Herpes Simplex Virus Glycoprotein D
Protective Immunity Against Murine Herpetic Keratitis

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The protective effect of glycoprotein D (gD) immunization against murine herpetic keratitis was investigated. gD was purified by affinity chromatography using anti-gD monoclonal antibodies. Prior immunization with gD was shown to be effective in protecting mice from both the development of stromal keratitis and the spread of the virus to the central nervous system. The level of serum antibodies for virus neutralization, as well as for complement-dependent cytolysis (CDC), was significantly elevated in gD-immunized animals. Cellular immunity, however, was not detected. These results indicate that two antibody-mediated defense mechanisms—virus neutralization and CDC—were responsible for the protective effect observed in our study. Invest Ophthalmol Vis Sci 31:411-418, 1990

One of the current problems in the management of ocular herpetic diseases is recurrent stromal keratitis, which ultimately causes severe visual disturbances due to scarring and vascularization of the corneal stroma. It is widely believed that recurrent keratitis results from the reactivation of herpes virus from the latent state in the regional sensory ganglia. Immunoprophylaxis of primary herpetic infection, therefore, is a rational approach to deal with recurrent stromal keratitis. Several attempts have been made to develop herpes vaccines; however, the possible latency and oncogenicity of viral DNA pose serious hazards in the development of these vaccine. To circumvent these potential difficulties, a subunit type of vaccine has been studied extensively.

Glycoproteins B, C, and D (gB, gC, and gD), which are present on an herpes simplex virus (HSV) virion envelope and a cell membrane infected with HSV, are the targets for a variety of immunologic reactions mediated by host defense mechanisms, including virus neutralization and cytotoxicity to virus-infected cells; these cytotoxicity mechanisms include CDC (complement-dependent cytolysis), ADCC (antibody-dependent cellular cytotoxicity), CTL (cytotoxic T lymphocyte), and DTH (delayed-type hypersensitivity).

Therefore, purified or synthetic HSV glycoproteins are good candidates for a herpes vaccine. The effects of prior immunization with these glycoproteins have been investigated and has focused on protection against lethal HSV infection. The effects on herpetic keratitis remain as yet unclear. In this study, we investigated the effects of purified gD on the development of protective immunity to murine herpetic keratitis.

Materials and Methods

Cells

Vero cells derived from African green monkey kidney, L929 cells derived from C3H mice, and 3T3 clone A31 cells derived from BALB/c mice were grown in Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (FCS; Hyclone, Logan, UT). HEp-2 cells derived from human epidermoid carcinoma were grown in 10% FCS-MEM. Sp-2 cells derived from BALB/c mouse myelomas were grown in Dulbecco's minimum essential medium (DMEM; Gibco) supplemented with 10% FCS.

Animals

Six- to eight-week-old female BALB/c mice were purchased from Oriental-Kobo (Osaka, Japan). All
investigations conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research.

Virus

The Fukuda strain, a laboratory strain, and the Kawamura strain, freshly isolated from a patient with recurrent herpetic stromal keratitis, were used throughout the study. Both strains are HSV type 1. The infectious titers of each virus strain were determined by the plaque titration method.

Determination of Serum-Neutralizing Antibody Titers

The neutralizing antibody titers were determined by the plaque reduction method. In brief, serial 2-fold dilutions of heat-inactivated sera collected from immunized or nonimmunized mice were incubated with an equal volume of virus suspension (2000 PFU/ml) for 1 hr at 37°C. Then, 0.1-ml units of this mixture were assayed for plaque-forming ability on Vero cells. Neutralizing antibody titers were expressed as the reciprocal of the dilution causing 50% plaque reduction.

Preparation of Anti-HSV Monoclonal Antibodies

Six-week-old, female BALB/c mice were immunized with a mixture of NP-40 lysate from HSV-infected Vero cells and complete Freund adjuvant (Difco, Detroit, Michigan). One month after the immunization, the spleen cells obtained from these mice were fused with Sp-2 cells by means of polyethylene glycol, cultured in hypoxanthine/aminopterin/thymidine (HAT; Gibco) medium, and cloned by the limiting dilution method. Cells producing antibodies to HSV were screened by the indirect immunofluorescence method using acetone-fixed HSV-infected Vero cells and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Cappel, West Chester, Pennsylvania) as a secondary antibody.

The antigen recognized by each monoclonal antibody was determined by the immunoprecipitation method. In brief, monolayers of Vero cells were infected with the Fukuda strain at a multiplicity of infection (MOI) of 0.1. Five hours later, 100 µCi 35S-methionine (Amersham, Tokyo, Japan) was added to the culture, followed by overnight incubation. The next day, the infected cells were harvested and solubilized with RIPA buffer (0.15 M NaCl, 1 mM EDTA, 1% Triton-X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 2 mM Tris-HCl; pH 7.2). After brief sonication, the lysate was ultracentrifuged at 30,000 rpm for 1 hr. Two hundred microliters of the supernatant was then incubated either with 100 µl of the culture medium of each antibody-producing clone or with 1 µl of ascitic fluid from hybridoma-injected mice, at 4°C for 12 hr. The mixture was incubated at 4°C for 4 hr with 10 µl rabbit anti-mouse IgG, followed by the incubation at 4°C for 1 hr with 3 mg protein-A sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden), with continuous agitation. The beads were washed seven times by centrifugation, and finally solubilized with 100 µl SDS-sample buffer. The samples were subjected to SDS polyacrylamide gel electrophoresis (SDS–PAGE) for antigen determination.

Purification of gD

gD was purified with affinity chromatography as described elsewhere. One clone of mouse anti-gD monoclonal antibodies, purified by sedimentation with 30% ammonium sulfate from mouse ascitic fluids, was coupled with 3 g CNBr-activated sepharose 4B (Pharmacia Fine Chemicals) and packed in a 10-ml disposable syringe (anti-gD column).

Monolayers of HEp-2 cells, infected with the Fukuda strain at a MOI of 0.01 PFU per cell for 2 days, were dissociated with 0.1 M phosphate buffer containing 0.5% N-P-tosyl-lysine-chloromethyl ketone (Sigma, St. Louis, MO) and 0.5 M NaCl (pH 7.0). Following ultracentrifugation (at 25,000 rpm for 1 hr), the supernatant was applied to an anti-gD column. The column was washed with 0.1 M phosphate buffer containing 0.5 M NaCl for 12 hr, and gD finally was eluted with 0.1 M diethylamine (Wako Junyaku, Osaka, Japan).

Immunization Protocol

BALB/c mice were immunized intraperitoneally with purified gD (20 µg/0.1 ml) three times at weekly intervals, without using adjuvants. One week after the last immunization, the mice were divided into three groups; the first group was sacrificed for the measurement of various immunologic parameters; the second group was used for the DTH assay; and the third group was challenged by way of the corneas with the Kawamura strain of HSV-1, for the clinical evaluation. Mice receiving lysate of uninfected HEp-2 cells served as controls.

Murine Herpetic Keratitis Model

After anesthetization by intraperitoneal injection of sodium pentobarbital (1.5 mg/0.15 ml), the corneas of BALB/c mice were sacrificed 20 times with a 26-gauge needle and infected with 1.7–8.5 × 105/10 µl of the Kawamura strain per eye. From day 1–10
and on day 14, the eyes were examined with a hand-slit lamp by the same masked observer.

Epithelial lesions were scored as follows: 0, no epithelial lesion or punctate epithelial lesion; 1, stellate keratitis or residue of the dendritica; 2, dendritic keratitis occupying less than one quarter of the cornea; 3, dendritic keratitis occupying one quarter to one half of the cornea; and 4, severe dendritic keratitis extending over more than one half of the cornea.

Stromal lesions also were scored as follows: 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 more than one half the diameter of the cornea; and 4, severe stromal opacity and edema, through which the iris is invisible.

**Tissue Sampling and Virus Titration**

Eyeballs, trigeminal ganglia and midbrains were excised aseptically from infected animals on days 3, 5, and 7 postinfection. The eyeball was divided into anterior and posterior segments. These tissues were homogenized in 1 ml MEM, sonicated for 30 sec, and centrifuged at 3000 rpm for 10 min. Virus titers of the supernantant were determined by plaque assay, as described above.

**Assay for the Latent Infection**

Trigeminal ganglia of surviving mice were excised 1 month after infection and cocultivated with Vero cells to assess the rate of latent infection. The cultures were monitored for cytopathic change for 4 weeks.

**CDC Assays**

L929 cells, infected with the Fukuda strain at a MOI of 1 PFU/cell for 2 hr, were labeled with 200 μCi 51Cr (Amersham) for 1 hr. After three washings, 50 μl (10⁸ cells) of labeled L-929 cells suspended in RPMI-1640 containing 7% FCS and 2 mM HEPES; 50 μl serially diluted mouse sera; and 100 μl of 51Cr-labeled, HSV-infected L929 cells (1 × 10⁴) were incubated in U-shaped wells of a microtitration plate for 6 hr. One tenth of 1 ml of the supernatant medium from each well was collected to assess the released radioactivity. Specific 51Cr release was calculated with the following formula: percent lysis = ([sample release — medium control release]/[maximum release — medium control release]) × 100.

**CTL Assay**

Spleen cells aseptically obtained from immunized and nonimmunized mice were suspended at a concentration of 2 × 10⁶ cells/ml in RPMI-1640 containing 10% FCS, 2 mM glutamine, and 50 μM 2-mercaptoethanol, and were mixed with X-irradiated, lipopolysaccharide-stimulated mouse spleen cells infected with the Fukuda strain at a MOI of 1 PFU/cell for the secondary stimulation. The cultures were incubated at 37°C in a 5% CO₂-humidified incubator for 5 days. A mixture of 100 μl cultured spleen cells (2.5 × 10⁵ cells/well) and 51Cr-labeled, HSV-infected 3T3 clone A31 cells (1 × 10⁴ cells/well), prepared as described above, was incubated in 96 U-shaped wells of a microtitration plate for 7 hr. 51Cr-labeled, HSV-infected L929 cells were used as H-2 mismatched target cells. The specific 51Cr release was calculated with the formula described for the ADCC assay. Spontaneous release was below 5% of maximum release.

**DTH Assay**

The Fukuda strain, grown in 3T3 clone A31 cells and harvested by freezing and thawing, was sonicated and then centrifuged at 3000 rpm. The supernatant medium, heat-inactivated at 56°C for 45 min, was used as the HSV antigen for the DTH assay. The control antigen was prepared from uninfected 3T3 clone A31 cells in the same manner as described above. Immunized or nonimmunized mice were injected intradermally, with 10 μl of the HSV antigen in the right ear pinna and the same amount of the control antigen in the left ear pinna, using a microsyringe (Hamilton, Reno, NV). 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 the two ear pinnas.

**Statistical Analysis**

The student t-test was used in the study. Wilcoxon's test was used for clinical scoring.

**Results**

**The Specificity of Anti-gD Monoclonal Antibodies and Purification of gD**

Six clones reacting with gD were obtained. The protein recognized by these monoclonal antibodies were considered gD based on the following findings: 1) its molecular weight was estimated to be 60 kD; 2) these monoclonal antibodies had neutralizing activity against HSV-1 and cytotoxic activity to HSV-1 infected cells; and 3) the cell surface of HSV-1-infected cells was stained positively by these monoclonal antibodies in an immunofluorescent test. Figure 1 shows the profile of SDS-PAGE, with the antigen precipitated by one clone of monoclonal antibodies. Figure 2 shows the purity of the antigen used for immunization.

**The Effects of gD Immunization Against Herpetic Keratitis in Mice**

Epithelial lesions in control mice reached a peak between days 2–4 postinfection, and gradually reduced in severity by day 7. Stromal lesions in these animals began to develop around day 6, and reached peak severity between days 10–14. As shown in Figure 3A, prior gD immunization did not inhibit the occurrence of epithelial keratitis. However, the development of stromal keratitis was inhibited markedly in gD-immunized animals, as compared with control animals (Figure 3B). On days 5–14, clinical scores of gD-immunized mice were significantly lower than those of nonimmunized mice. The representative clinical pictures of two groups of mice are shown in Figure 4. Corneal vascularization and blepharitis, which usually developed at the late stage of the infection, also were inhibited completely in the gD-immunized group.

In another experiment, virus growth in the anterior and posterior segments of the eyeballs, trigeminal...
Immunity Induced by gD Immunization

Several parameters of humoral and cellular immunity were compared in gD-immunized and control animals. As shown in Figure 5, the serum-neutraliz-
Table 1. Virus titers after HSV-1 corneal infection in gD-immunized and nonimmunized mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mice</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior segment of eyeball</td>
<td>gD-immunized</td>
<td>0.75 ± 1.4*</td>
<td>4.0 ± 8.0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>nonimmunized</td>
<td>24 ± 25</td>
<td>1.6 ± 2.8</td>
<td>9.0 ± 9.6</td>
</tr>
<tr>
<td>Posterior segment of eyeball</td>
<td>gD-immunized</td>
<td>170 ± 270</td>
<td>2.6 ± 5.6†</td>
<td>0 ± 0*</td>
</tr>
<tr>
<td></td>
<td>nonimmunized</td>
<td>110 ± 150</td>
<td>110 ± 150</td>
<td>380 ± 610</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>gD-immunized</td>
<td>0 ± 0</td>
<td>1.8 ± 4.5‡</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>nonimmunized</td>
<td>58 ± 140</td>
<td>130 ± 100</td>
<td>70 ± 160</td>
</tr>
<tr>
<td>Midbrain</td>
<td>gD-immunized</td>
<td>0 ± 0</td>
<td>0.67 ± 1.6†</td>
<td>1.5 ± 2.6‡</td>
</tr>
<tr>
<td></td>
<td>nonimmunized</td>
<td>0 ± 0</td>
<td>690 ± 580</td>
<td>110 ± 180</td>
</tr>
</tbody>
</table>

Six samples in each group were measured. Values are mean ± standard deviation. Significantly different from the value of the nonimmunized group:

*    $P < 0.05$
†    $P < 0.02$
‡    $P < 0.01$

Discussion

Several investigators have reported that gB-, gC-, or gD-immunized mice effectively tolerated a lethal challenge of HSV. Kino et al.\(^{21}\) reported that purified-gB-immunized mice survived a lethal challenge of HSV-1 and HSV-2 inoculated into the cornea. Ocular symptoms, however, were not precisely evaluated. Foster et al.\(^{25}\) reported that gD immunization inhibited the development of stromal keratitis in mice. This report, however, failed to characterize adequately the immunologic mechanisms by which gD immunization was effective.

In the current study, gD was selected as a possible HSV vaccine candidate for two reasons: 1) gD is a common antigen to both type 1 and 2 HSV, and polypeptide and carbohydrate structure appears to be the same in different strains of HSV, and 2) gD is a major target for virus neutralization. It was shown clearly\(^{27}\) that prior gD immunization inhibits almost completely the development of stromal keratitis. In our murine keratitis model, growth of the virus started in the corneal epithelium, subsequently invaded the stroma, and finally infected the stromal keratocytes. The host defense induced by gD immunization therefore was either able to halt the spread of the virus from the corneal epithelium to the stroma, or to efficiently eliminate the virus-infected keratocytes. In addition, this immunity was found also to halt the viral spread from the eye to the central nervous system. However, this immunity was insufficient to suppress the growth of the virus in the corneal epithelium with respect to clinical scores.

Further immunologic analysis clearly revealed that humoral immunity was responsible for the observed results. It was demonstrated that both CDC activity and neutralizing antibody titers were elevated in immunized mice. Neutralizing antibody titers were found to vary; therefore, the observed protection cannot be explained by virus neutralization alone. CDC may therefore play a role in the elimination of HSV-infected cells. Cappel et al.\(^{11}\) reported an elevation in CDC activity in humans after the administration of HSV envelope antigens. These results are in accordance with our own. The significance of the in vivo CDC observations requires further investigation.

![Fig. 5. Serum-neutralizing antibody titers in gD-immunized and nonimmunized mice. Open circles represent nonimmunized mice; closed circles, gD-immunized mice. Sera were collected 1 week after the last immunization.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933577/)
dent cytolysis (CDC)

immunization, virus neutralization, complement-depen-

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tradermal injection of gD induced a DTH reaction in

results may be due to differences in the immuniza-

Table 2. Immunologic parameters induced in

gD-immunized and nonimmunized mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>gD-immunized</th>
<th>nonimmunized</th>
<th>HSV-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC (%)</td>
<td>50 ± 14* (10)</td>
<td>0.7 ± 3.1 (10)</td>
<td>67 ± 7.7 (10)</td>
</tr>
<tr>
<td>ADCC (%)</td>
<td>−3.0 ± 6.1 (10)</td>
<td>−5.1 ± 8.0 (10)</td>
<td>15 ± 5.8 (10)</td>
</tr>
<tr>
<td>CTL (%)</td>
<td>−7.3 ± 13.0 (13)</td>
<td>6.5 ± 13.0 (15)</td>
<td>30 ± 8.8 (11)</td>
</tr>
<tr>
<td>DTH (mm)</td>
<td>0.013 ± 0.02 (10)</td>
<td>0.019 ± 0.02 (10)</td>
<td>0.098 ± 0.059 (9)</td>
</tr>
</tbody>
</table>

Values represent mean ± standard deviation.

* Significantly different from value of nonimmunized group (P < 0.01).

Although antibodies produced as a result of gD
immunization were mainly IgG (data not shown)
ADCC activity was not detected in gD-immunized
animals. The reason for this discrepancy has not yet
been found; a more sensitive assay system, however,
may be able to detect ADCC activity. It appears, how-
ever that in our system, the ADCC mechanism was
far less responsible for the protection than was the
CDC mechanism.

Insofar as cellular immunity is concerned, neither
DTH nor CTL was induced by gD administration in
our study. Schrier et al., however, reported that in-
tradermal injection of gD induced a DTH reaction in
mice. The discrepancy between these and our own
results may be due to differences in the immuniza-

route or adjuvant usage. In preliminary experi-

ments, we have already demonstrated the existence
of a DTH reaction with the subcutaneous injection
of gD.

Laush et al., reported that HSV-1 replication was
markedly depressed in the corneas of antibody-pro-
ducing, DTH-tolerant mice. Viral replication was
clearly evident, though, in the corneas of antibody-
depressed, DTH-responsive mice. These results,
along with our own, indicate that humoral immunity
was at least as important in eliminating HSV from
the cornea as was cellular immunity. Passive transfer
studies using anti-gD monoclonal antibodies are cur-

ently under way to further elucidate the significance
of humoral immunity in in-vivo protection.

In conclusion, gD is a strong candidate for a HSV
vaccine, but has been shown to be unable to suppress
completely epithelial keratitis or to inhibit viral la-
tency in the trigeminal ganglia after primary infec-
tion. This might be due to the fact that our prepara-
tion could not induce cellular immunity. To develop
a more effective vaccine, further investigations are
necessary on the use of an adjuvant or on modifica-
tion of the form of vaccine preparation.

Key words: murine herpetic keratitis, glycoprotein D (gD),
immunization, virus neutralization, complement-depend-
cytolysis (CDC)

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