Gender-Dependent HLA-DR-Restricted Epitopes Identified from Herpes Simplex Virus Type 1 Glycoprotein D

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In recent clinical trials, a herpes simplex virus (HSV) recombinant glycoprotein D (gD) vaccine was more efficacious in woman than in men. Here we report six HLA-DR-restricted T-cell gD epitope peptides that bind to multiple HLA-DR (DR1, DR4, DR7, DR13, DR15, and DRB5) molecules that represent a large proportion of the human population. Four of these peptides recalled naturally primed CD4+ T cells in up to 45% of the 46 HSV-seropositive, asymptomatic individuals studied. For the gD49-82, gD77-104, and gD121-152 peptides, the CD4+ T-cell responses detected in HSV-seropositive, asymptomatic women were higher and more frequent than the responses detected in men. Immunization of susceptible DRB1*0101 transgenic mice with a mixture of three newly identified, gender-dependent, immunodominant epitope peptides (gD49-82, gD77-104, and gD121-152) induced a gender- and CD4+ T-cell-dependent immunity against ocular HSV type 1 challenge. These results revealed a gender-dependent T-cell response to a discrete set of gD epitopes and suggest that while a T-cell epitope-based HSV vaccine that targets a large percentage of the human population may be feasible with a limited number of immunodominant promiscuous HLA-DR-restricted epitopes, gender should be taken into account during evaluations of such vaccines.

The vast majority of the world's human population is infected with herpes simplex virus type 1 (HSV-1) and/or HSV-2, which cause diseases in every stage of life, ranging from fatal disseminated disease in newborns to skin lesions (cold sores), genital ulcerations, blinding eye lesions, and fatal encephalitis in adults (21, 43, 47, 85). More than 450,000 people in the United States have a history of recurrent ocular herpes disease that can lead to sight-threatening herpetic stromal keratitis caused by an immunopathological response in the cornea (2, 25, 31, 66). Recurrent genital herpes disease also has an immunopathological course that leads to the development of genital lesions, ulcerations, and scarring (2). The rapid spread of genital herpesvirus infection, which occurs mostly during unrecognized or asymptomatic shedding, is reflected in more than one million new cases per year in the United States (44).

Although costly drug therapy (e.g., acyclovir and derivatives) can limit morbidity and mortality from active disease, it cannot cure infection (38). Thus, developing an effective immunotherapeutic vaccine against herpesvirus infection must be an important global health priority (52, 53, 55, 85, 86). Such a vaccine would be a cost-effective approach and would be a limited number of immunodominant HLA-DR-restricted epitopes, gender should be taken into account during evaluations of such vaccines.
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FIG. 1. Illustration of HSV-1 gD showing the relative positions of T-cell epitope peptides used in this study. HSV-1 (strain 17) gD regions carrying potential human class II-restricted T-cell epitopes predicted by the TEPITOPE computer-assisted algorithm based on known HLA-peptide-TCR interactions are shown. The amino acid sequence, in single-letter code, and the peptide positions based on the full-length sequence of gD are shown. The black box represents the transmembrane domain.

dicted 12 potential epitope peptides from HSV-1 gD that are specific to 10 HLA-DR molecules representing the majority of the world population (2). Here we demonstrated that 6 of the 12 potential epitope peptides bind in vitro with high affinity to 6 of the 10 HLA-DR molecules tested. Four gD epitope peptides recalled functional HSV-specific gamma interferon (IFN-γ)-producing CD4+ T cells in healthy, HSV-seropositive, asymptomatic individuals and were recognizable by virus-primed CD4+ T cells in HLA-DR*0101 and HLA-DR*0401 transgenic (Tg) mice following ocular infection with HSV. Interestingly, three gD peptides (gD49-82, gD77-104, and gD121-152) that recalled stronger CD4+ T-cell responses in HSV-seropositive women than in men also recalled stronger CD4+ T-cell responses in HSV-infected age-matched female compared to male HLA-DR*0101 and HLA-DR*0401 Tg mice. Finally, a cocktail of three immunodominant, gender-dependent gD peptide epitopes elicited CD4+ T-cell-dependent immunity against lethal herpesvirus infection in HLA-DR*0101 Tg mice.

MATERIALS AND METHODS

Study population. From August 2003 to October 2007, we screened 283 HSV-1- and/or HSV-2-seropositive individuals. Among these, a cohort of 87 immunocompetent individuals, with an age range of 18 to 63 (median, 31) years, who were seropositive or seronegative for HSV-1 and/or HSV-2 were enrolled in the present study. For the characteristics of this study population with respect to sex, age, and HSV serology, see Table 3. Fifty-seven individuals were white, 30 were nonwhite (African, Asian, Hispanic, or other), 43 were females, and 44 were males. All patients were negative for HIV and hepatitis B virus and had no history of immunodeficiency. Twenty-eight patients were HSV-1 seropositive and HSV-2 seronegative, 10 patients were HSV-2 seropositive and HSV-1 seronegative, 9 patients were both HSV-1 and HSV-2 seropositive, and 40 were HSV seronegative. All of the patients selected for this study were healthy and asymptomatic (i.e., seropositive with no history of recurrent ocular, orofacial, or genital herpes disease). All subjects were enrolled at the University of California Irvine under an institutional review board-approved protocol. All subjects provided written informed consent.

HSV-1 and HSV-2 seropositivity screening. The sera collected from 61 patients were tested for HSV-1 and HSV-2 status with HerpeSelect immunoglobulin G1 and G2 HSV enzyme-linked immunosorbent assay (ELISA) kits (Focus Diagnostics, Cypress, CA) as previously described (30). The sensitivities and specificities of these ELISAs were 91.2% (HSV-1) to 96.1% (HSV-2) and 92.3% (HSV-1) to 97% (HSV-2). Although this assay generally gives a clear-cut result, in some instances the serotyping was also validated by Western blotting as previously described (63).

Bioinformatic analysis. The full-length gD sequence (26) was loaded into the new prediction software TEPITOPE to predict promiscuous epitopes (19). The TEPITOPE algorithm is a Windows application that is based on 25 quantitative matrix-based motifs that cover a significant part of the human HLA class II peptide binding specificity (58). The algorithm permits the prediction and parallel display of ligands for each of the 25 HLA-DR alleles. The TEPITOPE prediction threshold was set at 5%, and 12 regions predicted to bind a minimum of 50% of the major histocompatibility complex (MHC) class II molecules were picked (Fig. 1). The HLA-DR-restricted peptide epitopes derived from OVA (OVA252-258) (45) and from gB (gB161-175) (ATMYYKDVTVSQVWF) were used for control of specificity. Peptide OVA252-258 (LPELSEPTNPATOLEAPE DPEDSA) from region gD257-264, and peptide gB161-175 (NAAMFAGLQFAFEYMNGLAVQMGGGYVV), which were not picked by the TEPITOPE program as containing potential T-cell epitopes, were synthesized and used as negative controls.

Peptides and immunization. A total of 12 gD peptides were selected by TEPITOPE as previously described (2). Each peptide, consisting of 27 to 34 amino acids, was synthesized as previously described (1, 3–7, 14, 27, 59). gB (ATMYYKDVTSQVWF) and OVA T helper peptides were used as control peptides. The purity of peptides was 96%, as determined by reversed-phase high-performance liquid chromatography ( Vyad C18) and mass spectroscopy (Voyager matrix-assisted laser desorption ionization-time of flight mass spectrometry system). Stock solutions were made at 1 mg/ml in water, except for peptide gD146-179, which was solubilized in 5% dimethyl sulfoxide. All peptides were aliquoted and stored at −20°C until assayed. All in vivo immunization studies were conducted with the immunogen emulsified in CpG1826 adjuvant, which was immediately injected subcutaneously into mice as recently described (10, 12, 53, 62).

Virus. The McKrae strain of HSV-1 and strain 333 of HSV-2 were used in this study. The virus was triple plaque purified and was prepared as previously described (54, 56). UV-inactivated HSV-1 and HSV-2 were made by exposing the live virus to a Philips 30-W UV bulb for 10 min at a distance of 5 cm. UV-inactivated virus was made by heating a virus solution at 100°C for 5 min. HSV inactivation was confirmed by the inability to produce plaques when tested on Vero cells as previously described (11, 52).

MHC-peptide binding assays. HLA-DR molecules were purified from homologous Epstein-Barr virus-infected cell lines by affinity chromatography with monomorph monomoclonal antibody (MAb) L243 coupled to protein A-Sepharose CL-4B...
gel (Amersham Pharma Biotech, Orsay, France) as described previously (70, 71). Binding to HLA-DR molecules was assessed by competitive ELISA as previously reported (71). HLA-DR molecules were diluted with an appropriate biotinylated peptide and serial dilutions of competitor peptides. Samples (100 µg/ml) were incubated in 96-well polypropylene plates (Nunc, Roskilde, Denmark) at 37°C for 24 to 72 h. After pH neutralization, samples were applied to 96-well Maxisorp ELISA plates (Nunc) previously coated with 10 µg/ml MAb L243 for HLA-DR molecules. Bound biotinylated peptides were detected by streptavidin alkaline phosphatase (Amersham, Little Chalfont, United Kingdom) with 4-methylumbelliferyl phosphate as the substrate (Sigma Chemical Co., St. Quentin Fallavier, France). Fluorescence (excitation at 365 nm, emission at 450 nm) was measured on a Wallac Victor2 1420 multilabel counter (Perkin-Elmer, Courbeaufl, France). Maximal binding was determined by incubating the biotinylated peptide with the MHC II molecule in the absence of competitor. Data were expressed as the concentration of peptide that prevented binding of 50% of the labeled peptide (IC50). Binding specificity for each HLA II was ensured by the choice of the biotinylated peptides as described previously (71). Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their IC50 variation did not exceed a factor of 3. Their sequences and IC50 are the following: HLA-DRB1*0401 (42 nM), HLA-DRB1*1001 (25 nM), and HLA-DRB1*1103 (16 nM). YKL (AAYAAKAALAA) for HLA-DRB1*0101 (6 nM); A3 152-162 (EAEEQLRAYLDGTGVE) for HLA-DRB1*1501 (13 nM); MT 2-16 (GAGTLTTLG) for HLA-DRB3*0101 (12 nM); and E2/E168 (AGDLLLAIETDKATI) for HLA-DRB4*0101 (10 nM).

Virus challenge. Ocular challenge was performed by dropping of 2 × 10^5 PFU of HSV-1 (McKrae) in 4 µl tissue culture medium on each eye, and gently rubbing the lid against the eye for 30 s.

PBMC isolation and HLA-DR typing. Peripheral blood mononuclear cells (PBMC) were prepared as previously described (5, 6, 27) by density gradient centrifugation of leukapheresis blood products provided by healthy individuals (PBMC) were prepared as previously described (5, 6, 27), by density gradient centrifugation of leukapheresis blood products provided by healthy individuals (PBMC) were prepared as previously described (5, 6, 27). Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their IC50 variation did not exceed a factor of 3. Their sequences and IC50 are the following: HLA-DRB1*0401 (42 nM), HLA-DRB1*1001 (25 nM), and HLA-DRB1*1103 (16 nM). YKL (AAYAAKAALAA) for HLA-DRB1*0101 (6 nM); A3 152-162 (EAEEQLRAYLDGTGVE) for HLA-DRB1*1501 (13 nM); MT 2-16 (GAGTLTTLG) for HLA-DRB3*0101 (12 nM); and E2/E168 (AGDLLLAIETDKATI) for HLA-DRB4*0101 (10 nM).

RESULTS

In vitro binding of potential CD4+ T-cell epitope peptides from selected gD to HLA-DR molecules. With the computer program TEPITOPE, we previously found 12 predicted DR-restricted epitope peptides from HSV-1 gD (2). The predicted peptides were synthesized and tested in vitro for binding to 10 soluble HLA-DR molecules (Table 1). This panel of available HLA-DR molecules consists of the most common HLA II haplotypes, regardless of ethnicity (32). The HLA-DRB1 locus encodes seven
of the HLA-DR molecules (HLA-DRB1*0101, HLA-DRB1*0301, HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101, HLA-DRB1*1301, and HLA-DRB1*1501). The HLA-DRB3, HLA-DRB4, and HLA-DRB5 molecules were encode by a second HLA-DRB locus. The relative binding capacity (nanomolar IC₅₀) for each peptide was calculated as the concentration of competitor peptide required to inhibit 50% of the binding of an allele-specific biotinylated peptide (indicator peptide) as described in Materials and Methods. Based on an upper threshold of 100 nM, which characterizes high-affinity peptide binders (17, 32), six peptides (gD1-29, gD49-82, gD96-123, gD121-152, and gD176-206 and gD300-334) bound to five or more different HLA-DR molecules, five peptides (gD22-52, gD77-104, gD146-179, gD226-257, and gD332-358) bound to four different HLA-DR molecules, and one peptide (gD228-257) did not bind to any of the HLA-DR molecules tested. This suggests that 11 of the 12 predicted peptides contain at least one universal T-cell epitope or several overlapping epitopes presented by multiple HLA-DR molecules.

The projected population coverage of each of the 12 gD epitope peptides was calculated on the basis of the phenotypic frequencies of the HLA-DR haplotypes (Table 2), assuming that these molecules are representative of all subtypes of the same haplotype. A combination of the six promiscuous epitope peptides that bound to five or more HLA-DR molecules would be predicted to cover a large proportion of the human population, regardless of ethnicity.

**Recall of gD epitope-specific CD4⁺ T cells in asymptomatic, HSV-seropositive individuals.** The cohorts of 55 (22 seronegative and 33 seropositive) individuals used to study T-cell proliferative responses (Tp-cell responses) to the 12 gD epitopes are shown in Table 3, column 2. All 33 seropositive individuals who had well-defined clinical histories with one or no episodes of recurrent disease/year (i.e., “protected asymptomatic” or “low-recurrent disease”) were healthy and hepatitis B virus and HIV seronegative. At the time of the study, none of them had had any active clinical ocular or genital herpes symptoms for at least 1 year. Based on their ethnic backgrounds, the 55 individuals were divided into 33 Caucasians (i.e., white) and 20 non-Caucasians (i.e., nonwhites or Asians, blacks, Hispanics, and members of other ethnic minorities).

The six highest Tp-cell responses were recalled by gD1-29, gD22-52, gD49-82, gD77-104, gD121-152, and gD176-206 (Fig. 2A). These six peptides also had medium to strong binding to multiple HLA-DR molecules. This suggests that each of these peptides bears a minimum of one promiscuous T-cell determinant or several overlapping determinants recognized by human T cells from multiple ethnicities. In contrast, gD96-123 and

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**TABLE 1. HSV-1 gD-derived human epitope binding to soluble HLA-DR molecules**

| Peptide     | HLA-DR1 | HLA-DR3 | HLA-DR4 | HLA-DR7 | HLA-DR11 | HLA-DR13 | HLA-DR15 | HLA-DRB3 | HLA-DRB4 | HLA-DRB5 |
|-------------|---------|---------|---------|---------|----------|----------|----------|----------|----------|----------|
|             | B1*0101| B1*0301| B1*0401| B1*0701| B1*1101  | B1*1301  | B1*1501  | B3*0101  | B5*0101  | B4*0101  |
|             | (12.9) | (11)   | (45.2)  | (29.0)  | (19.4)   | (6)      | (5)      | (51.4)   | (45.8)   | (23.9)   |
| gD1-29      | 58      | 79      | 54      | 57      | 64       | 68       | 53       | 70       | 59       | 69       |
| gD22-52     | 3       | 2,492   | 63      | 222     | 25       | 26       | 18       | 5,000    | 170      | 66       |
| gD49-82     | 3       | 1,249   | 93      | 173     | 120      | >100,000 | 18       | 5,000    | 170      | 66       |
| gD77-104    | 22      | 2,349   | ND      | 4       | 300      | >100,000 | ND       | >100,000 | ND       | 1        |
| gD96-123    | 3       | ND      | 61      | 37      | 598      | 4,762    | 167      | >100,000 | 1,672    | 102      |
| gD121-152   | 53      | 66      | 8       | 19      | 289      | 160      | 2        | >100,000 | 319      | 134      |
| gD146-179   | 54      | 1,0247  | 632     | 316     | 175      | >100,000 | 35       | 2,020    | 743      | 85       |
| gD176-206   | 4       | 307     | 40      | 200     | 44       | 2,049    | 13       | 31       | 3,742    | 68       |
| gD200-234   | 4       | 307     | 40      | 200     | 44       | 2,049    | 13       | 31       | 3,742    | 68       |
| gD228-257   | 1,162   | 2,392   | 9,920   | 20      | 39       | 1,587    | 22       | >100,000 | 1,163    | 22       |
| gD287-317   | 3,162   | 19,494  | 600     | 2,449   | 25,000   | >100,000 | 6,788    | 5,000    | 3,256    | 4,500    |
| gD332-358   | 150     | 1,643   | 5,872   | 274     | 5        | 56       | 950      | 2,307    | 703      | 31       |

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**TABLE 2. Expected and actual population coverages of HLA-DR binding gD epitope peptides**

| Peptide   | No. of DR molecules bound | Population coverage (%) |
|-----------|---------------------------|-------------------------|
|           | Projected | Actual |
| gD1-29    | 56.0 | 41.8 |
| gD22-52   | 53.8 | 28.4 |
| gD49-82   | 40.6 | 44.8 |
| gD77-104  | 52.8 | 39.9 |
| gD121-152 | 60.1 | 18.1 |
| gD146-179 | 72.8 | 34.5 |
| gD176-206 | 31.6 | 20.1 |
| gD200-234 | 64.6 | 32.7 |
| gD228-257 | 65.5 | 24.5 |
| gD287-317 | 52.5 | 22.2 |
| gD332-358 | 40.9 | 25.4 |

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* a Competitive binding of synthetic gD peptides bearing potential HLA class II epitopes to solubilized HLA-DR and HLA-DP molecules was tested in vitro. Results are expressed as means of HLA binding capacities determined from three independent experiments.

* b Shown in bold are IC₅₀ of <100 nM, which indicate gD peptides with high HLA binding capacities.

* c One of six MHC class II-restricted peptide sequences that bind to at least five HLA-DR and two HLA-DP4 polymorphic molecules.

* d Values in parentheses show percent population coverage (allelic frequencies in a Caucasian population). HLA-DP4 alleles (DP401 and DP402) are carried by 75% of individuals and are the most frequent HLA II alleles worldwide. Two gD peptides (gD211,152 and gD176,206) bind with high affinity to the HLA-DP401 molecule, whereas three peptides (gD228-257, gD300-334 and gD332-358) bind to HLA-DP402.

* e ND means binding of the peptide was not determined due to lack of its solubility.
gD200-234 peptides induced weaker Tp-cell responses, despite their high-affinity binding to four HLA-DR molecules. Ten of the 12 gD peptides induced similar Tp-cell responses in white and nonwhite populations. Only gD77-104 and gD176-206 appeared to induce higher Tp-cell responses in white than in nonwhite individuals (*P < 0.05*). No differences in Tp-cell responses were detected in individuals seropositive for HSV-1 compared to HSV-2 or compared to HSV-1/HSV-2 (data not shown). None of the 12 gD peptides produced induced positive proliferative response of CD4⁺ T cells from the 22 HSV-seronegative individuals, regardless of gender (Fig. 3D). As a control, CD4⁺ T cells from all 55 individuals produced positive responses to the mitogens LeuA and PHA (i.e., SIs of 32 to 45 and 10⁴ CD4⁺ T cells). Only gD77-104 and gD176-206 appeared to induce higher Tp-cell responses in white than in nonwhite individuals (*P < 0.05*). No differences in Tp-cell responses were detected in individuals seropositive for HSV-1 compared to HSV-2 or compared to HSV-1/HSV-2 (data not shown).

None of the 12 gD peptides produced induced positive proliferative response of CD4⁺ T cells from the 22 HSV-seronegative individuals, regardless of gender (Fig. 3D). As a control, CD4⁺ T cells from all 55 individuals produced positive responses to the mitogens LeuA and PHA (i.e., SIs of 32 to 45 and Δcpm values of 54 × 10³ to 62 × 10³). However, CD4⁺ T cells alone, without Ag stimulation, produced low background responses (i.e., SIs of 3.8 to 4.3 and Δcpm values of 3 × 10³ to 5 × 10³).

The HLA-DR dependence of the peptide-specific T-cell responses was confirmed in a representative HLA-DR blocking experiment, as shown in Fig. 2B. Blockage of HLA-DR molecules resulted in a >95% reduction (*P < 0.005*) in Tp-cell responses to either 10 or 1 µg/ml gD1-29 or gD49-82 peptide. However, blockage of HLA-DQ molecules did not affect Tp-cell responses induced by either gD1-29 or gD49-82 peptide (e.g., at 10 µg/ml, the gD1-29-specific Tp responses were Δcpm values of 32.9 × 10³ and 32.1 × 10³ in the presence and absence of anti-HLA-DQ MAb, respectively).

Gender-dependent CD4⁺ T-cell responses to three gD peptide epitopes in asymptomatic, HSV-seropositive individuals. IFN-γ secretion is a marker of T-cell activation that is frequently dissociated from the Tp-cell response (5, 6, 27). In addition, HSV-1 and -2 are susceptible to IFN-γ interference (2). To determine whether the T-cell response to the 12 gD peptides is gender dependent, gD epitope-specific IFN-γ-producing CD4⁺ T-cell responses were examined in a larger cohort of age-matched, HSV-seropositive, asymptomatic individuals composed 43 women and 44 men (Table 3, column 3). A preliminary analysis of IFN-γ-producing CD4⁺ T cells revealed that 95 or more spots/10⁴ CD4⁺ T cells gave a 97% probability of defining a positive response. Of the 960 separate ELISPOT wells used to screen 40 HSV-seronegative individuals, only 32 wells (3%) exceeded 95 spot-forming cells (SFC)/10⁴ CD4⁺ T cells. Of the 1,128 separate ELISPOT assays used to screen the 47 seropositive individuals, 192 (17%) had readings above 95 SFC/10⁴ CD4⁺ T cells. A value of 95 SFC/10⁴ CD4⁺ T cells was therefore used as the cutoff point for subsequent analyses.

Three epitope peptides (gD49-82, gD77-104, and gD121-152) induced significantly higher CD4⁺ Tp responses in HSV-seropositive women than in men (*P < 0.05*) (Fig. 3A). All three of the epitope peptides that induced higher Tp responses in women than in men and two additional epitope peptides (gD200-234 and gD228-257) induced significantly higher levels of IFN-γ-producing CD4⁺ T-cell responses in women than in men (*P < 0.05*) (Fig. 3B). The largest gender-dependent difference appeared to occur with gD77-104 (*P < 0.0001*). The levels of IFN-γ production in response to PHA were similar in men and women (data not shown). To ascertain the epitope specificity of gender-dependent CD4⁺ T cells detected in seropositive individuals, we used an irrelevant HLA-DR-restricted peptide epitope derived from ovalbumin (OVA), i.e., OVA23-33 (45). The geometric mean of T-cell proliferation in response to the OVA323-339 peptide was low and similarly not significant in both males and females. The IFN-γ response was also HSV epitope specific, since no IFN-γ production was induced when HSV-specific T-cell lines were incubated with the CD4⁺ T cell OVA323-339 peptide (Fig. 3C). Taken together, these results suggest that, compared to those from men, CD4⁺ T cells from HSV-seropositive, asymptomatic women responded to the gD49-82, gD77-104, and gD121-152 epitope peptides with higher frequency and magnitude.

Gender-dependent CD4⁺ T-cell responses confirmed in HLA-DRB1*0101 and HLA-DRB1*0401 Tg mice. Fourteen days after ocular infection with HSV-1 (2 × 10⁶ PFU/eye, strain McKrae) or intraperitoneal injection of vaccinia virus expressing HSV-1 gD (VVgD), splenocyte CD4⁺ T cells from HLA-DR1 and HLA-DR4 Tg mice were recalled in vitro with the 12 individual epitope peptides and IFN-γ ELISPOT assays as described above. Studies similar to those described above with humans determined that the cutoff for a positive response in mice was 50 spots/10⁴ cells (data not shown). The results were similar for HLA-DR1 and HLA-DR4 mice, as well as with HSV-1 and VVgD (Fig. 4). In all instances in which a positive IFN-γ response was detected, female mice had a higher response than age-matched male mice. In addition, the same eight peptides produced a positive IFN-γ response regardless of the mouse strain or HSV-1 versus VVgD infection. Similar to the case for humans, gender-dependent IFN-γ-producing CD4⁺ T-cell responses against the gD1-29, gD49-82, gD77-104, and gD121-152 peptides were detected in HLA-DR Tg mice (Fig. 4A and B). However, the HLA-DR Tg mice revealed a gender-dependent T-cell response against four additional peptides: gD96-123, gD146-179, gD176-206, and gD200-234. Interestingly, although a gender-dependent IFN-γ-producing CD4⁺ T-cell response to gD228-257 was seen in humans, this
FIG. 2. CD4⁺ T-cell proliferative response to HSV-1 gD-derived epitopes. (A) Fresh peripheral blood-derived CD4⁺ T cells were isolated and stimulated in vitro with 12 individual gD peptides in a 6-day proliferation assay as described in Materials and Methods. The Tp-cell responses of Caucasian (white) and non-Caucasian (nonwhite) ethnic groups against each peptide, assessed by a [³H]thymidine incorporation assay, were compared. Proliferative responses were expressed as Δcpm values (counts per minute of cells incubated with peptide − counts per minute of cells without peptide). The mean of each group is shown as a solid horizontal line. An analysis of T-cell responses in 22 seronegative individuals is shown. A positive T-cell proliferative response was defined on the basis of the responses of HSV-seronegative control individuals, with a Δcpm value of ≥1,000 and an SI (counts per minute of cells incubated with peptide/counts per minute of cells without peptide) of ≥2. The values at the bottom of each data set (i.e., 30/35) are the mean number of responders/total number of individuals tested. The value at the top of each data set (i.e., 11.5) is the mean SI of each of the two groups, i.e., white or nonwhite. The P values compare the Tp-cell responses between white and nonwhite groups by one-way ANOVA. An asterisk in parentheses indicates a peptide that induced higher T-cell proliferation compared with HSV-seronegative control individuals (i.e., significantly higher SI and Δcpm value). (B) Analysis of HLA-DR restriction and functional frequencies of HSV-1 gD peptide-specific CD4⁺ T cells. Blocking of PBMC proliferation in response to the gD₁₋₂₉ and gD₉₋₈₂ peptides by specific anti-HLA-DR MAbs.
epitope peptide did not induce any significant response in mice. The IFN-γ responses against gD peptides detected in HSV-1-infected HLA Tg mice were specific, since no IFN-γ production was observed when gender-dependent gD49-82-, gD77-104-, and gD121-152-specific T-cell lines were incubated in vitro with a heterologous HLA-DR-restricted T-cell epitope from HSV-1 gB (i.e., gB161-175) (Fig. 4C).

The IFN-γ production was abrogated by anti-HLA-DR, but not by anti-HLA-A2.1, MAbs (i.e., in HLA-DR1, IFN-γ ELISPOT response against gD49-82 peptide; 152 spot-forming cells [SFC] without MAb reduced to 33 SFC and 148 SFC when anti-HLA-DR and anti-HLA-A, -B, and -C were added, respectively). The IFN-γ response was gD peptide specific, since no IFN-γ production was observed in response to a heterologous gB CD4+ Th peptide (Fig. 4C). Overall these results confirm that, similar to humans, a gender difference in CD4+ T-cell responses was detected in age-matched HLA-DR1 and HLA-DR4 Tg mice against the gD1-29, gD49-82, gD77-104, and gD121-152 peptides following either HSV infection or VVgD immunization.

Immunization with a pool of immunodominant gD epitope peptides induced CD4+ T-cell-dependent protective immunity against lethal herpesvirus infection in HLA-DR*0101 Tg mice. Three epitope peptide pools (gD49-82, gD77-104, and gD121-152) were selected as the “best” overall on the basis of three criteria: (i) high-affinity binding to multiple HLA class II molecules, including HLA-DRB1*0101 (Table 1); (ii) strong recall of CD4+ Tp-cell and IFN-γ responses in HSV-seropositive, asymptomatic individuals (Fig. 2 and 3); and (iii) high recall of IFN-γ-producing CD4+ T cells in HLA-DRB1*0101 Tg mice (Fig. 4).

A pool of these three peptides was then used to immunize susceptible HLA-DR1 Tg mice against a lethal HSV-1 challenge. The previously described protective epitope gD1-29 (20, 21, 53, 68, 86), which exhibited similar properties, was excluded from these experiments. A pool of the gD146-179, gD287-317, and gD332-358 subdominant peptides was used as a control. Three groups of 20 HLA-DR*0101 Tg female mice were immunized with (i) a pool of the gD49-82, gD77-104, and gD121-152 peptides emulsified in CpG1826 adjuvant; (ii) a pool of the gD49-82, gD77-104, and gD121-152 peptides emulsified in CpG1826 adjuvant; or (iii) the CpG1826 adjuvant alone (mock-immunized control). Two weeks after the second immunization, all mice in each group were challenged ocularly with a lethal dose of 5 x 10^5 PFU of HSV-1 (McKrae strain). All of the mice that died following the challenge did so between days 8 and 14 postinfection. The HLA-DR*0101 Tg mice immunized with the immunodominant CD4+ T-cell epitope peptide pool were protected against death, compared to those immunized with the subdominant CD4+ T-cell epitope peptide pool (95% versus 25% survival, P < 0.05) or adjuvant alone (0% survival) (Table 4). When the mice immunized with the immunodominant CD4+ T-cell epitope peptide pool were depleted of CD4+ T cells prior to virus challenge, survival was significantly decreased (Table 4). In contrast, depletion of CD8+ T cells had no significant effect. Depletion of either T-cell type had no significant impact on the mock immunized or subdominant immunized groups. Thus, with this immunization regimen, CD4+ T cells were required and CD8+ T cells were not sufficient for protective immunity against lethal ocular HSV-1 challenge.

To assess a gender dependence in protective immunity induced by gD peptides, 10 age-matched HLA-DR*0101 Tg female and male mice were immunized with the individual gD49-82, gD77-104, and gD121-152 peptides emulsified in CpG1826 adjuvant. Two weeks after the second immunization, both genders were similarly challenged ocularly with a lethal dose of HSV-1 as described above. The HLA-DR*0101 Tg female mice immunized with the immunodominant CD4+ T-cell gD49-82, gD77-104, and gD121-152 peptides showed better protection against death compared to male mice immunized with the same epitopes (65% versus 35% survival for gD49-82, 60% versus 25% survival for gD77-104, and 55% versus 20% survival for gD121-152, respectively). This result indicates, similar to their T-cell immunogenicity, that there was also a gender-dependent protective immunity induced by the immunodominant CD4+ T-cell gD49-82, gD77-104, and gD121-152 peptides.

DISCUSSION

We report here the identification of a panel of HLA-DR-restricted epitope peptides derived from HSV-1 gD, a glycoprotein that produces protective immunity against herpesvirus infection and disease in animal models and humans (20, 21, 53, 68, 86). These epitopes previously identified by screening HSV-1 gD for peptides that contain the HLA-DR supertype binding motif (2), We demonstrated that 11 of the 12 predicted peptides bound with high affinity to three or more different types of HLA-DR molecules. Three of them (i.e., gD49-82, gD77-104, and gD121-152) recalled significantly higher responses in CD4+ T cells from asymptomatic, HSV-seropositive women than in those from men. A pool of these three epitope peptides induced a gender-dependent protective CD4+ T-cell-dependent immunity against HSV challenge in HLA-DR*0101 and HLA-DR*0401 Tg mice.

In humans, HSV-specific CD4+ T cells play a crucial role in controlling both primary and recurrent infections (23, 60). In the late 1980s and early 1990s, Zarling and coworkers generated several human CD4+ T-cell clones from HSV-1-seropositive, asymptomatic individuals by stimulating peripheral blood lymphocytes (PBL) with a recombinant vaccinia virus expressing HSV-1 gD (83, 84). Five of these CD4+ T-cell clones lysed autologous HSV-1-infected lymphoblastoid cell lines, and their cytotoxicity was restricted to HLA class II molecules. Later, it was demonstrated that some of these HSV-1-specific T-cell clones were directed against both gD-1 and gD-2 by using target cells infected with wild-type HSV strains, a gC deletion.
A  HSV-1 (McKrae)

B  Vaccinia expressing gD (VVGd)

C  

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TABLE 4. Immunization with HLA-DR-restricted gD epitope peptides in CpG1826-adjuvant confers protective immunity to a lethal HSV-1 challenge in HLA-DR*0101 Tg mice

| Immunization group | No. of survivors/no. challenged* (% survivors) | P value vs control |
|--------------------|-----------------------------------------------|-------------------|
| Untreated          | CD4 depleted† | CD8 depleted† |
| 1                  | 19/20 (95)   | 0/20 (0)       | 18/20 (90)      | <0.005 |
| 2                  | 5/20 (25)    | 3/20 (15)      | 3/20 (15)       | >0.005 |
| Control†           | 0/20 (0)     | 0/20 (0)       | 0/20 (0)        |       |

a HLA-DR*0101 Tg mice, 60 per group, were immunized subcutaneously on day 0 and day 21 with 100 μg each of immunodominant gD241-260, gD209-231, and gD228-257, pooled as a group of three peptides and emulsified in CpG1826 adjuvant (group 1) or with 100 μg each of subdominant gD146-179, gD233-256, and gD133-166 pooled as a group of three peptides (group 2). Control mice received CpG1826 adjuvant alone.

b Twenty immunized HLA-DR*0101 Tg mice were left untreated.

c Twenty immunized HLA-DR*0101 Tg mice were depleted of CD8+ T cells with a MAb specific to mouse CD8.

d Twenty immunized HLA-DR*0101 Tg mice were depleted of CD8+ T cells with an irrelevant isotype control MAb.

* The P values shown are for comparisons of immunized versus control mice by ANOVA.

The minimum requirements needed to utilize this approach are (i) existing sequence data for the proteins in question; (ii) access to the genome (DNA or RNA) of the pathogen for the purpose of generating recombinant vaccinia virus vectors; (iii) HSV-seropositive, asymptomatic individuals; and (iv) HLA Tg mice. Moreover, the present strategy accurately identified regions bearing all of the previously reported gD epitopes: gD14-23 (24, 28, 36), gD241-260 (34, 48, 80), and gD209-231 (24). Therefore, our approach to epitope identification, as well as testing epitope peptides for their T-cell antigenicity, immunogenicity, and protective efficacy, should be nearly universal, regardless of the protein studied.

Recently, we reported four immunodominant H2d-restricted CD4+ T-cell epitope peptides on HSV-1 gD (2). Among these, the peptide gD241-260, a naturally processed epitope, protects H2d mice against lethal oral HSV-1 challenge (2). We have also recently found that the gD146-179 epitope contains an 8-mer, gD153-161, that represents an immunodominant HLA-A2.1-restricted human CD8+ T-cell epitope (18). Therefore, a minimum of two human epitopes, the gD146-179, CD8+ T-cell epitope and the CD4+ T-cell epitope, are nested within the gD241-260 peptide and this epitope peptide represents an excellent candidate for inclusion in a CD4+ and CD8+ T-cell epitope-based human vaccine.

Humans are not immunologically naive and often develop memory T cells that cross-react with and respond to unrelated pathogens or Ags, a phenomenon termed heterologous immunity (reviewed in references 64 and 76). Interestingly, although an IFN-γ-producing CD4+ T-cell response specific to gD228-257 was detected in HSV-seronegative individuals, no significant T-cell responses specific to this epitope peptide were detected in HSV-infected HLA-DR mice. This result suggests that T cells specific to gD228-257 detected in humans might cross-react with and respond to a yet-to-be-identified, unrelated-pathogen-derived epitope, a recently described phenomenon termed heterologous immunity (reviewed in references 64 and 76). The processing and presentation to T cells of the gD228-257 epitope could also be different between HLA Tg mice and humans. Therefore, some apparently CD8-specific CD4+ T cells identified in this study might have arisen due to cross-reaction with an unrelated pathogen. Such scenarios have been reported in murine CD4+ T-cell responses to cytomegalovirus (22, 64, 76) and may be true for HSV (22, 64, 76, 77). To help ascertain whether the detected CD4+ T-cell responses against the gD epitope peptides were primed by HSV,
the epitope mapping was extended to pathogen-free HLA-DR1 and HLA-DR4 Tg mice that were infected with either HSV-1 or VVgD. The results suggested that CD4⁺ T cells from both HLA-DR1- and HLA-DR4-infected Tg mice also recognized five of the six epitopes that were reactive to human CD4⁺ T cells (gD₁-29, gD₉₉-₈₂, gD₇₇-1₀₄, gD₁₂₁-₁₅₂, and gD₂₀₀-₂₃₄). In addition, HLA-DRB₁*₀₁₀₁ and DRB₁*₀₄₀₁ Tg mice also mounted CD4⁺ T-cell responses against three epitope peptides not recognized by humans. This may reflect the high level of infection in this mouse system. The ability of epitope peptides not recognized by humans. This may reflect CD4⁺ T cells (gD₁-29, gD₉₉-₈₂, gD₇₇-1₀₄, and gD₁₂₁-₁₅₂) and gD₂₀₀-₂₃₄ epitope peptides to protect against HSV challenge in HLA-DRB₁*₀₁₀₁ and DRB₁*₀₄₀₁ Tg mice is also a critical parameter. These mouse studies suggest that it is very likely that the gD₁-29, gD₉₉-₈₂, gD₇₇-1₀₄, gD₁₂₁-₁₅₂, and gD₂₀₀-₂₃₄ epitope peptides detected in humans were not cross-reactive with nonherpesvirus Ags or pathogens but rather were induced following HSV-1 or HSV-2 infection. This does not exclude the involvement of other infectious agents in shaping herpesvirus CD4⁺ T-cell responses. It also remains to be determined (i) whether the gender-dependent T-cell responses to gD epitopes will translate in a serotype-specific protective immunity against HSV-1 and/or HSV-2 and (ii) whether a therapeutic immunization with gD epitopes will protect latently HSV-1- and/or HSV-2-infected HLA-DR Tg male and female mice.

gD is a leading herpes vaccine candidate Ag that has been successful in eliciting protective immunity in women but failed to protect men (8, 68, 69). However, the gD T-cell epitopes that might be targeted during this apparent gender-dependent protective immunity have not been reported. In this study, there were gender disparities in the CD4⁺ T-cell responses of HSV-seropositive individuals to a discrete set of gD epitopes. Indeed, HSV-seropositive women exhibited higher magnitudes of CD4⁺ T-cell responses to three gD epitopes (gD₉₉-₈₂, gD₁₂₁-₁₅₂, and gD₂₀₀-₂₃₄) compared to age- and ethnicity-matched, HSV-seropositive men. In addition, a gender- and T-cell-dependent protective immunity was induced in susceptible HLA-DR Tg mice by the same three immunodominant CD4⁺ T-cell peptide epitopes (gD₉₉-₈₂, gD₁₂₁-₁₅₂, and gD₂₀₀-₂₃₄). Thus, results reported here are in agreement with the gender-dependent protective immunity reported in the above-cited clinical trial and both suggest that sex factors may affect the host’s immune response to herpesvirus epitopes. Although the present study was mainly focused on CD4⁺ epitopes, the possible involvement of gD CD8⁺ T-cell epitopes in shaping this gender-dependent protective immunity has not been ruled out. Altogether, these data indicate that gender should be taken into account during the evaluation of T-cell epitope-based herpes vaccines. Boosting the recombinant gD immunization with a discrete set of gD T-cell epitopes may be considered particularly for women, while boosting the immunization of men with a different, nonoverlapping set of gD epitopes may be warranted.

The first indication of a gender difference in herpes immunity was reported by Cantin and coworkers, who showed that female mice have low-grade infection and disease compared with strain- and age-matched male mice (35). Besides a possible association with the IFN-γ molecule (35), the immune mechanisms contributing to gender-dependent susceptibility to herpesvirus infection and disease have not been reported. A recent study by Diamond and coworkers demonstrated a higher frequency of memory T cells specific to human cytomegalovirus—another virus of the herpesvirus family—that produce higher levels of Th1 cytokines in women than in men (74). On the contrary, Klein and coworkers showed that women maintain lower levels of varicella-zoster virus-specific memory T cells compared to men (37). Although the above studies suggest that gender differences in memory T-cell responses exist for herpesviruses other than HSV-1 and HSV-2, no specific gender-associated Ags or epitopes have ever been defined. Identification of the viral Ags or epitopes recognized by T cells from HSV-seropositive men and women―either vaccinated individuals or individuals with naturally acquired herpes immunity (i.e., seropositive, asymptomatic individuals)—would facilitate the process of designing an efficient vaccine. It is possible that gender-dependent CD4⁺ T-cell responses detected in this study reside at the Ag-presenting cell (APC) level rather than at the T-cell level. Indeed, in other systems, female mice that are more susceptible to experimental allergic encephalomyelitis preferentially mount Th1 immune responses and their APCs produce IL-12 but not IL-10 (78). In contrast, the APCs derived from resistant male mice produce IL-10 but not IL-12. Activation of T cells with APCs derived from the opposite sex demonstrated that these cytokines were derived from the respective APC populations (78). Other studies have demonstrated that gender differences in T-cell responses are mediated, in part, by differences in sex hormone-immune interactions (37, 42, 51, 57, 74, 78). Kovats and coworkers were the first to demonstrate that sex hormones such as estrogen play a crucial role in regulating dendritic cell phenotype and functional differentiation (42, 51, 57). Whether an increase in dendritic cell phenotypic and functional maturation leads to the strong gD epitope-specific CD4⁺ T-cell responses detected in seropositive women or to an increase in the function of gD-specific memory T cells remains to be determined. Nevertheless, regardless of the mechanisms, our findings are the first demonstrating that a discrete set of gD CD4⁺ T-cell epitope peptides are highly targeted in women compared to men and were able to elicit a protective immunity in female HLA-DR Tg mice but not in age-matched male mice. Altogether, these data suggest that gender restrictions to herpes vaccines may be overcome by focusing the immune response toward selected sets of epitopes.

The vast majority of the world’s human population is infected with HSV-1 and/or HSV-2 and may benefit from therapeutic vaccination approaches designed to induce vigorous HSV-specific cellular immunity at a higher magnitude and breadth than the suboptimal natural immunity. Although the primary goals of this study were to evaluate the antigenicity of the HSV-1 CD4⁺ T-cell epitope peptides and the gender-specific responses to these epitopes, a secondary but important goal is to develop the knowledge of protective herpesvirus T-cell epitopes required for the development of a lipopeptide-based vaccine. Focusing on the immune response toward such epitopes could be of value in the case of HSV-1 and HSV-2 infections, where T cells directed against the immunodominant epitopes might have been inactivated and T cells specific for subdominant epitopes might have escaped T-cell tolerance. A question of practical importance is the translation of the current immunological findings for the development of an
epitope-based lipopeptide vaccine for a genetically heterogeneous human population. (1, 4, 7, 53, 59, 85, 86). Accordingly, a herpes vaccine will need to induce responses against a number of promiscuous epitopes that are recognized in the context of many different HLA alleles. Several studies have demonstrated that the majority of HLA-DR molecules can be grouped into broad supertypes with overlapping peptide binding specificities (65). In the case of the HLA-DR molecules, a single superfamily encompassing DRB1 alleles that are expressed in the majority of humans has been defined (67). HLA-DR supertypes have been studied in detail, and several CD4+ T-cell epitopes that belong to these supertypes have been identified and validated (9). Broad population coverage can be established, provided that epitopes corresponding to multiple HLA supertype families are incorporated into the vaccine. From the standpoint of vaccine development, the panel of immunodominant gD peptides identified in this study has the potential to induce CD4+ T-cell responses in up to 92.9% of an average human population. The fact that each of the peptides was antigenic in the human cohort studied is most likely a result of focusing the screening exclusively on peptides capable of binding multiple HLA-DR alleles. Similar correlations have been found between promiscuous HLA-DR binding capacity and antigenicity for HIV peptides (79). Because of their apparent promiscuousness, these CD4+ T-cell epitope peptides should prove to be useful in vaccines for the heterogeneous human population. To maximize efficacy, a multi-epitope-based herpes vaccine should probably include several T-cell epitopes from several different structural glycoproteins and regulatory proteins, each chosen to represent the HLA supertypes known to provide recognition in a large proportion of the global population, regardless of race and ethnicity.

In the past 2 decades, there has been increasing recognition of a worldwide pandemic of herpesvirus infection despite the widespread use of antiviral drug therapies (reviewed in reference 39). Although drug therapy can limit morbidity and mortality from active herpes disease, it cannot cure infection. In addition, considering cost, toxicity, and viral escape issues, the usefulness of drug treatment might be limited, especially in underdeveloped regions such as parts of sub-Saharan Africa, where 70% of high-risk, HIV-negative individuals and 85% of HIV-infected individuals are seropositive for herpesvirus (46, 61, 75). Developing an effective immunophenotypic or immunotherapeutic vaccine against herpesvirus infection must be an important global health priority. The use of multivalent epitope vaccines is a promising approach to fighting herpesvirus infections—yesterday, today, and tomorrow. © 2008 by Springer-Verlag New York, Inc.

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