Preventive Effect of Local Plasmid DNA Vaccine Encoding gD or gD-IL-2 on Herpetic Keratitis

Tomoyuki Inoue,[1] Yoshitsugu Inoue,[1] Takao Nakamura,[1] Atsushi Yoshida,[1] Kenichiro Takahashi,[1] Yumiko Inoue,[1] Yoshikazu Shimomura,[2] Yasuo Tano,[1] Yukio Fujisawa,[3] Aki Aono,[3] and Kozaburo Hayashi[4]

Purpose. The goal of this study was to evaluate the effectiveness of a local plasmid DNA vaccine encoding herpes simplex virus (HSV) type 1 glycoprotein D (gD) or gD-interleukin (IL)-2 (chimeric gene of gD and human IL-2) in preventing murine herpetic keratitis.

Methods. Plasmids containing gD (pHSDneo1), gD-IL-2 (pHDLneo1), or vaccine vector (pHSGneo) were injected subconjunctivally with BALB/c mice on days 0 and 7 (90 μg $\times$ 2). Immunization was indicated by positive virus-neutralizing antibody titer, swollen pinna (due to delayed-type hypersensitivity [DTH] reaction), and release of ^{51}Cr from splenic and/or local cytotoxic effector cells on day 28. In another group of the immunized mice, corneas were challenged with HSV-1 (CHR3 strain, 10 μl of 3 $\times$ 10^6 plaque-forming units [PFU]/ml). Mice were evaluated for clinical signs of epithelial or stromal keratitis on days 1 through 8 and days 10 and 14 or measured on days 2, 4, or 6 for viral titers in the eyes, trigeminal ganglia, and brain.

Results. All gD-DNA–injected mice obtained specific immunity. Furthermore, gD-IL-2-DNA elicited a higher DTH reaction and more vigorous cytotoxic effector cell activity. Stromal keratitis scores were lower for all immunized mice compared with control mice, although the difference in epithelial keratitis scores was not statistically significant. Viral titers in eyes, trigeminal ganglia, and brains were suppressed in all immunized mice.

Conclusions. Local immunization with plasmid DNA encoding gD or gD-IL-2 induces humoral and cellular immunity against HSV-1 and inhibits development of stromal keratitis. gD-IL-2 DNA induces greater cell-mediated immunity than gD DNA alone. A plasmid encoding gD-IL-2 is therefore a promising candidate for a vaccine against HSV-1. (*Invest Ophthalmol Vis Sci.* 2000; 41:4209–4215)

Herpetic stromal keratitis, one of the most common vision-threatening diseases, is caused by recurrent attacks of herpes simplex virus (HSV) type 1.[1] The currently accepted theory regarding the origin of stromal opacification is that it involves cell-mediated immune responses against viral antigens elicited after inflammatory cytokine release.[2] Administering potent antiviral drugs such as acyclovir during an acute infection can improve the prognosis for vision, but an immunosuppressive agent must also be administered to keep the cornea clear. Thus, the risk of stromal keratitis would be decreased by preventing or at least eliminating recurrent viral attack before an immune response occurs.[3]

Previously, several HSV-1 glycoproteins[4] have been studied as a vaccine candidate.[5–24] Of these proteins, glycoprotein D (gD) has been found to be most efficient at conferring protection on immunized animals.[25,26] Nevertheless, these conventional immunization protocols using one of the viral components usually induce effective antiviral antibody; however, cytotoxic T-lymphocyte (CTL) response was often difficult to elicit.[27] Furthermore, when mice were immunized with purified gD protein, high antibody titers[28,29] and low delayed-type hypersensitivity (DTH) responses[28] were obtained with minimal CTL induction.[26] To gain further effective cellular immunity, we fused human interleukin (IL)-2 to gD to achieve a safe and effective adjuvant.[30–33] The combined protein, gD-IL-2, successfully induced strong humoral antibody and better cell-mediated immunity.[34,35]

Recently, injection of naked DNA opened a new era of vaccine.[36–38] This technique induced a long-lasting humoral and cell-mediated immunity to several viruses.[39,40]

In this study, we determined the efficacy of subconjunctival injections of naked plasmid DNA carrying a chimeric gD-IL-2 gene under the control of the simian virus (SV)40 early promoter. We constructed two plasmids for use in the study, encoding gD and gD-IL-2 (chimeric gene of gD and human IL-2), and compared the immune responses with these plasmids and their effectiveness in preventing herpetic stromal keratitis.

From the [1]Department of Ophthalmology, Osaka University Medical School; the [2]Department of Ophthalmology, Kinki University School of Medicine, Osaka; [3]Biotechnology Research Laboratories, Research and Development Division, Takeda Chemical Industries, Osaka; and the [4]Kobe Institute of Health, Hyogo, Japan.

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Corresponding author: Tomoyuki Inoue, Department of Ophthalmology, Osaka University Medical School, 2-2 Yamadaoka, Suita, 565-0871, Japan. tinoue@virus.kyoto-u.ac.jp

Materials and Methods

Mice

Eight-week-old female BALB/c mice (H-2^d) were used in this study. The mice were bred in our laboratory and treated humanely in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Virus

HSV-1 (CHR3 strain) was propagated in green monkey kidney (GMK) cells. At maximum cytopathic effect, the virus was harvested by thrice freezing and thawing. After centrifugation at 3000 rpm for 10 minutes, the supernatant was aliquoted and stored at −80°C before use.

The virus was titrated by the antibody-overlay method using GMK monolayers on 96-well microplates (viral titer = 3×10^6 plaque-forming units [PFU]/ml).

DNA Vaccine Preparation and Immunization Procedure

Preparation of DNA Plasmids. Two plasmids (gD expression vector and gD-IL-2 expression vector) were constructed as described previously,[35] and a vector plasmid was prepared as a control. Briefly, the gD plasmid was constructed by inserting the gD DNA fragment obtained from HSV-1 Miyama strain into the expression vector (pHSGneo), which yielded the truncated gD (277 amino acids) expression plasmid (pHSDneo1). The gD-IL-2 plasmid was constructed by binding the truncated gD fragment with a fragment of the mature human IL-2 gene, obtained from an IL-2 expression plasmid. The resultant plasmid (pHDLneo1) contained the truncated gD-IL-2 fusion gene (410 amino acids) under the control of murine leukemia virus (MuLV) long terminal repeat (LTR) and SV40 early promoter (Fig. 1). The vector plasmid (pHSGneo) was prepared for use as a control.

Preparation of Vaccine Solutions. These clones were transfected into *Escherichia coli* and stored at −40°C in a 15% saline-0.1% glycerol solution. Then the bacteria were grown in 2× YT medium and the plasmid was isolated using a kit (Plasmid Mega; Quiagen, Hilden, Germany).

The gD and gD-IL-2 vaccine solutions were purified and concentrated and tested for the presence of bacterial lipopolysaccharide using a commercial test (Limulus test; Wako, Osaka, Japan) and for a minor amount of viral protein (gD) by Western blot analysis using mouse anti-HSV gD monoclonal antibody (Chemicon, Temecula, CA). Both vaccine solutions tested negative, indicating that they were free of bacterial lipopolysaccharide and viral protein (gD).

Immunization Procedure. Mice were given two bilateral subconjunctival injections, each containing 45 μg of plasmid DNA, on days 0 and 7.

Assay Procedures

Neutralization Assay. During weeks 1, 2 and 4 after the first immunization, serial fourfold dilutions of mice sera were incubated with an equal volume of the virus (2×10^3 PFU/ml) for 1 hour at 37°C. Residual plaque-forming units of the infective virus were assayed on Vero cell monolayers. Virus-neutralizing antibody titer was determined as the reciprocal of the dilution, causing 50% plaque reduction.

DTH Assay. Three weeks after the second immunization, mice immunized with gD, gD-IL-2, or control plasmid received an intradermal injection in each pinna. The right pinna was injected with 10 μl of UV light-inactivated HSV antigen (10^7 PFU/ml before inoculation). The left pinna was injected with the same amount of supernatant of GMK cell lysate as a control. Forty-eight hours later, the thickness of each ear was measured with an engineer's micrometer. The DTH response in each mouse was expressed as the difference in thickness between left and right pinnas.

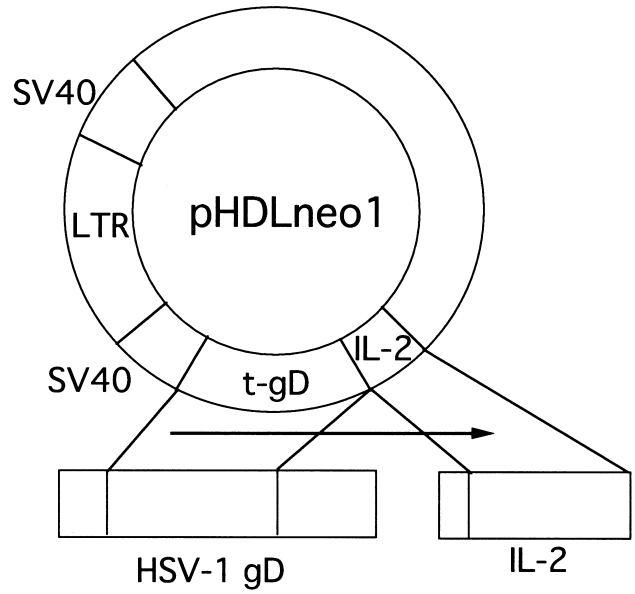

Figure 1. Construction of the plasmid encoding gD-IL-2. The fragment containing the truncated gD (amino acids 1-277) gene was ligated with the fragment containing the mature human IL-2 gene from the IL-2 expression plasmid, and this ligated fragment inserted into pHSGneo. The resultant plasmid contains the truncated gD-IL-2 fusion gene, which codes for the gD-IL-2 (410 amino acid) under the control of MuLV LTR and SV40 early promoter (pHDLneo1).

Mice that had received an intraperitoneal injection of live virus (1×10^4 PFU/ml) 2 weeks earlier were used as positive control subjects.

Cytotoxic Effector Cell Assay. The spleen and cervical lymph nodes were removed from gD-vaccinated, gD-IL-2–vaccinated, and vaccine vector–injected mice 3 weeks after the last immunization, and cells from each location in each mouse were suspended (4×10^6 cells/ml). The suspended cells were then mixed with partially purified virus (CHR3 strain of HSV-1 at a multiplicity of infection of 1.0 PFU/cell) and incubated for 5 days at 37°C in a humidified 5% CO_2-air incubator. The effectiveness of the vaccine in stimulating the development of cytotoxic effector cells that lysed HSV-1–infected cells was evaluated with a ^{51}Cr release. A total 100 μl mixture of cultured cells (1×10^6 cells/well) and ^{51}Cr-labeled, HSV-infected 3T3 clone A31 cells (H-2^d, 1×10^4 cells/well) were incubated in a microplate with 96 U-shaped wells for 4 hours at 37°C. Radioactivities released in the supernatant were counted by an auto-γ-spectrophotometer. ^{51}Cr-labeled, HSV-infected L929 cells (H-2^k) were used as H-2–mismatched target cells. The specific ^{51}Cr release was calculated with the following formula: percentage of specific lysis = ([sample release − control release]/[maximum release − control release]) × 100. Spontaneous release was less than 5% of the maximum release. Mice that had received an intraperitoneal injection of live virus

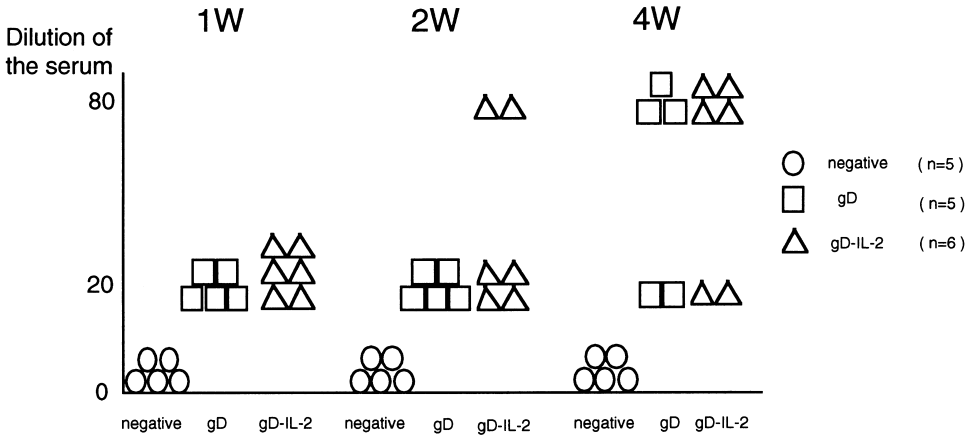

FIGURE 2. Serum-neutralizing antibody titers in gD-immunized mice (gD) and gD-IL-2–immunized mice (gD-IL-2) were elevated compared with titers in control plasmid-immunized (negative) mice at the end of weeks 1 (1W), 2 (2W), and 4 (4W) after first immunization (one-way ANOVA on ranks and Tukey's method, $P < 0.05$).

(1×10^4 PFU/ml) 2 weeks earlier were used as positive control subjects.

Viral Infectious Challenge of the Cornea and Evaluation of Results

Procedure for Viral Infectious Challenge. Three weeks after the second immunization, both corneas of mice were scarified crisscross-wise 10 times with a 27-gauge needle. Ten microliters of solution containing 3×10^6 PFU/ml of virus was instilled into the conjunctival sac of each eye.

Clinical Evaluation of Viral Infectious Challenge. Every day from day 1 through day 8 and on days 10 and 14 after instillation of the viral challenge solution into the eyes, the same observer examined the eyes with a hand-held slit-lamp biomicroscope and scored the severity of epithelial and stromal lesions using the following criteria:[26]

The scale for epithelial lesions was 0, no epithelial lesion or punctate epithelial erosion; 1, stellate keratitis or residue of the dendritic keratitis; 2, dendritic keratitis occupying less than one quarter of the cornea; 3, dendritic keratitis occupying one quarter to one half of the cornea; 4, dendritic keratitis extending over more than one half of the cornea.

The scale for stromal lesions was 0, normal; 1, slight edema or slight opacity of the stroma; 2, opacity and edema of the stroma confined to less than one half the diameter of the cornea; 3, opacity and edema of the stroma extending over one half the diameter of the cornea; 4, severe stromal opacity and edema, through which the iris is invisible.

Viral Titration in Eyeball, Trigeminal Ganglia, and Brain. The eyes, trigeminal ganglia, and brain of infected mice were excised by using an aseptic technique on day 2, day 4, or day 6 after viral infectious challenge. Each type of tissue from each mouse was homogenized with a mortar and pestle and diluted with solution to a final emulsion that contained 10% by volume. Each emulsion was centrifuged at 3000 rpm for 10 minutes, and the supernatant was assayed by an antibody overlay method.

Statistical Analyses

The Kruskal–Wallis one-way analysis of variance (ANOVA) with Tukey's method was used to test the statistical significance of differences in DTH reactions and cytotoxic effector cell assay results. One-way ANOVA on ranks with Tukey's method was performed to test for the statistical significance of differences in serum-neutralizing antibody titers, clinical scores, and virus titration assay results.[41]

RESULTS

Assay Results

Serum-neutralizing antibody titers were elevated significantly in both gD- and gD-IL-2–immunized mice, compared with negative control (control plasmid immunized) mice at the end of weeks 1, 2, and 4 after the first immunization (one-way ANOVA on ranks and Tukey's method, $P < 0.05$; Fig. 2).

DTH reactions occurred in mice immunized with plasmid gD or gD-IL-2 that were then challenged with UV-inactivated

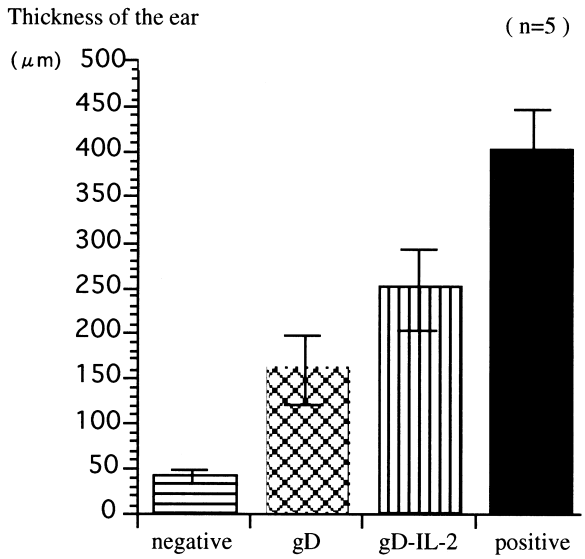

FIGURE 3. DTH reactions in control plasmid-immunized (negative) mice, gD-immunized (gD) mice, gD-IL-2–immunized (gD-IL-2) mice, and mice receiving an intraperitoneal injection of live virus (1×10^4 PFU/ml) 2 weeks before the assay (positive control subjects). Plasmid gD or gD-IL-2 elicited DTH responses in immunized mice when challenged with UV-inactivated HSV. Local injection of gD-IL-2 DNA resulted in a higher DTH response than did local injection of gD DNA (one-way ANOVA and Tukey's method, $P < 0.05$). Local injection of DNA encoding gD or gD-IL-2 could induce cell-mediated immunity. Data are the mean ± SD.

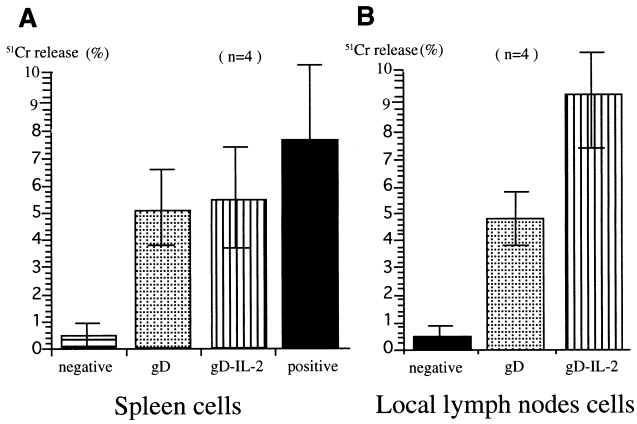

FIGURE 4. Cytotoxic effector cell activity in control plasmid-immunized (negative) mice, gD-immunized (gD) mice, gD-IL-2–immunized (gD-IL-2) mice, and mice receiving an intraperitoneal injection of live virus (1×10^4 PFU/ml) 2 weeks before the assay (positive control subjects). Cytotoxic effector cell activity was detected in plasmid gD- or gD-IL-2–immunized mice both systemically (**A**) and locally (**B**; one-way ANOVA and Tukey's method, $P < 0.05$). Cytotoxic effector cell activity was greater in mice immunized locally with gD-IL-2 DNA compared with gD DNA. Local DNA immunization encoding gD or gD-IL-2 could induce cell-mediated immunity. Data are the mean ± SD.

HSV. gD-IL-2 DNA elicited a more prominent DTH reaction than did gD DNA (one-way ANOVA and Tukey's method, $P < 0.05$; Fig. 3).

Systemic and local cytotoxic effector cell activities at statistically significant levels were detected in both gD-immunized and gD-IL-2–immunized mice (one-way ANOVA and Tukey's method, $P < 0.05$; Figs. 4A, 4B). In terms of local immune responses, immunization with gD-IL-2 DNA induced statistically significantly more cytotoxic effector cell activity than did immunization with gD DNA. The percentage of spontaneous release of ^{51}Cr from the HSV-1–infected major histocompatibility complex (MHC)-mismatched L929 cells or uninfected 3T3 clone A31 cells was less than 1% in any groups.

Results of Viral Challenge

In control mice (nonimmunized and control plasmid-immunized mice), epithelial lesion scores peaked on day 2 after viral challenge and then gradually declined. The epithelial lesion scores were not significantly lower in gD-immunized or gD-IL-2–immunized mice compared with control mice (Fig. 5A).

Stromal lesions started to develop in control mice on day 5, after viral challenge and stromal lesion scores in control mice rose to a plateau level between day 8 and day 14 after infection. In gD- or gD-IL-2–immunized mice, however, stromal keratitis was completely inhibited (Fig. 5B). From days 6 to 10 after viral challenge, stromal lesion scores in gD-immunized or gD-IL-2–immunized mice were significantly lower than those in control mice (one-way ANOVA on ranks and Tukey's method, $P < 0.05$). Figure 6 shows pictures of the corneas of control plasmid-immunized mice and gD-IL-2–immunized mice on day 10 after viral infectious challenge.

On day 14 after viral challenge, 80% of nonimmunized mice and control plasmid-immunized mice died of herpetic encephalitis. In contrast, all gD-immunized or gD-IL-2–immunized mice survived.

Viral titers in the eyeballs and trigeminal ganglia were significantly suppressed on days 2, 4, and 6 after infection, and titers in the brains were significantly suppressed on days 4 and 6 after infection in gD-immunized and gD-IL-2–immunized mice compared with control mice (one-way ANOVA on ranks and Tukey's method, $P < 0.05$; Fig. 7). These results suggest that the virus did not spread to the central nervous system in gD-immunized or gD-IL-2–immunized mice.

DISCUSSION

To our knowledge, this is the first report of the effects of local administration of a DNA vaccine to manage ocular disease—specifically, to prevent the development of herpetic stromal keratitis. In our study, mice that had been immunized against HSV-1 by subconjunctival injection of vaccine prepared from HSV-1 gD or gD-IL-2 DNA had negligible evidence of stromal keratitis and did not show development of stromal opacification. These results indicate that our novel DNA immunization protocol may induce sufficient immunity to halt the spread of HSV before the infection promotes a cytokine storm in the recipient.

Immunization with plasmid DNA that encodes for several viral antigens has been effective in inducing immunity against HSV. Many investigators have reported techniques for inducing systemic immunization, including intramuscular injection,

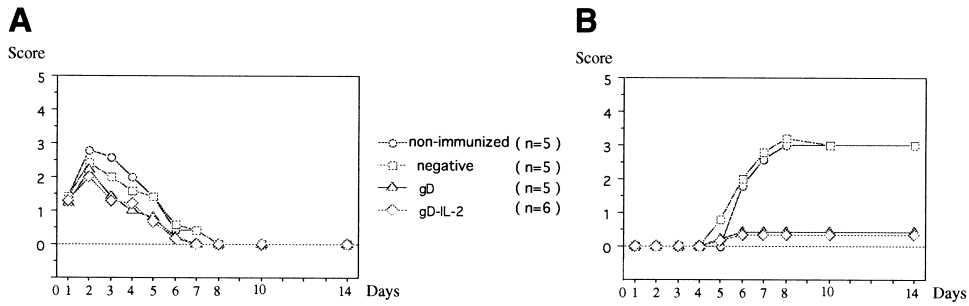

FIGURE 5. Clinical scores for severity of epithelial keratitis (**A**) and stromal keratitis (**B**) in nonimmunized mice, control plasmid-immunized (negative) mice, gD-immunized (gD) mice, and gD-IL-2–immunized (gD-IL-2) mice. Differences between control mice (nonimmunized and negative control mice) and immunized mice in severity of stromal keratitis were statistically significant (one-way ANOVA on ranks and Tukey's method, $P < 0.05$).

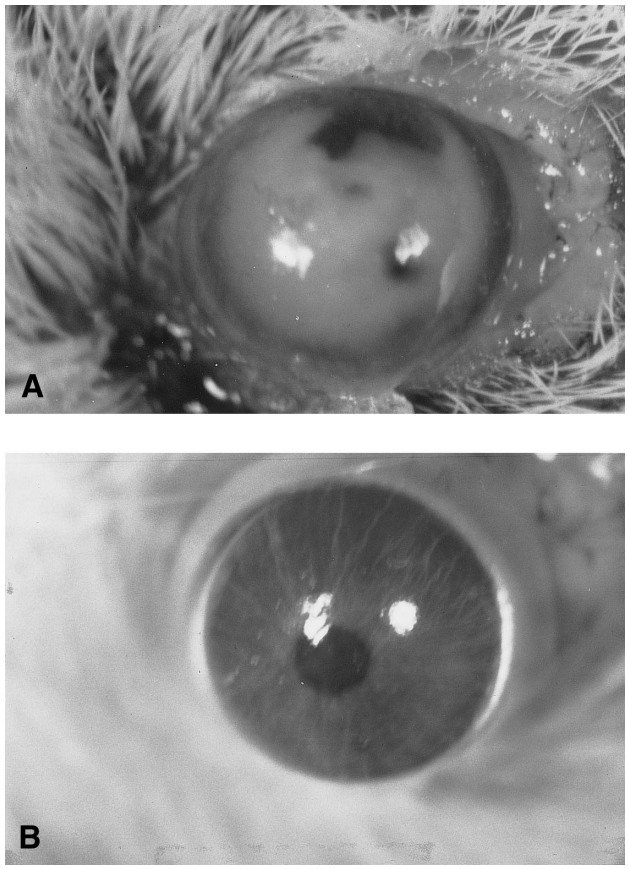

FIGURE 6. Clinical pictures of control plasmid-immunized and gD-IL-2–immunized mice. (**A**) Control plasmid-immunized mice on 10 day after viral challenge. The cornea shows severe stromal opacity and edema. (**B**) gD-IL-2–immunized mice on 10 day after viral challenge. The cornea appears normal. Stromal keratitis was completely inhibited in gD-IL-2–immunized mice.

gene gun delivery, or intradermal injection.[36–38] One study showed that intranasal administration of plasmid DNA encoding gB of HSV-1 was an effective means of inducing production of mucosal antibody. However, the intranasal route was inferior to the intramuscular injection route for delivery of DNA vaccine to protect against a lethal HSV challenge administered through the vaginal route.[42]

We constructed the chimeric gD-IL-2 DNA vaccine and demonstrated that immunization with a plasmid DNA encoding gD or gD-IL-2 inhibits the development of stromal keratitis and protects mice from lethal encephalitis. We found that immunization with either gD or gD-IL-2 can induce both humoral and cellular immunity against HSV. As we expected, however, vaccination with gD-IL-2 induced more potent DTH reactions and greater cytotoxic effector cell activity than did vaccination with gD. Our expectation was based on previous experiments in which a fusion protein consisting of HSV-1 gD plus human IL-2 induced a higher anti-HSV antibody response than did a single gD protein, and in fact, gD plus human IL-2 even induced cell-mediated immunity, and those immunized with gD-IL-2 survived longer.[34,35]

Many reports document the effects of immunization with DNA encoding gD of HSV. In one study, mice that had received an intramuscular injection of gD plasmid produced HSV-1–specific antibody and were protected against a lethal intraperitoneal challenge dose of HSV-1.[43] In another study of genital HSV infection, titers of virus in vaginal washings were significantly reduced by immunization with gD DNA,[44] and immunized animals experienced significantly fewer recurrences of viral infection.[45] In a previous study, intramuscular injection of plasmid DNA encoding gD did not induce CTL or lymphocyte-proliferative responses.[46] The ineffectiveness of the gD vaccine in that study may be attributable to the immunization route chosen. It has been reported that local immunization provides more local protection than systemic immunization.[47] This may be because expression of genetic immunity at the local level, where actual viral replication takes place, continues for a long time after immunization.

Manickan et al.[48] demonstrated that cell-mediated immunity after DNA immunization could be the result of the activity of $CD4^+$ T cells. They found that, after intramuscular injection of plasmid DNA encoding the immediate early protein ICP 27, immune splenocytes showed HSV-specific lymphoproliferation, CTL activity, DTH reaction, and type 1 cytokine production.[49] This vaccination protocol effectively inhibited the formation of herpetic zosteriform lesions on the murine skin. This finding further indicated the potential value of DNA immunization.

In summary, plasmid vaccines administered locally show promise in the prevention of recurrent infection and merit

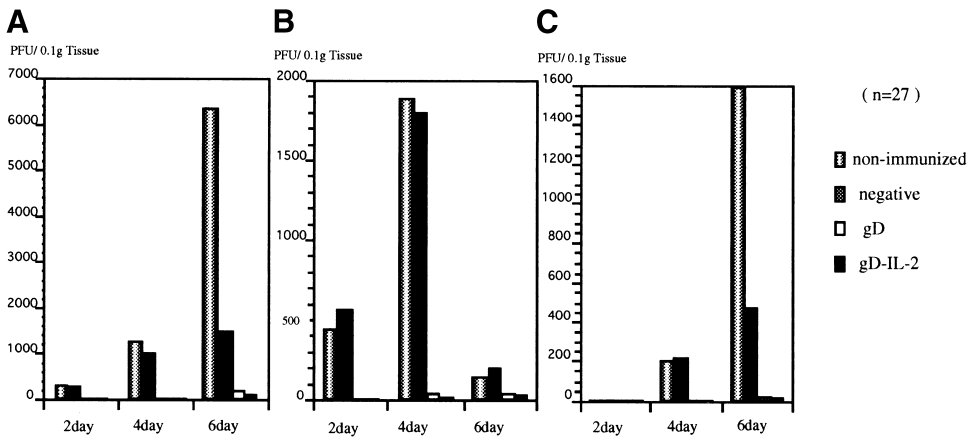

FIGURE 7. Viral titers after HSV-1 corneal infection in nonimmunized mice, control plasmid-immunized (negative) mice, gD-immunized (gD) mice, and gD-IL-2–immunized (gD-IL-2) mice. Viral titers in the (**A**) eyeballs and (**B**) trigeminal ganglia on days 2, 4, and 6 after viral challenge and in the (**C**) brains on days 4 and 6 after challenge were suppressed in gD- or gD-IL-2–immunized mice (one-way ANOVA on ranks and Tukey's method, $P < 0.05$), suggesting that the virus did not spread to the central nervous system in gD- or gD-IL-2–immunized mice.

further research. Identification of the type of functional cells or cytokines after local DNA immunization will facilitate development of more effective ways to prevent and eliminate herpetic stromal keratitis.

Acknowledgments

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