Protection Against Herpes Simplex Virus-Induced Eye Disease After Vaccination With Seven Individually Expressed Herpes Simplex Virus 1 Glycoproteins

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Purpose. To compare the efficacy of each of seven expressed herpes simplex virus 1 (HSV-1) glycoproteins as vaccines to protect against ocular disease after primary ocular HSV-1 infection.

Methods. Mice were vaccinated three times with equal amounts of each of seven individually expressed HSV-1 glycoproteins (gB, gC, gD, gE, gG, gH, and gl) and then ocularly challenged with McKrae, a corneal disease-producing strain of HSV-1. Viral clearance from the eye, blepharitis, keratitis, and neovascularization were determined at various times after infection.

Results. Mice vaccinated with gD or gB had the best protection against eye disease. Vaccination with gl, gC, or gE produced moderate protection against eye disease. Vaccination with gG produced less protection, and vaccination with gH produced no apparent protection against eye disease.

Conclusions. These results suggest that when used as vaccines, different HSV-1 glycoproteins provide different levels of protection against HSV-1-induced eye disease. Based on comparison with the authors' previously published results, the ability of each glycoprotein to protect against eye disease correlated with the ability of the glycoprotein to induce high serum neutralizing antibody titers and killer cell activity. Results suggest that the effectiveness of these seven glycoproteins in protecting against eye disease can be ranked as follows: gD > gB > gl > (gC = gE) > gG > gH. Invest Ophthalmol Vis Sci. 1995;36:1352-1360.

Herpes simplex virus (HSV) is a major cause of ocular infection in developing countries.1-3 In the United States, herpes simplex virus 1 (HSV-1) is the major cause of blindness from an infectious agent.4 After primary HSV-1 infection of the eye, virus ascends to the trigeminal ganglia, where it becomes latent in sensory neurons.5-8 At various times throughout the life of the latently infected individual, the virus may reactivate, travel back to the eye, and cause recurrent disease, potentially leading to keratitis and loss of sight.9-11 Thus, an ideal HSV vaccine against human ocular herpetic disease should prevent primary ocular infection, latency after primary infection, and eye disease.12,13

There are at least 10 antigenically distinct glycoproteins in HSV-1 virions.14-27 HSV-1 glycoproteins are the primary inducers and targets of the immune response during HSV infection.25 In addition, three HSV-1 glycoproteins (gC, gE, and gl) can directly interact with components of the immune system.24,25 Most immunologic studies and vaccine studies on HSV-1 glycoproteins have focused on gD, gB, or both because they were the first two HSV glycoproteins to be shown to provide protection against viral challenge in animals.12,13,26,27 Until our recent work on the expression of HSV-1 glycoproteins in baculovirus vectors,28-34 no laboratory had undertaken to express large quantities of numerous glycoproteins in the same type of expression vector so that meaningful comparative studies could be performed.

In the current study, we have evaluated the ability of vaccination with each of the first seven HSV-1 glycoproteins to be identified (gB, gC, gD, gE, gG, gl, and gH)14-19 to protect against ocular disease after ocular challenge with a stromal disease-producing strain of HSV-1 (McKrae). Mice were vaccinated using equal amounts of each of the seven individual HSV-1 glyco-
proteins expressed in a baculovirus expression system. The highest level of protection against eye disease was obtained by vaccination with gB. This was followed by vaccination with gB. Vaccination with gL, gC, or gE produced less protection against eye disease. Vaccination with gG or gH provided no apparent protection. These results represent the first study in which a large number of HSV-1 glycoproteins, all expressed with the same expression system, have been compared for their potential as vaccines to protect against HSV-1-induced eye disease.

MATERIALS AND METHODS

Virus

Plaque purified HSV-1 strains were grown in CV-1 cell monolayers in minimal essential medium containing 10% fetal calf serum, as described. McKrae, a stromal disease-causing, neurovirulent, HSV-1 strain was the challenge virus. KOS, a nonneurovirulent, nonstromal disease-producing strain was the positive control vaccine. Baculovirus recombinants were grown in Sf9 cells using TNM–FH media containing 10% fetal bovine serum, as described.

Mice

Six- to 8-week-old female Balb/c mice were used. Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Expressed Glycoproteins

Recombinant baculoviruses expressing gB, gC, gD, gE, gG, gH, or gI (all derived from KOS) were developed in our laboratory. The level of expression for each recombinant glycoprotein was sufficient to enable us to identify the expressed glycoproteins by Coomassie blue staining of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of total cell extracts. The relative proportion of each expressed glycoprotein was determined by scanning Coomassie blue-stained gels on a laser densitometer. Individual glycoproteins were expressed at relatively similar high levels representing at least 10% of the total cell protein.

Preparation of Cell Lysates for Immunization

Sf9 cells were infected with 10 plaque-forming units of baculovirus recombinants (gB, gC, gD, gE, gG, gH, or gI). The infected cells were collected after 72 hours, washed, suspended in phosphate-buffered saline and freeze-thawed for later use. For mock vaccination, we used Sf9 cells infected with wild type baculovirus under the same conditions and identically processed.

Immunization of Mice

Mice were vaccinated three times at 3-week intervals with freeze-thawed cell lysates containing an individual baculovirus recombinant expressed glycoprotein. All inoculations were performed using extract from 1 × 10^6 cells that contained 5 to 10 μg of the specified glycoprotein. Each vaccination consisted of a subcutaneous and an intraperitoneal inoculation administered simultaneously. Subcutaneous injections were performed using Freund's complete adjuvant on day 0 and a similar preparation with Freund's incomplete adjuvant on days 21 and 42. Intraperitoneal injections were performed on the same days using the same dose of infected Sf9 cells in phosphate-buffered saline. Negative control, mock-vaccinated mice were similarly inoculated with wild type, baculovirus-infected Sf9 cells. Positive control mice were inoculated according to the same schedule, but the inoculations were performed only intraperitoneally using 2 × 10^6 plaque-forming units of live KOS in tissue culture media.

Ocular Challenge

Ocular challenge was performed 3 weeks after the final vaccination; 2 × 10^5 plaque-forming units of HSV-1 strain McKrae in 5 μl of tissue culture media was placed in each eye without corneal scarification, and the lid was held closed and rubbed gently for 30 seconds.

Monitoring Eye Disease

Severity of ocular disease was scored on a scale of 0 to 4 in a masked fashion by examination with slit lamp biomicroscopy using 1% fluorescein sodium to delineate epithelial ulceration, iritis, and stromal keratitis, as we have previously performed in rabbits. Eyes were examined on days 1, 3, 7, 14, 21, and 28.

Viral Clearance From the Eye

To detect virus in the eyes of challenged mice, the eyes from three mice (six eyes) were swabbed with a Dacron swab (spectrum type 1; Spectrum Laboratories, Houston, TX), and the swabs were transferred to a 12 × 75-mm culture tube containing 0.5 ml of media. The presence of virus in the tear films over time was determined by analysis of tear films collected on days 1, 2, 3, 4, 5, 7, 10, 14, 21, and 28. Briefly, 100-μl aliquots were placed on confluent monolayers of CV-1 cells in 96-well plates. The cells were incubated at 37°C for 1 hour. The medium was removed, and the cells were overlaid with 1% methylcellulose. Plates were incubated at 37°C for 3 days, stained with 1% crystal violet, and observed for the presence or absence of viral plaques.

Statistical Analysis

Blepharitis, keratitis, and neovascularization results were analyzed by the Student's t test using Instat, a personal computer program. Results were considered statistically significant when P < 0.05.

RESULTS

Effect of Vaccination on Viral Clearance From the Eye

Ten Balb/c mice per group were vaccinated three times at 3-week intervals with one of the seven glyco-
**TABLE I. Detection of Virus in Tear Films of Mice Vaccinated With Individual HSV-1 Glycoproteins After Ocular Challenge**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Days After Ocular Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>gB</td>
<td>+</td>
</tr>
<tr>
<td>gC</td>
<td>+</td>
</tr>
<tr>
<td>gD</td>
<td>-</td>
</tr>
<tr>
<td>gE</td>
<td>-</td>
</tr>
<tr>
<td>gF</td>
<td>-</td>
</tr>
<tr>
<td>KOS</td>
<td>-</td>
</tr>
<tr>
<td>Mock</td>
<td>-</td>
</tr>
</tbody>
</table>

* Mice were vaccinated three times and challenged ocularly as described in Materials and Methods. 
† The presence of virus in the eyes of challenged mice was determined by titration of tear films as described in Materials and Methods. 

— = no virus in any eye; + = virus in one eye; ++ = virus in two to three eyes; +++ = virus in four to five eyes; and ++++ = virus in six eyes.

proteins, as described in Materials and Methods. A negative control group of 10 mice was similarly inoculated with wild type baculovirus expressing no HSV-1 glycoprotein. A positive control group of 10 mice was inoculated on the same schedule with 2 × 10⁵ plaque-forming units of live KOS (an avirulent HSV-1 strain). Three weeks after the final vaccination, all mice were challenged biocularly with a corneal disease-producing strain of HSV-1 (McKrae). Tear films were collected from six eyes per group on days 1, 2, 3, 4, 5, 7, 10, 14, 21, and 28 after challenge and cultured for the presence of infectious virus (Table 1). In the gC, gH, and mock-vaccinated groups, virus was detected in one or two of the six eyes as late as 10 days after infection. In contrast, after postinfection day 5, no virus was detected in any eyes in the gB-, gE-, gG-, or gl-vaccinated groups. In the gD-vaccinated group, no virus was detected after day 3, whereas in the KOS-vaccinated mice, no virus was detected in tear films at any time after infection. Thus, compared to mock-vaccinated mice, vaccination with gC or gH did not appear to affect clearance of virus from the eye. Of the seven glycoproteins tested, vaccination with gD appeared to produce the most rapid clearance of virus from the eye. Vaccination with gB, gE, gG, or gl also appeared to decrease the time required for virus clearance.

**Protection of Vaccinated Mice From Blepharitis After Lethal Ocular Herpes Simplex Virus 1 Challenge**

The vaccinated and ocularly challenged mice were monitored for blepharitis, neovascularization, and keratitis on days 1, 3, 7, 10, 14, 21, and 28 after challenge using a scale of 0 (no disease) to 4 (maximum disease), as described in Materials and Methods. Mean and standard deviation for each of the disease scores were shown in Figures 1 to 9, and raw data showing the incidence of disease are shown in Table 2. All groups initially contained 10 mice (20 eyes/group) (Figs. 1 to 9; also see Table 2). Consistent with what we have previously reported, numerous mice in the mock-, gG-, and gH-vaccinated groups did not survive past day 10 after ocular challenge. Thus, after this time, the gE, gH, and mock-vaccinated groups consisted of only 12 eyes each. Also consistent with our previous reports, the remaining groups were completely protected against HSV-1-induced death and consisted of 10 mice (20 eyes) each throughout.

Blepharitis was not detected in any eyes before day 3 (Figs. 1 to 9, closed bars; Table 2). For most groups, the highest level of blepharitis was seen on day 7 after challenge. The exceptions were gB, which had similar blepharitis levels on days 7 and 14 (Fig. 2); gC, which peaked on day 14 rather than on day 7 (Fig. 3); and KOS, in which blepharitis was never detected (Fig. 9). The peak blepharitis score in the mock-vaccinated group was 1.7 ± 0.4 (Fig. 1). The gD- and gl-vaccinated groups had average peak blepharitis scores of 0.6 ± 0.2 (Fig. 4) and 0.6 ± 0.3 (Fig. 8), respectively, and both were significantly less than the mock group (Student's t-test, \( P < 0.02 \)). In contrast, the mice vaccinated with gB (Fig. 2), gC (Fig. 3), gE (Fig. 5), gG (Fig. 6), and gH (Fig. 7) all had average peak blepharitis scores that were not significantly different from the mock-vaccinated group (\( P > 0.05 \)). Thus, vaccination with gD or gl provided significant protection against blepharitis, whereas vaccination with gB, gC, gE, gG, or gH did not. Interestingly, vaccination with KOS completely eliminated detectable blepharitis, and this protection was significantly more efficient than any of the individual glycoproteins (\( P < 0.003 \)).

**Protection of Vaccinated Mice From Herpes Keratitis After Lethal Ocular Herpes Simplex Virus 1 Challenge**

Keratitis in the form of geographic ulceration first appeared on day 3 and proceeded to corneal ulcer-
Vaccination With Seven Herpes Simplex Virus 1 Glycoproteins

FIGURE 1

1. Mock vaccinated

Days Post Ocular Challenge

FIGURE 2

2. gB vaccinated

Days Post Ocular Challenge

FIGURE 3

3. gC vaccinated

Days Post Ocular Challenge

FIGURE 4

4. gD vaccinated

Days Post Ocular Challenge

FIGURES 1 TO 4. Protection against eye disease in mice vaccinated three times with gB, gC, gD, gE, gG, gH, or gI. Ten mice per group (20 eyes) were vaccinated with individual glycoproteins and challenged ocularly, as described in Materials and Methods. Eye disease was examined on days 1, 3, 7, 10, 14, 21, and 28, as described in Materials and Methods. The numbers in parentheses indicate the number of eyes examined on the indicated day after challenge. Differences were caused by the differences in survival rates among the groups. Eye disease was scored on a scale of 0 to 4: 0 = no disease; 1 = 25% involvement of the lid or cornea; 2 = 50% lid or cornea involvement; 3 = 75% lid or cornea involvement; and 4 = 100% lid or cornea involvement. (shaded bars) = blepharitis; (diagonal bars) = keratitis; and (clear bars) = neovascularization. The bars below the zero line are shown as place holders and represent no disease.
5. gE vaccinated

6. gG vaccinated

7. gH vaccinated

8. gI vaccinated

Days Post Ocular Challenge

Days Post Ocular Challenge

Days Post Ocular Challenge

Days Post Ocular Challenge

FIGURE 5.

FIGURE 6.

FIGURE 7.

FIGURE 8.

ation and then corneal scarring by day 28 (Figs. 1 to 9, shaded bars; also see Table 2). In general, in eyes with keratitis, the scores peaked and were similar on days 21 and 28 after ocular challenge. Statistical analyses were, therefore, performed using the results from day 28. The surviving mice in the mock-vaccinated group had an average keratitis score of 0.8 ± 0.3 (Fig. 1). No protection was produced by vaccination with gH (Fig. 7) (1.0 ± 0.5, P = 0.38). The average keratitis score for the gC (Fig. 3) and gG (Fig. 6) groups was less than for the mock-vaccinated group, but the differences were not statistically significant (0.3 ± 0.2 and 0.3 ± 0.3, P > 0.3). In contrast, protection against keratitis was readily apparent in the groups vaccinated with gB (Fig. 2), gD (Fig. 5), gE (Fig. 5), gI (Fig. 8), or KOS (Fig. 9) (P < 0.01), and no keratitis was de-
Vaccination With Seven Herpes Simplex Virus 1 Glycoproteins

As with keratitis, in eyes with neovascularization, the complete protection against keratitis. glycoproteins (gB, gD, gE, or gI) appeared to produce significant protection against keratitis. Unlike blepharitis, which complete protection was provided only by vaccination with KOS, gB and gD each produced complete protection against keratitis.

Protection of Vaccinated Mice From Neovascularization After Ocular Challenge

As with keratitis, in eyes with neovascularization, the scores peaked and were similar on days 21 and 28 after ocular challenge (Figs. 1 to 9, open bars; also see Table 2). The mock- (Fig. 1) and gH- (Fig. 7) vaccinated mice had similar neovascularization scores, close to 1, that were not significantly different (P = 0.28). The gC- (Fig. 3), gE- (Fig. 5), gG- (Fig. 6), and gI- (Fig. 8) vaccinated groups all had peak scores lower than the mock-vaccinated group, but these differences were not statistically significant (P > 0.1). The gB (Fig. 2), gD (Fig. 4), and KOS (Fig. 9) groups had no detectable neovascularization, and this protection was highly significant compared to the mock-vaccinated group (P = 0.0002). Thus, gB and gD were the only individual glycoproteins that appeared to provide efficient protection against neovascularization.

DISCUSSION

We previously reported the construction of recombinant baculoviruses individually expressing the seven HSV-1 glycoproteins studied here.28-34 We found that vaccination of mice with gB, gC, gD, gE, or gI resulted in production of high neutralizing antibody titers to HSV-1 and protection against death after intraperitoneal or ocular challenge with lethal doses of HSV-1. This protection was similar to the protection provided by vaccination with live, nonviral HSV-1 (100% survival). In contrast, vaccination with gH produced low neutralizing antibody titers and no protection against lethal HSV-1 challenge. Vaccination with gG produced no significant neutralizing antibody titer and no protection against ocular challenge, but it did show modest protection against lethal intraperitoneal challenge. Compared to the other glycoproteins, gG and gH were inefficient in preventing the establishment of latency, whereas gD provided the best protection against the establishment of latency.

We also found that delayed-type hypersensitivity (DTH) responses to HSV-1 at day 3 were highest in gG-, gH-, and gE-vaccinated mice, whereas on day 6, gC-, gE-, and gI-vaccinated mice had the highest DTH responses. All seven glycoproteins produced lymphocyte proliferation responses, with the highest response seen with gC. The same five glycoproteins (gB, gC, gD, gE, and gI) that induced the highest serum neu-

9. KOS vaccinated

<table>
<thead>
<tr>
<th>Severity of Eye Disease</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N)</td>
<td>1/20</td>
<td>3/20</td>
<td>7/20</td>
<td>14/20</td>
<td>21/20</td>
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<tr>
<td>Days Post Ocular Challenge</td>
<td>(20)</td>
<td>(20)</td>
<td>(20)</td>
<td>(20)</td>
<td>(20)</td>
</tr>
</tbody>
</table>

Table 2. Incidence of Eye Disease in Mice Vaccinated With Individual HSV-1 Glycoproteins After Ocular Challenge

<table>
<thead>
<tr>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bla</td>
<td>Neo</td>
<td>Ker</td>
<td>Bla</td>
<td>Neo</td>
</tr>
<tr>
<td>gB</td>
<td>0/20</td>
<td>0/20</td>
<td>1/20</td>
<td>8/20</td>
</tr>
<tr>
<td>gC</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>10/20</td>
</tr>
<tr>
<td>gD</td>
<td>1/20</td>
<td>0/20</td>
<td>2/20</td>
<td>5/20</td>
</tr>
<tr>
<td>gE</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>10/20</td>
</tr>
<tr>
<td>gG</td>
<td>1/20</td>
<td>0/20</td>
<td>2/20</td>
<td>5/20</td>
</tr>
<tr>
<td>gH</td>
<td>1/20</td>
<td>0/20</td>
<td>0/20</td>
<td>10/20</td>
</tr>
<tr>
<td>KOS</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Mock</td>
<td>0/20</td>
<td>0/20</td>
<td>1/20</td>
<td>11/20</td>
</tr>
</tbody>
</table>

Bla = blepharitis; Neo = neovascularization; and Ker = keratitis.

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teralization titers and the best protection against lethal challenge also induced killer cell activity. These results suggested that protection against lethal HSV-1 challenge and the establishment of latency correlated best with high neutralizing antibody titers, although there also may have been a correlation with killer cell activity.

In this article, we have extended these findings to the analysis of protection against eye disease. Our results indicated that, regarding clearance of challenge virus from the eye, vaccination with gD was the most efficient glycoprotein, followed by gB, gE, gG, and gl, whereas gC and gH were ineffective. Regarding protection against blepharitis, gD and gl were most efficient, whereas the other five glycoproteins were ineffective. Regarding protection against keratitis, gD and gB were most efficient, followed by gE and gl, whereas gC, gG, and gH were ineffective. Regarding protection against neovascularization, gD and gB were most effective, followed by gC, gE, gG, gl, and gH. Thus, for all the ocular parameters examined, gD was always as effective or more effective than any of the individual glycoprotein vaccines.

Interestingly, vaccination with live KOS virus appeared to provide complete protection against all four parameters examined in this study. The KOS-vaccinated mice had no detectable replication of challenge virus in their eyes, no blepharitis, no keratitis, and no neovascularization. This is similar to the results reported by Tullo et al after ocular challenge of mice vaccinated in ear pinna. In contrast, although vaccination with gD completely eliminated keratitis and neovascularization, virus was detected in some eyes for up to 3 days after challenge, and a small amount of blepharitis occurred. The more efficient protection seen with KOS could be the result of vaccination with a live virus vaccine. It also could be the result of vaccination with multiple glycoproteins rather than vaccination with a single expressed glycoprotein.

The level of eye disease seen in mice vaccinated with gC or gE was higher than we expected because we found previously that each of these glycoproteins induced high neutralizing antibody titers and completely protected vaccinated mice from death after lethal ocular challenge. This apparently reduced protection may have something to do with the fact that gC and gE individually interact with specific components of the immune system. It is thought that these interactions may help the virus partially evade the immune response. Antibody to these glycoproteins induced by vaccination may reduce this evasion, resulting in more severe ocular disease than expected. Even though gl binds to a specific part of the immune system (the Fc receptor), it does so only in the presence of gE. Thus, antibody to gl protein does not block binding of the Fc receptor (by gE). This may be why gl vaccination (unlike gE and gC vaccination) does not appear to have less protection than expected.

Numerous reports have shown the possible role of DTH in corneal scarring and neovascularization. Several studies have also suggested that local expression of gamma interferon is correlated with higher levels of eye disease. Previously, we showed that vaccination with gC, gE, gG, gH, or gl induced DTH responses in mice, whereas vaccination with gB or gD did not. In the current study, we found that mice vaccinated with these five DTH-inducing glycoproteins also had more severe eye disease than mice vaccinated with gD or gB that did not induce DTH. Therefore, it is possible that either DTH, interleukin-2, or gamma interferon may be a major contributing factor in the eye disease seen in mice vaccinated with gC, gE, gG, gH, or gl.

Our results show that different expressed glycoproteins used as vaccines produce different patterns of protection against infection. Based on the results obtained in this study, different glycoproteins can be ordered as to ocular protectiveness as follows: gD > gB > gl > (gC, gE) > gG > gH. Because KOS produced better protection than any of the individual glycoproteins, experiments are under way to determine if a cocktail of expressed glycoproteins will be a more efficient vaccine than any individual glycoprotein.

Key Words
HSV-1, eye disease, glycoproteins, ocular, mice, vaccine

Acknowledgments
The authors thank Susan Slanina and Anita Avery for technical assistance.

References
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