A Therapeutic Vaccine That Reduces Recurrent Herpes Simplex Virus Type 1 Corneal Disease

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PURPOSE. To investigate the therapeutic efficacy of periocular vaccination with herpes simplex virus (HSV) recombinant glycoprotein D from HSV-1 (gD1) or HSV-2 (gD2) in decreasing HSV-induced recurrent dendritic keratitis and HSV-induced recurrent ocular shedding in rabbits latently infected with HSV-1.

METHODS. Rabbits latently infected with HSV-1 were vaccinated periocularly (by subconjunctival injection) with gD1 and adjuvant, gD2 and adjuvant, or adjuvant alone. Eyes were examined daily for 49 days for recurrent herpetic keratitis and for recurrent infectious HSV-1 shedding.

RESULTS. In both vaccinated groups, a significantly decreased number of eyes exhibited recurrences of herpetic keratitis compared with recurrences in adjuvant-treated control eyes (gD1 group, 27/1372, [2%]; gD2 group, 24/1274, [2%]; and control, 54/1274 [4%]; P < 0.005). Eyes in the gD1-vaccinated group (44/1308 [3.4%]; P = 0.01), but not those in the gD2-vaccinated group (71/1274 [5.6%]; P = 0.93), had significantly decreased viral shedding (positive cultures compared with total cultures) compared with eyes in the adjuvant-treated control group (69 of 1275 [5.4%]).

CONCLUSIONS. Recurrent HSV-1 corneal disease was significantly reduced by therapeutic local periocular vaccination. The vaccine may be more efficacious against HSV-1-induced recurrent corneal disease than against recurrent HSV-1 ocular shedding. Its efficacy against corneal disease appeared to be longer lasting than its efficacy against recurrent spontaneous shedding. The heterotypic gD2 vaccine was as efficacious as the homotypic gD1 vaccine against recurrent corneal disease, whereas the homotypic vaccine was much more efficacious than the heterotypic vaccine against recurrent HSV-1 shedding. This is the first report in any animal model of a successful therapeutic vaccine against recurrent HSV-1-induced corneal disease. These results support the concept that development of a therapeutic vaccine for ocular HSV-1 recurrence in humans may be possible. (Invest Ophthalmol Vis Sci. 1998;39:1163-1170)

Ocular herpes simplex virus (HSV) infection is the most frequent serious viral corneal infection in the United States. Recurrent HSV infection is a major cause of virus-induced blindness.1 Most HSV-induced corneal scarring leading to blindness is caused by recurrent HSV infection rather than by primary HSV infection.2,3 In the United States, approximately 400,000 people per year have recurrent episodes of ocular HSV that require doctor visits and medication. The development of a therapeutic vaccine—that is, a vaccine to reduce HSV ocular recurrences—would greatly alleviate the incidence of virus-induced blindness.

Many studies have shown that mice and rabbits can be protected against primary HSV-1–induced corneal disease if the animals are vaccinated before ocular viral challenge.4-13 Thus, some prophylactic HSV-1 vaccines protect against primary ocular HSV-1 infection. In addition, in some instances, prophylactic vaccines also protect against the establishment of latency by the challenge virus,5,7 a situation that obviously would result in reduced viral recurrences. It is likely that a prophylactic vaccine strategy could be developed that would protect against primary and recurrent ocular HSV-1 disease. However, such a strategy would require that the vaccine be given before primary exposure to HSV-1. This prophylactic vaccine approach would not help the majority of adults (70%-90%) who are already HSV-seropositive (and therefore harbor latent HSV) and who may experience HSV recurrences.

The ideal vaccine against ocular HSV-1 should be efficacious not only against primary ocular challenge, but also against recurrent ocular infection when delivered to patients who harbor a latent HSV-1 infection. The ideal vaccine should be effective whether delivered in a prophylactic or a therapeutic regimen. Because the immune responses most important in protecting against primary infection may differ from those most important in protecting against recurrent infection, two types of vaccines may ultimately be required.
In a previous study, we used a rabbit model, to demonstrate for the first time in an animal, a vaccine and adjuvant combination that produces a therapeutic reduction of spontaneous recurrent ocular HSV-1 shedding. This was done by using microparticle formulation 59 (MF59) combined with N-acetyl-muramyl-l-alanyl-d-isoglutaminyl-l-alanine-2-1,2-dipalmitoyl-sn-glycero-3-(hydroxyphosphoryl)ethanolamide (MTP-PE) as adjuvant, recombiantly expressed, and highly purified gD2 and glycoprotein B2 (gB2) as antigens administered by a local periocular route of inoculation. Vaccination resulted in a twofold to threefold decrease in spontaneously reactivated HSV-1 in tear films (i.e., the mock-vaccinated group had two to three times more shedding than did the vaccinated group). This vaccine is also efficacious against primary ocular infection when given prophylactically (Nesburn et al. unpublished observations, 1998).

In the present study, we examined the effect of a similar vaccine on spontaneous recurrent herpetic corneal disease (dendritic and geographic lesions) and on spontaneous shedding. Although it is logical to assume that a vaccine that reduces ocular virus shedding would also reduce virus-associated recurrent corneal disease, it had not been demonstrated previously. Therefore, in addition to determining the number of days that recurrent virus could be detected in tears, we also recorded daily the incidence and severity of recurrent herpetic corneal disease in the eyes throughout the 49-day test period.

In previous studies, we used heterotypic type 2 glycoprotein vaccines to treat recurrent HSV-1 shedding. To determine in the present study whether homotypic (type 1) glycoprotein would be a more efficacious therapeutic vaccine against recurrent HSV-1 shedding and HSV-1-induced eye disease, we compared the efficacy of a homologous type 1 glycoprotein vaccine (gD1) with that of the heterotypic type 2 glycoprotein vaccine (gD2). We report here that both therapeutic vaccines provided significant protection against recurrent corneal epithelial lesions and recurrent virus shedding. The magnitude of protection against recurrent herpetic corneal epithelial lesions was similar with both vaccines. However, the homologous gD1 vaccine appeared to provide more efficacious protection against recurrent virus shedding than did the heterotypic gD2 vaccine.

**Materials and Methods**

**Virus**

The HSV-1 strain McKrae, which produces severe ocular disease in rabbits, was used as the challenge virus in all experiments. The virus was triple-plaque purified and passaged twice in CV-1 cells to produce a virus stock with a titer of $1.2 \times 10^8$ plaque-forming units per milliliter.

**Rabbits**

New Zealand White female rabbits (Irish Farms, Norco, CA) were used for all experiments. Rabbits were 8 to 10 weeks old at the start of the procedure. All animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and with the guidelines of the American Association for Laboratory Animal Care and National Institutes of Health.

**Rabbit Model of Ocular Herpes Simplex Virus-1 Infection, Latency, and Spontaneous Reactivation**

To establish a cohort of rabbits with spontaneous recurrent ocular HSV-1 infections, naive rabbits were bilaterally infected without scarification or anesthesia by placing $2 \times 10^5$ plaque-forming units (HSV-1; McKrae strain) in a total volume of 50 μl into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye. Forty of 90 (44%) rabbits survived the acute infection. As we have previously described, all trigeminal ganglia in the surviving rabbits harbor a latent HSV-1 infection that results in a high group rate of spontaneous reactivation (approximately 5%-10% of tear film cultures are positive for HSV-1 during the 3 to 4 months after the establishment of latency). Acute ocular infection was confirmed by HSV-1-positive tear film cultures collected on days 3 and 4 after infection. Based on results of our previous experiments, by 21 days after infection all surviving rabbits harbor a bilateral HSV-1 latent infection of both trigeminal ganglia. To control for a possible bias introduced by the severity of the initial infection, the latently infected rabbits were divided into three similar groups according to the severity of corneal disease observed during the acute phase of HSV-1 infection. Thus, each group contained rabbits with a similar spectrum of severity of acute eye disease. The resulting groups were then randomly assigned to receive one of the three vaccine treatments (gD1 and adjuvant, gD2 and adjuvant, or adjuvant alone).

**Glycoproteins for Vaccination**

Herpes simplex virus-2 glycoprotein D (gD2) was prepared by expression of the carboxyl-terminal (C-terminal)-truncated gene in Chinese hamster ovary cells followed by purification to near homogeneity. In this process a series of traditional chromatographic steps was used, as previously described and as previously used by Chiron Corporation (Emeryville, CA) in clinical trials measuring protection from HSV-2 infection. Herpes simplex virus-1 glycoprotein D (gD1) was identically prepared at Chiron. It is a C-terminal truncation of the full-length protein comprising amino acids 1 through 290 of the extracellular domain of the mature protein after removal of the signal sequence. The entire extracellular domain comprises amino acids 1 through 314. Similarly, gD2 is a C-terminal truncation comprising amino acids 1 through 294 and three non-gD2 amino acids, Leu-Thr-Asn, at the C-terminus. The entire extracellular domain comprises amino acids 1 through 313. The gD1 gene was obtained from HSV-1 strain Patton. The gD2 gene was obtained from strain 333.

**Adjuvant and Vaccine Preparation**

The adjuvant, MF59 combined with MTP-PE, was prepared as follows. The MF59 emulsion contained 5% (vol/vol) squalene (E. Merck, Darmstadt, Germany), a natural metabolizable oil, and 0.5% (vol/vol) each of the surfactants polyoxyethylene sorbitan mono-oleate (Tweepon 80; ICI America, Wilmington, DE) and sorbitan trioleate (Span 85; ICI America). The mixture was emulsified by mixing at high pressure (approximately 10,000 psi) using an emulsifier (model 110Y; Microfluidics, Newton, MA). The resulting emulsion was sterile filtered and stored at 4°C. MTP-PE was obtained as a dry powder from Ciba-Geigy (Basel, Switzerland). It was dissolved in the aqueous phase of the emulsion mixture before homogenization. This adjuvant was previously used in human clinical trials.
against genital herpes. The vaccine, containing MF59 with 50 μg MTP-PE per dose (MF59–MTP-PE), was prepared just before immunization by mixing one volume of the desired antigen (gD1 or gD2) in 2 × phosphate-buffered saline with one volume emulsion.

**Periocular Vaccinations**

Before inoculation, one or two drops of a 1% solution of proparacaine was administered topically (as eye drops) for local anesthesia. The vaccine was delivered by subconjunctival inoculation in the upper cul-de-sac with a 30-gauge needle on a disposable insulin syringe. The subconjunctival route allows the use of adjuvant and assures that the vaccine is delivered and retained at the local site. Subconjunctival injection is routinely used in clinical ophthalmology. Because no evidence has shown that it induces reactivation of HSV, it would be an acceptable route of vaccination for patients with severe, recurrent ocular HSV infection. All eyes were administered three single-dose inoculations on days 28, 49, and 70 after infection, corresponding to three vaccinations at 3-week intervals. Control vaccinations were given on the same schedule.

**Inocula**

Each periocular (subconjunctival) vaccine dose contained 15 μg gD1 or gD2 in a total volume of 0.1 ml (0.05 ml adjuvant and 0.05 ml glycoprotein in 2X phosphate-buffered saline). Control vaccinations consisted of 0.05 ml of adjuvant and 0.05 ml 2X phosphate-buffered saline.

Recurrent eye disease was determined by examining the rabbit eyes once a day (7 days a week), beginning 3 weeks after the final vaccination. The examinations were performed in a blinded manner by slit lamp biomicroscopy, using fluorescein. The goal of the examination was to differentiate herpetic versus nonherpetic epithelial lesions. For this purpose, lesions were classified as dendritic-geographic keratitis, non-HSV, or clear. Spontaneous recurrent herpetic keratitis in rabbits infected with HSV-1 McKrae consists of small, single- or multiple-characteristic dendritic figures or small geographic figures, which usually clear in 1 to 2 days. Larger dendritic or geographic lesions, which are the hallmark of primary ocular infection, are rarely seen. New stromal scarring or edema was also tracked; but in this model, the incidence of scarring was insufficient to make significant comparisons between experimental groups of the size used here. Thus, all recurrent herpetic corneal disease recorded was caused by dendritic-geographic lesions. Corneal disease was scored on a scale of 0 to 4, with values of 0, 1, 2, 3, and 4 representing, respectively, no disease and disease involving 25%, 50%, 75%, and 100% of the corneal surface.

**Tissue Culture Assay for Herpes Simplex Virus-1 Ocular Shedding**

Beginning 3 weeks after the final vaccination, tear film specimens were collected daily from each eye using a nylon-tipped swab. To lessen the possibility that nonspecific lesions might be observed by slit lamp biomicroscopy, cultures were taken immediately after biomicroscopy each day. The swab was placed in 0.5 ml tissue culture medium containing antibiotics and antifungal agents, and the inoculated medium was used to infect rabbit skin cell monolayers. Cell monolayers were observed in blinded manner by phase contrast microscopy for HSV-1 cytopathic effects. All positive cultures were blind-passaged onto fresh rabbit skin cells for confirmation. Neutralizing antibody titers were obtained by a 50% plaque-reduction assay on CV-1 cells, as described previously.

**Percentage of Vaccine Efficacy**

The percentage of vaccine efficacy was calculated as 1 – (the fraction of symptomatic eyes [or rabbits] in the vaccine group)/(the fraction of symptomatic eyes [or rabbits] in the control group) × 100%.

**RESULTS**

**Ocular Vaccination of Rabbits with Pre-existing Herpes Simplex Virus-1 Latent Infection**

To establish cohorts with HSV-1 latent bilateral infections of the trigeminal ganglia, rabbits were infected in both eyes with McKrae strain HSV-1 as described in the Materials and Methods section. To control for possible bias introduced by severity of initial infection, the latently infected rabbits were divided into gD1 (13 rabbits), gD2 (12 rabbits), and adjuvant control (12 rabbits) groups, according to the severity of eye disease observed during the acute phase of HSV-1 infection. Thus, the control and vaccine groups were known to contain a range of rabbits each with a similar spectrum of severity of acute eye disease. All eyes were inoculated subconjunctivally with HSV-1 glycoprotein D in the adjuvant MF59–MTP-PE (gD1 group), HSV-2 glycoprotein D in the adjuvant MF59–MTP-PE (gD2 group), or the MF59–MTP-PE adjuvant alone (control group), as described in the Materials and Methods section. Three vaccinations were given at 3-week intervals.

**Vaccination-Induced Neutralization Titers**

To confirm that the above vaccinations induced an immune response in the latently infected rabbits, serum was collected from three to five rabbits per group, 3 weeks after the initial HSV-1 infection (just before the first vaccination) and 3 weeks after the third vaccination (just before the study period). Before the first vaccination, the HSV-1 neutralization titers for every rabbit in all three groups were less than 10 (the baseline of the assay, Table 1). Mock vaccination had no effect on the neutralization titers. Vaccination with gD1 induced an average neutralization titer of 237 ± 59. This was significantly higher than the mock-vaccinated group (P = 0.02) or in the mock-vaccinated group (P = 0.04). Vaccination with gD2 induced an average neutralization titer of 359 ± 182. This was also greater than that seen before vaccination of the gD1 group rabbits (P = 0.05). There was no difference in the neutralization titers induced by gD1 and gD2 vaccination (P = 0.4), because of the small number of animals per group.

**Recurrent Herpetic Keratitis Versus Total Corneal Readings**

Beginning 3 weeks after the third vaccination, all eyes were examined daily for HSV-1–induced recurrent herpetic corneal disease (dendritic or geographic lesions). The cumulative number of positive herpetic keratitis readings during the 49-day study period are shown in Figure 1. Because of the slight difference in the number of rabbits (12 or 13 per group) and...
TABLE 1. Neutralization Titers

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Prevaccination*</th>
<th>Postvaccination†</th>
<th>$P_{\text{d}}$ vs. Prevaccination</th>
<th>$P_{\text{d}}$ vs. Mock</th>
<th>$P_{\text{d}}$ vs. gD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>gD1§</td>
<td>&lt;100</td>
<td></td>
<td></td>
<td>$237 \pm 59$</td>
<td>0.02</td>
</tr>
<tr>
<td>gD2#</td>
<td>&lt;100</td>
<td></td>
<td></td>
<td>$359 \pm 182$</td>
<td>0.05</td>
</tr>
<tr>
<td>Mock#</td>
<td>&lt;100</td>
<td></td>
<td></td>
<td>&lt;100</td>
<td></td>
</tr>
</tbody>
</table>

* Serum was collected 21 days after infection, just before the first vaccination. Numbers represent the geometric means (± SEM) of the reciprocal of the serum dilutions that produced a 50% reduction in the number of HSV-1 plaques.
† Serum was collected 21 days after the final (third) vaccination.
‡ Mann-Whitney rank sum test, one sided. $P < 0.05$ was considered statistically significant.
§ Serum from five rabbits.
|| Assay baseline.
# Serum from three rabbits.

gD1, glycoprotein D herpes simplex virus-1; gD2, glycoprotein D herpes simplex virus-2; HSV-1, herpes simplex virus-1.

Recurrent Herpetic Keratitis

Recurrence of Herpetic Keratitis during a 49-day test period. Rabbits were ocularly infected with McKrae strain herpes simplex virus (HSV)-1, as described in the Materials and Methods section. Once latency had been established, groups of rabbits were vaccinated subconjunctivally with a glycoprotein D1 vaccine, a glycoprotein D2 vaccine, or a mock vaccine. Identical vaccinations were administered 21 and 42 days later for a total of three vaccinations. Twenty-one days after the final vaccination, rabbit eyes were observed daily for herpetic keratitis. In contrast, the gD1- and the gD2-vaccinated rabbits had, on average, approximately one positive eye reading per eye during the same period (1.21 and 0.92, respectively). Thus, both vaccines appeared to reduce the cumulative number of positive readings by approximately 50% (43% and 57%, respectively).

A statistical analysis of positive versus negative eye readings is shown in Table 2. In the control group, herpetic keratitis was observed in 55 of 1275 eye readings (4.3%). In contrast, only 29 of 1308 eye readings (2.2%) in the gD1 vaccine group and 24 of 1275 eye readings (1.9%) in the gD2 vaccine group were positive for herpetic keratitis. These were both significantly less than positive readings in the control group ($P = 0.004$ and $P = 0.001$; chi-square analysis) and were similar to each other ($P > 0.05$). Thus, by this analysis, the gD2 and the gD1 vaccines appeared to reduce by approximately 44% to 51% the number of days during which recurrent HSV-1 eye disease was detected. Vaccine efficacy against recurrent herpetic keratitis, determined as described in the Materials and Methods section, was 49% with gD1 and 56% with gD2 (Table 2).

Fraction of Herpetic Keratitis–Positive Eye Days Per Eye

The described analyses are commonly used for the evaluation of these types of experiments. However, these approaches do not take into account the number of eyes in each group and therefore do not distinguish among large numbers of eyes observed during a short period, moderate numbers of eyes observed during a medium period, or small numbers of eyes observed during a long period. Therefore, we analyzed the data by another approach. For each eye in each group, the fraction of herpetic keratitis-positive days was determined (total herpetic keratitis readings/total readings for each eye). The resulting fractions (one for each eye) were then subjected to statistical analysis (Mann–Whitney rank sum tests). Because each eye is now represented by the fraction of herpetic keratitis-positive days, this analysis takes into account the number of days of each group and is therefore more stringent. By this analysis, the decrease in recurrent corneal disease in both vaccinated groups was again significant (Table 2, "More Stringent").

Recurrent Viral Shedding in Tears during the 49-Day Collection Period

Beginning 3 weeks after the final vaccination, tear films were collected daily from all eyes and were individually plated on
TABLE 2. Recurrent Herpetic Keratitis

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Positive Readings/Total Eyes*</th>
<th>P vs. Mock (Less Stringent)†</th>
<th>P vs. Mock (More Stringent)‡‡</th>
<th>Vaccine Efficacy§</th>
</tr>
</thead>
<tbody>
<tr>
<td>gD1</td>
<td>29/1308 (2.2%)</td>
<td>0.004</td>
<td>0.03</td>
<td>49%</td>
</tr>
<tr>
<td>gD2</td>
<td>24/1274 (1.9%)</td>
<td>0.001</td>
<td>0.01</td>
<td>56%</td>
</tr>
<tr>
<td>Mock</td>
<td>55/1275 (4.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Eye readings for recurrent HSV-1 induced corneal disease (herpetic keratitis) were recorded daily for 49 days. The number of positive eye readings/total number of eye readings is shown for each group of rabbits.
† Chi-square test based on total disease positive and disease negative eye readings.
‡‡ The fraction of time each eye was positive for herpetic keratitis (number of positive/total readings) was determined, producing a fraction for each eye. The fractions for all the eyes in groups gD1 and gD2 were compared to the fractions for all the eyes in the mock group using the Mann-Whitney rank sum test.
§ Vaccine efficacy was determined as described in Materials and Methods section.

gD1, glycoprotein D herpes simplex virus-1; gD2, glycoprotein D herpes simplex virus-2; HSV-1 herpes simplex virus-1.

indicator cells to assay for the presence of reactivated (recurrent) virus. The cumulative number of positive cultures during the 49 days of collection is shown in Figure 2. The data were standardized to represent cumulative positive cultures per eye. The control group and the gD2 vaccine group had almost three positive cultures per eye for the observation period. In contrast, the gD1 vaccinated rabbits had, on average, only 1.6 positive cultures per eye.

A statistical analysis of positive versus negative tear film cultures is shown in Table 3. During the 49-day study period, 5.4% (69/1275) of the tear films from control eyes and 5.6% (71/1274) of the tear films from gD2-vaccinated eyes contained recurrent virus. In contrast, only 3.4% (44/1308) of the tear films from gD1-vaccinated eyes were positive for virus. This was significantly less than in the mock-vaccinated group ($P = 0.01$; chi-square analysis). As described earlier, we also analyzed these data by the more stringent method of using the fraction of time each eye was positive. For each eye in each group, the fraction of positive cultures was determined (total positive cultures compared with total cultures for each eye; Table 3). The resulting fractions (one for each eye) were then subjected to statistical analysis. By this approach, neither the gD2 nor the gD1 vaccine groups were significantly different from the mock-vaccinated group ($P = 0.94$ and 0.32, respectively; Mann-Whitney rank sum test). These analyses suggest that the gD1 vaccine, but not the gD2 vaccine, may have provided a small amount of therapeutic protection against recurrent viral shedding during the 49-day collection period. Vaccine efficacy during the 49-day period was 37% for gD1 and 0% for gD2.

Recurrent Viral Shedding during the First 21 Days of Tear Film Collection

During the first 21 days of the 49-day tear film collection period (Fig. 2), it appeared that the vaccines may have been efficacious in this 3- to 6-week period after the last vaccination. We therefore analyzed the first 21 days of tear film collection as above. The mock-vaccine group had an average of 1.3 positive cultures per eye during this period. The gD2 vaccine group had approximately 0.7 positive cultures per eye, whereas the gD1 vaccine group had an average of only 0.18 positive cultures during this period.

In the first 21 days, almost 6% of the tear films from mock-vaccinated eyes contained recurrent virus (Table 3; days 1-21). In contrast, less than 1% of the tear films from gD1-vaccinated eyes were positive for virus ($P < 0.0001$; Fisher's exact test), whereas 3.3% of the tear films from gD2-vaccinated eyes contained recurrent virus ($P = 0.03$, one-sided Fisher's exact test). Analysis by the more stringent method of using the fraction of time each eye was positive also indicated that the gD1 group was significantly different from the mock group ($P = 0.02$). However, the gD2 group was no longer different from the mock group ($P = 0.3$). Thus, during the first 21 days of collection (days 21 to 41 after vaccination) the therapeutic gD1 vaccine provided more than a sixfold decrease against recurrent viral shedding (5.9% in the mock group versus 0.9% in the gD1 group) that was significant even by the more
Table 3. Recurrent Ocular HSV-1 Shedding

<table>
<thead>
<tr>
<th>Days 1-49</th>
<th>Positive/Total Eye Cultures*</th>
<th>P vs. Mock (Less Stringent)†</th>
<th>P vs. Mock (More Stringent)‡</th>
<th>Vaccine Efficacy (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>gD1</td>
<td>44/1308 (3.4)</td>
<td>0.01</td>
<td>0.32</td>
<td>37</td>
</tr>
<tr>
<td>gD2</td>
<td>71/1274 (5.6)</td>
<td>0.93</td>
<td>0.94</td>
<td>0</td>
</tr>
<tr>
<td>Mock</td>
<td>69/1275 (5.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 1-21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gD1</td>
<td>5/588 (0.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gD2</td>
<td>18/546 (3.3)</td>
<td>0.03*</td>
<td>0.3</td>
<td>44</td>
</tr>
<tr>
<td>Mock</td>
<td>32/546 (5.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 22-49</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gD1</td>
<td>39/720 (5.4)</td>
<td>0.81</td>
<td>0.72</td>
<td>0</td>
</tr>
<tr>
<td>gD2</td>
<td>53/728 (7.3)</td>
<td>0.1</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td>Mock</td>
<td>37/728 (5.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tears were collected daily for 49 days from all eyes and were cultured on indicator cells for the presence of reactivated HSV-1. The number of HSV-1 positive cultures/total number of cultures is shown for each group of rabbits.
† Chi-square test based on total positive and total negative cultures in each group.
‡ The fraction of time each eye was positive for recurrent HSV-1 (number of positive tear film cultures/total number of cultures) was determined, producing a fraction for each eye. The fractions for all the eyes in groups gD1 and gD2 were compared to the fractions for all the eyes in the mock group using the Mann-Whitney rank sum test.
§ Vaccine efficacy was determined as described in Materials and Methods section.
¶ Fisher's exact test based on total positive and total negative cultures in each group.
* Single-sided.

rigorous statistical test. The gD2 vaccine may have provided a small amount of protection that appeared significant only when a less stringent statistical analysis was used. Thus, the homotypic therapeutic gD1 vaccine appeared to be more efficacious than the heterotypic gD2 vaccine against recurrent shedding. Vaccine efficacy for the period covering day 1 to day 21 was 85% for gD1 and 44% for gD2.

Recurrent Viral Shedding from Day 22 to Day 49 of the Study Period

To complement the 1- to 21-day analysis, we also analyzed the shedding results just for the period from days 22 to 49. Cumulative shedding for this period in response to both vaccines appears similar to that of the control group (Fig. 3). In fact, as shown in Table 3 (days 22-49) there were no significant differences among the spontaneous shedding rates of the control and vaccine groups. During this period, neither vaccine was efficacious against recurrent HSV-1 shedding. Thus, it appeared that neither vaccine provided long-lasting protection against recurrent HSV-1 shedding.

**DISCUSSION**

In a previous report, we showed that local ocular vaccination with glycoprotein B2 and gD2 and MF59-MTP-PE adjuvant produced protection against recurrent HSV-1 ocular shedding in the rabbit ocular model. We used recurrent ocular shedding, measured by a positive viral culture, as the end point in that study because it is a simple, objective, and unambiguous end point. In contrast, recurrent herpetic keratitis is much more difficult to measure, the event rate is lower, and it is a much more subjective end point. Although it seemed likely that a therapeutic vaccine that reduced recurrent ocular shedding would also decrease recurrent herpetic keratitis, it was possible that some recurrent herpetic keratitis could occur in the absence of detectable recurrent viral shedding. In addition, in humans detectable HSV-1 shedding is more common than detectable recurrent disease. Thus, in this study we examined recurrent herpetic keratitis and recurrent HSV-1 shedding. The experiments reported here were designed to answer two questions. First, would the ocular vaccination that reduced...
Vaccine for Ocular Herpes Simplex Virus-1


recurrent viral shedding also reduce recurrent herpetic keratitis? Second, would the homotypic recombinant gD1 be a more efficacious therapeutic vaccine against recurrent HSV-1 shedding and recurrent HSV-1-induced herpetic keratitis than the more easily obtained gD2, which we had used (in combination with gB2) in previous studies?

This study presents new information about periocular vaccination as a means of protecting against recurrent HSV disease. In this and our previous studies, we have shown that periocular vaccination can reduce ocular HSV shedding. In addition, for the first time, we demonstrated that periocular vaccination of latently infected rabbits can produce a statistically significant decrease in recurrence of herpetic keratitis. The gD1 and gD2 vaccines decreased recurrences of herpetic corneal disease during the 49 days of this study. In fact, the vaccines appeared to be more efficacious against recurrent lesions than against recurrent viral shedding.

Surprisingly, the homotypic gD1 vaccine and the heterotypic gD2 vaccine appeared to be equally efficacious against recurrent corneal disease. In contrast, and as expected, the homotypic gD1 vaccine provided greater efficacy than the heterotypic gD2 vaccine against recurrent viral shedding. Because the gD1 and gD2 vaccines were identically produced, the increased efficacy of the gD1 vaccine supports the notion that the local ocular vaccine effect (at least regarding protection against recurrent viral shedding) was caused by an HSV-1-specific immune response rather than by nonspecific stimulation of protective substances such as interferon or cytokines.

In our previous therapeutic vaccine study using gD2 and gB2 and the same adjuvant used here, protection against recurrent HSV-1 ocular shedding lasted throughout the entire 43-day study period. In contrast, in the present study, protection by the gD1 vaccine against recurrent shedding appeared much shorter lived, lasting only approximately 21 days after the last vaccination. In addition, protection against recurrent HSV-1 shedding by the gD2 vaccine was minimal. The previous study was identical with the present study, except for the glycoproteins contained in the vaccines. The total amount of glycoprotein was the same in both studies (7.5 μg gB2 and 7.5 μg gD2 for each inoculation in the previous study, compared with 15 μg gD1 or gD2 in the present study). Thus, in providing protection against recurrent HSV-1 shedding, the gD2-gB2 vaccine not only appeared to be more efficacious than the gD2 vaccine, but also more efficacious than the homotypic gD1 vaccine. Whether the increased efficacy of gD2-gB2 is because of an additive or synergistic effect is unknown. Regardless, these results suggest that gB would be a valuable component of a therapeutic vaccine against recurrent ocular HSV-1. In addition, because the homotypic gD1 was much more efficacious than the heterotypic gD2, it is likely that a combination of gD1 and gB1 will be even more efficacious than gB2-gD2 against recurrent HSV-1.

The limited duration of therapeutic vaccine efficacy seen here (during the first 3 weeks of the study period, which correspond to weeks 4 to 6 after the final vaccination) may parallel the findings of a recent report on therapeutic vaccine efficacy against recurrent genital herpes in humans using a similar subunit vaccine. In that report, the duration and severity of the first study outbreak was significantly reduced by vaccination. However, despite significant increases in vaccination-induced HSV-1 serum-neutralizing antibody, vaccination did not decrease the overall rate of genital herpes recurrences. The apparent limited duration of the vaccine efficacy in this clinical trial and in our rabbit model could be caused by poor memory T-cell stimulation by subunit vaccines.

As in the clinical trial against genital HSV-2 recurrence, we found no correlation between serum neutralizing antibody and therapeutic vaccine efficacy of the vaccines against recurrent ocular shedding. The gD1 and the gD2 vaccines induced significant and similar levels of serum-neutralizing antibody, but only the gD1 vaccine showed therapeutic efficacy against spontaneous recurrent ocular shedding. The mechanism by which recurrent shedding and recurrent corneal disease are decreased remains unknown. However, we have recently found that the therapeutic vaccine efficacy exhibited in this report after periocular vaccination, could not be achieved with systemic vaccination (manuscript submitted). This strongly suggests that local or mucosal immune responses such as secretory IgA may be essential for efficient therapeutic vaccine efficacy against recurrent ocular HSV-1.

Other than the present report and our previous report, there have been only two other reports, both very recent, of therapeutic vaccine efficacy against ocular HSV-1 in an experimental model. In the first, latently infected mice were injected intraperitoneally with a gD2 subunit vaccine, and reactivation was induced by UVB. There was no decrease in the number of mice induced to shed virus, although the duration of the induced shedding was reduced. One explanation for the poor vaccine efficacy of the vaccine is that the subunit vaccine was delivered systemically rather than locally, as in the present study. In a second study by the same group, latently infected mice were inoculated with a live virus vaccine and efficacy was determined against induced virus shedding. In this study, the number of mice induced to shed virus was reduced, and the investigators speculated that vaccine efficacy may have been related to strong stimulation of T-cell responses. Because of the short-term nature of induced shedding, the duration of the vaccine efficacy was not addressed.

The adjuvant used here, MTP-PE was selected because at the time this study was begun, it appeared to be the most powerful adjuvant likely to be approved for human use. Unfortunately, additional human studies revealed unacceptable local side effects with the MTP-PE adjuvant, and it is now no longer considered to be a candidate for use in humans.

Although there is a report that subconjunctival injection of buffer can induce HSV-1 reactivation in the mouse eye, our experience in the rabbit differs. We have found that in the rabbit, subconjunctival injection of the adjuvant used in the present study does not result in an increase in recurrent ocular shedding compared with subcutaneous injection of the same adjuvant on the back (Nesburn et al., manuscript submitted, 1998). In addition, subconjunctival injections are routinely used clinically for drug delivery with no evidence that such injections induce ocular herpetic recurrences in humans.

CONCLUSIONS

Our results show that recurrent HSV-1 corneal disease in latently infected rabbits can be significantly reduced by a therapeutic local periocular vaccination. To our knowledge, this is the first report in any animal model of a successful therapeutic vaccine against recurrent HSV-1-induced herpetic keratitis. In
addition, our results suggest that the vaccine efficacy against recurrent herpetic keratitis may be greater than that against recurrent HSV-1 ocular shedding, because efficacy against herpetic keratitis appeared to be longer lasting than that against recurrent spontaneous shedding. In addition, the heterotypic gD2 vaccine was as efficacious as the homotypic gD1 vaccine against recurrent herpetic keratitis, whereas the homotypic vaccine was much more efficacious than the heterotypic vaccine against recurrent HSV-1 shedding. These results support the concept that development of a therapeutic vaccine for ocular HSV-1 recurrence in humans may well be possible.

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