Herpes Simplex Virus DNA Vaccine Efficacy: Effect of Glycoprotein D Plasmid Constructs

J. E. Strasser, R. L. Arnold, C. Pachuk, T. J. Higgins, and D. I. Bernstein

The impact of vaccination with plasmid DNA encoding full-length glycoprotein D (gD) from herpes simplex virus (HSV) type 2 (gD2), secreted gD2, or cytosolic gD2 was evaluated in mice and guinea pigs. Immunization with plasmids encoding full-length gD2 or secreted gD2 produced high antibody levels, whereas immunization with DNA encoding cytosolic gD2 resulted in significantly lower antibody titers in both species \( (P < .001) \). Vaccination with DNA encoding full-length or secreted gD2 significantly reduced acute disease in mice and guinea pigs (both \( P < .001 \)) and subsequent recurrent disease in guinea pigs \( (P < .05) \). In guinea pigs, immunization with DNA encoding cytosolic gD2 did not protect from acute or recurrent disease, whereas in mice it did protect, but not as well as DNA encoding full-length or secreted gD2. None of the vaccines resulted in improved virus clearance from the inoculation site, and none significantly reduced recurrent disease when used as a therapeutic vaccine in HSV-2-infected guinea pigs.

Herpes simplex virus type 2 (HSV-2) infections are common and increasing with seroprevalence rates now >20% [1]. Acute infection at the mucosal surface is followed by the establishment of a latent infection in the innervating sensory neurons (reviewed in [2]). Reactivation can be accompanied by herpetic lesions, but asymptomatic shedding is even more common [3, 4]. Vaccines can either prophylactically for uninfected persons or therapeutic for persons already infected with HSV-2 [5]. The goals of a prophylactic vaccine are to limit acute viral replication, prevent or modify primary disease, and decrease subsequent symptomatic and asymptomatic recurrences. The goals of a therapeutic vaccine are to decrease clinical recurrences and asymptomatic recurrences in those already infected with HSV [5–7].

Immune protection from HSV-2 infection is complex. HSV-2 antibodies provide some protection from primary disease and limit the establishment of latent infection but are neither necessary nor sufficient to clear viral infection [8, 9]. We [9–11] and others [8] have demonstrated that immunity to vaginal viral replication is primarily mediated by HSV-specific CD4 T cells. Thus, depletion of CD4 T cells in mice previously infected with HSV-2 eliminated protection of the vaginal mucosae, despite the continued presence of local and systemic antibody [9]. However, the exact role of CD4 or CD8 T cells in the protection is far from clear [12–14]. Protection of the sensory ganglia appears to be mediated by either HSV-specific CD4 or CD8 T cells [9]. Other studies have mainly implicated CD8 T cells in protection of the nervous system from HSV infection [15–17].

Immune control of recurrent HSV disease is even less clear. Recent data have correlated recurrences with the number of HSV-specific CD8 T cells [18]. However, the best correlate to the number of recurrences in the guinea pig model of genital herpes was interleukin (IL)-2 production, a CD4 T cell-mediated response [19]. Furthermore, CD4 T cells are the first cells found in recurrent lesions [20–22]. Clearly, the role of T cell subsets in HSV infection has not been well defined.

Many approaches to HSV vaccines have been evaluated, including killed whole virus, subunit, live attenuated, vectored, and DNA vaccines (reviewed in [5, 6, 23]). Because they contain neutralizing and T cell epitopes [23], the HSV-2 glycoproteins, especially glycoprotein D (gD), have been major targets for vaccine development, including DNA vaccines [24]. DNA vaccination produces humoral and CD4 and CD8 T cell responses [25–27]. To evaluate the effect of antigen delivery to different cellular locations, we developed a series of plasmids that encode full-length, secreted, and cytosolic gD2 [28]. Expression of the full-length gD2 molecule resulted in gD2 becoming inserted into the plasma membrane and exposed on the outer surface of the cell. Removal of the glycoprotein’s membrane anchor resulted in a secreted protein that could be internalized, degraded, and presented by neighboring cells, which increased the likelihood of presentation by major histocompatibility complex (MHC) class II. Removal of the signal sequence of gD2 prevented endoplasmic reticulum insertion and caused the protein to remain in the cytosol. Cytosolic localization causes a diminished CD4 T cell response and can ablate the generation of antibodies [29]. The
removal of the signal sequence could also result in enhanced MHC class I presentation, because cytoplasmic retention and high turnover may allow for more efficient loading of MHC class I, which results in improved CD8 T cell-mediated responses.

We postulated that improving the CD4 T cell response might have its greatest impact on a prophylactic vaccine and that improving the CD8 HSV response might have more effect on a therapeutic vaccine. We previously determined the immune response to immunization with these plasmids [28]. As predicted, secretion of gD2 resulted in an increased antibody response and a Th2 dominant response, whereas retention of gD2 in the cytosol diminished the antibody response [28]. Here we describe the relative efficacies of vaccination with DNA encoding full-length, cytosolic, and secreted gD2 in outbred mice and guinea pigs.

Materials and Methods

Plasmids. The plasmids used in this study are described elsewhere [28]. In brief, the full-length gD2 construct, pAPL-gD2, contains the entire gD2 coding sequence from HSV-2 strain 12 in a 2-kb insert [30]. The secreted version of gD2, pTMRI, encodes the intact amino terminus of the protein but lacks the 66 amino acid at the carboxy terminus, comprising the transmembrane and cytosolic domains. The cytosolic gD2, pSS, encodes the mature protein but lacks the signal sequence. The control plasmid encodes human immunodeficiency virus env instead of gD2. The expression vector was identical in all constructs, containing a human cytomegalovirus immediate early promoter and an SV40 polyadenylation signal. Plasmid DNA was purified by column chromatography and formulated in bupivacaine HCl (Ceres Chemical, White Plains, NY) in a buffered sodium chloride solution.

Swiss Webster mice (15 mice/group) were vaccinated once intramuscularly (im) in the quadriceps with 25 µg of DNA in 0.25% bupivacaine. Animals were vaccinated with plasmid encoding either full-length gD2, secreted gD2, cytosolic gD2, or control plasmid. Fourteen days after vaccination, mice were treated with 3 mg of medroxyprogesterone acetate (Upjohn, Kalamazoo, MI). Twenty days after vaccination, animals were bled and were treated with 3 mg of medroxyprogesterone (1 day before viral challenge). The following day, mice were inoculated intravaginally with 5 × 10^3 pfu of HSV-2 strain 333, as described elsewhere [11]. Mice were followed daily for the severity of herpetic disease and were scored using a continuous scale of 0 (no disease) to 4 (central nervous system [CNS] involvement or death).

Guinea pig model of genital herpes. We injected (im) female Hartley guinea pigs (300–350 g; 15 mice/group) in the quadriceps with 100 µg of the DNA in 0.25% bupivacaine. Animals were vaccinated with plasmid encoding either full-length gD2, secreted gD2, cytosolic gD2, or control plasmid. Animals were boosted with an identical injection 3 weeks after the first injection. Sera were collected by toenail clip 3 weeks after the boost. Researchers were blinded to the identity of the vaccine. Animals were intravaginally inoculated 50 days after the boost by instillation of 5 × 10^3 pfu of HSV-2 strain MS into the vaginal vault, as described elsewhere [31, 32]. Animals were then scored daily using a scale of 1–4, as described elsewhere [31]. The total lesion score is the sum of the scores from days 1–14. After recovery from the acute disease on day 14, animals were scored daily for 46 days for the presence or absence of recurrent herpetic lesions [24, 31, 32].

For studies of immunotherapy, 60 Hartley guinea pigs were intravaginally inoculated with HSV-2 strain MS, as described above, and were followed daily for the severity of acute disease. Animals that developed acute disease then were randomized into 1 of 4 groups to receive immunization with plasmid encoding either full-length gD2, secreted gD2, cytosolic gD2, or control plasmid, as described above, on days 21 and 42 after HSV inoculation. Animals then were followed daily for recurrent lesions, until 90 days after HSV inoculation.

ELISA for HSV-2 antibody. The ELISA protocol has been described elsewhere [32]. In brief, 96-well ELISA plates (Costar, Cambridge, MA) were coated with HSV-2 glycoproteins. Nonspecific binding was blocked with bovine serum albumin. Sera were diluted in blocking buffer. Phosphatase-labeled anti–guinea pig IgG (Jackson ImmunoResearch, West Grove, PA) was diluted in blocking buffer and visualized by development with O-phenylenediamine. End points were read on a plate reader (Molecular Dynamics, Sunnyvale, CA). Results are expressed as relative units, compared with sera from animals infected with HSV-2, and subsequently were boosted with HSV-2 glycoproteins.

Mouse ELISA. Isotype ELISAs for HSV-2 antibody were done as described elsewhere [28]. In brief, baculovirus-derived gD2 was used to coat 96-well plates. Plates were subsequently blocked and were incubated with diluted serum, and isotypes were detected with isotype-specific monoclonal antibodies.

Virus swab titration. To quantify the amount of virus in the vaginal vault, guinea pigs were swabbed on days 1, 2, 4, and 6 after viral challenge. Swabs were diluted and were plated on Vero monolayers, and viral plaques were quantified, as described elsewhere [32].

Statistical analyses. Continuous variables were analyzed by 1-way analysis of variance, followed by Tukey posttest probability analysis for between-group comparisons. We used Fisher’s exact test for mortality comparisons. P < .05 was considered significant.

Results

Our previous data [28] confirmed that plasmid encoding the transmembrane deletion of gD2 resulted in secretion of gD2, whereas deletion of the signal sequence increased the turnover rate of the soluble cytosolic gD2 and prevented expression on the surface of cells or secretion of gD2.

Mouse

Initial evaluations determined the antibody response to immunization. As seen in table 1, immunization with the full-length gD2 construct induced an IgG2a dominant HSV-2 antibody response indicative of a Th1 response. An antibody response was also seen in all mice immunized with the secreted gD2 plasmid, but this was a more balanced IgG1 and IgG2a response. The antibody titer in animals receiving plasmid ex-
Table 1. Mouse herpes simplex virus (HSV) antibody isotypes.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>IgG1, ng/mL</th>
<th>IgG2a, ng/mL</th>
<th>IgG1 IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length</td>
<td>69 ± 29</td>
<td>5563 ± 1418</td>
<td>0.01</td>
</tr>
<tr>
<td>Secreted</td>
<td>2284.1 ± 562</td>
<td>11,780 ± 5410</td>
<td>0.19</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>0.00</td>
<td>242.2 ± 82</td>
<td>0.00</td>
</tr>
<tr>
<td>HIV</td>
<td>1.02 ± 1.02</td>
<td>46.7 ± 31</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE. Female Swiss Webster mice were vaccinated once intramuscularly with 25 μg of plasmid DNA, as described in Materials and Methods. Twenty days after the initial vaccination, blood was collected by orbital bleed, and levels of HSV IgG isotypes were determined by ELISA, as described in Materials and Methods. HIV, human immunodeficiency virus.

pressing cytosolic gD2 was significantly less than in the animals receiving DNA encoding secreted gD2 ($P < .001$).

To correlate altered immune response with protection from disease, Swiss Webster mice were given a lethal vaginal challenge of HSV-2 and were monitored daily for disease. None of the animals vaccinated with an irrelevant plasmid (controls) survived. As seen in figure 1, DNA encoding full-length and secreted gD2 both provide complete protection from death ($P < .001$), whereas DNA encoding cytosolic gD2 gave intermediate protection (7 of 15 surviving; $P < .001$ vs. control). Similarly, significant protection from disease symptoms (as assessed by total lesion score) was observed after immunization with the full-length gD2 (figure 2; mean total lesion score, 9.3 ± 1.1; $P < .001$) or the secreted gD2 (mean total lesion score, 9.4 ± 1.1; $P < .001$), compared with that of the controls (mean total lesion score, 13.0 ± 3.2). DNA encoding cytosolic gD2 provided no protection from disease symptoms (excluding viability; mean total lesion score, 15.7 ± 2.3).

Guinea Pig

Prophylaxis. The guinea pig model of genital HSV more closely resembles human disease, because it is not a lethal model and is the only small animal model that permits evaluation of spontaneous recurrences. Therefore, we evaluated the effects of immunization with plasmid DNA encoding full-length gD2, secreted gD2, cytosolic gD2, or control plasmid lacking gD2 on genital HSV-2 infection in this model. HSV-2 antibody was induced in all guinea pigs receiving the full-length or secreted gD2 but was not detected in guinea pigs immunized with the cytosolic gD2 (figure 3). Antibody levels were highest in the group receiving secreted gD2 ($P < .001$, vs. full-length gD2).

As seen in figure 4, total lesion scores were significantly diminished during acute disease (postinoculation days 4–14) after immunization with DNA encoding the secreted (1.2 ± 0.3) or full-length gD2 (2.6 ± 0.7), compared with those of controls vaccinated with an irrelevant plasmid (5.9 ± 0.5; $P < .001$ for each). There was no significant difference between groups receiving full-length or secreted gD2. Immunization with the DNA encoding cytosolic gD2 did not significantly decrease the total lesion score (4.8 ± 0.9), compared with that of the control group.

To determine the impact of vaccination on virus clearance from the vagina, we compared virus titers in vaginal secretions on days 1, 2, 4, and 6. There was no significant difference in symptoms (excluding viability; mean total lesion score, 15.7 ± 2.3).

Figure 1. Survival of mice after genital herpes simplex virus type 2 (HSV-2) challenge. Female Swiss Webster mice (15 mice/group) were vaccinated once intramuscularly with 25 μg of plasmid DNA, as described in Materials and Methods. At day 21 after vaccination, mice were inoculated vaginally with $5 \times 10^7$ pfu of HSV-2 strain 333. Animals were monitored daily for mortality.
Figure 2. Severity of genital disease in female Swiss Webster mice (15 mice/group) vaccinated once intramuscularly with 25 µg of plasmid DNA, as described in Materials and Methods. At day 21 after vaccination, animals were inoculated vaginally with $5 \times 10^7$ pfu of herpes simplex virus type 2 strain 333. Animals were monitored daily for disease on a scale of 0 (no disease) to 4 (central nervous system involvement or death) for 21 days after viral challenge. gD, glycoprotein D; HIV, human immunodeficiency virus.

Figure 3. ELISA antibody levels after vaccination of female Hartley guinea pigs (15 mice/group) vaccinated twice intramuscularly with 100 µg of plasmid DNA. Blood was collected 21 days after the second vaccination. Sera were normalized to guinea pig herpes simplex virus (HSV) type 2 hyperimmune sera, as described in Materials and Methods. gD, glycoprotein D; HIV, human immunodeficiency virus.

Discussion

In the studies described, we found that both full-length and secreted HSV gD2 plasmid prophylactic vaccines protected the animals vaccinated with DNA encoding cytosolic gD2 was least severe.

**Therapeutic.** Although prophylactic vaccination is ideal, a large percentage of the human population is already infected with HSV-2. Protection from reactivation in previously infected persons is therefore important for quality of life and to prevent further spread of the disease. The ability to negatively impact recurrent disease by vaccinating latently infected guinea pigs with protein vaccines has been well documented [33–36]. To examine the impact of vaccination with the various gD2 constructs on recurrences, HSV-2-infected guinea pigs were immunized with the plasmids described above after recovery from acute disease (days 21 and 42 after inoculation). None of the vaccines significantly reduced recurrences when given therapeutically (data not shown). Of interest, although not significant, recurrent disease virus titer on any day for the 4 groups (data not shown), suggesting that the impact of immunization reflects protection of the epithelium as virus exits the nerve endings or at the ganglia rather than at the vaginal mucosae.

Diminished acute disease may or may not impact the establishment of latency and subsequent recurrent disease. Animals were followed for recurrent disease, to determine the impact of prophylactic immunization on recurrences. As seen in figure 5, the effect on recurrent disease parallels that seen against acute disease. The number of recurrent lesion days was decreased significantly in animals immunized with either secreted (4.8 ± 1.4) or full-length gD2 (4.3 ± 0.6; $P > .001$, for either vs. control plasmid [7.3 ± 1.0]), whereas the effect of immunization with DNA encoding cytosolic gD2 (6.9 ± 1.1) was similar to that in controls.

In the studies described, we found that both full-length and secreted HSV gD2 plasmid prophylactic vaccines protected...
against genital HSV-2 inoculation in mice and guinea pigs. In contrast, the cytosolic gD2 plasmid vaccines provided only minimal protection in the mouse model and no protection in guinea pigs. None of the vaccines reduced recurrent disease when used as an immunotherapeutic vaccine (i.e., when given to animals already infected with HSV-2).

The mouse and guinea pig data presented here and elsewhere [28] indicate that vaccination with DNA encoding the cytosolic gD2 produces a minimal antibody response. In contrast, immunization with the plasmid encoding the secreted form of gD2 produces the highest antibody levels. Furthermore, immunization with the secreted form induced a more balanced Th1:Th2 response, whereas the full-length gD2 construct induced a predominant Th1 response. In the mouse, immunization with the full-length gD2 induced a predominantly IgG2a response and interferon-γ, with little evidence of the type 2 cytokines IL-4 or IL-5. Immunization with the secreted form increased IgG1 and IL-4 and IL-5 levels [28].

There are increasing reports of antigen secretion resulting in higher levels of IgG1 [37–44]. Conversely, the removal of an antigen’s signal sequence (resulting in a cytosolic location) decreases antibody levels [29, 45, 46]. Our data concur with that of others and suggest that secretion of antigen results in a stronger Th2-mediated response, with a concomitant increase in antibody titer. This increased antibody titer may alone be sufficient for any increase in protection. However, whereas the Th2 markers increase with secreted antigen, the Th1 markers persist, suggesting a mixed Th1:Th2 response. It may be that this combined response is optimal for protection, at least for HSV-2.

The current data indicate that protection against acute disease is mediated by CD4 T cells [8–11]. This led us to predict that the secreted form of gD2 would result in better protection than would the full-length gD2, whereas the cytosolic form would furnish little protection. Vaccination with the secreted form of gD2 did result in the most effective protection from acute disease (figures 1, 2, and 4). This may be mediated at least, in part, by the increased antibody titer seen in these animals, by other CD4 T cell–mediated responses, or by both. Although antibodies are neither necessary nor sufficient to clear viral infection [8, 9], they provide some protection from primary disease and limit the establishment of latent infection or degree of reactivation [8, 9].

The animals in this study were injected im, which suggests that most of the DNA will be taken up by (and expressed in) muscle cells. Because MHC class II is expressed only by professional antigen-presenting cells (APCs), presentation by MHC class II following im injection therefore requires at least 1 of 3 things to happen: (1) APC(s) take up and express the DNA, (2) the muscle cell(s) expressing the antigen dies and is scavenged by APCs, or (3) the antigen is secreted by the expressing cells and subsequently

Figure 4. Severity of acute genital disease in female Hartley guinea pigs (15 mice/group) vaccinated twice intramuscularly with 100 μg of plasmid DNA. At day 50, after the second vaccination, animals were inoculated with 5 × 10⁷ pfu of herpes simplex virus (HSV) type 2 strain MS. Animals were monitored daily for disease symptoms. Disease was scored on a scale of 0 (no disease) to 4 (100% coverage of perianal region by HSV lesions). HIV, human immunodeficiency virus.
taken up by APCs. Therefore, it is not surprising that secretion of the expressed antigen enhances the MHC class II–mediated immune response(s). It has long been postulated anecdotally that an abundance of antigen increases the humoral response and decreases the cell-mediated response. Plasmid-encoded secreted antigen may be an example of such a phenomenon.

We were somewhat surprised to find that none of the vaccines affected the rate of virus clearance from the viral mucosae. In a previous study with the full-length construct, 3 immunizations (as opposed to 2 used here) significantly reduced vaginal virus titers [32]. Nonetheless, others have also found that vaccines that effectively reduce HSV disease may not reduce vaginal virus clearance [47]. This distinction suggests that vaccines can affect HSV disease by decreasing the amount of virus entering or exiting nerve endings, to cause the primary lesions without affecting local vaginal replication.

None of the DNA vaccines significantly reduced recurrences when used as a therapeutic vaccine in infected animals. Although not significant, the DNA vaccine that most reduced recurrences, when used therapeutically, encoded cytosolic gD2, which suggests that perhaps CD8 T cells may be involved in control of recurrent disease. To enhance therapeutic vaccine efficacy, the CD8 T cell–mediated response may need to be enhanced by increasing MHC class I presentation of HSV-2 antigen, by giving a second boost, or by including other HSV antigens that are CD8 T cell targets. It is also possible that CD4 and CD8 T cells are both important in reactivation from latency and that a combination of the secreted and cytosolic forms of gD2 would prove preferable to either alone. Given the complexity of herpetic infection and its ability to become latent, a combination of immune responses resulting from differentially targeted antigens probably will result in optimal protection.

References


