Protective Immunity against Ocular Herpes Infection and Disease Induced by Highly Immunogenic Self-Adjuvanting Glycoprotein D Lipopeptide Vaccines

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PURPOSE. An important phase in the development of an ocular herpes simplex virus type 1 (HSV-1) subunit vaccine is the identification of an efficient, safe, and adjuvant-free antigen delivery system capable of inducing and sustaining long-term memory T-cell protective immunity. This study was conducted to test the hypothesis that immunization with self-adjuvanting lipopeptide bearing HSV-1 glycoprotein D (gD) T-cell epitopes would elicit long-term HSV-specific T cells and decrease infection, disease, or both in a ocula r herpes mouse model.

METHODS. Five immunodominant CD4 T-cell peptide epitopes (gD1-29, gD200-292, gD146-179, gD228-257, and gD332-358), recently identified from HSV-1, gD, were covalently linked to a palmitic acid moiety (lipopeptides) and delivered subcutaneously in adjuvant-free saline. The primary and memory T cells induced by these molecularly defined lipopeptides and their protective efficacy were assessed, in terms of virus replication in the eye, ocular disease, and survival.

RESULTS. Three gD lipopeptides, that drive dendritic cell maturation in vitro, induced long-term, virus-specific, IFN-γ-producing CD4 Th1 responses, associated with a reduction in ocular herpes infection and disease. Immunization with a cocktail of these three highly immunogenic Th1 lipopeptides increased survival, lowered the peak of ocular virus titer, and cleared the ocular disease.

CONCLUSIONS. Vaccination with a mixture self-adjuvanting lipopeptides containing novel HSV-1 immunodominant gD T-cell epitopes protected mice from ocular herpes infection and disease. The strength of protective immunity induced by these lipopeptides together with their safety provide a molecularly defined vaccine formulation that could combat ocular herpes infection and disease in humans. (Invest Ophthalmol Vis Sci. 2007;48:4643–4653) DOI:10.1167/iovs.07-0356

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ease, or both. We found that three palmitoyl-tailed gD lipopeptides, as opposed to their nonlipidated peptide analogues, induced potent HSV-specific IFN-γ-producing CD4+ T cells and were associated with a reduction in ocular infection and disease. Of interest, immunization with a mixture of these three highly immunogenic gD lipopeptides afforded better protection than any individual lipopeptide. Finally, the mixture of the highly protective lipopeptides induced the maturation of dendritic cells (DCs). This preclinical study provides a first proof-of-concept that the lipopeptide approach is a safe and efficient antigen delivery system for protection against ocular herpess.

**Materials and Methods**

**Antigens and Immunogens**

The HSV-1 glycoprotein gD peptides used in this study are shown in Figure 1A and have been extensively described.10,15,31 The lipidation was performed using the chemoselective ligation chemistry coupling method32 as detailed in the Results section.

**Herpes Simplex Virus Type 1**

HSV-1 (strain McKrae) was used in this study,35,54 and was grown and titrated on RS (rabbit skin) cells. Heat-inactivated HSV-1 was generated by incubating live virus at 65°C for 8 minutes. HSV inactivation was confirmed by the inability to produce plaques when tested on RS cells. Heat-inactivated HSV-1 was generated and titrated on RS cell monolayers, as described previously.33,34

**Immunization and Virus Challenge**

Five-week-old female BALB/c mice (H2d; The Jackson Laboratories, Bar Harbor, ME) were immunized subcutaneously (subcutaneously) at the base of the tail, on days 0 and 21, with 100 µg/dose of each gD lipopeptide. Control mice were injected with an equimolar amount of parental-peptide alone or with saline alone. Before HSV-1 challenge, mice were examined to exclude those with ocular disease. For ocular infection and disease, the corneas were inoculated without scarification using 5 M streptomycin and 2% newborn calf serum) and frozen at −80°C until titrated on RS cell monolayers, as described previously.35,54

**Generation of Bone Marrow–Derived Dendritic Cells**

Murine DCs were generated from naïve BALB/c mice by feeding bone marrow–derived cells with fresh medium supplemented with 25 ng/mL granulocyte-monocyte–colony-stimulating factor (GM-CSF) and 25 ng/mL interleukin (IL)-4 every 72 hours, as we previously described.56 The phenotype of DCs was confirmed by flow cytometry using CD11c (clone HL3) and DEC-205 (clone MG38) surface markers (PharMingen, San Diego, CA).

**T-Cell Assays**

**T-Cell Proliferation.** Cells isolated from the draining inguinal lymph nodes (DLNs) or spleen (SP) were cultured at 5 × 10⁶ cells/well in serum-free HL-1 medium supplemented with 15 mM HEPES, 5 × 10⁻⁷ M β-mercaptoethanol, 2 mM glutamine, 50 IU penicillin, and 50 µg streptomycin (Invitrogen-Gibco, Grand Island, NY) (complete medium). The cells were stimulated with immunizing peptides at 100, 30, 10, 3, 1, or 0.3 µg/mL or with heat inactivated HSV-1 at 10, 3, 1, or 0.3 multiplicities of infection (MOI; equivalent to 10⁻⁷, 5 × 10⁻⁷, 10⁻⁶, 5 × 10⁻⁶, or 10⁻⁵ pfu/mL of the original titer of the virus before heat inactivation). One µCi of ³H-thymidine (Dupont NEN, Boston, MA) was added to each well 16 to 20 hours before harvest, and the incorporation of ³H-thymidine into DNA was determined by liquid scintillation counting.

**Figure 1.** Illustration of the HSV-1 gD T-cell epitope lipopeptides used in this study. (A) The amino acid sequence in a single letter code and position of five HSV-1 (strain 17) gD lipopeptides bearing immunodominant CD4+ T-cell epitopes. Black box: transmembrane domain of gD. (B) The structure of HSV-1 gD1042C palmitoyl-Hipidated peptide (lipopeptide). The first NH₂-terminal amino acids of the gD1042C peptide backbone are extended with a lysine (K) spacer that is covalently coupled to a single molecule of palmitic acid using the chemoselective ligation.
added to each well during the last 16 hours of culture. The incorporated radioactivity was counted on a direct ionization counter (Matrix 96; PerkinElmer Biosystems, Meriden, CT).13,30-37

**Cytokine Assay.** The amounts of IFN-γ, IL-2, IL-4, and IL-12 produced by SP-derived CD4⁺ T cells stimulated with either immunizing parental peptide (10 μg/mL) or with heat-inactivated HSV-1 were determined in supernatants of cell cultures by using sandwich ELISA kits (PharMingen).

**ELISPot Assay.** An IFN-γ ELISPot assay was performed as previously described.31 CD4⁺ T cells were purified by incubation of SP cells for 45 minutes with anti-CD4-coated magnetic beads (PharMingen) and positively sorted on a MACS column. CD4⁺ T cells were cocultured with DCs preincubated with 10 μM of immunizing peptide or with heat-inactivated HSV-1 (MOI = 5 or 3 × 10⁶ pfu/mL), irradiated (3000 rad from a 137Cs source) at a T-cell/DC ratio of 4 to 1.

**Flow Cytometry**

The following mAbs were used in single-color flow cytometry analysis: PE-CD80 (clone 10-10A1, IgG1), PE-CD86 (clone GL1, IgG2a), and FITC-MHC-II (clone FL18.26, IgG2b) (all from PharMingen). The cells were acquired at 20,000 events of flow cytometry (FACScalibur, analyzed by CellQuest software; BD Biosciences, San Jose, CA).

For in vivo depletion of T-cells, immunized mice were injected intraperitoneally on days -7, -1, 0, 2, and 5 after injection with six doses of 0.1 mL of PBS containing 0.1 mg of GK1.5 mAb (anti-CD4), 2.43 mAb (anti-CD8), or hamster IgG control mAb (NCCC, Minneapolis, MN). Treatment with these mAbs resulted in depletion of >98% of the T cells.

**Statistical Analysis**

Each experiment was performed at least twice; the number of animals used in each test is specified in the figure legends. Wherever specified, data were analyzed for statistical significance by a two-tailed standard Student’s t-test and the Fisher exact test (Statview II statistical program; Abacus Concepts, Berkeley, CA).

**RESULTS**

**Construction and Physicochemical Characteristics of Lipopeptide Vaccines Derived from HSV-1 gD Protective Regions**

Five nonoverlapping, 26- to 33-amino-acid synthetic lipopeptides were derived from the extracellular region of HSV-1 gD and one from the intracellular region. These lipopeptides encompass the previously described gD1-29,31,38 epitope and four new peptides—gD49-82, gD146-179, gD228-257, and gD352-358—found to contain at least one CD4⁺ T-cell epitope (Fig. 1A).13,39 Each of the five peptides was covalently linked to a single Nε-succinimidyl-hylysin residue via a functional base lysine ε-amino group (Fig. 1B). This linking was performed by using the newly described chemosynthetic ligation that allows synthesis of molecularly pure, high-yielding and water-soluble lipopeptides.32 Unlike the first-generation lipopeptides constructed using the classic solid-phase method, which introduces the fatty acyl moiety to the crude peptide backbone before its purification,37,38,40,41 the lipopeptides used in this study were constructed in two steps. A first step of synthesis and purification of the peptide backbone was tailed by a second step of ligation of the lipid moiety site-specifically introduced in solution.32 The physicochemical properties of each lipopeptide are compatible with multidimensional analysis, using reversed-phase (RP)-HPLC, two-dimensional nuclear magnet resonance (2D-NMR), Edman sequencing, and electrospray mass spectrometry (not shown). The novel process of lipopeptide synthesis is also compatible with cysteinyl pep-

tides, such as the gD49-82, with full solubility obtained when lipopeptides were formulated in water or in PBS solutions at concentrations as high as 3 mg/mL. After multiple freeze-thaw cycles, over a period of 1 year, the physicochemical properties of each lipopeptide were preserved. Detailed physicochemical analyses of the lipopeptides’ impurity profiles led to the detection of a molecularly defined formulation with relatively small proportions of by-products (not shown).

**Primary T-Cell Immunogenicity of gD Lipopeptides Delivered in Adjuvant-Free Saline**

We evaluated the primary T-cell responses induced after subcutaneous delivery in H2b mice of individual gD1-29, gD49-82, gD146-179, gD228-257, and gD352-358 lipopeptides in adjuvant-free saline. In an initial experiment, we performed a dose–response study using 50-, 100- or 200-μg doses of each lipopeptide delivered subcutaneously. Nonlipidated peptide analogues, also subcutaneously delivered in an adjuvant-free saline, were used as the control. All three doses induced a similar magnitude of T-cell responses (not shown). There were no obvious vaccine-related severe side effects with any of the lipopeptides at any dose, as evaluated by weight loss or injection-site local reactions. Accordingly, subsequent experiments were performed using the middle dose of 100 μg. Two weeks after the second injection, four of the five gD lipopeptide-elicited T cells that recognized both the immunizing peptide epitopes (Fig. 2A) and heat-inactivated HSV-1 particles (Fig. 2B, P < 0.05). The highest T-cell responses were induced by lipopeptides gD1-29, gD49-82, gD146-179, and gD352-358. There were no significant HSV-specific T-cell responses in control mice injected with the parental nonlipidated peptides in saline, indicating the absolute requirement of the attached lipid moiety (Figs. 2A, 2B; P > 0.05). The induced primary T-cell responses were abrogated in vitro by an mAb against CD4 molecules, but not by an mAb against CD8 molecules. An example is shown in Figure 2C.

**In Vivo Delivery of gD Lipopeptides in Adjuvant-Free, Saline-Induced, Virus-Specific Long-Term Memory Th1 Cell Responses**

Next, we evaluated whether gD lipopeptide immunization is sufficient to induce peptide- and virus-specific long-term memory CD4⁺ T cells. Ten groups of 10 mice each were immunized with individual gD1-29, gD49-82, gD146-179, gD228-257, or gD352-358 lipopeptides in saline or with nonlipidated parental peptide in saline (control). Three months after the final immunization, bulk cultures of SP-derived CD4⁺ T cells were established after 5 days of in vitro stimulation with the immunizing peptides or with heat-inactivated HSV-1 particles. Responding CD4⁺ T cells were then quantified in an IFN-γ ELISPot assay, as described in the Materials and Methods section. Peptide-specific IFN-γ-producing memory CD4⁺ T cells were detected in mice immunized with each of the five lipopeptides (Fig. 3A; P < 0.005). Virus-specific IFN-γ-secreting CD4 T cells were also detected in gD1-29, gD49-82, gD146-179, and gD352-358 lipopeptide-immunized mice, but fewer IFN-γ-positive CD4 T cells were detected in gD228-257 lipopeptide-immunized mice (Fig. 3B; P < 0.005), which suggests that this epitope is not well presented in the native gD protein.

To characterize the quality of induced memory CD4⁺ T cells, we determined the Th₁ versus Th₂ cytokine profile induced by each gD lipopeptide. gD1-29, gD49-82, gD146-179, and gD352-358 induced IL-2, IL-12, and IFN-γ cytokines but only a low level of IL-4 (Fig. 3C). The level of cytokines induced by gD228-257 lipopeptide was not significant (P > 0.05, ANOVA
test; Fig. 3C) compared with unstimulated cells (control). Overall, three of five lipopeptides—gD 49-82, gD 146-179, and gD 332-358—induced significant production of IL-2, IL-12, and IFN-γ, indicating that these synthetic, self-adjuvivanting lipopeptides delivered in adjuvant-free saline, preferentially elicited a polarized Th1-type response.

**FIGURE 2.** The magnitude of peptide- and virus-specific CD4+ T-cell responses induced by five gD lipopeptides. (A) Groups of age- and sex-matched BALB/c mice (n = 10) were immunized subcutaneously with the indicated gD lipopeptide (filled triangles) or with corresponding nonlipidated peptide in saline (open triangles). Draining lymph nodes were harvested 2 weeks after the second immunization, and in vitro T-cell proliferative responses against the immunizing peptide were assessed in a [3H]-thymidine incorporation assay. The background of nonstimulated cells ranged from 2396 to 3000 cpm. Experiments were repeated twice, and a T-cell proliferative response was considered positive when stimulation index (SI) > 2 and Δ cpm > 1000. (B) Virus-specific T-cell responses obtained from gD lipopeptides immunized mice or from mice immunized with gD 49-82 nonlipidated parental peptide, after in vitro stimulation with heat-inactivated HSV-1 particles. (C) T-cells from gD 49-82 lipopeptide or gD 49-82 nonlipidated peptide immunized mice (control) were stimulated with the gD 49-82 peptide alone (none) or in the presence of anti-CD4 mAbs, anti-CD8 mAbs, or an isotype control irrelevant mAb, added during the 3 days of culture. Shown are the mean SI ± SD obtained from groups of five mice each. The probabilities compare the maximum T-cell response to gD 49-82 lipopeptide in CD4 mAb–treated cultures compared with CD8 mAb or isotype control–treated cultures, according to Student’s t-test.

**FIGURE 3.** HSV-1 gD lipopeptide immunization preferentially generated Th1-cell responses. Groups of BALB/c mice (n = 10) were immunized subcutaneously with the indicated gD lipopeptides or nonlipidated parental peptides in saline and 12 weeks after the second immunization CD4+ T cells isolated from the spleen (SP) were stimulated with (A) the corresponding parental gD peptides or (B) heat-inactivated HSV-1 particles. (A) IFN-γ production by SP-derived CD4+ T cells were assessed by ELISpot assay. Data are the mean number of peptide-specific IFN-γ spot-forming cells (SFCs) from gD lipopeptides-immunized mice and control mice injected with nonlipidated parental peptides. (B) Number of IFN-γ SFCs after in vitro stimulation of SP-derived CD4+ T cells with heat-inactivated HSV-1 particles. (C) Cytokine profile produced by splenocytes CD4+ T cells from groups of mice immunized with five individual gD lipopeptides. The amounts of IFN-γ, IL-2, IL-4, and IL-12 cytokines secreted into the culture media of peptides, by stimulated or control unstimulated T cells, were quantified by a specific sandwich ELISA. Data are the mean ± SD of results in a group of 10 mice each in two independent experiments. The probabilities compare data from gD lipopeptide–immunized mice to nonlipidated parental peptide–injected control mice, according to Student’s t-test.
Protective Efficacy of gD Lipopeptide CD4⁺ T-Cell Epitopes against Ocular HSV-1 Infection and Disease

Six groups of 20 H2d mice each were immunized with gD1-29, gD49-82, gD146-179, gD228-257, or gD332-358 lipopeptide in saline or with saline alone (mock immunization). Two weeks after the second immunization, the mice in each group were challenged ocularly with 5 × 10⁶ pfu per eye of HSV-1 (strain McKrae). All animals were then monitored for (1) signs of blepharitis, observed daily for 10 days after challenge and of stromal disease, observed daily for 4 weeks after challenge; and (3) mortality recorded for up to 4 weeks after challenge.

Protection against Overt Signs of Ocular Herpes Disease. As shown in Figure 4A, gD49-82, gD228-257, and gD332-358 lipopeptide-immunized mice developed significantly fewer signs of blepharitis, than did nonimmunized control animals (P < 0.005). However, gD1-29 and gD146-179 lipopeptide-immunized mice developed blepharitis similar to nonimmunized control animals. Fourteen days after challenge, at the peak of severe stromal disease, only 45% of animals immunized with gD49-82 lipopeptide showed herpetic ocular disease compared with up to 73% of mice immunized with gD1-29, gD146-179, gD228-257, and gD332-358 lipopeptides (Fig. 4B).

Interference with Virus Replication in the Eye. Accordingly, immunization with gD1-29, gD49-82, gD146-179, and gD332-358 lipopeptide afforded a significant inhibition of virus replication in the eyes (Table 1). However, similar to nonimmunized control animals, in the gD228-257 lipopeptide-immunized group, the virus was detected as early as day 5.

Protection from Death after Ocular Infection. Only mice immunized with gD1-29 or gD49-82 lipopeptide had a significantly higher survival rate (50% and 30%, respectively) than in the control group (0% survival rate; P < 0.005; Table 1).

Table 1. gD Lipopeptide-Induced Virus-Specific IFN-γ-Producing CD4⁺ T-cell Responses Associated with a Reduction of Virus Replication in Eyes and Protection from Death

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>Peak Virus Titers (Day 5)</th>
<th>Survival Rates (Day 28)</th>
<th>IFN-γ (SFC/10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gD1-29</td>
<td>0.07 × 10⁻⁵ a</td>
<td>10/20 (50%)*</td>
<td>38.50 ± 3.5*</td>
</tr>
<tr>
<td>gD49-82</td>
<td>0.05 × 10⁻⁵ a</td>
<td>6/20 (30%)*</td>
<td>35.50 ± 0.5*</td>
</tr>
<tr>
<td>gD146-179</td>
<td>0.14 × 10⁻⁵ a</td>
<td>2/20 (10%)</td>
<td>14.30 ± 1.7*</td>
</tr>
<tr>
<td>gD228-257</td>
<td>0.45 × 10⁻⁵ a</td>
<td>2/20 (10%)</td>
<td>6.50 ± 1.5</td>
</tr>
<tr>
<td>gD332-358</td>
<td>0.15 × 10⁻⁵ a</td>
<td>4/20 (20%)</td>
<td>29.50 ± 2.5</td>
</tr>
<tr>
<td>Control</td>
<td>0.62 × 10⁻⁵</td>
<td>0/20 (0%)</td>
<td>2.50 ± 1.5</td>
</tr>
</tbody>
</table>

Groups of mice (n = 20) were immunized twice with gD1-29, gD49-82, gD146-179, gD228-257, or gD332-358 or PBS (control). Two weeks after the second immunization, mice in each group were inoculated ocularly with HSV-1 (McKrae strain). All animals were monitored daily for 4 weeks after the challenge, for virus replication in the eyes and for survival. The number of IFN-γ-producing CD4⁺ T cells from each group is also shown.

* P < 0.005.
Effect of Immunization with Multiligopeptides on Ocular Viremia and Disease and Protection from Lethal HSV-1 Infection

Since immunization with individual gD49-82, gD146-179, and gD332-358 lipopeptides did not by themselves protect against lethal herpes infection, we sought to determine whether immunization with a cocktail of several lipopeptides would provide increased protection. To investigate just some of the newly discovered gD epitopes, we excluded the previously known gD1-29 epitope from this cocktail. Groups of 20 mice each were immunized with (1) a cocktail of gD49-82, gD146-179, and gD332-358 lipopeptides (100 μg each) and (2) corresponding individual lipopeptides (100 μg each). For assessment of the protective efficacy of nonlipidated parental peptides, groups of 20 mice each were immunized with (3) a cocktail of gD49-82, gD146-179, and gD332-358 nonlipidated parental peptides in saline (100 μg each) or (4) corresponding individual nonlipidated parental peptides in saline (100 μg each). All animals were then challenged ocularly with 5 × 10^5 pfu of HSV-1. Survival was improved significantly in the group of mice immunized with the cocktail of gD lipopeptides compared with the other three groups (Fig. 5A; P < 0.005, Fisher exact test). At 28 days after infection ~70% of mice immunized with the cocktail of lipopeptides survived the infection compared with only 0% to 30% in the other groups. Of note, both the peak ocular virus titer and stromal disease were reduced approximately twofold in mice immunized with the cocktail of lipopeptides compared with corresponding individual lipopeptides (Figs. 5B, 5C; P < 0.005, Fisher exact test), and the lipopeptide cocktail-immunized mice had a two- to fivefold increase in IFN-γ-producing CD4^+ T-cell responses compared with the individual lipopeptides (Fig. 5D). There was no significant protection against either infection or diseases in control mice injected with the parental nonlipidated peptides in saline, either alone or as a cocktail, indicating the requirement of the attached lipid moiety.

To assess directly the involvement of CD4^+ T-cell subsets in protection, in vivo depletion of either CD4^+ or CD8^+ T cells was performed in lipopeptide-immunized mice before virus challenge. Depletion of CD4^+ T cells, but not of CD8^+ T cells, significantly abrogated protection against death (Table 2; P < 0.005), suggesting that protection was CD4^+ T-cell mediated.

The Mixture of Protective Lipopeptides Drive DC Maturation

As a step toward elucidating the cellular mechanisms of gD lipopeptide T-cell immunogenicity, we sought to determine the effect of gD lipopeptides on DC maturation. Immature DCs were incubated in vitro with a mixture of gD49-82, gD146-179, and gD228-257 lipopeptides and then monitored for the expression of cell surface major histocompatibility complex (MHC) class II, CD80 and CD86 costimulatory markers indicative of DC maturation. Untreated immature DCs and LPS stimulated DCs were included as negative and positive controls, respectively. Incubation of immature DCs with gD49-82, gD146-179, and gD228-257 lipopeptides induced significant upregulation of MHC class II, CD80, and CD86 costimulatory molecules compared with untreated immature DCs (Fig. 6A). Moreover, exposure of DCs to the cocktail of gD lipopeptides was associated with a dose-dependent increase in the production of IL-12 and TNF-α proinflammatory cytokines (Fig. 6B; P < 0.005). The gD49-82 lipopeptide labeled by Alexa Fluor 488 (Invitrogen Molecular Probes, Eugene, OR) and its cellular membrane loading in primary cultures of bone marrow–derived DCs was visualized using confocal microscopy, and their cytoplasmic delivery was tracked for 30 minutes after incubation (Fig. 6C).

Within the first 5 minutes of incubation, gD49-82 lipopeptide was quickly loaded on the surface of the DCs. At 15 minutes of incubation, gD49-82 lipopeptide appeared to have accumulated within vesicular compartments under the plasma membrane. After 30 minutes, a massive cytosolic delivery of gD49-82 lipopeptide that excluded the nucleus was observed. The cytoplasmic uptake of gD49-82 lipopeptide by the DCs was inhibited...
at 4°C, indicating an active intracellular delivery mechanism (data not shown). Together, these results indicate that the ability of a cocktail of gD lipopeptides to induce T-cell protective immunity is reflected by its ability to be taken up by the DCs, to cross the cell membrane quickly into the cytoplasmic compartment, and to stimulate DC maturation.

**DISCUSSION**

Despite multiple approaches to drug therapy and prevention, ocular herpes infections are still prevalent with no vaccine against ocular herpes having even entered clinical trial. In the late 1990s, two glycoproteins, gB and gD, along with the MF-59 adjuvant were tested against genital herpes in a large phase III clinical trial. However, this vaccine induced antibody levels that exceeded those resulting from natural infection, and it was abandoned because it failed to meet any of the end point measures of efficacy. Another recent large phase III clinical trial used a similar gD vaccine strategy with the monophosphoryl lipid/alum adjuvant. This trial showed significant protection from acquisition of herpes disease among seronegative women with seropositive partners. However, similar protec-

**TABLE 2. Protective Immunity against Ocular Herpes Induced by gD Lipopeptides**

<table>
<thead>
<tr>
<th>Immunized Mouse Treatment</th>
<th>Spleen Cells (%) CD4+</th>
<th>CD8+</th>
<th>Protected/Tested (n) P vs. Lipopeptide-Vaccinated Untreated Mice</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>19.3</td>
<td>6.1</td>
<td>18/20</td>
</tr>
<tr>
<td>Anti-CD4 mAb</td>
<td>0.5</td>
<td>5.9</td>
<td>1/20</td>
</tr>
<tr>
<td>Anti-CD8 mAb</td>
<td>17.8</td>
<td>0.1</td>
<td>19/20</td>
</tr>
<tr>
<td>IgG control</td>
<td>18.8</td>
<td>5.9</td>
<td>19/20</td>
</tr>
</tbody>
</table>

The immunity was abrogated after depletion of CD4+ but not of CD8+ T-cell subsets. The gD lipopeptide-vaccinated BALB/c mice were left untreated (None) or depleted of CD4+ or CD8+ T cells by i.p. injections of corresponding mAbs. Control mice received intraperitoneal (IP) injections with a rat IgG. Following the second dose of gD lipopeptides, and before challenge with HSV-1 (McKrae), mice were injected IP with six doses of 100 µL PBS containing anti-CD4 or anti-CD8 or isotype control mAbs. Flow cytometry analysis of peripheral blood mononuclear cells confirmed that after an mAb treatment, there was a consistent decrease in circulating CD4+ and CD8+ T cells in the treated mice to less than 2%. The probabilities compare protection achieved in mAb-treated versus untreated mice according to Student’s t-test. The results are representative of two independent experiments.

**FIGURE 6.** DC uptake and phenotypic maturation after exposure to immunogenic gD lipopeptides. (A) Immature bone marrow–derived DCs were left untreated or cultured for 48 hours with a cocktail of gD49-82, gD146-179, and gD332-358 lipopeptides and assayed by flow cytometry for cell surface expression of MHC (class I and class II) and B7 (CD80 and CD86) molecules. (B) DCs exposed to the cocktail of gD49-82, gD146-179, and gD332-358 lipopeptides were associated with a dose-dependent increase in the production of both IL-12 and TNF-α proinflammatory cytokines. Immature DCs were left untreated or exposed to a combination of gD49-82, gD146-179, and gD332-358 lipopeptides at either 50 or 100 µg/mL concentrations. Supernatants were taken and assayed for IL-12 and TNF-α in a sandwich ELISA. (C) Uptake by immature DCs and cytoplasmic delivery of gD49-82 lipopeptide labeled by Alexa Fluor 488 visualized by confocal microscopy for 30 minutes after incubation. Data are the results of three independent experiments.

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tion was not found for seronegative men with seropositive partners, nor for seropositive men or women. The immune mechanism and the protective gD epitopes behind the apparent superiority of the later gD vaccine is unknown, but it was hypothesized that it might induce a more protective Th1-cell response. These studies as well as the recently identified T-cell epitopes in gD prompted us to evaluate the T-cell-mediated protective efficacy of gD lipopeptides. The results reported herein showed that immunization with three highly immunogenic gD T-cell epitopes induced virus-specific primary and long-term memory IFN-γ-producing CD4+ Th1 responses.

Peptide epitope-based vaccines are molecularly defined and highly purified antigenic moieties that offer potential advantages over traditional vaccines, including the safety and the capacity of eliciting highly specific immune responses. Despite these advantages, however, many peptide vaccines tend to be poorly immunogenic in vivo and often require coadministration with external immunoadjuvants. However, concerns about using toxic adjuvants, which are especially critical for T-cell immunogenicity, still remain.

In the present study, we demonstrated that immunization with totally synthetic self-adjuvanting lipopeptides induced a strong and long-lasting Th1-cell-dependent response against ocular HSV-1 infection. Of interest, in contrast to the individual lipopeptides, immunization with a mixture of three highly immunogenic lipopeptides afforded better protection against lethal infection and resulted in lower levels of virus in the eye. These molecularly defined lipopeptide vaccines can be reproducibly synthesized, are safe, and can be easily handled for vaccination programs. Together with the strength and longevity of the protective T-cell immunity they induce, these or similar lipopeptide vaccines are therefore a safe and powerful immunomodulatory vaccine formulation that could be tested in humans.

Our and others previous findings in both experimental animal models (mice and primates) and in humans have demonstrated the safety of lipopeptide vaccines, derived from HSV, HIV-1, HBV, influenza virus, HCMV, streptococci, and malaria, constructed in a similar way as the lipopeptides reported in this study. The present study therefore extends those studies by confirming that HSV-1 gD lipopeptide formulations were well tolerated in naïve mice. No local or systemic manifestations were observed. However, the safety of these formulations still remains to be further validated, especially in HSV-infected animals, before the widespread clinical use of CD4+ T-cell epitope-based lipopeptide vaccines. Indeed, bearing in mind the high percentage of the adult population that is already infected with herpes, there is the concern that an immunotherapeutic vaccine against HSV, even if it is efficacious against primary challenge, may induce a CD4+ T-cell-mediated immunopathologic response that could exacerbate the HSK associated with ocular HSV recurrences. For this reason, all ocular HSV immunotherapeutic vaccines, including lipopeptide vaccines, should be tested in a recurrent eye model (i.e., rabbit), before any widespread clinical application. In addition, extensive pharmacotoxicological testing of GMP-grade molecularly defined lipopeptide vaccines is necessary to confirm their purity before any clinical application.

Another presumed advantage of lipopeptide immunogens is the possibility of production of multiepitope vaccine formulations by a simple physical mixture of lipopeptides, each bearing immunodominant T-cell epitopes from one or diverse herpes glycoproteins, rather than chemical covalent association of T-cell epitopes in one molecule. Such strategy is backed by our findings that immunization with a cocktail of three HSV-1 gD lipopeptides, each bearing immunodominant epitope(s) afforded better protection against lethal challenge. Our results also concur with those in a recent clinical trial of an HIV-1 vaccine, which showed that up to six T-cell lipopeptides derived from several proteins and delivered simultaneously as a cocktail are strongly immunogenic in humans. Thus, the concept of selecting the “best” HSV epitopes, not only from gD but also from other structural or regulatory proteins, to form a multivalent lipopeptide vaccine might be of great benefit and a practical way to generate broader multiepitopic protective T-cell responses.

Although gD228-257, lipopeptide, which failed to induce significant peptide- and HSV-specific T-cell proliferation (Figs 2A, 2B), induced peptide-specific IFN-γ-producing T cells (Fig. 3A), it induced only a negligible amount of HSV-specific IFN-γ-producing T cells (Fig. 3B), as detected by ELISPOT assay. It is not uncommon for a peptide epitope to induce IFN-γ-producing T cells in the absence of any T-cell proliferation and vice versa. The lack of HSV-specific IFN-γ-producing T cells, however, might suggest that the CD4+ T-cell epitope(s) harbored by gD228-257 lipopeptide may not be processed and presented from naïve herpes gD protein. Also, gD149-82 and gD332-358 both induced HSV-specific IFN-γ-producing T cells (Fig. 3B) and protected from blepharitis, although gD228-257 did not induce IFN-γ, yet afforded protection from blepharitis (Fig. 4B). There was also a lack of correlation between IFN-γ production and preventing stromal disease, since only gD149-82 protected against stromal disease. Thus, although IFN-γ may play a role in some instances, other immune effectors induced by some of these lipopeptide vaccines appeared to play a role in protecting against both blepharitis and stromal disease.

Although it is generally accepted that the resolution of ocular herpes infection is T-cell-dependent and can be accomplished in the absence of antibodies, the nature of the protective epitopes is not completely understood. In the present study, a cocktail of gD149-82, gD166-179, and gD332-358 lipopeptides bearing newly discovered CD4+ T-cell epitopes induced CD4+ T-cell-dependent protection in the mouse model of ocular herpes. These lipopeptides also induced IL-2, IL-12, and IFN-γ-producing CD4+ T cells. The present results support recent data showing a crucial role of Th1 responses in protecting against ocular infection in mice. Whereas the role of CD8+ T cells in herpes immunity has been extensively investigated, few reports have described the involvement of CD4+ T cells. This is a significant omission, because generation and maintenance of CD8+ T-cell responses often requires CD4+ T-cell activation. In addition, CD4+ T cells play an important role by secreting Th1 cytokines, such as IFN-γ, that by itself downregulate virus replication. The recurrent herpes increase in severity and frequency in HIV-positive patients as the CD4+ cell counts decline and immunosuppression worsens, suggests a role of CD4+ T cells in herpes immunity. Immunization with gD lipopeptides elicited IFN-γ-producing CD4+ T cells associated with protection from HSV-1 infection and disease. The lipopeptides used in this study also induced serum peptide-specific IgGs (not shown), but these antibodies failed to neutralize the virus in vitro, suggesting a lack or limited involvement of antibodies in the observed protection.

It has become increasingly clear that induction and modulation of T-cell immunity against intracellular pathogens, such as HSV-1, require immunogenic formulations that allow efficient targeting and maturation of DCs. The professional Ag-capturing and Ag-presenting cells, with a unique ability to prime naïve T cells, are critical to this function as a program of maturation that enhances DCs Ag-presenting and costimulatory capacity. We recently suggested that among the cellular and molecular mechanisms behind the immunogenicity of lipopeptides is the probable exertion of the adjuvant effect by the lipid moiety.
which interacts with and stimulates DC populations. Lipopeptides are also taken up more efficiently by DCs than by monocytes/macrophages. The present report extends these findings by showing that in vitro incubation of immature DCs with a cocktail of newly discovered HSV-1 lipopeptides increased cell surface expression of MHC class II and CD80/CD86 costimulatory molecules, resulting in mature DCs with an ability to produce high levels of proinflammatory IL-12 and TNF-α cytokines. The effect of gD49-82, gD146-179, and gD352-358 lipopeptides on DCs maturation, together with their strong T-cell immunogenicity in vivo, underscores the potential of this set of gD lipopeptides against herpes infection and disease.

A question of practical importance is the translation of the current immunologic findings in a single mouse strain for the development of an epitope-based vaccine for a genetically heterogeneous human population. Although the high degree of HLA polymorphism is often pointed to as a major hindrance to the use of epitope-based vaccines, this constraint can be dealt with through the inclusion of multiple supertype-restricted epitopes, recognized in the context of diverse related HLA alleles, and by designing cocktails of peptide- or lipopeptide-based vaccines with higher epitope densities. A broad population coverage can be established, providing that epitopes corresponding to multiple HLA supertype families are incorporated into the vaccine. Sette et al. recently defined nine HLA supertypes that provide an almost perfect coverage (>99%) of the entire repertoire of HLA molecules. A multi-epitope-based herpes vaccine could also include several T-cell epitopes present not only in one herpesvirus glycoprotein, such as gD, but also in several different structural glycoproteins and regulatory proteins targeted by CD4 T-cells from diverse HLA supertypes known to provide recognition in a large proportion of the global population, regardless of race and ethnicity. Hence, bearing in mind the particular properties that would be required in a prospective human lipopeptide vaccine. Studies are conceived in our laboratory to identify HLA-pronamicous T-cell epitopes in HSV structural glycoproteins and regulatory proteins targeted by CD4 T-cells from HLA class II supertype seropositive humans of diverse ethnicity. Of interest, the CD4+ T-cell lipopeptide epitopes identified in this study recalled naturally primed CD4+ T cells in up to 45% of HSV seropositive individuals. In addition, after HSV-1 infection of HLA-DRB1*0101 and HLA-DRB1*0401 transgenic mice, every mouse developed HLA-DR-restricted T-cell responses directed at the same epitopes that were identified in naturally infected humans (Zhang et al., manuscript submitted). CD4+ T-cell epitopes identified in this study, along with similarly identified HLA class I supertype-restricted HSV CD8+ CTL epitopes (Chentouf et al., manuscript submitted), would therefore provide the database needed to develop a human multiepitope Th-CTL chimeric peptide or lipopeptide vaccine, as we recently demonstrated in the mouse model of ocular herpes infection. Such a multiepitope Th-CTL chimeric peptide vaccine would be broadly effective in most outbred racial and ethnic populations.

In summary, a herpes lipopeptide vaccine formulation that contains three peptide epitopes derived from the sequence of HSV-1 glycoprotein D (gD) has been described. We demonstrated that totally synthetic HSV-1 gD lipopeptides delivered in H24 mice without exogenous adjuvant-stimulated, virus-specific long-lasting IFN-γ-producing CD4+ T cells. Immunization with a cocktail of three immunogenic Th1 lipopeptides, that stimulates in vitro maturation of DCs, protected against lethal infection and resulted in the decreased levels of virus replication in the eye and a reduction in overt signs of herpes stromal disease. Overall, this preclinical study in mice illustrated the feasibility of a molecularly defined lipopeptide-based vaccine, engineered by the newly described chemoselective ligation method, as a quick and relatively low-cost means to provide material for future herpes clinical trials.

References


