The Effect of Immunization with Herpes Simplex Virus Glycoprotein D Fused with Interleukin-2 against Murine Herpetic Keratitis

Tomoyuki Inoue*,
Yoshitsugu Inoue*, Takao Nakamura*,
Atsushi Yoshida*, Yumiko Inoue*, Yasuo Tano*,
Yoshikazu Shimomura[†], Yukio Fujisawa[‡], Aki Aono[‡] and Kozaburo Hayashi[§]

*Department of Ophthalmology,
Osaka University Medical School, Suita, Japan;
[†]Department of Ophthalmology, Kinki University School of Medicine,
Osaka Sayama, Japan; [‡]Biotechnology Research Laboratories, Research and Development
Division, Takeda Chemical Industries, Osaka, Japan; [§]Kobe Institute of Health, Kobe, Japan

Purpose: To evaluate the preventive effect of vaccination using fusion protein (gD-IL-2) consisting of herpes simplex virus type 1 (HSV-1) glycoprotein D (gD), and human interleukin-2 (IL-2), and plasmid DNA encoding gD-IL-2 against murine herpetic keratitis.

Methods: Plasmid containing gD-IL-2 (pHDLneo1) was constructed, and gD-IL-2 peptide was purified. BALB/c mice were injected hypodermally or subconjunctivally twice with 1 μg/0.1 mL of gD-IL-2 peptide, or subconjunctivally twice with 90 μg/0.05 mL of gD-IL-2 plasmid DNA. Neutralizing antibody titer and delayed-type hypersensitivity (DTH) against HSV-1 were measured. Immunized mice were challenged with CHR3 strain of HSV-1 into the cornea. Clinical manifestations of the epithelial and stromal keratitis were scored.

Results: Stromal keratitis was inhibited in gD-IL-2 peptide- or DNA-immunized mice; however, epithelial keratitis was not. It was confirmed that plasmid gD-IL-2 elicited significant virus neutralizing titer in sera and DTH response.

Conclusion: Vaccination with gD-IL-2 was effective against murine herpetic keratitis. **Jpn J Ophthalmol 2002;46:370–376** © 2002 Japanese Ophthalmological Society

Key Words: DNA vaccination, gD-IL-2, herpetic keratitis, murine model, peptide vaccination.

Introduction

Herpes simplex virus type-1 (HSV-1) is a leading cause of virus-induced corneal disease. One of the current problems in the management of ocular herpetic diseases is the prevention of severe visual loss resulting from recurrent stromal keratitis. Characteristic features of this disease are scarring and neovascularization in the corneal stroma. The current thesis on the etiology theorizes that acute viral replication in the cornea elicits cell-mediated immunity, followed by the inflammatory cytokine storm, which devastates the corneal stroma.[1] Nowadays, potent antiviral drugs, such as acyclovir, are available, and corneal inflammation is empirically controlled by corticosteroids. However, steroid treatment also triggers a viral reactivation, and so fundamental suppression of the development of stromal keratitis is still unattainable. Therefore, the ultimate goal of current therapy is the prevention and elimination of recurrent attack of the virus before it induces blinding immune responses.

Vaccination has been used to inhibit the diseases induced by HSV. It is preferable to use the subunit vaccine rather than the live vaccine to avoid tumorgenesis and latent infection.[2] Envelope glycoproteins of HSV-1 have been investigated as components for the HSV vaccine. They are expressed on the virions and the surface of HSV-infected cells, reported as the main targets for neutralizing antibody response, delayed-type hypersensitivity (DTH), and cytotoxic T lymphocyte (CTL).[3–7] Especially, glycoprotein D (gD) has been considered as one of the potent vaccine components, because it has conferred the most efficient protection to the immunized animals.[8] Inoue et al[9] have reported that corneal stromal keratitis was inhibited by vaccination with gD protein. However, multiple injections and the combination with an adjuvant are imperative to attain the proper immunogenicity with this vaccine.[9] Therefore, Hinuma et al contrived to utilize the strong immune-inducing character of interleukin-2 (IL-2) by producing a chimera protein, gD-IL-2 (a fusion protein consisting of HSV-1, gD, and human IL-2), which induced a high antibody titer and T-cell–mediated immunity against HSV-1.[10,11] In this report, we evaluated the preventive effect of vaccination using gD-IL-2 protein in the murine herpetic keratitis model.

Recently, the injection of naked DNA has opened up a new field of vaccinology.[12,13] It can induce sufficient cell-mediated immunity as well as humoral immunity against several viruses.[14,15] This genetic immunization has several advantages in vaccinology.[16] These include the ease of manipulation, resistance to interference by any pre-existing immune response and prolonged expression of antigens that may induce immunity similar to that obtained by the replicating agents. Therefore, to determine if this novel approach would be useful in preventing herpetic keratitis, we measured the acquired immunity by the vaccination of plasmids containing gD-IL-2, and estimated the preventive effect of this DNA vaccine against herpetic keratitis.

Materials and Methods

Mice

Eight-week-old female BALB/c mice were used. They were treated according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

In the experiment of clinical scoring employing hypodermal vaccination with protein, 5 mice were used in each group (nonimmunized group and gD-IL-2-immunized group). In the experiment of clinical scoring using subconjunctival vaccination with protein, 6 mice were used in each group (nonimmunized group and gD-IL-2-immunized group). In the experiment of clinical scoring using subconjunctival vaccination with DNA, there were 5 mice in the nonimmunized group, 5 mice in the control plasmid-immunized group, and 6 mice in the gD-IL-2-immunized group. In the experiment of neural antibody titer using subconjunctival vaccination with DNA, there were 5 mice in the control plasmid-immunized group, and 6 mice in the gD-IL-2-immunized group. In the experiment of DTH using subconjunctival vaccination with DNA, there were 5 mice in the control plasmid-immunized group, 6 mice in the gD-IL-2-immunized group, and 6 mice in the positive control group.

Virus

HSV-1 (CHR3 strain) was propagated in green-monkey-kidney (GMK) cells. At maximum cytopathic effect (CPE), the virus was harvested by freezing and thawing three times. After centrifugation at 2×10^3 g for 10 minutes, the supernatant was aliquoted and stocked at $-80°C$ until use. The virus was titrated using GMK monolayers on 96-well microplates by the antibody overlay method (virus titer = 3×10^6 plaque-forming units [PFUs]/mL).

gD-IL-2 DNA and Protein Preparation

Plasmid containing gD-IL-2 (pHDLneo1) was constructed, and gD-IL-2 peptide was purified. These methods were described in detail previously.[10,11] Briefly, gD DNA fragment obtained from HSV-1 Miyama strain was ligated with the mature human IL-2 gene fragment. The resulting plasmid (pHDLneo1) contains the truncated gD-IL-2 fusion gene (410 amino acids) under the control of murine leukemia virus long terminal repeat (LTR) and Simian virus 40(SV40) early promoter (Figure 1). Vector plasmid (pHSGneo) was prepared for control. Furthermore, the mouse myeloma cell line Sp 2/0-Ag 14 was transformed with pHDLneo1, and gD-IL-2 protein was purified from the culture supernatant of the gD-IL-2-expressed cells using affinity chromatography.

Immunization Protocol

pHDLneo1, containing 90 µg/10 µL of DNA, was administered topically to the conjunctival sac bilaterally on days 0 and 7.

The BALB/c mice were administered by injection to the hypodermal space or to the subconjunctival sac bilaterally twice on days 0 and 21 with 1 µg/0.1 mL of
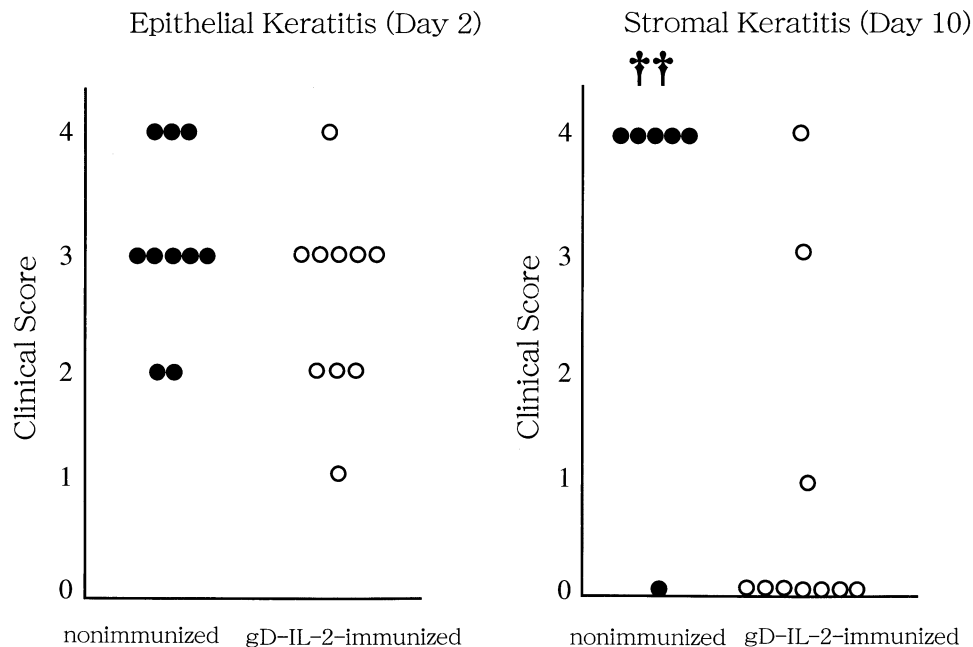
Figure 1. Effect of hypodermal vaccination with gD-IL-2 (a fusion protein consisting of herpes simplex virus type 1, glycoprotein D, and human interleukin-2) in BALB/c mice. Left: Epithelial lesions on day 2 postinfection (PI). Right: Stromal lesions on day 10 PI. Each group consisted of 5 mice (10 eyes). Scores of stromal lesion were significantly lower in immunized mice (○) than in nonimmunized animals (●) (Mann-Whitney U-test, $P < .05$). [†]Mouse died.

gD-IL-2 peptide, or to the subconjunctival sac bilaterally twice on days 0 and 7 with 90 μg/0.05 mL of gD-IL-2 plasmid DNA (pHDLneo1). In DNA vaccination, mice immunized with control plasmid (without insert; pHSGneo) served as a negative control.

Murine Herpetic Keratitis Model

Three weeks after the last immunization, mice were challenged with CHR3 strain of HSV-1 via the cornea, which was scarified crisscross-wise 10 times with a 27-gauge needle. Ten microliters of the virus was instilled into the conjunctival sac. Every day from day 1 through day 10 and on day 14, the eyes were examined with a hand-slit lamp by the same observer. Clinical manifestations of epithelial and stromal keratitis were scored according to the same clinical scoring used in our previous report.[9]

Epithelial lesions were scored as follows: (0) no epithelial lesion or punctate epithelial erosions, (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 one-half of the cornea.

Stromal lesions were also scored as follows: (0) no opacity and edema, (1) slight edema or opacity of the stroma, (2) stromal opacity and edema confined to less than one-half the diameter of the cornea, (3) stromal opacity and edema extending over one-half the diameter of the cornea, (4) severe stromal opacity and edema, through which the iris is not visible.

Neutralization Assay

This assay was performed in control plasmid-immunized and gD-IL-2 DNA-immunized groups. The virus neutralizing antibody titer was determined by the plaque reduction method. In brief, serial 4-fold dilutions of heat-inactivated (56°C, 30 minutes) murine sera obtained from the orbital venous plexus at 3 weeks after the first immunization were incubated with an equal volume of the virus (2×10^3 PFUs/mL) for 1 hour at 37°C. Residual PFUs 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.

Delayed-type Hypersensitivity Assay

Mice that had been infected intraperitoneally with live virus (1×10^4 PFUs/mL) 2 weeks earlier were used as positive control.[6] Three weeks after the first immunization of gD-IL-2 DNA, these mice were injected intradermally with 10 μL of the ultraviolet-

inactivated HSV antigen (1×10^7 PFUs/mL before inactivation) in the right ear pinna and the same amount of the control antigen (supernatant of GMK cell lysate) in the left ear pinna. Twenty-four hours later, ear thickness was measured with an engineer's micrometer. The degree of DTH response was expressed as the difference in thickness between bilateral ear pinnas.

Results

The Effect of gD-IL-2 Immunization Against Herpetic Keratitis

Epithelial lesions in control mice (nonimmunized mice) reached a peak around day 2 postinfection (PI), and gradually reduced. Stromal lesions started to develop around day 5, and reached a peak between day 8 and day 14 PI.

In the clinical findings observed from day 1 to day 14 in the group hypodermally immunized with gD-IL-2 protein, stromal keratitis was significantly inhibited; however, epithelial keratitis was not. Figure 1 shows the results when each lesion reaches the peak of severity. Scores of epithelial lesion on day 2 PI were not different between two groups (Mann-Whitney U-test, $P > .05$). On the other hand, stromal lesions on day 10 PI in immunized mice were significantly fewer than in nonimmunized animals (Mann-Whitney U-test, $P < .05$). The representative clinical pictures of gD-IL-2-immunized mouse and nonimmunized mouse are shown in Figure 2.

In the immunized group injected subconjunctivally with gD-IL-2 protein, similar results were obtained.

Epithelial lesions on day 2 PI were not inhibited (Mann-Whitney U-test, $P > .05$). However, stromal lesions on day 10 PI were significantly suppressed (Mann-Whitney U-test, $P < .05$) (Figure 3).

There was no significant difference between the scores of control plasmid-immunized mice and those of nonimmunized mice in both epithelial and stromal lesions (one-way analysis of variance [ANOVA][17], $P > .05$). However, stromal lesions in mice immunized with plasmid encoding gD-IL-2 were significantly suppressed on day 10 PI compared to those of nonimmunized and control plasmid-immunized mice one-way ANOVA and Tukey test, $P < .05$, although epithelial lesions on day 2 PI were not different among gD-IL-2 plasmid-immunized mice, control plasmid-immunized mice and nonimmunized mice one-way ANOVA, $P > .05$) (Figure 4).

Immunity Induced by gD-IL-2 DNA Immunization

Serum-neutralizing antibody titers were elevated significantly in gD-IL-2-immunized mice compared to control plasmid-immunized mice at 3 weeks after the first immunization (Mann-Whitney U-test, $P < .05$) (Figure 5). We found that immunization with gD-IL-2 DNA can induce humoral immunity against HSV.

DTH response was elicited in gD-IL-2-immunized mice 24 hours after challenge with UV-inactivated HSV (one-way ANOVA and Tukey test, $P < .05$) (Table 1). These results indicated that gD-IL-2 DNA can also induce cell-mediate immunity against HSV.
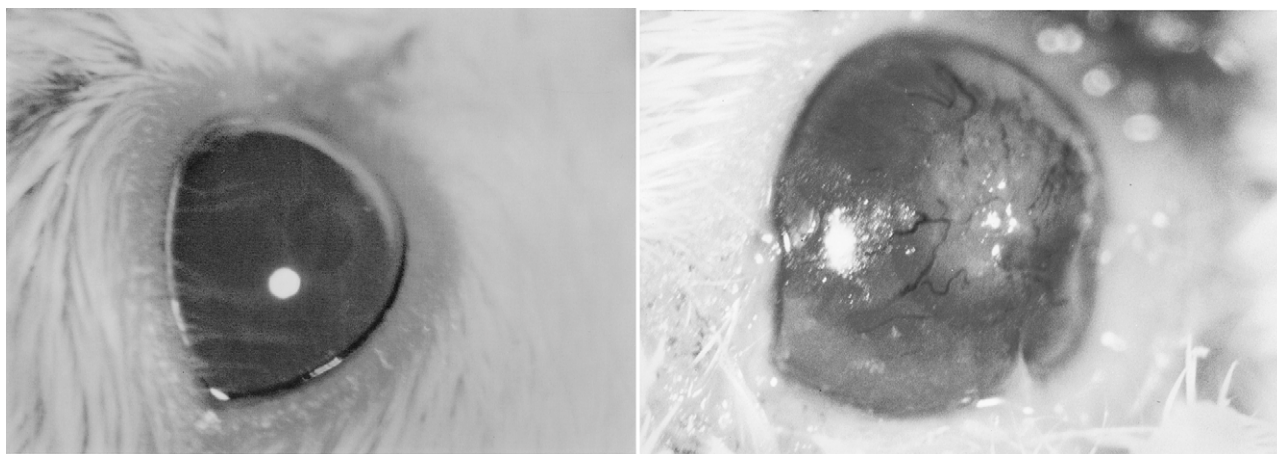
Figure 2. Clinical pictures of nonimmunized mouse eye and gD-IL-2 (a fusion protein consisting of herpes simplex virus type 1, glycoprotein D, and human interleukin-2) -immunized mouse eye. Left: gD-IL-2 protein-immunized mouse eye on day 10 postinfection (PI). Cornea has normal appearance. Right: Nonimmunized mouse eye on day 10 PI. Cornea shows severe stromal opacity and edema.

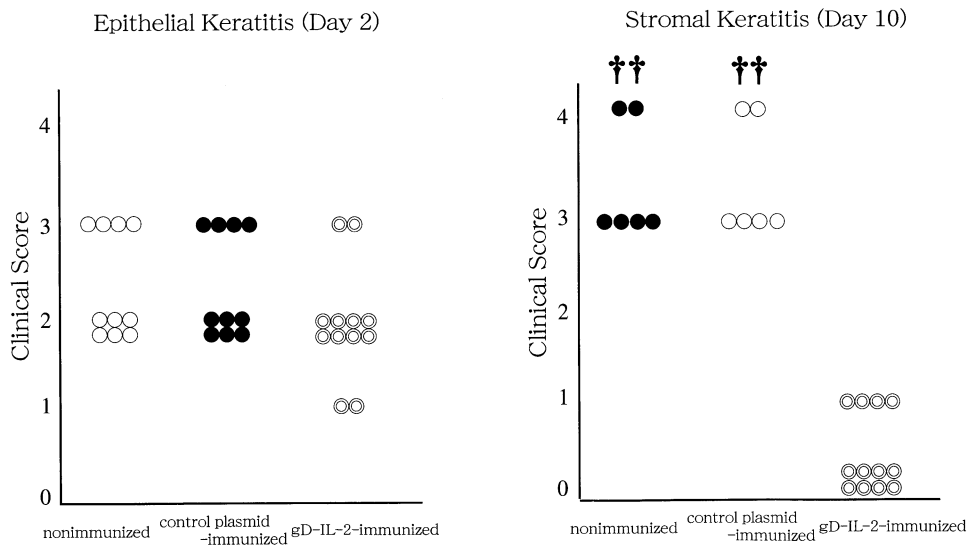Epithelial Keratitis (Day 2)   Stromal Keratitis (Day 10)
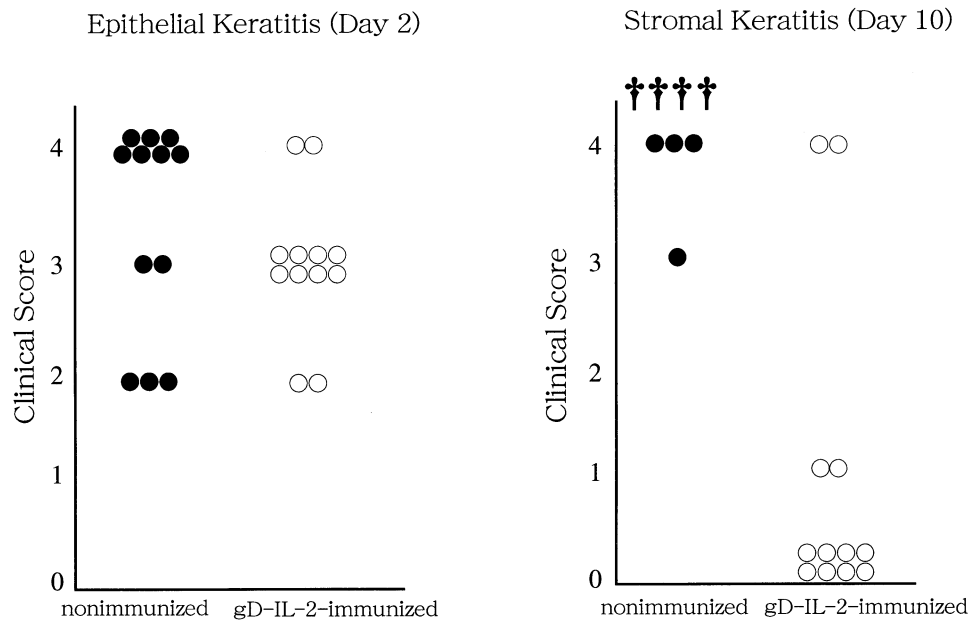
Figure 3. Effect of subconjunctival vaccination with gD-IL-2 (a fusion protein consisting of herpes simplex virus type 1, glycoprotein D, and human interleukin-2) in BALB/c mice. Left: Epithelial lesions on day 2 postinfection (PI). Right: Stromal lesions on day 10 PI. Each group consisted of 6 mice (12 eyes). Scores of stromal lesion were significantly lower in immunized mice (○) than in nonimmunized animals (●) (Mann-Whitney U-test, $P < .05$). [†]Mouse died.

Discussion

gD peptide has been studied as a potential HSV vaccine. A previous paper showed that gD protein distinctly inhibited the development of stromal keratitis. Inoue et al reported that gD peptide-immunized mice acquired humoral immunity; however, cellular immunity including DTH and CTL was not detected.[9] These results agree with the general theory:

Epithelial Keratitis (Day 2)   Stromal Keratitis (Day 10)

Figure 4. Effect of subconjunctival vaccination with plasmid-encoded gD-IL-2 (a fusion protein consisting of herpes simplex virus type 1, glycoprotein D, and human interleukin-2) in BALB/c mice. Left: Epithelial lesions on day 2 postinfection (PI). Right: Stromal lesions on day 10 PI. Nonimmunized group and control plasmid-immunized group consisted of 5 mice (10 eyes), and gD-IL-2 plasmid-immunized group consisted of 6 mice (12 eyes). Stromal lesions of gD-IL-2 plasmid-immunized mice (double circle) on day 10 PI were significantly suppressed compared to those of nonimmunized (●) and control plasmid-immunized mice (○) one-way analysis of variance and Tukey test, $P < .05$. [†]Mouse died.
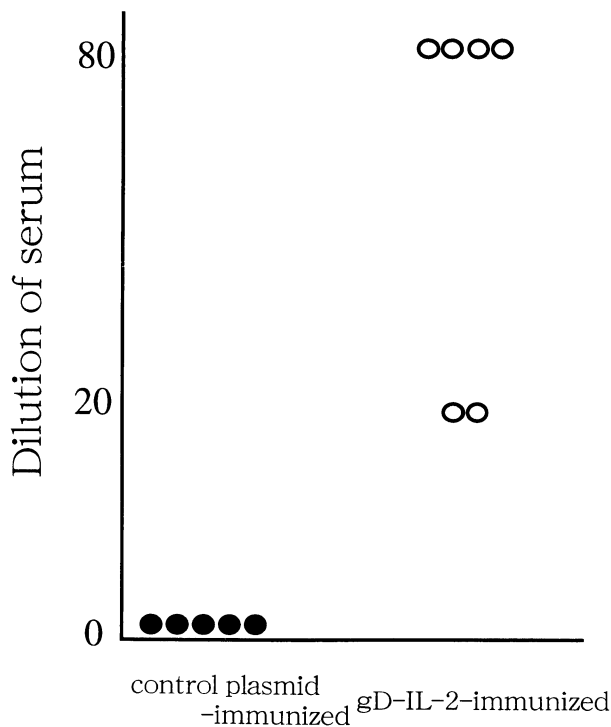
Figure 5. Serum neutralizing antibody titers. Serum-neutralizing antibody titers were elevated in gD-IL-2 (a fusion protein consisting of herpes simplex virus type 1, glycoprotein D, and human interleukin-2) plasmid-immunized mice (○) compared to control plasmid-immunized mice (●) at 3 weeks after the first immunization (Mann-Whitney *U*-test, $P < .05$). Control plasmid-immunized group consisted of 5 mice, and gD-IL-2 plasmid-immunized group consisted of 6 mice.

Table 1. Delayed-type Hypersensitivity

	Mean ± SD (mm)
HSV-infected group	0.495 ± 0.105* (n = 6)
GD-IL-2-immunized group	0.294 ± 0.037* (n = 6)
Control plasmid-immunized group	0.026 ± 0.011 (n = 5)

HSV: herpes simplex virus, gD-IL-2: glycoprotein D-interleukin-2.

Delayed-type hypersensitivity (DTH) responses were elicited in gD-IL-2 plasmid-immunized mice compared to control plasmid-immunized mice at 24 hours after challenge with ultraviolet-inactivated HSV (one-way analysis of variance and Tukey test, $P < .05$).

*Statistical significance compared to control plasmid-immunized group. Each group consisted of 6 mice. Mice in positive control group were infected intraperitoneally with live HSV (1×10^4 plaque-forming units/mL) 2 weeks before DTH assessment.

vaccination using protein can induce humoral immunity, but not cell-mediated immunity. However, ideal effective protection against HSV-1 keratitis should be mediated by both antibody and T cells because of the persistence of the infection in the cells.

In subunit vaccine, antigenic activity is usually weak without immunological adjuvant. Only aluminum hydroxide is licensed for human usage; however, it is far from ideal because its ability to induce cellular immunity is very slight, and it requires frozen storage.[10] In the studies on safe and effective adjuvants, IL-2 has been shown to enhance the immunity.[18,19]

In our previous study, it required hypodermal administration of 20 μg protein three times to obtain an effective immune response without adjuvant; while in this report, hypodermal administration of only 1 μg gD-IL-2 protein twice was needed. These results demonstrated that immunogenic activity was enhanced by the addition of IL-2. In a previous report, immunization with gD-IL-2 protein could induce DTH and CTL as well as neutralizing antibody.[10] In the study in the murine herpetic keratitis model, stromal lesions were completely inhibited using hypodermal administration of gD-IL-2 protein.

We selected subconjunctival injection as the administration route of gD-IL-2, because the acquisition of stronger local immunity can be expected if the injection point is close to the actual viral replicating site. Actually, it has been reported that a local immunization could confer more local protection than a systemic one.[20] As the result of the subconjunctival administration of gD-IL-2 protein, stromal lesions were suppressed. Taking our results together, the vaccination using gD-IL-2 protein was the efficient way for both systemic and local administration.

As the next step, the immunization with plasmids encoding gD-IL-2 provided the preventive effect against herpetic keratitis similar to that with gD-IL-2 protein. In this study, the same results were obtained with gD-IL-2 DNA immunization as in our previous report.[21] Generally, immunization with plasmid DNA encoding antigenic proteins elicits both humoral and cellular immune responses. In our data, this DNA immunization induced DTH reaction as well as neutralizing antibody against HSV, as expected.

Our findings demonstrated that mice vaccinated with protein as well as DNA in gD-IL-2 were completely free of stromal opacification. It was indicated that the high immunity induced by these forms of vaccine is strong enough to halt the spread of the virus prior to the occurrence of cytokine storm. Ideally, it is desirable that epithelial lesions should also be suppressed. In the primary infection model, because of the direct instillation of high-titer HSV after corneal scraping, it might be very difficult to immunologically control the onset of epithelial keratitis.

However, epithelial lesions can be treated using conventional antiviral drugs. Moreover, the prognosis for vision is mainly related to the stromal lesion, and the halt of the development from epithelial to stromal keratitis is the most important issue. In this sense, gD-IL-2 could be a strong candidate for HSV vaccine. In addition, the main problem of herpetic infection is not viremia, but the persistent infection in the cell. Therefore, the vaccine with the ability to induce cell-mediated immunity against infected cells like gD-IL-2, is preferable as an HSV vaccine. gD DNA could induce cell-mediated immunity,[21] but gD protein could not. In contrast, both gD-IL-2 DNA[21] and protein[11] could induce cell-mediated immunity. In this sense, both types of gD-IL-2 are preferable for vaccination against herpetic keratitis.

In the future, we are planning to estimate the preventive effect of gD-IL-2 in recurrent HSV keratitis, and thereby, to study further the mechanisms of infection in order to obtain the ideal vaccine.

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