Prevention of Herpes Keratitis by Monoclonal Antibodies Specific for Discontinuous and Continuous Epitopes on Glycoprotein D

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Seven monoclonal antibodies (mAb) specific for defined discontinuous and continuous epitopes on glycoprotein D of herpes simplex virus type 1 (HSV-1) were surveyed for their capacity to protect against virus-induced corneal disease in a murine ocular infection model. A known amount of purified mAb was transferred passively to BALB/c mice 24 hr after topical infection with HSV-1 on their scarified corneas. At high doses (50–136 µg), all seven mAbs protected against the development of persistent necrotizing stromal keratitis. Significant protection was also observed at low doses (20 µg) with two mAbs to discontinuous epitopes and two mAbs to continuous epitopes. Selected high-dose mAbs also were able to reduce the severity of blepharitis. These results indicated that at least seven different antigenic sites on glycoprotein D can serve as targets for effective antibody therapy in the murine model of HSV-1 ocular infection. Invest Ophthalmol Vis Sci 32:2735–2740, 1991

Glycoprotein D (gD) is a major structural component of the herpes simplex virus (HSV) envelope and is required for virus replication in tissue culture.1 Recent studies identified a critical site on this molecule that is involved in virus penetration into cells.2,3 The gD is expressed on the surface of virus-infected cells and on the virus particle; it is an important antigen, inducing both cellular4 and humoral5–7 immune responses in humans. The potent immunogenicity of gD led to its evaluation as a vaccine candidate. Many studies established that immunization to gD, or its peptides, can protect against acute infection and reduce the incidence of latency and recurrent disease.8–13

The antigenic nature of gD is complex, and it still is not defined completely. Analysis of its antigenic sites was investigated principally through use of monoclonal antibodies (mAbs) specific for this molecule. These antibodies were arranged into groups that recognize distinct common and type-specific sites on HSV type 1 (HSV-1) and HSV type 2 (HSV-2) gDs.14,15 Several mAb groups recognize discontinuous epitopes that are dependent on proper tertiary structure; others recognize continuous epitopes that are present in both the denatured and native forms of the molecule.

The availability of mAbs to HSV antigens stimulated interest in whether they might be effective as antiviral agents in vivo. Initially, it was shown by several laboratories that passive transfer of mAbs to the major glycoproteins could protect mice against the development of a fatal encephalitis.16–19 Metcalf and co-workers20 were the first to show that mAbs to gB, gC, and gD also could protect animals against corneal disease when passively transferred after ocular infection. Subsequently, we conducted studies with a purified anti-gD mAb and found that concentrations as low as 10 µg could protect against necrotizing stromal keratitis.21

Previous passive immunization studies were conducted with mAbs whose reactivity with specific antigenic sites on the HSV glycoproteins was unknown. We exploited the availability of mAbs to defined discontinuous and continuous epitopes on gD to investigate whether certain antigenic sites on this important molecule were more effective than others as targets for antibody therapy. Our experimental approach was to test whether known concentrations of purified mAbs to selected epitopes would be capable of preventing blinding corneal disease in mice infected topically with HSV-1.
Materials and Methods

Animals

Four-week-old female BALB/c mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). The animals were treated and housed in accordance with the ARVO Resolution on the Use of Animals in Research.

Virus and Cells

The virus used for corneal infection was HSV-1 strain RE. It was plaque purified three times, and then virus stocks were grown and titrated on Vero cells as previously described.22 The HSV-1 strain KOS, used in the neutralizing antibody tests, also was grown and titered on Vero cells. The latter were grown in Dulbecco’s modified minimal essential medium containing 5% calf serum.

Monoclonal Antibodies

For this study, seven anti-gD mouse mAbs were used. These were 8D2, DL11, DL2, BD78, DL6, ID3, and BD66. The epitope specificity of each is summarized in Table 1 and discussed subsequently. Before purification, each mAb-ascites fluid preparation was filtered (0.22 μm) to remove particulate matter. The filtered samples then were diluted 1:2 with phosphate-buffered saline (PBS) and purified using an immobilized protein G column (Genex, Gaithersburg, MD) according to the manufacturer’s instructions. After elution from the column, the immunoglobulins were concentrated using a Centriprep 30 ultrafiltration cell (Amicon, Beverly, MA). The resulting concentrated mAbs were dialyzed against PBS, and the protein content was determined using the BCA* protein assay (Pierce, Rockford, IL).

Immunoglobulin isotypes were determined using an enzyme-linked immunosorbent assay (ELISA) kit obtained from Southern Biotechnology (Birmingham, AL). The titration end point was designated as that at which the optical density was threefold higher or more than the control optical density.

Table 1. Properties of Purified Anti-gD mAbs

<table>
<thead>
<tr>
<th>mAb</th>
<th>Antibody Group</th>
<th>Type Epitope</th>
<th>IgG Isotype</th>
<th>ELISA Titer</th>
<th>HSV-1 Neutralizing Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>8D2</td>
<td>III</td>
<td>Discontinuous</td>
<td>2a</td>
<td>10^6</td>
<td>10^3</td>
</tr>
<tr>
<td>DL11</td>
<td>Ib</td>
<td>Discontinuous</td>
<td>2a</td>
<td>10^5</td>
<td>10^3</td>
</tr>
<tr>
<td>DL2</td>
<td>VI</td>
<td>Discontinuous</td>
<td>2b</td>
<td>&lt;10^5</td>
<td>10^3</td>
</tr>
<tr>
<td>BD78</td>
<td>II</td>
<td>Continuous</td>
<td>1</td>
<td>10^5</td>
<td>10^3</td>
</tr>
<tr>
<td>DL6</td>
<td>II</td>
<td>Continuous</td>
<td>2a</td>
<td>10^5</td>
<td>10^3</td>
</tr>
<tr>
<td>ID3</td>
<td>VII</td>
<td>Continuous</td>
<td>2a</td>
<td>10^5</td>
<td>10^3</td>
</tr>
<tr>
<td>BD66</td>
<td>XI</td>
<td>Continuous</td>
<td>1</td>
<td>10^5</td>
<td>&lt;10^3</td>
</tr>
</tbody>
</table>

Corneal Infection

For corneal infection, the mice were anesthetized with 0.2 ml of a 1:10 dilution of sodium pentobarbital (50 mg/ml stock solution), and one eye was scarified by three twists of a 2-mm corneal trephine. A 2-μl volume containing the desired concentration of HSV-1 RE was dropped onto the corneal surface and gently massaged into the eye with the eyelids. The eyes were examined two to three times a week for the first 2 weeks after infection and then weekly thereafter using a stereo microscope with a fiberoptic light source. Stromal keratitis and blepharitis were graded on a scale of 0 (no disease) to +5 (most severe disease) as previously described.20,23 All clinical scoring was done in a masked fashion. The data were analyzed using the Mann-Whitney U test.

Serologic Tests

Each purified monoclonal antibody was tested for its capacity to neutralize HSV-1. Neutralizing tests were conducted by incubating equal volumes of diluted antibody with strain KOS and guinea pig complement (1:15 dilution). After 30 min incubation at 37°C, the residual infectious virus was assayed on Vero monolayers in 24-well plates. Appropriate positive and negative controls were included in each test.

Antibody binding to HSV-1 RE strain-infected Vero cells and uninfected cells were assayed in the ELISA. The protocol used was that previously described,24 except that 0.01% poly L-lysine was used to pretreat the plates. Target cells were fixed with 0.5% glutaraldehyde and blocked with 1.0% bovine albumin in borate-buffered saline.

Results

Specificity and Properties of Anti-GD mAbs

The gD present in HSV-1 (gD-1) is 85% homologous with gD-2 found in HSV-2.15 The former (in mature form) consists of 369 amino acids; the latter has 368 amino acids. The mAbs that recognize discontinuous epitopes on gD were arranged into four groups, one of which was subdivided. We selected mAbs 8D2, DL11, and DL2 that recognize discontinuous epitopes III (RJE and GHC, unpublished observations), Ib,25 and VI,15,26 respectively, for study (Table 1).

Four additional mAbs studied recognized continuous epitopes (Table 1). Specifically, BD78 and DL6 recognized overlapping epitopes at antigenic site II.27 The BD78 binds to residues 264–275; DL6 binding was mapped to amino acids 272–279. The MAb BD66 recently was shown to bind to amino acids 284–301, an antigenic site designated XI.27 Finally,
ID3 is a group VII mAb that recognizes a continuous epitope mapped to residues 1–23.15,28

All seven mAbs (after purification) were tested for their capacity to bind to HSV-1 RE-infected cells and neutralize HSV-1 KOS infectivity. Table 1 shows the isotype, the ELISA titer against Vero cells infected with HSV-1 strain RE, and the HSV-1 neutralization titer of each mAb. Two of the mAbs, DL2 and BD66, did not show detectable neutralizing activity. Each of the antibodies recognized a type common epitope except for DL2 which was gD-1 specific.26

Protective Activity of mAbs Specific for Discontinuous Epitopes on gD

Previous studies found that purified mAb 8D2 was protective when passively transferred in the 10–50-µg dose range.21 For our initial studies, we tested whether mAbs to discontinuous epitopes Ib and VI also could prevent the development of virus-induced stromal keratitis. Four-week-old BALB/c mice were infected on the right trephined cornea with $2 \times 10^4$ plaque-forming units of HSV-1 strain RE. Twenty-four hours later, the animals were divided into groups of six to eight each and given a single intraperitoneal inoculation of high-dose purified mAb. The control animals received nonimmune globulin of the immunoglobulin G2a isotype. The mice then were followed for the development of corneal disease.

Figure 1A shows the mean corneal opacity score 4 weeks postinfection. All eight control mice had severe necrotizing stromal keratitis that persisted through the remainder of the 7-week observation period. Each of the three mAbs to discontinuous epitopes was strongly protective ($P < 0.005$).

Next, the protective capacity of low-dose mAbs was evaluated (Fig. 1B). Passive transfer of 20 µg of 8D2 was highly protective (in agreement with previous observations).21 At the 20-µg dose, DL11 resolved corneal opacity in 60% of the recipients; DL2 was not protective ($P > 0.05$).

Protective Activity of mAbs Specific for Continuous Epitopes on gD

Four mAbs that recognized continuous epitopes on gD were tested for their capacity to protect against stromal keratitis. Figure 2A shows that, at high antibody doses, all four were strongly protective ($P < 0.005$) compared with the control group. However, treatment with ID3 and DL6 mAbs appeared to be more efficacious than that with BD78 and BD66 mAbs. This impression was confirmed by low-dose testing, ie, at the 20-µg test dose, only ID3 and DL6 gave significant ($P < 0.05$) protection (Fig. 2B).

Capacity of Anti-gD mAbs to Protect Against Blepharitis

The HSV-1 infection of the mouse eye consistently induces blepharitis and corneal disease. The severity of eyelid disease usually peaks during the first week of infection and then gradually diminishes. The capacity of the passively transferred mAbs to limit the development of this disease was examined. Figure 3 shows the mean blepharitis score 7 and 14 days postinfection in animals given high-dose antibody. Selected mAbs to discontinuous epitopes (8D2 and DL11) and continuous epitopes (ID3 and DL6) were able to reduce the severity of eyelid disease significantly, but the protective effect was relatively modest. On days 7 and 14, none of the mAbs given at the 20-µg concentration was protective (data not shown). Overall, the antibodies most effective in preventing or reducing the severity of stromal keratitis were the same as those that ameliorated blepharitis.

Discussion

We conducted this study to determine whether mAbs specific for discrete antigenic sites on gD could
protect against the development of HSV-1-induced stromal keratitis. Antibodies specific for six distinct regions on the molecule were tested, and it was found that each could protect if a sufficient concentration of immunoglobulin was administered. Thus, both continuous epitopes II, VII, and XI and discontinuous epitopes Ib, III, and VI could serve as effective targets on gD. Others previously reported that a mAb specific for antigenic site Ib would protect mice against intracranial challenge with HSV-1. The mAbs reactive with site Ia were shown to protect against lethal footpad challenge with HSV-1 or HSV-2 and against zosteriform spread of HSV-1 in the flanks of mice.

In our study, the protective effect of high-dose antibody was independent of the antibody isotype; immunoglobulin G2a, G2b, and G1 antibodies all were therapeutically active. Also, mAbs DL2 and BD66 (which did not display any detectable neutralizing activity against HSV-1) were able to prevent stromal disease. The absence of a correlation between neutralizing antibody titers and levels of protection also was observed in other studies with HSV.

Previous work showed that 8D2 mAb could reduce the severity of blepharitis but not prevent the disease. Our study confirmed this finding and extended it to show that both continuous and discontinuous epitopes can function as protective target antigens in cutaneous eyelid disease. The observation that protection was modest and mediated by only four of the seven mAbs tested supports the view that humoral immunity directed against gD is therapeutically more effective against corneal than HSV-induced eyelid disease.

When immunoglobulin was transferred passively at low doses (20 μg) only four of the seven mAbs protected significantly against corneal opacity. The reason for this is unclear. It may be more than a coincidence that all four protective mAbs were of the immunoglobulin G2a isotype; the three nonprotective antibodies had a different isotype. Studies by others found that immunoglobulin G2a antibodies are synthesized preferentially by mice after viral infection, and of the four murine immunoglobulin G isotypes, G2a is the most efficient at mediating antibody-dependent cellular cytotoxicity. It is also possible...
that the affinities of the protective antibodies were higher than those of the nonprotective mAbs. This could explain why DL6 and BD78, which recognize overlapping epitopes, differ in their therapeutic potential.

It is not known how a single intraperitoneal inoculation of mAb at doses as low as 10–20 μg is able to prevent corneal blindness. The host immune response is thought to play an important role in the development of HSV-induced stromal keratitis. Previous histologic studies found that, in contrast to untreated infected corneas, there is little or no mononuclear cell infiltrate of the corneas of antibody-treated hosts. Thus, we originally postulated that antibody might protect by suppressing the host immune response. However, this explanation is not possible because additional studies showed that immunoglobulin treatment does not inhibit antibody formation, and it accelerates the development of delayed hypersensitivity.

In our model, antibody treatment did not enhance significantly infectious virus clearance from the eye. Thus, it seems unlikely that the passively transferred immunoglobulin protected primarily by promoting lysis of virus infected cells. Others also found that passively transferred antibody did not reduce HSV titers at the inoculum site, even when the antibody was given 1 day before corneal infection. Their provocative hypothesis is that the antibody protects against severe ocular damage by restricting virus spread from the nervous system back into the eye. Whatever the correct explanation, it is likely that these antibodies exert their therapeutic effect through collaboration with other antiviral defense mechanisms.

In summary, antibodies specific for seven different antigenic sites widely distributed on the gD molecule could protect against HSV-1-induced corneal blindness. Our results indicate that humoral immunity can facilitate resolution of HSV infection and minimize cellular infiltration of the cornea. Thus, these findings provide a rational basis for investigating whether monoclonal or polyclonal antibodies would be useful in the treatment of HSV corneal infection in humans.

Key words: herpes simplex virus, monoclonal antibodies, stromal keratitis, glycoprotein D, blepharitis

Acknowledgments

The authors thank Marty Mayo for excellent technical assistance, Rita Thompson for typing the manuscript, and Gary Siebert, Becton Dickinson Research Laboratories, Sunnyvale, California, for mAbs BD66 and BD78.

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