Immunodominant Epitopes in Herpes Simplex Virus Type 2 Glycoprotein D Are Recognized by CD4 Lymphocytes from Both HSV-1 and HSV-2 Seropositive Subjects

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Primary genital herpes occurs in individuals without preexisting Abs who acquire HSV1 or HSV2 de novo. Recurrent episodes of genital herpes occur despite the presence of circulating neutralizing antiviral Abs (1). Such episodes of infections are of a shorter duration and less severity than primary infection. Preexisting immunity to HSV1 reduces the severity of genital herpes caused by HSV2 infections (2). After virus reacts from latency in the neurons of the dorsal root ganglion, it is transported anterogradely to the axon terminus and then transmitted to the epidermal keratinocytes. A sequence of viral and immunologic interactions occurring both in the dorsal root ganglion and the recurrent herpetic lesion follows (3).

In humans and/or murine models, HSV-specific CD4 and CD8 T lymphocytes play a central role in controlling primary and recurrent HSV infections, in recovery from infection, and in restricting HSV spread in the nervous system (3–5). They are recruited to sites of productive HSV infection or reactivation in the dorsal root ganglion and skin (3). In skin, the immunoreactive cells responsible for controlling the transmitted HSV include the normal constituents of the squamous epidermis, keratinocytes and Langerhans cells, and infiltrating cells: first predominantly monocytes/macrophages and CD4 lymphocytes, and later predominantly CD8 lymphocytes, as shown by immunohistochemistry and direct T cell cloning from lesions (6, 7). Infection of epidermal keratinocytes induces the secretion of a sequence of chemokines and cytokines, which is reflected in the whole lesion in vivo, that is, first IFN-α and β-chemokines and then IL-12 followed by IL-1 and IL-6 (8). The β-chemokines may assist in chemotaxis of monocytes, CD4, and CD8 lymphocytes into lesions. IFN-α and IFN-γ synergize to inhibit infection of keratinocytes after transmission from axon termini (9). HSV1 or HSV2 down-regulates MHC class I expression by epidermal keratinocytes, and this is reversed by IFN-γ mainly secreted by CD4 lymphocytes infiltrating the lesion (10–12). The CD8 lymphocytes do not recognize the infected keratinocytes until MHC class I is restored on their surface by IFN-γ secreted by CD4 lymphocytes. Both CD4 and CD8 CTLs have been isolated from genital lesions ex vivo and shown to have cytotoxic activity (13). The CD8 lymphocyte infiltrate appears to correlate with virus eradication from the skin (14). CD4 CTLs were also shown to recognize HSV2 tegument proteins especially VP16 and VP22 (13). These CD4 CTLs probably act early, and CD8 CTL late, in controlling HSV (3, 6).

Previous work from our laboratory has shown that both human CD4 and CD8 T lymphocytes recognize IFN-γ-stimulated
HSV1-infected keratinocytes. Using vaccinia virus recombinants expressing HSV2 proteins and blood CD4 lymphocytes restimulated in vitro, we showed that CD8 T lymphocytes recognized immediate early/early proteins, whereas CD4 T lymphocytes recognized late HSV1 or HSV2 structural proteins, especially glycoprotein D (gD)3 (8, 15, 16), complementing earlier studies by Zarling et al. demonstrating that gD can stimulate human CD4 helper cells (17). Parallel studies in mice have also showed CD4 lymphocyte specificity for gD (18, 19).

Successful trials of gD of HSV2 (gD2) immunization in mice and guinea pigs (20) preceded human trials of immunization with recombinant gD2 vaccine mixed with the adjuvants alum and deacetylated monophosphoryl lipid A. The latter were shown to substantially induce protection (>70%) (2) against genital herpes disease in HSV1 and HSV2 seronegative but not in HSV1 seropositive women. Prior natural HSV1 infection reduced development of HSV2 genital herpes disease (2), gD2 has also been shown to induce IFN-γ secretion from the PBMC of similarly immunized patients when stimulated in vitro (21).

In view of the importance of gD2 as an immunogen, the objective of this study was to identify the immunodominant peptides of gD2 recognized by bulk human CD4 lymphocytes in most HSV2 seropositive subjects by screening a gD2 peptide library. We also determined their MHC II restriction and whether such peptides were also recognized by HSV1+ subjects. Furthermore, we correlated these empirically defined CD4-MHC II-restricted epitopes with those predicted by the algorithm TEPITOPE (22).

Previous similar human studies in the literature have been limited to those defining a single peptide or a preliminary scan of gD of HSV1 (gD1) with large peptides using older insensitive T cell proliferation assays, defining relatively few epitopes. MHC II restriction or HSV1/2 cross-reactivity was not examined (23, 24).

These studies provide an empirical basis for cross-reactive and possibly cross-protective epitopes between gD1 and gD2 suspected from the vaccine studies. A vaccine effective against both genital HSV1 and HSV2 infection and disease is required in view of the recent increasing incidence of genital HSV1 disease, especially in adolescents (25).

Materials and Methods

Patients and HSV type-specific serotyping

Blood was obtained from 16 HSV2 seropositive (HSV1+/HSV2+ or HSV1-/HSV2+ ) patients usually 1–4 mo after recurrences of genital herpes and 8 patients who were only HSV1 seropositive (HSV1+/HSV2−), usually 1–4 mo after recurrence of oral herpes. In 9 HSV 2+ patients follow-up bleeds were taken at 6–8 mo and in 3 a further 12–15 mo. None had an episode of recurrent genital herpes within the previous 4 mo. Informed consent was obtained from all the blood donors, and the study was approved by the Western Sydney Area Health Service Research and Ethics Committee. HSV2 type-specific serology was performed by ELISA (Focus Diagnostics) and confirmed by Western blot. HSV1 type-specific serology was performed by approved by the Western Sydney Area Health Service Research and Ethics Committee. HSV2 type-specific serology was performed by

Herpes simplex viruses and peptides

HSV2 strain 186 was grown and titrated in Vero cells for subsequent use as control.

Initial screening was performed using a library of gD2 20-mer peptides provided by Dr. Martine Denis of SmithKline Beecham (SKB). Recombinant gD2 was also kindly provided by SKB. Each of these 39 peptides was 20 aa long and had 10 residue overlaps. They were dissolved in DMSO to a final volume of 2 mg/ml and stored at −80°C.

For the second screening, the selected peptides based on the result of the first screening were truncated into nine serial 12-mers within the four most frequently recognized 20-mer peptides (see peptides 2, 24, 30, and 34 in Fig. 1). The selected immunodominant 20-mers and serial 12-mers were produced by Mimotopes. They had an 11-aa sequence overlap with adjacent peptides. The peptides were dissolved in DMSO at a concentration of 10 nM and stored at −80°C.

Preparation of HSV2-specific CD4 effector T lymphocytes

PBMC prepared by Ficoll-Hypaque gradient were stimulated with UV-inactivated HSV2 (12) and then cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Invitrogen), 2 mM glutamine (Sigma-Aldrich, RF10), and 20 U/ml recombinant IL-2 (Roche). CD4 effector T lymphocytes were enriched by CD8 heads (Milenyi Biotech) immediately before the cytotoxicity experiment. The efficacy of CD8 T cell depletion was checked routinely by flow cytometry using anti-Leu 2a + 2b Ab (BD Biosciences) and showed <1% of CD8 T cell contamination. Peptide-specific CD4 effector T lymphocytes were, if necessary, restimulated with gamma-irradiated (5000 rad) and peptide-sensitized (2 μg/ml for 1 h at 37°C) autologous PBMC.

Preparation of target cells for cytotoxicity assays

B-lymphoblastoid cell lines (LCLs) were established by EBV transformation of peripheral B cells as described previously (8) and used as target cells for 51Cr-release assay, as well as the HLA-DR and HLA-DQ blocking assay.PHA blasts prepared as described previously (15) were used to examine the specificity of gD2 peptide-specific CD4 effector T cells. For each of gD2 peptides tested, 105 LCLs were sensitized with 3 μg/ml of peptide and 1 μCi of sterile 51Cr (Amersham Pharmacia Biotech) added for 90 min at 37°C in 5% CO2. After sensitization, the cells were washed three times by centrifugation for 7 min at 270 × g with lukewarm RF10 before coculture with effector cells. The two positive control tubes included target cells sensitized with the same concentration of gD2 Ag and target cells infected with 10 PFU/tube of HSV2 instead of peptide. The controls were cell control (nonsensitized targets with effectors), spontaneous release (targets with no effectors), and total release control tubes containing only target cells labeled with 51Cr sodium solution.

Chromium release cytotoxicity assays

A total of 106 peptide-sensitized target cells were cocultured with CD4 T cell effectors in each well of a Lumaplate (PerkinElmer Life and Analytical Sciences) at E/T ratios of 5:1 and 25:1 for 14 h at 37°C in 5% CO2 in triplicate. The plates were prepared for analysis on a Packard TopCount gamma counter (24, 29). The amount of 51Cr released was quantified as cpm using a gamma counter, and the percentage of specific cytotoxic activity was calculated using the following equation: specific lysis ((total release − spontaneous release) × 100)/ (total release − spontaneous release). SEs of experimental cpm (triplicates) were <3%. Differences between the percentage of specific 51Cr release obtained with peptides or controls were assessed for statistical significance by Student’s t test, with p < 0.05 indicating recognition of peptide epitopes.

IFN-γ ELISPOT assays

To examine the immune response of CD4 T lymphocytes to truncated gD2 peptides, CD4 lymphocytes were depleted from isolated PBMC using Miltenyi CD8 microbeads (Miltenyi Biotech) according to the manufacturer’s instruction. IFN-γ production was measured as the immune response following stimulating CD8-depleted PBMC with 10 μM peptide by ELISPOT assay as described below.

A Millicopore plate with Immunobion-P Polyvinylidene difluoride membrane was coated with purified IFN-γ capture Ab (1D1K, Mabtech) to a final concentration of 5 μg/ml in sterile PBS. The plate was washed three times with sterile PBS and blocked with RF10 for 2 h. After washing the plate three times with PBS, 5–7 × 105 CD8 lymphocyte-depleted PBMC
were added to each well in 100 μl of RF10 supplemented with 10 ng/ml IL-12. Peptides, UV-inactivated HSV1 and HSV2, and PHA were added at a final concentration of 10 μM, 0.5 multiplicity of infection, and 0.5 μg/ml, respectively. After incubating the cells for 40 h at 37°C, the plate was washed three times with PBS and then three times with PBS containing 0.05% Tween 20 (PBST). Biotinylated IFN-γ detection Ab (Mabtech, 7-B6-1) diluted to 1 μg/ml in PBST containing 1% BSA was added to each well. The plate was incubated for 2 h at room temperature and washed six times with PBST. Streptavidin-alkaline phosphatase enzyme conjugate (Bio-Rad) diluted in 1/1000 was added to each well. After incubating for 45 min at room temperature, the plate was washed three times with PBST, and then four times with PBS. BCIP (5-bromo-4-chloro-3-indolyl phosphate/NBT (Bio-Rad) was added as substrate according to the manufacturer’s instructions followed by incubation for ~5 min at room temperature in the dark. The reaction was stopped by extensive rinsing of the plate, under running water with underdrain removed. The plate was dried overnight in the dark. The spots were counted after scanned by KS ELISPOT system (Zeiss) and counted manually (30).

In vitro HLA-DR peptide binding assays

 FREQUENTLY targeted peptides were tested for in vitro binding to 10 common HLA-DR molecules. HLA-DR molecules were purified, and binding assays were performed essentially as previously described (31). Purified human HLA-DR molecules were incubated with unlabeled gD peptides and 0.1–1 nM 125I-labeled peptide for 48 h. MHC binding of the radiolabeled peptide was determined by capturing MHC peptide complexes on LB31 (anti-HLA-DR)-Ab-coated Lumitar 600 plates (Greiner Bio-One) and measuring bound cpm using the TopCount (Packard Instrument) microscintillation counter. The binding data were analyzed and IC50 (nanomolar) was determined as previously described (31, 32).

T cell epitope prediction

The whole gD2 sequence was loaded into the peptide prediction software (TEPITOPE) to predict promiscuous epitopes. The prediction threshold was set at 5% and all the available MHC II molecules were selected to match with predicted epitopes (33).

Statistical analysis of type-specific peptide epitopes

The statistical software package SPSS for Windows version 14 was used to analyze the data. For each of the four peptides (2, 24, 30, and 34), the CD4 lymphocyte responses by IFN-γ production to nine internal peptides were available on each subject. The distributions of these results clearly departed from normality for each peptide. We therefore decided to compute the rank scores (one to nine) of the internal peptides for each peptide within each subject. Statistical analyses were performed separately for each peptide using these rank scores.

There were four subjects positive only for HSV2 and eight subjects positive only for HSV1. Repeated-measures ANOVA was used to test whether there was a significant interaction between the effect of internal peptide number (one to nine) and HSV status (HSV1 only or HSV2 only) on the rank scores for each peptide. Internal peptide number was treated as a within-subject factor and HSV status as a between-subject factor. Statistical tests of interaction typically require larger sample sizes to achieve a within-subject factor and HSV status as a between-subject factor. Statistical tests of interaction typically require larger sample sizes to achieve.

Results

Subjects

Overall, 16 patients with a history of genital herpes and 8 patients with oral herpes were studied in two stages: general screening for gD2 20-mer peptide recognition in 12 patients with genital herpes and then specific studies in 4 with genital herpes and 8 with oral herpes. A history of recent or current active lesions was noted and then their blood was screened by ELISA and/or Western blot for HSV1 and HSV2 Abs. In this study, all patients with genital herpes were HSV2-seropositive and all patients with oral herpes were HSV1-seropositive. In studies of HSV1/2 cross-reactive T cell epitopes, only HSV1+/HSV2− or HSV1−/HSV2+ patients were used; after screening, HSV1+/HSV2− patients were excluded to avoid ambiguity in interpretation of HSV1/2 cross-reactive epitopes (Tables I and II).

Definition of gD2 peptide epitopes by human CD4 lymphocytes

The entire sequence of gD2 and the key peptide 20-mer epitopes identified in this study are shown in Fig. 1a. The amino acid sequences of the key 20-mer peptides of gD2 are compared with those of gD1 in Fig. 1b. Reactivity of CD4 lymphocytes from HSV2-seropositive subjects with a history of a recurrent genital herpes was tested against the full panel of 20-mer peptides using the 51Cr-release cytotoxicity assay and confirmed by IFN-γ ELISPOT assays. Initially autologous LCLs from 12 patients with recurrent genital herpes were sensitized with each peptide of the entire gD2 library and tested against patients CD4 lymphocytes in a bulk cytotoxicity assay at E:T ratios of 5:1 and 25:1. Patient CD4 lymphocytes were tested against each peptide individually but, for logistical reasons, usually in two overlapping sets of 20 or 19 consecutive 20-mer peptides as odd- or even-numbered peptides: 1, 3,…, 33 or 2, 4,…, 34 per bleed for each patient.

Experimental results for E:T ratios of 5:1 and 25:1 were found not to be significantly different (p > 0.5) from each other (data not shown), so only the results for selected experiments with an E:T ratio of 5:1 are presented. In the 12 patients initially tested (shown in Table I) and another 4 (in Table II), there was CD4 lymphocyte recognition of at least some gD2 peptides varying from two to six in the 20-mer peptides tested in one batch. The peptides 2, 24, 30, and 34 were the most frequently recognized even-numbered peptides, followed by peptides 10, 12, and 26 (Fig. 2 and Table I). This was confirmed by IFN-γ ELISPOT assay. Where one of these even-numbered peptides was recognized, either or both flanking odd-numbered peptides were often recognized (e.g., peptides 1 and 3 for peptide 2 or peptides 33 and 35 for peptide 34; Table I). For follow-up studies at 6–8 mo in nine subjects and at 12–15 mo in three subjects after the first bleed, CD4 cytokine responses to the

Table 1. gD2 peptide recognition by CD4 lymphocytes of HSV1- or HSV2-seropositive patients and correlation of MHC II type: recognition of 20-mer peptides by CD4 lymphocytes of 12 HSV2-seropositive patients with recurrent genital herpes

<table>
<thead>
<tr>
<th>Patient</th>
<th>HLA Type</th>
<th>Peptides Recognized (Highest to Lowest)</th>
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<tr>
<td>1</td>
<td>DRB1<em>01, 11, DQB1</em>05, 07</td>
<td>10 &gt; 34 &gt; (34)(5), 25 &gt; 26 &gt; 27</td>
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<td>2</td>
<td>DRB1<em>01, 04, DQB1</em>05, 08</td>
<td>34 &gt; 22, 2</td>
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<tr>
<td>3</td>
<td>DRB1<em>07, 07, DQB1</em>02, 02</td>
<td>12 &gt; 24 &gt; 30</td>
</tr>
<tr>
<td>4</td>
<td>DRB1<em>04, 11, DQB1</em>07, 08</td>
<td>2 (1), (33)(34)(35)</td>
</tr>
<tr>
<td>5</td>
<td>DRB1<em>01, 15, DQB1</em>05, 06</td>
<td>35 (34)(36) &gt; 2</td>
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<tr>
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<td>DRB1<em>01, 15, DQB1</em>06, 10</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>7</td>
<td>DRB1<em>01, 10, DQB1</em>02, 05</td>
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</tr>
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<td>24 &gt; 30 &gt; 34</td>
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<td>33, 34 &gt; 24 &gt; 1, 2</td>
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<tr>
<td>12</td>
<td>DRB1<em>01, 15, DQB1</em>03, 07</td>
<td>1, 2, 3 &gt; 35, 36</td>
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</table>

*Peptide 1 = aa 1–20; peptide 2, aa 11–30; peptide 3, aa 21–40, and so forth. Peptide x > peptide y indicates that peptide x was more immunostimulatory than peptide y. Peptide x = peptide y indicates that peptide x exhibited the same immunogenicity as peptide y.
peptides declined to minimal (by $^{51}$Cr-release assay), although good responses to gD2 and whole HSV2 Ag were retained (data not shown).

**Definition of peptide epitopes within the native viral protein gD2 and their MHC II restriction**

To confirm that these peptides truly contained epitopes recognized by gD2-specific CD4 lymphocytes, autologous effector T cell lines from three HSV2-seropositive patients were restimulated in vitro with peptides 2 or 12 and the effectors were tested initially by $^{51}$Cr-release assay against peptide-sensitized target autologous PHA blasts and, as controls, target cells infected with HSV2, and recombinant gD2-incubated targets. These CD4 effector T lymphocytes when stimulated by peptides 2 or 12 showed high levels of specific activity only against either peptide 2- or 12-sensitized target cells, respectively, and, in each case, also against gD2-sensitized and HSV2-infected target cells. Fig. 3 shows a representative experiment for peptide 12.

**MHC II specificity and its nature were determined by incubation of peptide-sensitized target cells with anti-HLA-DR and anti-HLA-DQ Abs. As shown in Table III, CD4 lymphocyte recognition of peptides 1 and 33 was ablated by anti-HLA-DR Abs. Peptide 24-4 appeared to be restricted only by HLA-DR (Table III). However, with peptide 30-5 there was inhibition by both anti-HLA-DR and anti-HLA-DQ Abs to similar degrees (data not shown). MHC II typing of all patients and the predominant peptides recognized by these patients are shown in Tables I and II.

**Fine mapping of the minimal epitopes within peptides 2, 24, 30, and 34**

Serial 12-mers within the 20-mers 2, 24, 30, and 34, selected as the most frequent immunodominant epitopes, were used for fine mapping of CD4 lymphocyte epitopes.

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**Table II. gD2 peptide recognition by CD4 lymphocytes of HSV1- or HSV2-seropositive patients and correlation of MHC II type: gD2 12-mer peptide recognition by HSV1- and HSV2-seropositive patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>HSV Serotype</th>
<th>HLA Type</th>
<th>Recognized gD2 Peptides (Highest to Lowest)*</th>
<th>Parental 20-mer Peptide</th>
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<td>30-5</td>
<td>30</td>
</tr>
<tr>
<td>24</td>
<td>HSV1+/2-</td>
<td>DRB1<em>01011, 04051, DQB1</em>0202, 0501</td>
<td>34-6</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2-2</td>
<td>24</td>
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<td></td>
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<td></td>
<td>24-3, 24-4</td>
<td>24</td>
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<td></td>
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<td>30-4, 30-1</td>
<td>30</td>
</tr>
</tbody>
</table>

*34-1 refers to the first of the nine 12-mers within 20-mer peptide 34.
In some patients definition of minimal epitopes was clear, as shown in Fig. 4A, where there is an increasing recognition of peptides 2.1–2.4 and no recognition thereafter within peptide 2 (patient 13, Fig. 4A). However, in other patients it was very difficult to define minimal epitopes within the 20-mers (patient 14, Fig. 4B). Furthermore, as for the 20-mers it was impossible to assign recognition of individual 12-mers to specific MHC II (HLA-DR) alleles. Indeed, comparisons of the patterns of recognition between patients of different HLA-DR and HLA-DQ types indicate cross-recognition of specific 12-mer and 20-mer peptide epitopes across different MHC II alleles.

In completely asymptomatic HSV1-seropositive patients, responses to the 20- and 12-mer peptides were lower and variable, with only 33% positive responding to six major immunodominant peptides (data not shown).

Comparison of empirically defined epitopes with those predicted by the TEPITOPE algorithm

According to the TEPITOPE algorithm, there was a very high density of predicted epitopes in gD2 for the most common MHC II alleles, whether the predictive levels were set at 2% or 5% (e.g., four epitopes recognized by at least 10 alleles were predicted within peptide 2 when set at the 5% level) (data not shown). The algorithm also predicted cross-recognition and binding of different epitopes within 12-mer or 20-mer peptides according to different MHC II alleles (22, 33) (e.g., peptide epitopes within the 20-mer peptide 2 for HLA DRB1*0101, *0301, *0402, *0701, *1101/1104/1106, *1305, and *1501/1502). In some cases, there was a good correlation between predicted and empirically determined epitopes (e.g., within peptides 2 and 10) whereas in others the

FIGURE 1. The amino acid sequences of gD2, as well as key gD1 and gD2 epitopes for induction of CD4 lymphocyte responses. Each 20-mer peptide analog had a 10-aa overlap with adjacent peptides. Nine 12-mers were synthesized from each 20-mer of peptides 2, 24, 30, and 34 for fine mapping. Each 12-mer overlapped by 11 aa with adjacent 12-mer peptides (a). The differences in amino acid sequences between HSV1 (strain 17) and HSV2 (clinical isolate 356.2038) for key peptides tested are shown in b.

FIGURE 2. gD2 peptides recognized by CD4 lymphocytes from two HSV2-seropositive patients with recurrent genital herpes. CD4 lymphocytes were enriched by negative selection as outlined in Materials and Methods and stimulated with a UV-inactivated HSV2 Ag. Target cells were LCLs incubated with each of the individual peptides or gD2 and with 51Cr. Exogenous recombinant gD2 was used as the positive control. Effectors and targets were mixed in a ratio of 5:1. Each peptide was tested in triplicate, and histograms represent means. Dashed line represents mean of no peptide controls and 3 SDs. Key peptide recognition was later checked by IFN-γ ELISPOT (see Materials and Methods). Two representative results (A and B, patient 7 and 8 in Table I, respectively) are shown.

MHC II alleles (22, 33) (e.g., peptide epitopes within the 20-mer peptide 2 for HLA DRB1*0101, *0301, *0402, *0701, *1101/1104/1106, *1305, and *1501/1502). In some cases, there was a good correlation between predicted and empirically determined epitopes (e.g., within peptides 2 and 10) whereas in others the

FIGURE 3. Verification of specificity of peptide-specific T cell lines. A CD4 (cytotoxic) lymphocyte cell line from patient 3 was restimulated with peptide 12 through two cycles and then specificity of the cell lines was tested against autologous target PHA blasts with a range of peptides including the peptide stimulator (12), the overlapping flanking peptides and, as positive controls, target cells incubated with gD2 or infected with HSV2. The E:T ratio was 20:1. Experiments were conducted in triplicate. The representative results of three experiments are shown.
cross-reactive recognition of HSV2 peptides by CD4 lymphocytes for HSV1-seropositive subjects

Several of the peptides were cross-recognized by patients who were HSV1\(^+\)/HSV2\(^+\) (Fig. 4, C and D). In some cases, their CD4 lymphocytes responded to these peptides at lower levels than those by patients who were HSV1\(^+\)/HSV2\(^-\), whereas in other cases CD4 lymphocyte responses were as strong. The comparison of the four key selected 20-mer peptides and their serial overlapping 12-mers for cross-reactivity between HSV2 and HSV1 are shown in Fig. 6. All were recognized by HSV1\(^+\)/HSV2\(^+\) patients. Because there was promiscuous recognition of peptide epitopes across different MHC II alleles, it was possible to conduct a comparative quantitative study of recognition of the nine internal serial 12-mers for each 20-mer peptides 2, 24, 30, and 34 by CD4 lymphocytes from eight HSV1\(^+\)/HSV2\(^+\) and four HSV1\(^-\)/HSV2\(^+\) patients. As shown in Fig. 6, the patterns of recognition were very similar between CD4 lymphocytes of HSV1\(^+\)/HSV1\(^+\) and HSV2\(^+\)/HSV2\(^+\) patients for peptide 30 and mostly similar for peptides 2, 24, and 34. Peptide 34 showed the greatest differences in predominant recognition of internal peptides at the N- and C-terminal regions for HSV1\(^+\)/HSV2\(^+\) patients. For peptides 2 and 24, recognition of the 12-mers in the amino half of the 20-mer was dominant. Peptides 30 and 24 have the greatest degree of homology between the HSV1 and HSV2 sequences (Fig. 1). The slight differences in responses to some of the 12-mers in peptides 2 and 34 may be explained by unlike amino acid substitutions at positions 4, 14, and 15 in peptide 2 and positions 3, 6, 8, and 10 in peptide 34, especially prolines at positions 3 and 10. No statistically significant interaction between the effects of internal peptide number and HSV status was found for peptide 30 (p = 0.239), nor were there any major amino acid differences between HSV1 and HSV2 in this 20-mer. As was therefore anticipated, none of the Mann-Whitney tests of the rank scores by HSV group were statistically significant for any of the nine internal peptides.

FIGURE 4. Recognition of gD2 12-mer peptides by CD4 lymphocytes of HSV1\(^+\) and HSV2\(^+\) patients. IFN-γ production by CD4 lymphocytes of two HSV1\(^+\)/HSV2\(^+\) patients (patient 13 and 14; A and B, respectively) and two HSV1\(^+\)/HSV2\(^-\) patients (patient 19 and 20; C and D, respectively) was measured by IFN-γ ELISPOT after stimulation with gD2 peptides. The dashed line indicates the threshold for recognition, which was 3 SDs above the mean value of nonstimulated CD4 lymphocyte response (as in Fig. 2).
However, peptides 2, 24, and 34 demonstrated a statistically significant interaction between the effects of internal peptide number and HSV1/2 status (p-value for interaction 0.098, 0.057, and 0.048, respectively). For peptide 2, there were major amino acid differences between internal peptide 4 and, to a lesser extent, for internal peptide 3 of HSV1 and HSV2. Mann-Whitney tests of the rank scores by HSV1/2 status showed a statistically significant difference in recognition by HSV1 and HSV2 patients for internal peptide 3 (p = 0.048) but not for internal peptide 2 (p = 0.461).

For peptide 24, there was no amino acid difference between internal peptides 2–8 of HSV1 and HSV2. Nevertheless, Mann-Whitney tests of the rank scores by HSV1/2 status showed a statistically significant difference in 12-mer recognition according to HSV1/2 status (HSV1 only or HSV2 only) on the rank scores. Considering the total number of internal peptides (36), for the nine in which major amino acid differences were identified, statistically significant differences in 12-mer recognition (4 of 27, p = 0.016). Thus, HSV1/2 sequence comparisons correlated with observed 12-mer peptide recognition (4 of 9 vs 2 of 27, p = 0.014, Fisher’s exact test), providing validation for the experimental results.

### Binding of peptides to HLA-DR molecules

We next assessed whether the set of epitopes identified showed promiscuous HLA-DR binding affinity. Each of the 20-mer peptides was tested for its capacity to bind to a panel of 10 common DR molecules. As shown in Table IV, peptides 2 and 24 were found to be degenerate binders, binding 50% or more of the molecules tested with high affinity (IC$_{50}$ of <1000 nM). These
epitopes also bound several other specificities with intermediate affinity (IC50 1000–5000 nM). Peptide 34 was less degenerate, but still bound 4 of the 10 DR molecules tested with high affinity. By contrast, peptide 30 bound only DRB1*0101 and DRB1*1501. The peptide 35 20-mer, which was less immunogenic than the other four, also bound to DRB1*0101, *0404, *0701, and *1302 (unpublished observation).

Analysis of the pattern of binding of the corresponding 12-mer truncations of each epitope was also undertaken. The data, shown in Table IV, revealed in several cases that binding of the 20-mer peptide may be achieved by use of more than one core region. For example, in the case of peptide 2, binding to DRB1*0101, DRB1*0701, and DRB1*1501 was observed with truncations incorporating either the N- or C-terminal regions of the peptide. In most other cases, optimal binding appeared to be more concentrated in one region of the 20-mer peptide. However, because the binding groove of the DR molecule allows for multiple core alignments even in the context of a 12-mer peptide, the possibility that each of these regions represents multiple closely nested epitopes cannot be excluded.

Taken together with the Ab blocking data, these binding data support DR restriction in the majority of cases for peptides 2, 24, and 34 and suggest that binding in several contexts may involve more than one core region. These data also suggest that, at least in

FIGURE 5. gD2 peptides recognized by HSV-infected patients. Each bar indicates the recognized 12-mer or 20-mer peptide.

FIGURE 6. Immune response profiles of HSV1+ or HSV2+ patient to nine serial gD2 12-mer peptides within immunodominant 20-mers (peptides 2, 24, 30, and 34). The Mann-Whitney U test has been performed to obtain y values, which have been represented as estimated marginal means (Materials and Methods). Triangle symbols represent HSV1+/HSV2+ patients and square symbols represent HSV1+/HSV2− patients.
some cases, peptide 30 is DQ restricted (recently confirmed by HLA-DQ binding assay, data not shown). In most cases, there was a good correlation between peptide binding to HLA-DR and T cell responses to the peptides in subjects of similar HLA-DR types (e.g., 8 of 10 HLA DRB1*0101-positive subjects recognize epitopes in peptide 2).

Discussion

In this work, we have demonstrated that CD4 lymphocytes of all HSV2-positive patients with genital herpes recognized at least two to six of an overlapping library of peptide 20-mers spanning the whole glycoprotein gD2 sequence, including the leader sequence. Bulk CD4 lymphocytes were used to identify specific epitopes in an effort to minimize the bias induced by using T cell clones. However this did reduce the magnitude of response, requiring the use of IFN-γ ELISPOT or 51Cr-release assay for maximum sensitivity. The latter was a valid screening assay, as previously gD-specific CD4 lymphocytes have shown to be cytolytic by this assay (12). This is consistent with the relatively low frequency of 0.2–0.4% of gD peptide-specific CD4 lymphocytes in vivo shown by intracellular cytokine staining for IFN-γ (34, 35). This low frequency of responder in HSV-seropositive patients contrasts with those in the other herpesvirus infections, CMV (1.2%), and EBV (35). In nine HSV2+ patients with recurrent genital herpes, their CD4 lymphocyte responses (by 51Cr-release assay) to gD2 peptides but not whole gD2 or HSV2 declined to undetectable when re-tested 6–8 and 12–15 mo later. The kinetics of these enhanced memory CD4 lymphocyte responses to gD2 peptides soon after clinical recurrences show similarities to the kinetics of IFN-γ responses from PBMC after recurrences of orolabial herpes (6). However, the detection of IFN-γ secretion by CD4 lymphocytes responding to all peptides in some asymptomatic patients indicates the importance of also considering asymptomatic oral shedding in boosting responses. Identification of key 20-mer peptide epitopes (shown in Table I) was confirmed by both 51Cr-release assays using LCLs as targets and ELISPOT assays, and the specificity of gD2 peptide-specific T cell lines was verified by 51Cr-release assay using PHA blasts as target cells to reduce nonspecific lysis.

Serially truncated 12-mers within the initially identified 20-mer peptide epitope were used to define the minimal T cell epitopes in most patients, but in others this was impossible, suggesting clustering or overlapping of more than one epitope in the 20-mer. It was also difficult to assign minimal 12-mer peptides to an individual HLA-DR or HLA-DQ allele. However, in three patients tested for peptides with HLA-DR- or HLA-DQ-blocking Abs, they were HLA-DR restricted and all appeared to be recognized in the context of several HLA-DR alleles. This was clearer in some of the patients who were homozygous for the HLA-DR state. However, in one patient recognizing peptide 30-5, anti-HLA-DR and anti-HLA-DQ Abs were equally inhibitory, suggesting there were also HLA-DQ-specific peptides within gD2.

Peptide-MHC binding data showed that peptides 2 and 24, and to a lesser degree peptide 34, bind multiple DR types with high affinity. The peptide binding data also support the proposition that in some cases there may be multiple epitopes within the 20-mers studied. This is especially apparent in the case of peptide 2, where binding of both N- and C-terminal-truncated peptides was observed to DRB1*0101, DRB1*0701, and DRB1*1501. The presence of overlapping epitopes obscures identification of the exact specificity of recognition for each HLA-DR allele. However, while the exact minimal epitope has not been elucidated in each context, we think that the present data will be of value to those studying T cell recognition of gD2 and the correlates of HSV immunity.

In some instances, differences were observed between the binding assays and the CD4 lymphocyte response in that a peptide binding to a specific DR type may be recognized in one patient expressing that molecule, but not in another. These differential responses may reflect the unique T cell repertoire of each donor, or differences in peptide processing or differences in fine specificity of HLA-DR types.

The TEPITOPE program, which predicts MHC II-specific peptide epitopes within a protein, suggested possible reasons for the difficulty in defining individual 20-mer epitopes in some patients (22). At the most stringent levels, several overlapping or clustered MHC II-restricted epitopes were predicted to occur (within 20-mers) at various places within the gD2 molecule. They were also predicted to be promiscuously recognized by a number of MHC II alleles (33). Thus, our T cell response and HLA-DR binding data were mostly consistent with that predicted by the TEPITOPE algorithm in terms of densely clustered epitopes within 20-mers and their promiscuous recognition. Nevertheless, there was an incomplete correlation of empirical and predicted epitopes (e.g., the consistent T cell recognition and binding of epitopes in 330–350 regions by patients with a broad range of HLA-DR alleles). TEPITOPE is clearly one of the best of the MHC II algorithms and may be useful to quickly identify some epitopes within a given protein. However, it does not identify all of them and clearly the results of the algorithms should be checked experimentally.

Cross-recognition of each of four major 20-mer peptide epitopes (peptides 2, 24, 30, and 34) and of the nine serial overlapping minimal T cell epitopes within each 20-mer was examined in patients who were seropositive only for HSV1 or for HSV2. All four 20-mer peptides were cross-recognized. Because of the promiscuity of recognition of the serial internal 12-mer epitopes across different HLA-DR alleles, consensus recognition patterns among eight HSV1-seropositive and four HSV2-seropositive patients became apparent. For one of these 20-mers (peptide 30), the pattern of recognition along the 20-mer was very similar by HSV1- or HSV2-seropositive patients, reflecting the sequence homology in those regions of D1 and D2. In another two (peptides 2 and 34), there was similarity in recognition in one but not in another region of the 20-mer. These differences in the two 20-mers were correlated with more marked differences in HSV1/2 sequence homology (Fig. 1). In peptide 24, there was a slight difference in recognition patterns for HSV1 and HSV2, but this was not obvious from inspection of sequences. Differences in binding of the 12-mer peptide to the key binding pockets of different HLA alleles or to the TCR might account for such differences. Nevertheless, overall statistical modeling suggested a high degree of correlation between sequences predicted and observed CD4 lymphocyte responses.

Our results provide a broader picture of the HSV structural peptides recognized by human and CD4 lymphocytes than hitherto published. In humans using HSV2-specific clones from genital lesions, Koelle et al. identified HSV type-specific epitopes in the tegument proteins VP16, VP22, and JU7Pase (UL49 and UL50), as well as UL21, by expression cloning, thus extending the range of CD4 epitopes beyond gD, gB, and gH (13, 16).

In mice, a recent thorough study determined four new immunodominant regions in gD, recognized by three murine strains of different MHC II types, using 12 regions predicted to be immunodominant from the TEPITOPE algorithm as their starting point (36). Allowing for their numbering system of mature gD (which excludes the 25-aa leader sequence of immature gD that was included in our studies) and that their studies focused on gD1 rather than gD2, there was still relatively little overlap with the four major 20-mer peptide epitopes found in our study, perhaps not
surprising in view of the species difference. Nevertheless, this murine and human study showed the presence of multiple epitopes scattered throughout the gD epitope and which can be cross-recognized by humans or mice of different MHC types. Importantly, these epitopes induced Th1 rather than the Th2 responses of some other gD epitopes, and when the Th1 epitopes were used to immunize mice, they protected against viral challenge. The major gD epitopes in our study were detected by cytotoxicity and IFN-γ ELISPOT assays, indicating they were also Th1 epitopes and may be useful as immunogens in future.

An earlier murine study also showed a marked diversity and lack of immunodominance of peptide epitopes within gD1 recognized by mice after infection but a single immunodominant epitope after 240–260 aa after immunization with gD (18, 19), which was confirmed by another study that employed immunization with gD1 molecules lacking the C-terminal 93 aa (37). Brynestad et al. (29) also showed immunogenicity of peptide 1–23 amino acids of glycoprotein D1 that was later enhanced by palmitic acid conjugation (36).

Only two reports have defined gD epitopes recognized by human T lymphocytes. Neither addressed HLA restriction of the human CD4 lymphocyte responses, HLA-DR binding, or HSV1/2 cross-reactivity (using acceptable modern type-specific serology). Damhof et al. used overlapping synthetic peptides from gD1 to examine the response by T lymphocyte proliferation from 10 healthy HSV seropositive patients and found marked differences in individual responses to peptides. They defined broad immunodominant regions of 1–54, 110–214 and 290–314 of mature gD molecules (23). As in mice, this study emphasized the diversity and lack of immunodominance in human recognition of gD peptides also shown in our studies. DeFreitas et al. examined the response of human peripheral blood T lymphocytes to the mature gD1 peptide amino acids 1–23, indicating immunogenicity particularly in the N-terminal half of the peptide (24). However, there were also responses induced in HSV-seronegative patients who could have been nonspecific or, less likely, due to primary immunization.

There are similarities and differences between our results and these human and murine studies. We examined the whole of the gD2 (rather than gD1) molecule, including the leader sequence (resulting in a 25-aa shift in numbering systems between the studies) and the transmembrane region of gD2. We reasoned that gD2 was clinically a more important molecule and secondly that the leader and transmembrane sequences might still be important in generating CD4 lymphocyte responses, especially following up-take of apoptotic or necrotic debris by APCs (although usually inducing an MHC I-restricted response). In support of this hypothesis (and the TEPITOPE algorithm), an in vitro binding assay indicated the presence of potential broadly recognized MHC II epitopes in the region 10–30 of the immature gD molecule. Furthermore, the serial internal 12-mer peptide studies showed recognition by HSV1-seropositive patients in regions of peptide 2 overlapping with those detected by DeFreitas et al. (24). Peptide 34 (aa 331–350) in our study includes the transmembrane region, which was not tested by Damhof et al. (23). This region showed the greatest difference in recognition between HSV1+ and HSV2+ patients with clear separation of epitopes in the N- and C-terminal regions. The detection of broad human responses (across HLA-DR alleles) to peptides 24 (aa 231–250) and 30 (aa 291–310) in our study, and not in theirs, probably reflects the enhanced sensitivity of ELISPOT assay over T lymphocyte proliferation for CD4 lymphocyte responses. These responses were confirmed with the serial 12-mer studies, which also showed peptide 34 to be the most divergent in recognition by HSV1- and HSV2-seropositive patients of the four key peptides epitopes.

The predominant restriction of gD peptide recognition by CD4 lymphocytes according to HLA-DR and broad recognition across different HLA-DR alleles can be added to the short list of other published studies of CD4 lymphocyte recognition of pathogens, including Plasmodium falciparum, hepatitis C, and HIV (32, 38–40).

The high density of CD4 lymphocyte epitopes and their cross-recognition by different HLA-DR alleles is consistent with our previously published studies, which showed that glycoprotein D was recognized by all 18 patients studied using gD recombinant vaccinia viruses (16). Presumably these results underlie the high rate of protection (73–74%) of HSV1- and HSV2-seronegative women against genital herpes disease in two trials of a gD2 vaccine with a deacetylated monophosphoryl lipid A adjuvant, which induces CD4 lymphocyte responses oriented toward a Th1 pattern of cytokine production, including IFN-γ (2). In this study all patients tested developed T lymphocyte reactivity to the vaccine (G. Dubin, M. Denis, A. L. Cunningham, S. Spruance, L. Stanberry, D. I. Bernstein, A. Mindel, S. Sacks, S. Tying, F. Y. Aoki, and M. Sloumi, unpublished results). The vaccines produced by Chiron and GSK that differed in their adjuvants and in their efficacy despite both inducing neutralizing Ab has been widely interpreted to mean that the Th1 CD4 lymphocyte response induced by the GSK vaccine is likely to be highly significant (2, 41). Furthermore, the gD2 vaccine afforded no additional protection against disease to those women who were already HSV1-seropositive. Such data strongly suggested that T lymphocyte- (especially CD4 lymphocyte and Th1 cytokino) mediated immunity was responsible for the protection against disease in the HSV-seronegative women and also that there are cross-reactive (and cross-protective) epitopes in gD1 and gD2. Furthermore, in a separate analysis of the placebo recipients, HSV1+ patients appear to be better protected against genital herpes disease than those who are HSV1-seronegative (2). There are other data to suggest that HSV1-seropositive patients have milder initial genital herpes disease to support such a hypothesis (42). This study was designed to provide the first data to examine and possibly support these hypotheses. Herein, we have clearly demonstrated that there are indeed such cross-reactive epitopes in gD2 and that they are actively recognized by patients naturally infected with HSV1, inducing a Th1 response. In some peptides, this was because of their sequence similarities. In others, there were two different epitopes within the one 20-mer, each recognized by either HSV1+ or HSV2+ subjects. This suggests that a gD2/deacylated monophosphoryl lipid A vaccine might also be effective against genital HSV1 disease in HSV1+/HSV2- women or even a future childhood vaccine against severe diseases caused by both HSV1 and HSV2.

However, recent studies of serial infection by certain immunologically related viral species in animal models show that reactivation of cross-reactive CD8 lymphocyte memory responses by the second infection can alter the normally naive CD8 lymphocyte responses to the second virus by heterologous immunity in various ways, such as enhancement, inhibition, or immunopathology. Although only cross-protection has been observed in natural serial infection with HSV1 and HSV2 (for disease), these unpredictable T cell responses indicate the need for appropriate clinical and immunologic observations when such anti-HSV1/2 vaccine strategies are used in trials. However, our results do show a marked contrast between the broadly recognized MHC II-restricted gD2 epitopes and the variable but usually narrow spectrum of recognition of MHC I-restricted peptide epitopes (43).

These results lay the foundation for future studies in naturally infected patients and those immunized against HSV and for future development of vaccines. For example, future studies defining
HSV1/2 type-specific and/or type common epitopes for both CD4 and CD8 lymphocytes in key HSV immunogenic proteins other than gD (e.g., tegument proteins) should assist in designing more effective vaccines, especially in men and HSV1-seropositive women. Lessons from murine studies, demonstrating potentially deleterious Th2-inducing gD epitopes, should also be applied to such human studies. The definition of a number of (promiscuous) HLA-DR epitopes will also enable the construction of MHC II tetramers (although this is often difficult and variable results can be obtained).

These studies have shown the important epitopes recognized soon after recurrent diseases. However, as a follow-up to these studies, carefully planned, longitudinal, well-controlled clinical studies are required to test the importance of CD4 lymphocytes in general and to each of these epitopes in particular in the control of initial symptomatic HSV1/2 disease. Such studies require prospective lymphocyte collections and epitope-specific studies during primary disease or asymptomatic serocconversion, probably during placebo-controlled vaccine studies in seronegative partners of patients with genital herpes. Furthermore, responses to HSV1/2 non-cross-reactive vs cross-reactive epitopes needed to be tested during first HSV2 infections in HSV1-seropositive subjects.

In general, these studies extend the relatively small number of pathogens for which MHC II restricted promiscuous epitope recognition by CD4 lymphocytes, and they provide a mechanism and a rationale for the use of safe viral protein vaccine candidates, especially where CD4 lymphocyte responses are important. Proteins with dense numbers of epitopes each binding to multiple DR and DQ types should be useful. The results also provide further data on discrepancies between murine and human CD4 responses and data to compare with predictive algorithms, and they emphasize the importance of developing peptide-DQ binding assays.

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References


