Improved Protection from Primary Ocular HSV-1 Infection and Establishment of Latency Using Multigenic DNA Vaccines

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Purpose. To compare the effectiveness of immunization with “naked” DNA corresponding to the genes encoding five HSV-1 glycoproteins, gB, gC, gD, gE, and gI (5gP DNA), with immunization with the five glycoproteins (5gP protein). Also, to compare immunization of 5gP protein in Montanide ISA 720 (SEPPIC, Paris, France), an adjuvant recently approved for use in humans, with immunization of 5gP protein in Freund’s adjuvant.

Methods. BALB/c mice were vaccinated with 5gP DNA or 5gP protein emulsified in ISA 720 or Freund’s adjuvant. Neutralizing antibody titers were determined by plaque-reduction assays. IL-2, -4, and -12 and IFN-γ levels were determined by ELISA after in vitro stimulation of spleen cells. After ocular challenge with 2 × 10³ plaque-forming units [pfu] per eye of HSV-1 strain McKrae, virus replication in the eye, survival, blepharitis, corneal scarring, and latency were determined.

Results. Neutralizing antibody titers (approximately 1:800–1:1200), corneal scarring (trace) and survival (100%) were similar for all vaccine groups, including 5gP DNA. Compared with the other vaccine groups, the 5gP DNA group had less ocular virus replication, as judged both by maximum virus titer and time of viral clearance. ISA 720 appeared more effective than Freund’s against ocular virus replication and eye disease. The 5gP DNA-vaccinated mice had less blepharitis and latency than any other group and had the highest levels of IL-12 and IFN-γ. All vaccine groups had similar levels of IL-2.

Conclusions. The 5gP DNA vaccine appeared to be more effective than the corresponding protein subunit vaccine, regardless of adjuvant. Emulsification of the 5gP protein in ISA 720 appeared to be more effective than emulsification in Freund’s adjuvant. (Invest Ophthalmol Vis Sci. 2004;45:506–514) DOI: 10.1167/iovs.03-0828

Herpes simplex virus (HSV) infections are among the most prevalent serious viral eye infections in the United States and are a major cause of virus-induced blindness.1–4 Ocular infection with HSV-1 can cause eye disease ranging in severity from blepharitis, conjunctivitis, and dendritic keratitis to disform stromal edema and necrotizing stromal keratitis.1,5 HSV-1-induced corneal scarring can lead to blindness, making this virus the leading cause of corneal blindness by an infectious agent in developed countries.5 In the United States, approximately 500,000 people annually have recurrent ocular HSV episodes requiring doctor visits, medication, and, in severe cases, corneal transplants. Despite the seriousness of recurrent ocular herpes, the only approved treatments are for acute primary and acute recurrent infections. No drug has yet been approved for the prevention of ocular recurrences. Thus, developing a subunit vaccine that greatly decreases HSV-1 replication in the eye is the most efficient method of controlling HSV-1 infection that leads to loss of vision.

Protective immunity against HSV infection involves both the innate immune system, including macrophages and NK cells, and the adaptive immune system, including neutralizing antibodies and cytotoxic T lymphocytes (CTLs).6–11 Immunization of a host with purified proteins generally induces antibody production and CD4+ T cell responses, but not CD8+ major histocompatibility complex (MHC)-I-restricted CTL or innate responses. In contrast, injection of mice with plasmid DNA encoding the influenza virus nucleoprotein (NP) was observed to induce high titers of neutralizing antibody against the NP, as well as CTL responses against influenza virus.12 Furthermore, immunization with DNA has been shown to stimulate innate immune responses, in addition to humoral and CTL responses, leading to improved protection.13–15 Thus, immunization with DNA may induce a more complete immune response than immunization with protein, in that the former can induce a strong CD8+ T cell response12,16,17 and an innate immune response13–15 in addition to a CD4+ T-cell response.

Previous work from this laboratory showed that immunization with the HSV-1 gB, gC, gD, gE, or gI protein, cloned into baculovirus and expressed in vitro, protects mice against lethal HSV-1 infection,18 but does not eliminate eye disease or the establishment of latency.5,18 More recently, we showed that a cocktail of all five HSV-1 glycoproteins emulsified in Freund’s adjuvant provides more effective immune protection than each baculovirus-expressed protein alone.6,16,18 However, concerns have been raised about the use of Freund’s adjuvant to induce an immune response. In addition, we10 and others12,24 have shown that a DNA vaccine is not as effective as the corresponding protein vaccine in protecting mice against HSV infection. The availability of improved plasmid vectors for DNA immunization and new water in oil (W/O) adjuvants prompted us to reexamine this question. We therefore compared immunization with “naked” DNA, as represented by the genes encoding each of the five HSV-1 glycoproteins, gB, gC, gD, gE, and gI, with immunization with a vaccine containing the five glycoproteins. In addition, we compared the adjuvant Montanide ISA 720, which was recently approved for use in humans, with Freund’s adjuvant when used to emulsify our baculovirus-expressed protein vaccines.

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**Materials and Methods**

**Viruses and Cells**

Triple plaque-purified wild-type McKrae HSV-1 was prepared as described previously. Rabbit skin (RS) cells, which were used to prepare the virus stocks, culture the mouse tear films, and determine the growth kinetics, were grown in Eagle’s minimum essential medium (EMEM) supplemented with 5% fetal calf serum (FCS). Spleen cells were grown in RPMI 1640 medium supplemented with 10% FCS. Baculovirus recombinants were grown in S9 cells with TNM-FH medium (Invitrogen, San Diego, CA) containing 10% fetal bovine serum (FBS). Culture media, supplements, and FCS were purchased from Invitrogen-Gibco (Grand Island, NY). The RS cells were obtained from Richard Thompson (University of Cincinnati) and S9 cells were purchased from Invitrogen.

**Mice**

Female BALB/c mice, aged 4 to 6 weeks, were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**DNA Immunization**

The complete open reading frame (ORF) for each of the five HSV-1 glycoproteins (gB, gC, gD, gE, and gI) was cloned into the VR-1055 expression vector and grown in bacteria. Plasmid DNA encoding each glycoprotein was purified on a cesium chloride gradient. In each experiment, 10 mice per group were inoculated intramuscularly (IM) with 10^6 gDNA in a total volume of 100 μL using a 27-gauge needle. As the negative control, we used mock-vaccinated mice, which were similarly injected with vector alone.

**Protein Immunization**

S9 insect cells were infected with 10 plaque-forming units (pfu)/cell of each baculovirus recombinant, expressing gB, gC, gD, gE, or gI. The infected cells were grown for 72 hours, collected, washed, suspended in PBS, and freeze-thawed. Each expressed glycoprotein was affinity purified using its specific antibody. The purified glycoproteins were emulsified 1:1 in Montanide ISA 720, complete Freud’s adjuvant (CFA), or incomplete Freud’s adjuvant (IFA). On day 0, 10 mice per group were vaccinated subcutaneously (SC) in the shoulder with a glycoprotein cocktail containing 10 μg of each protein (50 μg total) in Montanide ISA 720 or CFA. On days 21 and 42, the mice were vaccinated with the same glycoprotein cocktail in Montanide ISA 720 or IFA. As the negative control, mice were similarly injected with adjuvants alone.

**Lymphokine ELISAs**

The secretion of IL-2, -4, and -12 and IFN-γ by spleen cells was measured in vitro 3 weeks after the third immunization. One mouse per group from four separate experiments was killed, and single cell suspensions of spleen cells were prepared. Spleen cells were cultured for 72 hours at a density of 1 x 10^6/well in 24-well plates in a humidified 5% CO_2 atmosphere. Lymphocytes were cultured in medium alone or in medium containing 10 pfu per cell of UV-inactivated HSV-1 strain McKrae. The supernatants were collected and stored at -80°C, and the concentrations of IL-2, -4, and -12 and IFN-γ were determined with ELISA kits specific for each cytokine (BD PharMingen, San Diego, CA). The concentration of each cytokine in the supernatants was estimated by comparing the optical densities of the unknowns to a range of standards and is presented as mean picograms per milliliter ± SEM.

**Efficacy of DNA Versus Protein Immunization**

**Serum Neutralizing Antibody Titers**

Serum was obtained from immunized mice 3 weeks after the final vaccination, just before virus challenge. The sera from 10 individual mice per group were heat inactivated for 30 minutes at 56°C, diluted in EMEM, mixed with 200 pfu of HSV-1 strain McKrae, and incubated for 1 hour at 37°C. Samples were added to RS cells in six-well microtiter plates and incubated at 37°C for 1 hour. The medium was removed, and the cells were overlaid with 1% methylcellulose. The plates were incubated at 37°C for 72 hours and stained with 1% cresyl violet, and the plaques were counted. The mean of each antibody titer (50% plaque reduction) was expressed as the reciprocal of the serum dilution.

**Ocular Infection**

Ocular challenge was performed 3 weeks after the final immunization. An inoculum of 2 x 10^6 or 2 x 10^5 pfu of HSV-1 strain McKrae in 5 μL tissue culture medium was placed in each eye without anesthesia and without corneal scarification, and the lid was gently rubbed for 30 seconds.

**HSV-1–Induced Eye Disease**

The severity of blepharitis and corneal scarring in each group of 10 mice was assessed by examination with a slit lamp biomicroscope after addition of 1% fluorescein dye as eye drops. The examination was performed by investigators blinded to the treatment regimen of the mice and scored according to a standard 0 to 4 scale: 0, no disease; 1, 25%; 2, 50%; 3, 75%; and 4, 100% staining or involvement. Eyes were examined on days 1 to 14 for blepharitis and corneal involvement and on day 28 for corneal scarring.

**Monitoring Replication and Clearance of HSV-1 from the Eye**

This procedure was performed by swabbing the eyes of 10 mice (20 eyes) once daily on days 1 to 10 with a dacron swab and transferring each swab to a 12 x 75-mm culture tube containing 1 mL of media. After eyes were swabbed, 100 μL aliquots of 10-fold serial dilutions were placed on confluent monolayers of RS cells in 24-well plates, incubated at 37°C for 1 hour and overlaid with medium containing 1% methylcellulose. The plates were incubated at 37°C for 3 days and stained with 1% cresyl violet, and the viral plaques were counted.

**Detection of Latent Virus in Trigeminal Ganglia**

Mice surviving 30 days after ocular infection were killed. Both trigeminal ganglia (TG) were removed and individually explanted onto RS cell monolayers, which were monitored for 10 days for the presence of infectious virus.

**Statistical Analysis**

Protective parameters were analyzed by Student’s t-test and the Fisher exact test (Instat, GraphPad, San Diego, CA). Results were considered to be statistically significant at P < 0.05.

**Results**

**Induction of HSV-1–Neutralizing Antibody**

Groups of 10 BALB/c mice from two separate experiments were vaccinated either once or three times with a DNA cocktail containing equal amounts of naked DNA corresponding to the HSV-1 gB, gC, gD, gE, and gI genes. Other groups of 10 mice were immunized with mixtures of the five purified HSV-1 proteins, gB, gC, gD, gE, and gI, each cloned into a baculovirus vector, grown in insect cells, purified, and emulsified in Freund’s adjuvant or ISA 720. With some immunogens, DNA immunization followed by protein immunization (booster) has been shown to be more effective in inducing an immune response.
response than protein immunization followed by DNA immunization or DNA immunization alone.\textsuperscript{25–26} Therefore, in parallel with these experiments, additional groups of mice were injected with DNA followed by proteins or with proteins followed by DNA, emulsified in Freund’s or ISA 720. Three weeks after the final immunization, sera were collected from 10 mice per group and neutralizing antibody titers were determined by a 50% plaque-reduction assay.

After the first immunization, the average titer in mice immunized with DNA was approximately the same as that in mice immunized with proteins, emulsified in either CFA or ISA 720 (Fig. 1A; \( P > 0.05 \), Student’s \( t \)-test). All three groups had significantly higher neutralizing antibody titers than the mock-vaccinated group (\( P < 0.0001 \), Student’s \( t \)-test).

After three immunizations, the average titer in mice immunized with DNA was similar to that in mice immunized with proteins, either in Freund’s or ISA 720 adjuvant (Fig. 1B; \( P > 0.05 \), Student’s \( t \)-test). In addition, mice initially immunized with proteins and given booster injections of DNA had titers similar to those in mice initially immunized with DNA and receiving protein booster injections. Moreover, there was no difference in antibody titer when the proteins were emulsified in ISA 720 or Freund’s adjuvant (Fig. 1B; \( P > 0.18 \)). Mice injected with protein-DNA or DNA-protein had lower titers of neutralizing antibody than mice immunized with DNA or protein alone, except for those mice initially immunized with DNA and with booster injections of proteins in Freund’s adjuvant (Fig. 1B; \( P < 0.05 \)). Thus, none of the vaccine regimens tested (i.e., proteins alone, proteins-DNA, DNA-proteins) induced significantly higher neutralizing antibody titers than DNA immunization alone (Fig. 1B; \( P > 0.05 \)). All immunized groups had significantly higher neutralizing antibody titers than mock-vaccinated mice (Fig. 1B; \( P < 0.0001 \)). These results suggest that the DNA vaccine induced neutralizing antibodies as effectively as the protein vaccine. Furthermore, mice immunized with proteins emulsified in ISA 720 had neutralizing antibody titers similar to those of mice immunized with proteins emulsified in Freund’s adjuvant (Fig. 1; \( P > 0.05 \)).

**Protection of Vaccinated Mice from Lethal Ocular Infection**

Three weeks after the third immunization, 10 mice per group were intraocularly infected with \( 2 \times 10^5 \) pfu per eye of HSV-1 strain McKrae. In all vaccine groups, 10 (100%) of 10 of the mice survived, whereas only 15 (21%) of 70 (all seven mock groups showed similar survival patterns after ocular infection; thus, we combined the data from all seven groups) of the mock-vaccinated mice survived (\( P < 0.0001 \), Fisher exact test; not shown).

We then sought to determine whether higher infectious doses of HSV-1 would differentiate between the DNA and protein subunit vaccines in terms of protection. Three weeks after the third immunization, 10 mice per group were intraocularly infected with \( 2 \times 10^6 \) pfu per eye of HSV-1 strain McKrae. Although none of the 70 mock-vaccinated mice survived intraocular infection at this dosage, 10 (100%) of 10 mice vaccinated with the DNA and/or protein subunit vaccines survived the 10-fold higher infection (\( P < 0.0001 \), Fisher exact test; not shown). These results indicate that all immunization regimens tested completely protected immunized mice against lethal ocular infection.

**Clearance of Virus from Eyes**

Mice vaccinated once or three times as just described were intraocularly infected with \( 2 \times 10^5 \) pfu per eye of HSV-1 strain McKrae. Tear films were collected daily from 20 eyes per group on days 1 to 10, and the amount of virus in each eye was determined by standard plaque assays (Fig. 2). After both one (Fig. 2A) and three (Fig. 2B) immunizations, the peak virus titer per eye was significantly lower in mice immunized with DNA alone than in any of the other groups (Fig. 2; \( P < 0.05 \), Student’s \( t \)-test), as was the range of virus titers (\( 0–320 \) pfu per eye vs. \( 0–4800 \) pfu per eye; not shown). All immunized mice had significantly lower peak virus titers per eye than mock-vaccinated mice (Fig. 2; \( P < 0.01 \)). In addition, mice immunized with DNA showed complete clearance of virus by day 6 after infection, whereas mice immunized with all other com-
combinations did not show complete clearance until 7 days after infection, and mock-immunized mice took 9 days (not shown). These findings suggest that the DNA vaccine was more effective than any of the other regimens (protein alone, protein-DNA, or DNA-protein) in decreasing both the amount and duration of HSV-1 in mouse tears.

**Protection of Vaccinated Mice from Blepharitis and Corneal Scarring**

Herpetic blepharitis is an inflammation of the lid margin after intraocular HSV-1 infection, and in the mouse, increased blepharitis correlates with increased HSV-1 replication.27,28 Therefore, the eyes of the groups of mice infected with $2 \times 10^5$ pfu per eye of HSV-1 strain McKrae were examined for blepharitis on day 7 after infection, with disease scored on a scale of 0 to 4. Mice vaccinated with DNA had less blepharitis than any of the other groups (Fig. 3; $P < 0.05$, Student’s t-test). Although Freund’s adjuvant generally has been thought to be most effective at inducing antibody titers, we found that emulsification of the five HSV-1 proteins in ISA 720 induced a greater degree of protection against blepharitis than emulsification in Freund’s adjuvant (Fig. 3; $P < 0.05$, Student’s t-test). All immunized mice had significantly lower blepharitis than mock-vaccinated mice (Fig. 3; $P < 0.01$).

When we examined the eyes of the immunized, intraocularly infected mice on day 28, we found an absence of corneal scarring in all groups of vaccinated mice (score, 0), whereas mock-vaccinated mice had considerable amounts of corneal scarring (score, $2.0 \pm 0.5$; $P < 0.0001$, Student’s t-test; not shown).

**Effect of Immunization on Establishment of Latent Infection**

Surviving mice that were immunized three times and intraocularly infected with $2 \times 10^5$ pfu per eye of HSV-1 McKrae were killed 30 days after infection. TG were removed and analyzed individually for the presence of latent HSV-1 by explant cocultivation. Mice vaccinated with DNA had less latent infection than mice immunized with protein, protein-DNA, or DNA-protein (Fig. 4; $P < 0.05$, Fisher exact test). All immunization protocols resulted in latency in fewer TG than mock immunization (Fig. 4; $P < 0.01$, Fisher exact test), indicating that these seven vaccine regimens provided some degree of protection against the establishment of latency.

**In Vitro Cytokine Production**

Three weeks after the third immunization, splenocytes were isolated from the vaccinated mice, cultured, and stimulated with UV-inactivated HSV-1 strain McKrae. The levels of IL-2, -4, and -12 and IFN-γ in the culture media were analyzed by ELISA. Splenocytes from vaccinated mice, regardless of the type of vaccine, secreted similar levels of IL-2, which were significantly lower than those in the mock-vaccinated group (Fig. 5; $P < 0.01$, Student’s t-test).
higher than the amount of IL-2 secreted by splenocytes of mock-vaccinated mice (Fig. 5A; \( P < 0.0001 \), Student’s \( t \)-test). The DNA-vaccinated mice showed the highest IL-2 levels, although the differences did not reach statistical significance (Fig. 5A; \( P > 0.25 \)). All the vaccinated groups had significantly lower IL-4 levels than mock-vaccinated mice (Fig. 5B; \( P < 0.0006 \)).

Splenocytes from all groups immunized with DNA (with or without protein) secreted significantly higher IFN-\( \gamma \)-levels than splenocytes from mice immunized with protein alone (Fig. 5C; \( P < 0.0001 \), with the highest IFN-\( \gamma \)-levels in the DNA-alone group. All the vaccinated groups had significantly higher IFN-\( \gamma \)-levels than mock-vaccinated mice (Fig. 5C; \( P < 0.0001 \)). Splenocytes from mice vaccinated with DNA alone secreted higher levels of IL-12 than did splenocytes from other groups of vaccinated mice (Fig. 5D; \( P < 0.001 \)), and all groups of vaccinated mice secreted higher levels of IL-12 than splenocytes from the mock-vaccinated group (Fig. 5D; \( P < 0.002 \)). These results, showing that DNA immunization increased secretion of both IFN-\( \gamma \) and IL-12 from splenocytes, may explain the higher vaccine efficacy demonstrated by this immunization protocol.

**DISCUSSION**

Occurrence of infection with HSV is widespread. It is estimated that 70% to 90% of people in the United States over the age of 18 have antibodies to HSV-1 and/or HSV-2 and carry the latent virus, with approximately 25% showing clinical symptoms. During the life of the latently infected individual, the virus can occasionally reactivate, travel back to the eye and cause recurrent disease. A major cause of corneal scarring is that induced by HSV-1 after reactivation from latency. Because of the preexisting immune response, corneal scarring is more likely to occur after recurrent, rather than primary, HSV infection. Thus, a vaccine to control HSV infection should have both prophylactic and therapeutic potential. Although it is possible that an effective prophylactic vaccine against ocular
HSV-1 infection may not prevent HSV-1 recurrences, it is unlikely, as we have shown that recurrent HSV-1 infection is significantly reduced by therapeutic vaccination of latently infected rabbits with gD2 subunit vaccine.33 These results and the human vaccine study reported recently34 support the concept that development of a vaccine with both prophylactic and therapeutic efficacy against HSV infection in humans is possible.

We have shown that a cocktail of seven baculovirus-expressed HSV-1 glycoproteins (gB, gC, gD, gE, gG, gH, and gI) provides better overall protection against primary intraocular HSV-1 infection in mice than any individual glycoprotein, but is not as effective as vaccine prepared from our live avirulent HSV-1 strain KOS.19 We later found that a cocktail of five baculovirus-expressed HSV-1 glycoproteins (gB, gC, gD, gE, and gI) provides better overall protection against eye disease than both the seven-glycoprotein cocktail and the live KOS vaccine.35 In response to the results of the Chiron (Irvine, CA) phase 3 clinical trial, in which a gB+gD protein vaccine was relatively ineffective against acquisition of genital HSV,36 we compared a cocktail of five naked DNAs, corresponding to the gB, gC, gD, gE, and gI genes, with our five-glycoprotein cocktail. In addition, we compared each of these with a regimen consisting of primary immunization with the five-DNA cocktail, followed by a booster injection of the five-glycoprotein cocktail, and with a regimen consisting of primary immunization with the five glycoproteins followed by a booster injection of the five DNAs. The five glycoproteins used in this study were isolated from the KOS strain of HSV-1 and may not be as effective in controlling infections associated with different forms of HSV-1. Because of the significant amino acid homology of these five glycoproteins among different strains of HSV-1,37 however, we do not expect any interstrain genetic variations to reduce the efficacy of our vaccine cocktail in effectively controlling other isolates of HSV-1. Thus, antigenic similarities among the different forms of HSV-1 make design of an effective vaccine against HSV-1 infection less problematic. In addition, the use of a cocktail of five glycoproteins, each of which individually produces significant protection against HSV-1 infection, increases the effectiveness in controlling different strains and variants of HSV-1.

Most immunizations with protein emulsified in adjuvant induce antibody production, but little or no CTL response,38 whereas DNA immunization produces both antibody and CTL responses.1,2 Theoretically, DNA vaccines against HSV-1 should be more effective than protein vaccines, because CTL appears to be important for protection against HSV-1.39 However, we20 and others1,2,42 have found that, although naked DNA immunization induces immunity, immunization with the corresponding protein(s) is usually more effective.20,40 The lower effectiveness of DNA vaccines may be caused by problems with delivery and/or expression levels. The development of new vectors that greatly improve uptake and expression efficiency in vivo, however, seems to have eliminated this drawback.17,23,44

In this study, mice immunized with a DNA cocktail had HSV-1–neutralizing antibody titers similar to those of mice immunized with the corresponding expressed protein. Furthermore, antibody titers in mice immunized with the protein cocktail were independent of adjuvant, because proteins emulsified in Freund’s and ISA 720 induced similar titers. We also found that immunization with DNA was more effective than immunization with protein, protein plus DNA, or DNA plus protein in significantly reducing blepharitis, viral replication in the eye, and latency. The effectiveness of DNA immunization even exceeded that previously observed with live HSV-1 strain KOS immunization19,35 (not shown).

Thus, this DNA cocktail appears to be the most effective vaccine against HSV-1 we have yet tested. Similar to our results, it has been shown recently that immunization with HSV-1 gD DNA induces humoral and cellular immunity against HSV-1 infection and inhibits the development of stromal keratitis, but not epithelial keratitis, in challenged mice.42 In this study, however, we have produced complete protection against all forms of eye diseases in DNA-immunized mice. The discrepancy between these findings and those reported earlier may be due to differences in the route of immunization, the amounts of antigen injected, or our use of a cocktail of five genes rather than one gene. Each of these discrepancies could affect the effectiveness of the vaccine. In addition, when we used the five-glycoprotein DNA cocktail vaccine as an eye drop, we obtained only partial protection against death and eye disease in challenged mice (not shown). This reduced protection compared with IM injections was probably due to the lower quantity of antigen (5 µg/eye compared with 50 µg/quadriceps).

An immunization regimen consisting of primary and booster injections of DNA has been shown to induce both CD4+ and CD8+ T-cell responses.12,16,17 It has been reported, however, that primary immunization with DNA followed by booster injections of protein can induce higher levels of CMI and antibody responses than either an all-protein or all-DNA regimen.5,36 The results reported herein suggest that neither a primary injection with protein followed by a boost with DNA nor a primary injection with DNA followed by a boost with protein had any advantage over an all-DNA regimen. The discrepancy between our results and those of other studies25,26,45,46 may be due to our use of a different DNA vector—one with improved uptake and expression efficiency in vivo17,23,41—or to our use of a cocktail of five genes rather than a single gene. In support of the latter, it has been reported recently that DNA vaccination with a mixture of four genes induces better protection against lethal vaccinia virus challenge than vaccination with any individual gene.47

Although Freund’s adjuvant has been used extensively to immunize animals,48 adverse reactions make it unacceptable for human use.50 We therefore tested a new water-in-oil (W/O) adjuvant, Montanide ISA 720, which has been shown to induce strong immune responses in vaccinated animals.51–53 In addition, ISA 720 has been tested successfully, with no side effects, in phase I human trials against HIV,54 metastatic melanoma,55 and malaria56,57 and has been approved for use in humans. Our results suggest that ISA 720 is even more effective than Freund’s adjuvant in protecting mice against blepharitis and in reducing viral replication in the eye.

The absence of IL-4 production by immunized mice, despite the occurrence of a strong humoral response, was unexpected. This finding, however, is consistent with a previous study showing that T-cell stimulation of HSV-seropositive individuals results in a prominent Th1 response with little IL-4 production.58 Moreover, it has been shown that, in the presence of antibodies to HSV-1, IL-4 was not detectable in the corneas of mice on recurrent HSV-1 infection.59 IL-2 and -4 are pleiotropic lymphokines involved in the development of cellular and humoral immunity, respectively.60,61 They may, however, play an antagonistic role in protection against viral infection. For example, we have previously shown that IL-2 suppresses, but IL-4 enhances, HSV-1 replication in the eye.27,62,63 Detrimental effects on host animals were also observed in experiments in which IL-4–expressing transgenic mice were infected with respiratory syncytial virus64 and in which animals infected with influenza virus infection were treated with recombinant IL-4.65 In addition, IL-4 expression by a recombinant vaccinia virus has been found to exacerbate infection.66 Thus, as expected, none of the vaccinated mice had detectable levels of IL-4, whereas
mock-vaccinated mice produced IL-4 and had higher amounts of ocular virus replication and eye disease. In contrast to IL-4, all vaccinated mice produced high levels of IL-2, and we observed no significant differences in IL-2 expression between protein- and DNA-vaccinated mice. Previously, using protein vaccines and live HSV-1 (KOS) immunization, we found that increased expression of IL-2 in the eye correlated with decreased HSV-1 replication in the eye and death.77,20 More recently, our experiments with IL-2, IL-4, STAT4, and STAT6-deficient mice revealed a functional relationship,62,63 in that HSV-1 replicated more in the eyes of IL-2- and STAT4-deficient mice than in IL-4- and STAT6-deficient mice. Similarly, it has been shown that greater efficacy of a gD vaccine against genital herpes in women than in men is due to the higher Th1 (i.e., IL-2, IFN-γ) response in women.34 Thus, our finding, that splenocytes of mice vaccinated with either DNA or protein produced enhanced levels of IL-2 but not IL-4, suggests that the presence of IL-2 correlates with vaccine efficacy.

We also found that mice immunized with a DNA cocktail produced higher levels of IL-12 and IFN-γ than mice immunized with a protein cocktail, suggesting that IL-12 and IFN-γ may be involved in enhancing the efficacy of DNA vaccines over protein vaccines. Similarly, it has been shown that immunization of mice with CpG oligodeoxynucleotide confers protection against malaria infection by inducing higher levels of IL-12 and IFN-γ.67 IL-12 is a regulatory cytokine produced by activated monocytes, macrophages, neutrophils, dendritic cells, and B cells, but not T cells.68,69 IL-12 may enhance the Th1 response by promoting the differentiation of Th0 to Th1 cells.70,74 Thus, IL-12 may act as a cusp between the innate and the adaptive immune responses. IFN-γ is a cytokine secreted by activated T cells and NK cells.72,73 The IFN-γ antiviral defense mechanism occurs very early during the course of infection, by interfering with the early steps of virus invasion and replication.74–77 Consequently, our results suggest that DNA vaccine induces a rapid antimicrobial host defense by stimulating innate immune responses, in addition to inducing strong cellular immune responses.

In this study we have shown positive correlations between faster viral clearance, decreased intraocular viral titers, and increased IFN-γ and IL-12 responses in vaccinated mice compared with the protein-vaccinated groups. Because corneal stroma contains large numbers of resident dendritic cells and macrophages,79,80 increased stimulation of these cells in the cornea after DNA immunization may play a role in the decreased viral replication observed in the eyes of DNA-vaccinated mice compared with protein-vaccinated mice.

Our results also showed that inclusion of DNA in an immunization regimen, either by primary immunization with DNA followed by a boost with protein or by primary immunization with protein followed by a boost with DNA, enhanced IFN-γ secretion by splenocytes. This result is supported by a recent finding, showing that IFN-γ levels are elevated in mice after DNA immunization against malaria infection.81 The production of higher levels of IFN-γ could have been due to higher NK or CTL activities in DNA-immunized mice.

In summary, a DNA vaccine appeared superior to the corresponding protein vaccine, and multiple immunizations with DNA were superior to a single immunization with DNA followed by a boost with protein or primary injection of protein followed by a booster injection of DNA, both in conferring immunity against HSV-1 and in inducing IL-12 and IFN-γ. Finally, Montanide ISA 720, a new adjuvant recently approved for human use, is at least as effective as Freund’s adjuvant, and, in protecting against blepharitis, is superior to Freund’s adjuvant.

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