Antibody Response and Protective Capacity of Plasmid Vaccines Expressing Three Different Herpes Simplex Virus Glycoproteins

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Plasmid expression vectors were constructed that contained the genes encoding herpes simplex virus 1 (HSV-1) glycoproteins C (gC), D (gD), and E (gE). Mice receiving two intramuscular injections of expression plasmid (50 μg) produced a specific HSV-1 antibody response. Mice receiving the gD plasmid were protected against a lethal intraperitoneal challenge of HSV-1 (5 × 10⁴ pfu) but not against more demanding challenge doses. Protection with gC or gE plasmid vaccination could be demonstrated only if the inoculating dose of DNA was increased to 250 μg. In contrast, all mice immunized with vaccinia recombinants expressing either gC or gE survived HSV-1 challenge. Analysis of the HSV-1 antibody isotype produced by plasmid immunization revealed a response dominated by IgG2a. Combination delivery of all three glycoprotein expression plasmids provided better protection against lethal challenge, but mice receiving the combination were still not able to withstand increased challenge doses of virus.

Most vaccine strategies for herpes simplex virus (HSV) have focused on the development of subunit vaccines that consist of one or more of the HSV glycoproteins. Vaccination of mice with purified glycoprotein D (gD) provides protection against a lethal intraperitoneal (ip) challenge of HSV, and vaccination of guinea pigs with recombinant gD or gB protects against intravaginal HSV-2 infection [1, 2]. To date, clinical trials indicate that purified gD has induced neutralizing antibody responses in previously uninfected persons and boosted the antibody response in patients with genital herpes [3]. However, a clinical trial designed to determine the efficacy of a therapeutic vaccine in patients with genital herpes found only a modest effect on the frequency of HSV recurrences [4], and there is no evidence yet to indicate that gD is effective as a prophylactic vaccine. These vaccines may be less than ideal for either prevention or therapy against genital HSV infection in humans for several reasons. First, effective resolution of viral infection and control of virus spread after reactivation from latency requires an efficient cell-mediated immune response that may not be stimulated by protein subunit vaccines. Second, it is quite likely that a successful immune response to a complex virus such as HSV, which codes for >75 different proteins, will require both cellular and humoral immune responses to multiple viral antigens.

DNA vaccines have several potential advantages as subunit vaccines. These include the elimination of time-consuming purification of recombinant proteins, the ability to stimulate both cell-mediated and humoral immunity, and the perceived safety advantages of subunit vaccines over live vaccines (for recent reviews, see [5, 6]). For complex pathogens, direct inoculation of plasmid DNA encoding viral antigens also offers an efficient means by which to evaluate multiple potential genes for their inclusion in a subunit vaccine [7].

Several reports have now described the use of plasmid DNAs encoding herpesvirus proteins to evoke a protective immune response in animal models. Plasmid expression of HSV-1 gB or ICP27 protected mice against HSV challenge in a zosteriform model of recrudescence [8, 9], and expression of HSV-1 gD protected mice against lethal ip challenge and induced low levels of neutralizing antibody [10]. Immunization with plasmids encoding HSV-2 gD or a truncated form of gB protected mice and guinea pigs from vaginal challenge with HSV-2 [11–13]. However, it is not yet clear which viral antigens would be most effective in formulation of a candidate vaccine, and it seems likely that many candidate viral genes will need to be evaluated individually and in combination. Toward this goal, we inoculated mice with DNA constructs expressing three different HSV glycoproteins (gD, gC, and gE) and characterized the resulting antibody response to each. Further, we evaluated whether simultaneous delivery of all three glycoprotein expression plasmids increased protection over any one alone.

Materials and Methods

Plasmids. The HSV-1 KOS gD, gC, and gE genes were isolated by polymerase chain reaction (PCR) using primers comple-
For Biologics Research and Evaluation (CBER), fed autoclaved pression plasmids were constructed containing the genes ... serology analysis and partial DNA sequencing of both the 5' and 3' ends of the glycoprotein reading frame. In vitro expression of the specific HSV-1 glycoprotein was verified by transfection of Vero cells with the appropriate expression plasmid followed by Western blotting and/or immunohistochemical staining. Routine procedures were used for PCR, cloning, and DNA sequencing [14]. Large amounts of plasmid DNA were prepared using Qiagen columns (Qiagen, Chatsworth, CA) and endotoxin-free buffers. Plasmid DNA was resuspended in low-endotoxin PBS (≤0.1 ng/mL; BioWhittaker, Walkersville, MD) for immunization.

Viruses. HSV strains KOS and McKrae were grown and titrated in Vero cells. The vaccinia virus containing gC has been described [15]. A vaccinia virus containing the HSV-1 gE gene under control of the vaccinia growth factor promoter was constructed in a similar manner. Vaccinia virus stocks were titered on CV-1 cells. All virus titers were calculated and expressed in plaque-forming units per milliliter.

Animals and immunizations. Specific-pathogen-free, male BALB/cByJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used at 6–12 weeks of age. All mice were housed in sterile microisolator cages in a barrier environment at the Center for Biologies Research and Evaluation (CBER), fed autoclaved food and water ad libitum, and routinely tested for common murine pathogens by a diagnostic service provided by the Division of Veterinary Services (CBER).

Groups of 5 BALB/cByJ mice received 100-μL injections of purified plasmids into the quadriceps muscles of the hind legs. In most cases, injections were 3 weeks apart; blood samples were taken from the tail vein 1 day before injection or challenge, and approximately equal volumes of blood from individual mice were pooled within a group. Three weeks after the final injection, mice were challenged ip with 5 × 10^4 pfu of HSV-1 McKrae, which was ~10 LD_{50}, as determined in BALB/cByJ mice. LD_{50} was calculated by the method of Reed and Muench as discussed by Lennette [16]. Mean time to death (days) was calculated by arithmetic mean ± SD for all mice within a group that died; surviving mice were not included in this calculation. The statistical significance of differences in mean time to death was assessed using Student’s t test.

HSV glycoprotein ELISA. HSV glycoproteins were purified from HSV-1 KOS–infected Vero cells by lentil-lectin chromatography, as described by Pachl et al. [17]. Immulon 1 plates (Dynatech, Chantilly, VA) were coated at 37°C overnight with 1 μg/mL glycoprotein preparation diluted in PBS (pH 7.2) and blocked the next day with 10% horse serum in PBS for 30 min at 37°C. Serum samples were applied in serial 2-fold dilutions and incubated for 2 h at 37°C. Plates were washed throughout with PBS-Tween (0.05%). After the plates were washed, enzyme-labeled antibodies (goat anti–mouse immunoglobulin or goat anti–mouse IgG1, IgG2a, IgG2b, or IgG3) directly conjugated to horseradish peroxidase (Southern Biotech, Birmingham, AL) were added for detection of specific antibodies. Optimal concentrations for specificity and sensitivity of the various lots of enzyme-labeled antibodies were determined in separate experiments using a panel of myeloma proteins with known heavy- and light-chain compositions. For color development, ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added; the reaction was stopped at 30 min by the addition of 1.5% KF (in H_2O), and the optical density (OD) was determined at 405 nm (reference OD = 490 nm). End-point titer was defined as the highest dilution of serum that gave an A_{50} value that was 2-fold greater than that of the matched dilution of normal prebleed mouse serum and was also >0.050. The sensitivity of the ELISA for HSV-1 glycoproteins was confirmed using monoclonal antibodies for gD, gE (Advanced Biotechnologies, Columbia, MD), and gC (Biodesign International, Kennebunk, ME).

HSV neutralization. Serial 2-fold dilutions of pooled serum samples were incubated with 5 × 10^3 pfu of HSV-1 and rabbit complement (1:100) in 96-well plates for 3 h at 37°C. Vero cells (5 × 10^3) were added, and the monolayers were incubated for 2 days at 37°C before being fixed and stained with 20% ethanol/7.5% formaldehyde/0.3% crystal violet solution. Neutralizing end-point titers were defined as the highest dilution that reduced cytopathic effects by 50%.

Results

To evaluate the antibody response to HSV glycoproteins expressed during plasmid DNA immunization, eukaryotic expression plasmids were constructed containing the genes coding for HSV-1 gC, gD, and gE. Recent reports [10–13] have indicated that plasmids expressing gD elicit an antibody response when injected intramuscularly into animals, and vaccination with these expression plasmids will protect animals against viral challenge. Therefore, our initial experiments utilized the gD expression plasmid to optimize several parameters of DNA inoculation. Initial results indicated that the specific antibody response was very low or absent for up to 6 weeks after a single injection of plasmid DNA; a second injection of plasmid DNA given 3 weeks after the initial injection boosted the specific antibody response; no advantage was conferred by waiting 6 weeks for a second injection of plasmid DNA; and a third injection of plasmid DNA resulted in a still higher antibody titer but did not lead to increased protection against a lethal ip viral challenge of ~10 LD_{50} (data not shown). Therefore, we selected two injections of plasmid DNA given 3 weeks apart and followed by challenge 3 weeks later as a schedule for vaccination in subsequent experiments.

Immunization of mice with an HSV-1 gD expression plasmid. On the basis of these initial results, we performed a dose titration of the gD expression plasmid and evaluated the antibody response that was elicited. Mice were injected intramuscularly two times at 3-week intervals with different amounts of gD expression plasmid. Sera were obtained 3 weeks after the second injection of plasmid DNA and evaluated for the production of antibody by an HSV-1 glycoprotein–specific ELISA. The results indicated that inoculation with 25 μg, 50 μg, and 100 μg elicited HSV-specific antibody, whereas inoculation with 1
Figure 1. HSV glycoprotein antibody titers in sera of mice inoculated with glycoprotein D (gD) expression plasmid. Mice were inoculated 2 times (3 weeks apart) with indicated amount of gD expression plasmid. Pooled sera from groups of 5 mice were assayed for HSV-specific antibody using HSV glycoprotein ELISA.

and 10 μg did not (figure 1). To further characterize and compare the nature of the elicited antibody response, we analyzed the sera from the HSV-1 antibody-positive groups of mice for their isotype specificity. In this experiment, all 3 dosage groups contained IgG2a-specific gD antibody with titers of 1:160 for the 25-μg group, 1:160 for the 50-μg group, and 1:320 for the 100-μg group. Mice in the 50- and 100-μg groups also had IgG2b antibody with titers of 1:320 and 1:80, respectively, and mice in the 100-μg group also further had IgG1 antibody at a titer of 1:80. The sera from these 3 groups of mice contained neutralizing antibody at titers of 1:4 for the 25-μg group, 1:16 for the 50-μg group, and 1:64 for the 100-μg group.

Plasmid-immunized and control mice (from experiments shown in figure 1) were then challenged by ip injection of HSV-1 McKrae. As shown in figure 2, all of the control immunized mice died within 9 days of challenge; none of the mice that had received 50 μg of gD plasmid DNA died over the course of the experiment (>25 days after challenge). Some mice that received 1 μg or 25 μg of plasmid DNA survived challenge, but most died, with a mean time to death similar to that of the control group. Of interest, 2 mice from the 100-μg group also died after challenge, although later than mice from the control group.

In addition to intramuscular (im) plasmid administration, several other routes of plasmid delivery were studied, including intradermal (id) and intravenous. HSV-1–specific antibody was detected in mice inoculated by all three routes, and all 3 showed some degree of protection. Comparison of id versus im delivery of gD expression plasmid was studied in some detail (table 1). Injection via either route resulted in HSV-1–specific antibody and afforded similar levels of protection. In repeated experiments, im and id inoculation resulted in similar quantities and isotypes of glycoprotein-specific antibodies, and therefore, im inoculation was studied in subsequent experiments.

To assess the strength of protection afforded by plasmid vaccination, mice were inoculated with gD expression plasmid (two im injections of 50 μg, 3 weeks apart) and challenged ip with 10-fold and 100-fold higher doses of HSV-1 McKrae (5 × 10^5 and 5 × 10^6 pfu, respectively). All mice died from these higher challenge doses, and there was no significant extension of the mean time to death compared with that of the plasmid-immunized groups (data not shown). Thus, DNA immunization protects against a lethal challenge of 5 × 10^4 pfu but not against more demanding challenge doses. Indeed, survival of mice challenged with this dose of virus ranged from 20% to 100% in repeated experiments (mean = 63%), indicating the variability of the protective response.

In addition, we compared the isotype profiles of the antibody response to 2 injections of 50 μg of gD plasmid in multiple experiments (figure 3). While IgG2a of relatively similar amounts was detected in all experiments, the titers of the other subclasses were quite variable. These results further underscore the variability of the response to im plasmid vaccination.

Immunization of mice with gC and gE expression plasmids. Plasmids expressing either HSV-1 gC or gE were also evaluated for their ability to elicit HSV-1–specific antibody. Mice were injected with two 50-μg doses of either plasmid, and the sera were evaluated by ELISA. Low titers of HSV-1–specific antibody were detected, but the sera were not neutralizing (table 2). To determine whether the low antibody titers to these two viral glycoproteins was an inherent characteristic of the antigens themselves, vaccinia vectors expressing HSV-1 gC or gE were also used to vaccinate mice. Infection with 5 × 10^7 pfu of either vacinia vector resulted in a substantial HSV-1–specific
antibody response, although only the vaccinia-gC–infected group produced detectable neutralizing antibody (table 2). Isotyping of sera from mice injected twice with 50 μg of gC plasmid detected IgG2a and IgG2b antibody (1:320 each) and low titers of IgG1 and IgG3 (table 2).

Although two injections of 50 μg of gC or gE plasmid produced a detectable antibody response, this was not sufficient to provide protection against a lethal ip challenge of HSV-1 (table 2, figure 4). However, protection could be achieved if mice were inoculated twice with 250 μg of plasmid DNA (figure 4). In contrast, vaccinia vectors expressing either gC or gE protected 100% of mice that were challenged ip with a similar dose of HSV-1 (table 2).

Inoculation of mice with combinations of glycoprotein expression plasmids. Since all three glycoprotein expression plasmids elicited a specific antibody response when injected 2 times at a 50-μg dose, it was of interest to determine whether vaccination with a combination of the three expression plasmids would provide greater protection than any individual plasmid. Mice were therefore inoculated twice with a mixture of all three plasmid DNAs (50 μg each). Isotyping of sera from mice injected with the combination of plasmids indicated predominately IgG2a antibody to HSV glycoproteins (data not shown). The combination of plasmids provided better protection than any plasmid alone (experiment 1, table 3). However, vaccination with the combination of glycoprotein expression plasmids did not provide protection against 10-fold and 100-fold challenge doses of HSV (experiment 2, table 3).

Discussion

While there are several potential advantages to DNA vaccines, evaluation of their effectiveness is still in the early stages.
Figure 3. Isotype analysis of antibody response to glycoprotein D (gD) plasmid immunization in multiple experiments. Mice were immunized by 2 injections of 50 μg of gD expression plasmid (3 weeks apart). Serum was obtained 3 weeks after second injection, pooled for groups of 5 mice, and analyzed for antibody isotype using HSV glycoprotein–specific ELISA and goat anti–mouse IgG1, IgG2a, IgG2b, or IgG3 horseradish peroxidase–conjugated antibodies.

However, since the ideal HSV subunit candidate is not known, DNA delivery may be an efficient way to screen multiple potential gene products for inclusion in either a traditional subunit vaccine or a DNA vaccine. As an initial step in this evaluation and screening process, we characterized and compared the antibody responses to three different HSV glycoproteins expressed by DNA vaccination and evaluated the protective capacity obtained by codelivery of all three expression plasmids.

The im inoculation of mice with plasmids expressing HSV-1 gD, gC, or gE resulted in a specific HSV-1 antibody response. Dose-response experiments indicated that as little as two injections of 25 μg of gD plasmid, given 3 weeks apart, elicited an antibody response. We were not able to detect HSV-1 antibody when lower amounts of plasmid were used, and for practical reasons, most of our experiments used two 50-μg doses of expression plasmid. It might be possible to enhance the immune response to lower doses of plasmid DNA either by use of a gene gun for immunization [18] or by pretreatment of muscle with bupivacaine [19]. Both techniques have been reported to increase plasmid DNA uptake by muscle cells.

It has been demonstrated [11] that two im injections of only 0.8 μg of a gD-2 expression plasmid induce a detectable antibody response. Although it is not clear what might account for the different amounts of plasmid required to elicit an antibody response in the two studies, there are several possible explanations that can be considered. One possibility is that the previously reported study utilized more sensitive antibody detection assays, although this seems unlikely to be the complete explanation, since we were also not able to obtain protection from lethal ip challenge at lower doses of gD plasmid. Another possibility is that during PCR cloning of the HSV-1 glycoprotein genes, certain critical epitopes were lost or altered. Although we think this is unlikely on the basis of restriction enzyme analysis and partial DNA sequencing of the individual clones, the possibility has not been formally eliminated. It is also possible that glycoprotein genes derived from the same HSV-1 strain as the challenge virus (HSV-1 McKrae) would have elicited better protection in this challenge model. Finally, it is possible that actual amounts of protein expressed from different expression plasmids vary greatly, and/or that different plasmid vectors are immunostimulatory to different degrees [20].
Although the majority of our experiments utilized im injection of plasmid DNA, we found that other routes of delivery were also effective in eliciting an antibody response, as has been reported for plasmid delivery of other antigens [18, 21]. A recent study reported that im injection of plasmid expression vector resulted in a predominately IgG2a antibody response, whereas id or gene-gun delivery resulted in a predominately IgG1 response [22]. These results suggested that im and id routes of delivery elicit qualitatively different types of antibody responses. In all of our experiments in which antibody isotype was determined, im delivery of gD expression plasmid resulted in IgG2a gD antibody, consistent with previous reports. However, IgG1 was also sometimes detected, as was IgG2b (Figure 3). In a more limited number of id delivery experiments, both IgG1 and IgG2a were detected. Therefore, in contrast to previous results, we did not observe a shift in the isotype profile following different routes of DNA delivery.

HSV gC and gE also represent good candidate antigens for vaccines. gC is involved in the initial interaction of the virus with the cell [23] and in immune evasion by binding the C3b component of complement [24]. gE is necessary for cell-to-cell spread of the virus [25] and also is likely involved in immune evasion by complexing with gI to bind immunoglobulin Fc receptor [26]. Both glycoproteins, when expressed in heterologous systems and used to vaccinate mice, have been shown to induce protective responses [15, 27, 28]. Although 50-μg doses of gC and gE expression plasmids elicited low antibody responses in vaccinated mice, neither group survived a lethal ip challenge. It is quite likely, however, that a replicating virus will infect many more cells in vivo than can be transfected by plasmid injection. In addition, unlike plasmid vector transfection, vaccinia vectors elicit a broad vigorous immune response during infection. A similar comparison between plasmid-delivered gD and a vaccinia-gD recombinant has been reported [10]. In that study, vaccination with the plasmid gD resulted in lower antibody titers than vaccination with vaccinia-gD, but both methods provided similar low level protection. Nevertheless, the results from such comparative experiments raise questions

**Table 3.** Combination delivery of HSV-1 gD, gC, and gE expression plasmids.

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge (pfu)</th>
<th>No. deaths/total no.</th>
<th>Mean time (days) to death + SD</th>
</tr>
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<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vector DNA</td>
<td>$5 \times 10^4$</td>
<td>5/5</td>
<td>11.6 ± 3.5</td>
</tr>
<tr>
<td>gC/gD/gE plasmids</td>
<td>$5 \times 10^4$</td>
<td>0/5</td>
<td>13.8 ± 5.3</td>
</tr>
<tr>
<td>gC plasmid</td>
<td>$5 \times 10^4$</td>
<td>5/5</td>
<td>9.5 ± 1.9</td>
</tr>
<tr>
<td>gD plasmid</td>
<td>$5 \times 10^4$</td>
<td>2/5</td>
<td>9.5 ± 1.9</td>
</tr>
<tr>
<td>gE plasmid</td>
<td>$5 \times 10^4$</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
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<td>11.5 ± 1.7</td>
</tr>
<tr>
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</tr>
<tr>
<td>gC/gD/gE plasmids</td>
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<tr>
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<td>13 ± 2.0</td>
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<td>gC/gD/gE plasmids</td>
<td>$5 \times 10^4$</td>
<td>3/5</td>
<td>8 ± 1.7</td>
</tr>
</tbody>
</table>
about whether sufficiently robust immune responses can routinely be generated by plasmid vaccination.

Simultaneous delivery of gC, gD, and gE expression plasmids at 50 μg protected mice against lethal challenge, but in multiple experiments, this protection was only marginally better than delivery of gD plasmid alone. In another report, simultaneous delivery of gD and gB expression plasmids protected guinea pigs against vaginal challenge with HSV-2, but there was no comparison made between the protective effect of the individual plasmids and the combination [11]. In our experiments, mice that received all three expression plasmids were not able to withstand increased challenge doses of virus, suggesting that the inclusion of gC and gE plasmids at the 50-μg dose did not augment the protective response. On the other hand, both gC and gE were immunogenic, and it may be that protective effects resulting from their delivery might be more apparent in other challenge models.

While an ip challenge may be a suitable model for an initial assessment of the antibody response to various glycoproteins produced by DNA inoculation, complete characterization of a particular antigen’s potential for inducing protective immunity will require evaluation in several model systems. There are several distinct stages in the interaction of HSV with its host, including infection and replication at a mucosal surface, establishment and maintenance of latency, and reactivation and spread from latency. Protection against infection and disease may require an immune response that targets critical antigens at several of these stages, and other models may reveal effects of plasmid vaccination at different stages in infection. Nonetheless, the heterogeneity of the immune response and the level of the protective response in this model is of concern. However, plasmid DNA vaccination may still be an efficient method to screen multiple viral antigens, individually and in combination, for their inclusion in a subunit vaccine.

References