the early stages of the lesions as determined by immunohistology and direct cloning of T lymphocytes from the lesions (Cunningham et al., 1985; Koelle et al., 1994a, b). B lymphocytes are scarce in the mononuclear infiltrates in recurrent herpes lesions and no correlation has been established between the herpes simplex virus (HSV) antibody titres or neutralizing antibody titres and frequency of recurrence (Reeves et al., 1981). However, high levels of pre-existing neutralizing antibody may still play a role in the prevention of spread of HSV, especially in the prevention of viraemia.

The persistence of herpes lesions in AIDS and transplant patients where the immune defect is predominantly, but not solely, in CD4 T lymphocytes and macrophages also suggests a major role for these cells (Siegal et al., 1981; Fauci, 1988). The presence of high levels of IFN-γ in vesicle fluid and the correlation of titres of IFN-γ in vesicle fluid or that secreted by blood T cells with the frequency of recurrence also supported the importance of CD4 cells in the control of recurrent herpes simplex lesions (Cunningham & Merigan, 1983, 1984; Torseth & Merigan, 1986). High titres of IFN-α and IFN-β in vesicular fluid, probably released from epidermal cells and/or macrophages, also suggest an important role of these cytokines in the prevention of spread, especially in the early stages before re-stimulation of immune T cells (Overall et al., 1981). Another function of IFN-γ within the lesions is the induction of major histocompatibility complex (MHC) class II antigens [human leukocyte antigens (HLA)-DR, not -DQ] on the surface of epidermal cells within the first 2 days of the appearance of lesions (Cunningham et al., 1985; Cunningham & Noble, 1989).
Hence, the early infiltrating CD4 T lymphocytes may exert immune control through the secretion of cytokines such as IFN-γ or possibly through CD4 T-lymphocyte cytotoxicity. Although the latter is controversial, there are two reasons to consider that CD4 T-lymphocyte cytotoxicity may be important in recurrent herpes simplex lesions. The first is the timing of events within the lesions. The initial infiltration of CD4 T lymphocytes correlates with the reduction of virus titres within vesicle fluid, whereas on average the later infiltration of CD8 T lymphocytes occurs after 2 days when vesicle fluid titres of virus have already declined (Cunningham et al., 1985; Spruance et al., 1977). Secondly, although it has been known for many years that human CD8 T lymphocytes exert very weak cytotoxicity against HSV-infected fibroblasts (Posavac & Rosenthal, 1992; Posavac et al., 1993; Koelle et al., 1993), only recently has the mechanism been established. HSV proteins, including ICP47, complex with the translocator protein associated with antigen presentation (TAP) preventing the reconstitution of MHC I with antigenic peptides and leading to trapping of MHC class I in the endoplasmic reticulum (York et al., 1994; Fruh et al., 1995; Hill et al., 1995). However, we have recently demonstrated that preincubation of keratinocytes with IFN-γ partially reverses this effect on HSV-infected keratinocytes and that MHC class II antigen expression was unaffected by HSV infection, enabling interaction between CD4 T lymphocytes and epidermal cells (Mikloska et al., 1996). Previously, we have demonstrated that T lymphocytes re-stimulated in vitro from the blood of HSV-immune patients are cytotoxic for HSV-infected keratinocytes which have been stimulated to express HLA-DR by IFN-γ pretreatment (Cunningham & Noble, 1989; Mikloska et al., 1996). CD4 T-lymphocyte cytotoxicity was always greater than CD8 T-lymphocyte cytotoxicity despite the partial restoration of MHC class I antigen expression on the surface of these cells. The predominant targets of CD4 T-lymphocyte cytotoxicity were late viral proteins (Mikloska et al., 1996). Here we have further defined the targets for CD4 T-lymphocyte cytotoxicity using a battery of vaccinia virus–HSV glycoprotein recombinants expressing the major HSV glycoproteins, gB, gC, gD and gH. We have also examined cytotoxicity for one of the two major tegument proteins, VP16, which is also a late protein (Ward & Roizman, 1994). These studies are highly relevant to the current trials of recombinant glycoproteins as candidate HSV vaccines for primary prophylaxis and therapeutic efficacy.

Methods

Patients and epidermal cultures. The tissues were obtained from HSV-1 seropositive patients undergoing split skin grafting for surgical procedures only after informed consent and under approval from the Western Sydney Area Health Service Research and Ethics Committee. Immediately after the operation, skin specimens were aseptically transferred into minimum essential medium (MEM) (Gibco) containing Earle’s salt and other ingredients as described previously (Cunningham & Noble, 1989; Mikloska et al., 1996) and epidermis was isolated as described. One to two months after recovery 60–100 ml of heparinized blood was drawn for isolation of peripheral blood mononuclear cells (PBMC).

The skin cultures were grown in growth medium (MEM) as described previously (Cunningham & Noble, 1989). Viability was determined using trypan blue exclusion and the viable cells were subcultured in 96-well flat-bottom plates (Nunc) at a density of 10⁶ cells per well in growth medium for 18 h at 37 °C in 5% CO₂ prior to the addition of IFN-γ and/or infection with HSV-1. The virus used were the RP1 KOS strain, RP2, RP3, or RP4 VP16 deletion mutant viruses of HSV KOS, wild-type vaccinia virus or vaccinia virus–HSV glycoprotein recombinants. For immunofluorescence experiments the explants or MRC-5 were grown in four-well tissue culture chambers (Nunc) to confluency.

HSV, HSV antigen and vaccinia virus–HSV glycoprotein recombinants. The wild-type HSV strains used in all cytotoxicity experiments were the F strain and a low passage clinical isolate of HSV-1 (WM2) (Cunningham & Noble, 1989). The RP1 KOS strain (which is HSV-1 KOS, with a BamHI site in the 3' region of the VP16 sequence) and the derived VP16 mutants RP2 (RP1, with the VP16 gene from HSV-2), RP3 (RP1, with deletion of amino acids 456–490) and RP4 (RP1, with deletion of amino acids 413–490) were all kindly provided by S. Trienzeben (Michigan State University, USA) and constructed in his laboratory by Rath Pichyangkura (Cress & Triezenberg, 1990; Weinheimer et al., 1992). These strains were used to infect human keratinocyte cultures. HSV antigen was prepared by UV inactivation of supernatants of HSV-1-infected HeP-2 cells and therefore includes both viral (structural) and non-structural (infectious) cellular proteins. Mock (control) antigen was produced by omitting HSV infection.

WR wild-strain vaccinia virus was used as a control and was kindly provided by I. Ramshaw (Australian National University, Canberra). Vaccinia recombinants VvgB (expressing HSV glycoprotein B) (Cantin et al., 1987), VvgC5 (Martin et al., 1993) and VvgD13 were kindly donated by B. Rouse (University of Tennessee, Knoxville, USA) and VvgH (Forrester et al., 1991) by A. Minson (University of Cambridge, UK) who also provided monoclonal antibody to gH. VvgC and VvgD were under the control of early promoters, VvgB was under late promoter control and VvgH was under early and late promoter control. WR strain vaccinia virus and vaccinia virus–HSV recombinants were grown in CV-1 cells. The same cells were used for quantification of vaccinia virus titre by the plaque assay.

Infection of keratinocytes with viruses and quantification of HSV protein expression by immunofluorescence. Keratinocytes and HEF monolayers were infected with the WM2 clinical strain or the RP1 strain or the RP2, RP3 or RP4 VP16 mutant viruses at 10 p.f.u. per cell of HSV-1, also with 15 p.f.u. per cell of vaccinia virus–HSV glycoprotein recombinants or wild-strain vaccinia virus in 96-well tissue culture plates (for cytotoxicity experiments) or four-well tissue culture chambers (for immunofluorescence staining). For cytotoxicity experiments cells were incubated with the virus for 1 h at 37 °C and then the growth medium was added. For immunofluorescence staining, keratinocytes infected with HSV VP16 deletion mutants or RP1 control virus for 24 h were fixed with methanol and incubated for 1 h with monoclonal antibody to gD1 (Cymbus). After double washing, cells were labelled with FITC-conjugated Fab'1 rabbit anti-mouse immunoglobulin (Dakopatts). The proportion and the intensity of gD staining within cells were estimated (see below). The expression of gB, gC, gD and gH by keratinocytes infected with vaccinia virus–HSV glycoprotein recombinants was checked in a kinetic study. Keratinocytes grown in tissue culture chambers were pretreated with 100 IU/ml IFN-γ for 4 days, infected with VvgB, VvgC, VvgD or VvgH at an m.o.i. of 15 p.f.u. per cell for 1 h,
washed as for a cytotoxicity assay and fixed at 0, 6, 8, 10, 12, 14, 18 and 24 h after infection. The slides were incubated with antibody for FITC-labelled anti-gC1 (Syva Microtrak), unlabelled anti-gB1 (Chemicon), unlabelled anti-gD1 (Cymbus) and unlabelled anti-gH at saturating concentrations. Rabbit FITC-labelled F(ab')2 to mouse immunoglobulin was then incubated with cells for a further 30 min. Controls using irrelevant monoclonal antibodies and uninfected keratinocytes were included in each experiment. One hundred cells were counted on each of 10 randomly chosen fields of the infected monolayer. The number of positive (infected) cells was recorded and the mean and the SE for control and IFN-γ-pretreated cells were calculated. The overall intensity of staining was estimated semi-quantitatively on a scale of 1 to 4 in comparison with keratinocytes infected with HSV at an m.o.i. of 10 p.f.u. per cell (4+ intensity).

**PBMC and lymphocyte subset preparation.** Approximately 1 month after initiation of the keratinocyte cultures, autologous blood was drawn. PBMC were isolated from heparinized blood samples over Ficoll–Hypaque density gradients. After 6 days of incubation with HSV antigen, PBMC were depleted from CD4 and natural killer (NK) cell (PBMC-CD8 cells) and CD4 T-lymphocyte (PBMC-CD8-NK cells) subsets using murine monoclonal antibodies and complement-dependent cytotoxicity as previously described (Cunningham & Noble, 1989). The number of CD4 lymphocytes increased by 30–40% due to the 6 day stimulation with HSV antigens whereas CD8 cells remained the same or declined slightly. Monoclonal antibodies to CD4 (anti-human Leu 3a and 3b), CD8 (anti-human Leu 2a) and NK cells (anti-human Leu 16) were obtained from Becton Dickinson. Residual contaminating cells in samples of PBMC and lymphocyte subsets were quantified by indirect immunofluorescence with OKT4 and OKT8 (Orthoimmune), respectively, and flow cytometry as described previously (Cunningham & Noble, 1989) and found to be < 5% (data not shown).

**Cytotoxicity assays.** Keratinocytes were subcultured in 96-well flat-bottom plates (as described above) and pretreated for 4 days with growth medium or growth medium containing 100 IU/ml of recombinant IFN-γ (Boehringer Mannheim). The cells were infected with HSV-1 as described previously (Cunningham & Noble, 1989; Mikloška et al., 1996), using RP1, RP2, RP3 or RP4 mutant viruses at 10 p.f.u. per cell, or with vaccinia virus control or vaccinia virus–HSV glycoprotein recombinants at an m.o.i. of 15 p.f.u. per cell for 1 h at 37 °C, washed twice with Hank’s balanced salt solution and incubated with 5 µCi per well of sterile ³¹Cr sodium solution (Amersham) for 90 min. In control wells virus was omitted. To determine the dependence of lysis on HLA-DR expression after infection, some keratinocytes were incubated with 5 µg/ml of anti HLA-DR antibody (Becton Dickinson) for 2 h. After washing and subsequent incubation with 5 µCi per well of sterile ³¹Cr for 90 min they were mixed with CD4 lymphocytes in an effector to target ratio of 25:1, 50:1, 80:1 and 100:1. Anti-HLA-DR antibody was maintained at 5 µg/ml for the duration of the 18 h incubation.

Mononuclear cells were stimulated with control or HSV-1 antigen for 6 days. On the day of experiment, cells were depleted of CD8 and/or CD16 cell subsets as described above, and were incubated (with targets at an effector to target ratio of 80:1) for 18 h, which was found to be optimal (for HSV-1, RP1–RP4 and also vaccinia virus control/vaccinia virus–HSV recombinants) in past and further preliminary experiments (see Results and Table 3) (Cunningham & Noble, 1989; Mikloška et al., 1996).

Supernatant radioactivity was quantified in a gamma-radiation counter (Beckmann) and the percentage of specific cytotoxic activity was determined by the following equation: % specific ³¹Cr release = [(mean experimental c.p.m. – mean spontaneous c.p.m.)/(mean maximal c.p.m. – mean spontaneous c.p.m.]) × 100.

Some of the cytotoxicity results were expressed as the mean net specific lysis: mean net specific lysis = (mean specific lysis for vaccinia virus–HSV glycoprotein recombinant) – (mean specific lysis for vaccinia virus control), where mean refers to the means of replicate experiments.

**Statistics.** Standard errors of experimental c.p.m. (triplicates and quadruplicates) were usually less than 10%. Differences between the percentage of specific ³¹Cr release obtained with different treatments were assessed for statistical significance by Student’s t-test with modification of the degrees of freedom to allow for unequal variances (Cunningham & Noble, 1989).

### Results

**Expression of HSV glycoproteins in keratinocytes infected with vaccinia virus–HSV glycoprotein recombinants**

The proportion and the intensity of gB, gC, gD and gH glycoprotein staining in infected cells at an m.o.i. of 15 p.f.u. per cell was examined by direct immunofluorescence at 0, 6, 8, 10, 12, 14, 18 and 24 h after infection, using murine monoclonal antibodies to each of the glycoproteins (Table 1). A high proportion of keratinocytes had high-intensity cytoplasmic and membrane glycoprotein expression after infection with the same m.o.i. and for the same time as for the cytotoxicity assay (Table 1). However, the proportion of cells expressing gH was consistently lower (significant in two out of three experiments) than for the other glycoproteins despite the use of the same m.o.i. The proportion of cells which stained at 18 h when

<table>
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<tr>
<th>Glycoprotein</th>
<th>% Positive cells (± SE)</th>
<th>Intensity</th>
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<tr>
<td>gB</td>
<td>83 ± 12</td>
<td>3±</td>
</tr>
<tr>
<td>gC</td>
<td>95 ± 6</td>
<td>3±</td>
</tr>
<tr>
<td>gD</td>
<td>87 ± 11</td>
<td>3±</td>
</tr>
<tr>
<td>gH</td>
<td>61 ± 22</td>
<td>3±</td>
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<table>
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<tr>
<th>Virus strain</th>
<th>% Positive cells (± SE)</th>
<th>Intensity</th>
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<tbody>
<tr>
<td>KOS</td>
<td>81 ± 12</td>
<td>4±</td>
</tr>
<tr>
<td>RP3</td>
<td>75 ± 7</td>
<td>4±</td>
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infected with the HSV-1 control was $90 \pm 3\%$. Negative and time 0 controls showed no staining.

As shown in Table 2, the proportion of keratinocytes expressing HSV gD1 in cells infected with the VP16 mutants RP3 or with the RP1 KOS control was similar. Similar results were also obtained after infection of keratinocytes with RP2 and RP4 mutants. There was also no difference in the (very high) intensity of staining.

**Comparison of PBMC, CD4 and NK and CD4 T cells as effectors in cytotoxicity for keratinocytes infected with vaccinia virus–HSV glycoprotein recombinants or VP16 deletion mutants**

Cytotoxicity for vaccinia virus–HSV glycoprotein recombinants was compared between PBMC, CD4 and NK and CD4 T effectors in four patients. These comparisons were carried out in one of the patients on six different occasions. In addition, variability in recognition of different HSV glycoproteins or VP16 was examined by using keratinocyte targets and mononuclear cell effectors from a number of patients: five different patients for PBMC, six for the CD4 and NK cell subset and 20 for the CD4 T-cell subset (seven for glycoproteins and 13 for the VP16 protein target). Experiments were usually repeated on two separate occasions for each patient.

In several preliminary experiments the optimal effector to target ratio and incubation time were examined with the vaccinia virus–HSV glycoprotein recombinants and the VP16 deletion mutants. The optimal effector to target ratio, probably determined by target cell type and size, was 80:1 (Table 3) and incubation time was 18 h (data not shown) as previously reported (Cunningham & Noble, 1989).

In four patients, mononuclear cell cytotoxicity for gB, gC, gD and gH was slightly greater (10–12% for gB, gC, gD and 5% for gH) than for CD4 and NK (data not shown) or CD4 T-cell cytotoxicity (Fig. 1). However, the latter two were almost identical (Fig. 1 and Table 3) indicating the reproducibility of the experiments and the lack of recognition of these glycoproteins by NK cells, as shown in a previous report (Cunningham & Noble, 1989). Cytotoxicity for negative and positive controls (vaccinia virus and HSV-1) was not significantly different for mononuclear cells, CD4 and NK and CD4 cells.

**PBMC cytotoxicity for IFN-γ-pretreated and vaccinia virus–HSV recombinant-infected keratinocytes**

In four of five patients, mononuclear cells lysed IFN-γ-pretreated and VvgD-infected keratinocytes (specific lysis of 61%) to a significantly greater extent than VvgB (45%) followed by VvgC (29%) and VvgH (18%) infected cells (Fig. 1). In one patient, lysis of VvgC-infected targets was the greatest (68%) in comparison with VvgD (46%), VvgB (38%) and VvgH (22%), but this hierarchy was not maintained for CD4 and CD4 and NK cytotoxicity.

**Table 3.** CD4 T-cell cytotoxicity against IFN-γ-pretreated keratinocytes infected with vaccinia virus–HSV glycoprotein recombinant: determination of optimal effector to target ratio

Results are expressed as % specific lysis of keratinocytes (mean±SE).

<table>
<thead>
<tr>
<th>Target</th>
<th>Effector to target ratio</th>
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<tbody>
<tr>
<td></td>
<td>25:1</td>
</tr>
<tr>
<td>HSV-1</td>
<td>45±0.6</td>
</tr>
<tr>
<td>Vaccinia control</td>
<td>12±1.3</td>
</tr>
<tr>
<td>VvgB</td>
<td>37±0.6</td>
</tr>
<tr>
<td>VvgC</td>
<td>34±0.9</td>
</tr>
<tr>
<td>VvgD</td>
<td>42±0.8</td>
</tr>
<tr>
<td>VvgH</td>
<td>20±0.8</td>
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**Fig. 1.** Comparison of PBMC (a) and CD4 (b) cell cytotoxicity for IFN-γ-pretreated and vaccinia virus–HSV glycoprotein recombinant-infected keratinocytes in the same person. Histograms represent mean of four replicates ±SE. Similar results were obtained in three other subjects. Effector to target ratio was 80 :1. VvgB, vaccinia virus–HSV glycoprotein B recombinant-infected targets; VvgC, vaccinia virus–HSV glycoprotein C recombinant-infected targets; VvgD, vaccinia virus–HSV glycoprotein D recombinant-infected targets; VvgH, vaccinia virus–HSV glycoprotein H recombinant-infected targets.

Reproducibility was tested by repeated experiments (six times) with the same patient’s PBMC as effectors and keratinocytes as targets. No significant differences in the above hierarchy of results were observed on six separate occasions ($P < 0.05$).
Table 4. CD4 cytotoxicity for IFN-γ-stimulated keratinocytes infected with vaccinia virus–HSV glycoprotein recombinants (mean net specific lysis)

Effector to target ratio was 80:1. Mean net specific lysis = (mean specific lysis for vaccinia virus–HSV glycoprotein recombinant)—(mean specific lysis for vaccinia control). Means are derived from the means of replicate experiments for each of 13 patients.

<table>
<thead>
<tr>
<th>Target</th>
<th>CD4 and NK cells</th>
<th>CD4 T cells</th>
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<tbody>
<tr>
<td>VvgB</td>
<td>41</td>
<td>38</td>
</tr>
<tr>
<td>VvgC</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>VvgD</td>
<td>53</td>
<td>48</td>
</tr>
<tr>
<td>VvgH</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>HSV-1 control</td>
<td>76</td>
<td>65</td>
</tr>
<tr>
<td>Vaccinia control</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

CD4 and NK and CD4 T-cell cytotoxicity for IFN-γ-pretreated and vaccinia virus–HSV recombinant-infected keratinocytes

There was little difference between CD4 and NK cell and CD4 T-cell cytotoxicity, hence they will be considered together.

With both CD4 and CD4 and NK populations, cytotoxicity for keratinocytes infected with VvgD1 was usually the highest (mean net specific lysis 53% for CD4 and NK; 48% for CD4 T-cell cytotoxicity) (Table 4) of all recombinants tested under conditions of equal m.o.i. and similar HSV glycoprotein expression (see above). In seven out of nine patients, including five out of seven with CD4 lymphocytes as effectors, there was a hierarchy in cytotoxicity for gD > gB > gC > gH at all effector to target ratios (Tables 3 and 4).

After VvgD1, the mean net specific lysis and % specific lysis for keratinocytes infected with VvgB was next highest (mean 41% for CD4 and NK cells, 38% for CD4 T cells) followed by VvgC (mean 35% for CD4 and NK cells, 32% for CD4 T cells), and VvgH (mean 30% for CD4 and NK cells, 26% for CD4 T cells) (Table 4 and Fig. 2). In the other two of seven patients with CD4 as effectors, there was a slightly different hierarchy of antigen recognition. Cytotoxicity targeted at the VvgD1 recombinant was highest followed by...
PBMC, CD4 and NK and CD4 T-cell cytotoxicity for IFN-γ-pretreated keratinocytes infected with HSV-1 (positive) and vaccinia virus (negative) controls

PBMC were used as effectors in five different donors. In these experiments, mean specific lysis for HSV-1-infected keratinocytes was 72% compared with mean specific lysis of 16% for vaccinia virus-infected keratinocytes.

In all six patients in whom CD4 and NK cells were used as effectors there was marked and significantly (P < 0.05) higher lysis for HSV-1-infected than for vaccinia virus control-infected, IFN-γ-pretreated keratinocytes (Table 4). Mean net lysis for HSV-1 was 76% and for vaccinia virus controls 18%. In seven patients with CD4 cells as effectors the mean net lysis for HSV-1-infected keratinocytes was 65% and for the vaccinia virus control 18%. In all but one patient, CD4 and NK-cell cytotoxicity for gH was significantly greater than the vaccinia virus control.

CD4 T-lymphocyte cytotoxicity for VP16 mutants
CD4 T-lymphocyte cytotoxicity for IFN-γ-pretreated keratinocytes infected with RP2 (HSV-2 VP16 exchange mutant), RP3 or RP4 (VP16 partial deletion mutants) was compared with that for the RP1 control under conditions of equal m.o.i. and similar HSV glycoprotein expression (see above). In two of nine patients a significant reduction in cytotoxicity for the RP3 mutant compared with the parental control was observed: 49% and 56% for the RP1 control and 32% and 39% for the RP3 VP16 mutant (results for one shown in Fig. 4a). In the other seven there was no significant difference in cytotoxicity between the RP3 mutant and control (results for one shown in Fig. 4b). Another four patients were tested for specific cytotoxicity against RP2 and RP4 in comparison with RP1. No reduction in specific cytotoxicity was observed. This indicates that the epitope bound by the RP3 (and larger RP4) deletion is occasionally important as a CTL epitope in some patients.

Discussion
In this autologous, human in vitro system, HSV antigen-restimulated cytotoxic T cells, within mononuclear populations depleted of CD8 T lymphocytes and/or NK cells, consistently recognized and lysed IFN-γ-stimulated, vaccinia virus–HSV glycoprotein recombinant-infected keratinocytes expressing the HSV glycoproteins gB, gC and gD with lesser and variable recognition of gH. These cells also recognized the abundant HSV-1 tegument protein VP16 expressed in keratinocytes stimulated with IFN-γ in a minority of patients as shown by infection with the VP16 partial deletion mutant RP3. In all three cell populations (mononuclear cells, CD4 and NK cells and CD4 T cells) from HSV-seropositive patients there was almost always a similar hierarchy of recognition of the proteins. In the CD4 T-lymphocyte subset, cytotoxicity was usually greatest for gD, followed by gB, gC with lesser and variable
recognition of gH and an epitope in VP16 (at all effector to target ratios). In two of seven patients cytotoxicity for gC was greater than for gB.

A significant decrease in CD4 T-lymphocyte cytotoxicity was detected in two of 13 patients when their keratinocytes were infected with the RP3 or RP4 mutants with the partial deletions of 7% and 16% of the VP16 protein (amino acids 456–490 for RP3 and 413–490 for RP4) instead of the parental KOS strain. Therefore, recognition of this VP16 region as an important epitope occurs in a minority of human subjects. In future experiments, use of vaccinia virus VP16 recombinants may demonstrate wider recognition of VP16 epitopes.

The similarity in hierarchy of recognition of glycoproteins with all subsets consisting of CD4 lymphocytes and NK cells or total PBMC, reflected both the predominance of CD4 lymphocyte cytotoxicity in this system, and also the predominant recognition of late HSV protein targets in IFN-γ-stimulated, HLA-DR antigen-expressing keratinocytes which we recently reported (Mikloska et al., 1996). CD4 T lymphocytes recognized HSV late proteins predominantly and consistently. CD8 T-lymphocyte cytotoxicity was only detectable with IFN-γ-stimulated keratinocytes and predominantly targeted early proteins. In these experiments, NK-cell cytotoxicity was also predominantly directed at early proteins, as previously reported by others (Fitzgerald-Bocarsly et al., 1991). Macrophage cytotoxicity was negligible. We demonstrate here a predominance of CD4 T-lymphocyte cytotoxicity for major late structural proteins of the virion, including the HSV glycoproteins gB, gC, gD and gH and occasionally the major tegument protein VP16. These results suggest that there is a variety of immunodominant epitopes within each of these proteins recognized by different MHC class II types thus resulting in recognition of each protein by a large proportion of the population. These results will be enhanced by more focused future studies of CD4 T-cell clones for individual peptide epitopes in HLA-DR-typed individuals. The difference between CD4 and CD8 lymphocyte specificity for HSV glycoproteins (and IE/E proteins) is probably due to differences in intracellular processing and subsequent binding to HLA-DR of the two classes of proteins, as reported for the glycoprotein and nucleoprotein of lymphocytic choriomeningitis virus (Oxenius et al., 1995). The slightly higher levels of cytotoxicity by mononuclear than CD4 cells and one case of greater recognition of gC than gD in the current experiments may reflect the lower level and variable inconstant recognition of late HSV proteins by CD8 cytotoxic T lymphocytes, as previously reported (Mikloska et al., 1990). The low recognition of gH could be due to several factors including low levels of transport to the cell membrane and hence to the MHC class II processing pathway, in the absence of gL. This is being investigated.

A potential confounding factor in establishing the hierarchy of glycoprotein recognition could be differences in the proportions of keratinocytes infected with recombinant vaccinia virus expressing the different HSV glycoproteins. To ensure a lack of bias in target protein recognition, the vaccinia virus–HSV glycoprotein recombinants were added at the same input m.o.i. as each target epidermal cell monolayer, and the proportion and intensity of cells staining for the target protein were assessed by immunofluorescence at different times. Flow cytometry using permeabilized keratinocytes with intact antigen expression was found to be difficult, mainly because of the obstacles in dissociating the cells tightly bound by desmosomes. However, similar high proportions of keratinocytes stained for the glycoproteins gB, gC and gD at similar high intensities of staining at the relevant time for cytotoxicity, 14–18 h post-infection. The proportion of cells expressing gH was lower than for the other glycoproteins (despite the same m.o.i.) and may partially, but not completely, explain the lower levels of cytotoxicity for this glycoprotein. Therefore the results presented in Table 1 do not account for the hierarchy in glycoprotein recognition and, given the high proportion and intensities of staining, the conclusions are unlikely to be changed by the more accurate quantitative flow cytometric techniques.

In the restimulation process, inactivated whole HSV antigen was added to PBMC. Therefore any bias to recognition of different proteins would be determined by the relative proportions of the proteins themselves within the virus-infected cells and differences in their processing within antigen-presenting cells (APC) such as macrophages (reflecting the same processes and biases in recurrent lesions in vivo). Furthermore, although it is true that this type of restimulation skews the response towards CD4 CTL as opposed to restimulation with HSV-infected (IFN-γ-stimulated) keratinocytes, this is the likely scenario in the stages of early lesions in vivo as shown by the following sequence. HSV infects keratinocytes from axon termini, down-regulating MHC class I before cell lysis and release of the full repertoire of viral proteins. Uptake and presentation of HSV antigens by MHC class II-expressing resident dendritic cells and resident/infiltrating macrophages to infiltrating CD4 cells leads to induction of cytotoxicity and IFN-γ production. Only after restoration of MHC class I expression on keratinocytes by IFN-γ can they act as stimulators to memory CD8 T cells.

As discussed in our previous papers, bulk cytotoxicity assays combined with lymphocyte subset deletion have been used in these initial studies because of the impracticality of using cytotoxic T-cell precursor limiting dilution assays (Cunningham & Noble, 1989; Mikloska et al., 1996). The number of cultured keratinocytes which can be obtained from split skin grafts in patients undergoing minor operations is insufficient to allow appropriate numbers of replicates for these limiting dilution assays.

Furthermore, the use of IFN-γ-stimulated, HSV-infected keratinocytes as stimulators as well as targets is technically very difficult and probably relevant only to the later stages of the lesion. Also, T-cell cloning is selective and hence we would
not obtain sufficient breadth in our sampling of the human outbred population to determine whether the results on HSV protein target recognition are representative.

Despite the marked down-regulation of MHC class I antigen expression on cells by HSV, partial reconstitution by IFN-\(\gamma\) (which also up-regulates TAP expression in infected cells) (Fruh et al., 1995; Hill et al., 1995) is a host cell counter to this mechanism of immune evasion (Mikloska et al., 1996). Hence CD8 T-lymphocyte cytotoxicity may still play an important role in recurrent herpes simplex lesions of at least some patients (Posavad et al., 1996) although this may vary in importance as, on average, HSV titres in lesions have already declined when CD8 T-cell influx is maximal, after 2–3 days (Spruance et al., 1977; Cunningham et al., 1985). Nevertheless, further attention should be paid to the herpes simplex protein target recognition by CD8 T lymphocytes and T-lymphocyte clones in mice (Lawman et al., 1980; Martin et al., 1988; Schmid & Rouse, 1992). It will be interesting to compare the relative target recognition of human CD8 cytosolic T lymphocytes in IFN-\(\gamma\)-stimulated targets with these results in mice. HSV does not appear to down-regulate MHC class I in mice because HSV ICP47 fails to complex with murine cell TAP (York et al., 1994; Fruh et al., 1995).

Nash et al. (1987) also demonstrated the importance of CD4 lymphocytes in primary HSV infection of mice using monoclonal antibody depletion.

The in vitro model used in these experiments contains the most appropriate target cell for cytotoxicity, the HSV-1-infected keratinocyte, which is stimulated by IFN-\(\gamma\) to express HLA-DR (Basham et al., 1984, 1985) and enhanced MHC class I antigens. This reflects what actually occurs in the lesion in vivo (Cunningham et al., 1985). It would be ideal, but extremely difficult, to use the CD4 lymphocytes infiltrating the lesion as the effector cells and also HSV-infected autologous HLA-DR antigen-expressing epidermal cells as targets in these assays. Koelle et al. (1994a, b) cloned HSV-2-specific CD4 T lymphocytes from the lesions of recurrent herpes (with PHA) but used Epstein–Barr virus-transformed lymphoblastoid cells as APC or targets. Somewhat surprisingly they found evidence of limited T-cell reactivity to the glycoproteins gB, gC and gD, which differed from our results and those of Zarling et al. (1986), probably partly due to lack of recognition of common epitopes between gC1 and gC2 and the nature and concentrations of recombinant antigens used. However, the most important differences were probably in target cells and also in HSV antigen-processing in lesions compared with that in blood cells.

The results of this study have considerable implications for the development of herpes simplex vaccines. The concept of therapeutic vaccines has been established experimentally in guinea-pig models. Stanberry (1992) has demonstrated that the immunization of guinea-pigs with recombinant gB, gD or both gB and gD after the experimental establishment of recurrent genital herpes reduces the frequency of genital herpes lesions. Although Straus et al. (1994) also reported a significant overall reduction in lesions of 20% after immunization of humans with a recombinant gB/gD vaccine, whether this can be repeated with recently developed adjuvants which increase the stimulation of T-lymphocyte activity and/or cytotoxic T-cell activity ([MF59], monophosphoryl lipid A (MPL) awaits the results of current clinical trials. However, the addition of MPL to an HSV gD vaccine candidate has clearly shown a marked increase in CD4 lymphocyte reactivity to the candidate immunogen (Bastin et al., 1994).

If there is indeed a difference in HSV antigen specificity between restimulated blood and lesional CD4 cytotoxic lymphocytes directed at infected epidermal cells and if these blood lymphocytes home to recurrent herpetic lesions this may explain the variability in the response to therapeutic immunization and help in prediction of responses to future prophylactic vaccines.

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References


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**HSV-1 targets for human CD4 cytotoxicity**