Previous Immunization of Mice with Herpes Simplex Virus Type-1 Strain MP Protects against Secondary Corneal Infection

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Herpes simplex virus (HSV)-induced ocular disease is occurring in epidemic proportions throughout the world, and is the number one cause of unilateral corneal blindness in all developed countries. We have found, in a mouse model of herpes simplex keratitis (HSK), that products encoded by the Igh-1 locus on chromosome 12 exert a profound influence on the immune/inflammatory response in the cornea after HSV inoculation in the cornea. Thus, mice with Igh-1a or Igh-1b phenotype routinely develop extreme keratopathy and loss of corneal clarity after HSV encounter in the eye, while congenic strains expressing other Igh-1 phenotypes develop substantially less keratopathy. We examined the effect of previous subcutaneous immunization with the mutant, less virulent, MP strain of HSV on the development of keratitis and encephalitis after secondary corneal inoculation with strains MP, mP, F, and KOS. AJ mice (Igh-1a), 5–6 weeks old, were injected sc with live HSV-1 strain MP. Controls were injected with culture media without virus. Three weeks later both immunized and control nonimmunized animals were challenged in the cornea with HSV-1, strains MP, mP, F, and KOS. The animals were clinically scored for keratitis and encephalitis at regular intervals for 21 days following corneal challenge. None of the immunized animals challenged in the cornea with strain MP, 5 × 10^6 plaque-forming units (PFU), developed clinical signs of encephalitis compared to 86% of unimmunized controls. Of the immunized animals challenged in the cornea with strain MP, 5 × 10^6 PFU, only 18% developed a mild keratitis, while 96% of unimmunized controls developed severe keratitis. Mice immunized subcutaneously with MP and subsequently challenged corneally with other HSV-1 strains (mP, F, or KOS) were also protected from development of severe keratopathy.

INTRODUCTION

Herpes simplex virus keratitis is a major cause of corneal blindness today, despite the availability of excellent antiviral agents effective in treating active herpes simplex virus (HSV) infections in the cornea. Herpes simplex virus differs from many viruses by neurotrophic properties and by its capacity to establish a state of latency in the ganglion. To date no antiviral agent has been shown to affect this latent state of infection and/or to prevent recurrent active disease.

We have studied a murine model of herpes simplex keratitis (HSK) and have discovered, using congenic strains of mice, that the Igh-1 gene locus on chromosome 12 of the mouse influences the clinical expression of HSK after HSV-1

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Corneal inoculation (1). A/J mice and other inbred strains having Igh-1c or Igh-1d allotypes develop a severe keratopathy after HSV corneal inoculation, while inbred strains possessing Igh-1a and Igh-1b allotypes develop a less severe keratitis. Compared to resistant mice, susceptible mice exhibit impaired immune responses to HSV as measured by delayed-type hypersensitivity and lymphocyte proliferation assays (1).

Stimulation of the host's immune response to HSV is one therapeutic strategy to prevention and therapy of infection by herpes simplex virus that has not been fully exploited, though previous studies have established that prior inoculation of the ear pinna with live HSV-1 protected mice from severe eye disease and signs of central nervous system (CNS) infection after corneal challenge (2).

In these studies, we have utilized strains mP, MP, F, and KOS of HSV-1. Strain MP, which causes fusion in mammalian cell lines, arose spontaneously in FL cells infected with the parent strain, mP (3). Strain MP, in addition to fusing cells, fails to express functional glycoprotein C (gC). Recent studies (4) indicate that the inability of MP to produce functional gC is the result of a frameshift mutation in the gC coding region of the HSV-1 genome. The parent strain, mP, is identical to MP except mP expresses a functional gC and does not fuse infected cells in vitro (5, 6). In addition to mP and MP, we have also inoculated mice via the corneal route with F and KOS strains, both of which express functional gC and do not fuse cells in vitro. This report details the effect of prior subcutaneous immunization of HSV-1 strain MP on subsequent keratopathy after corneal inoculation with strains MP, mP, F, and KOS of HSV-1.

MATERIALS AND METHODS

Animals. A/J female mice (H-2a, Igh-1'). 6 weeks old, were obtained from Jackson Laboratories (Bar Harbor, Maine).

Virus. HSV-1 strain MP stock, \(6.4 \times 10^7\) plaque-forming units (PFU)/ml; strain mP stock \(5.5 \times 10^7\) PFU/ml; strain F \(3.3 \times 10^7\) PFU/ml; and strain KOS, \(3.5 \times 10^7\) PFU/ml were used in this study. Virus stocks were produced by infecting Vero cell (ATCC, CCL 81) monolayers with each HSV-1 strain.

Experimental design. A/J mice were inoculated subcutaneously with 0.25 ml Eagle's minimum essential media with 5% fetal calf serum containing \(2.5 \times 10^4\) PFU of HSV-1 strain MP. Control animals were inoculated sc with 0.25 ml of media without virus. The same procedure was repeated 14 days later. Twenty-one days after the first inoculation, the mice were challenged in the right corneas with HSV-1 strain MP. Control animals were inoculated sc with 0.25 ml of media without virus. The same procedure was repeated 14 days later. Twenty-one days after the first inoculation, the mice were challenged in the right corneas with HSV-1 and followed clinically.

Corneal inoculation and clinical observation. The mice were anesthetized with ether and 0.5% proparacaine hydrochloride was applied to the right cornea of each mouse. Under binocular microscopy, the right cornea of each mouse was scratched with a 23-gauge needle (four horizontal and four vertical scratches). Five microliters of HSV-1 suspension was deposited onto the ocular surface. The lids were gently held closed for 5 sec. The animals were clinically evaluated and microscopically examined in a masked fashion at Days 2, 4, 7, 9, 11, 16, and 21 after corneal inoculation.

The geographical area of the lesions of the eyelids, corneal epithelial surface,
and corneal stroma were scored from 1 to 4. A clinical score of 1 was consistent with less than 25%, 2 with less than 50%, 3 with less than 75%, and 4 with 75-100% of the geographical area of the epithelial surface, the corneal stroma, and the eyelids, respectively. The thinning of the corneal stroma due to necrosis was graded from 1 to 100%, a score of 1 consistent with less than a 25% decrease in corneal thickness, and so on, with a score of 4 consistent with a 75% or more decrease in corneal thickness. Clinical signs compatible with CNS infection, such as the mouse leaning to the right and spinning if held by the tail, were recorded.

**Histopathology studies.** Randomly selected animals were sacrificed at Days 11 and 21 after corneal inoculation. The corneas were removed surgically, fixed in Karnovsky’s fixative, embedded in LKB Historesin plastic (LKB Inc.), sectioned at 2 μm, and stained with hematoxylin and eosin for histopathological study.

**Determination of HSV-1 neutralizing antibodies.** Blood was collected from immunized and control animals at Day 21 after the first immunization and at Days 7 and 11 after corneal inoculation. Serum was isolated from the blood following clot retraction at 4°C. Virus neutralizing antibodies were determined by mixing serially diluted aliquots of serum with a standard amount of HSV-1 MP strain and incubating for 30 min at 37°C. The serum–virus mixtures were then adsorbed onto Vero cell monolayers for 60 min at 37°C. Overlay media consisting of Eagle’s minimum essential media with 5% fetal calf serum and 0.1% human IgG (165 mg/ml, Gammar, Armour Pharmaceutical Co.) was then added to the monolayers and viral plaques were allowed to develop for 4 days at 37°C. Serum dilutions which resulted in a 50% reduction of the number of viral plaques compared to control values are reported as approximate titers of neutralizing antibodies. All virus neutralization assays were performed in triplicate.

**RESULTS**

**Pathological Studies**

**Corneal Inoculation with HSV-1 Strain MP**

Immunized animals did not develop signs of encephalitis following corneal inoculation with MP strain of HSV-1, 3 × 10⁵ PFU (Fig. 1). Three control (unimmunized) mice challenged corneally with MP, 3 × 10⁵ PFU, developed encephalitis by Day 9 (Fig. 1) and two were dead by Day 11.

Two of the five immunized mice challenged in the cornea with MP, 3 × 10⁵ PFU, developed mild stromal keratitis which totally resolved within 9 days. All control mice developed severe stromal keratitis during the first 7 days after corneal challenge with MP, 3 × 10⁵ PFU. However, the clinical course of the keratitis could not be followed for a prolonged period of time in unimmunized animals because of the fatal encephalitis.

Nineteen of twenty-two control animals (86%) developed clinical signs of encephalitis by Day 9 after corneal challenge with MP, 5 × 10⁴ PFU, and four of these died by Day 14 (Table 1). None of the immunized animals developed encephalitis following corneal challenge with MP, 5 × 10⁴ PFU, within an observation period of 21 days.
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Fig. 1. Accumulative percentage of immunized and nonimmunized mice with clinical signs of encephalitis after corneal inoculation with HSV-1 strain MP, $3 \times 10^5$ PFU, and strain mP, $3 \times 10^5$ PFU, respectively.

Eighteen of twenty-two controls (82%) developed lid lesions with an onset at Day 4 and with a maximum mean clinical score of 1.9 at Day 16 after corneal challenge. (Fig. 2). None of the immunized animals developed lid disease. All animals in both immunized and unimmunized groups had clinical signs of epithelial keratitis at Day 4 after corneal challenge; this keratitis cleared by Day 7, but

<table>
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<th>Strain</th>
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<tr>
<td></td>
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<td>%</td>
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$^a$ Incidence of stromal keratitis up to Day 21 after corneal inoculation.
control mice developed a recurrent epithelial keratitis. Immunized mice showed total epithelial healing without recurrent keratitis.

Twenty-one of twenty-two (96%) unimmunized control mice challenged in the cornea with MP, \(5 \times 10^4\) PFU, developed deep stromal keratitis with neovascularization and ulceration of the corneal stroma. The peak of the mean clinical score occurred at Day 14 after corneal challenge (Fig. 3). Eighteen of the twenty-two control mice developed clinical signs of mydriasis in the ipsilateral eye but no sign of mydriasis in the contralateral eye.

Only four of twenty-two immunized animals (18%) developed stromal keratitis. This keratitis totally resolved in one animal. Two animals developed a mild keratitis which went into remission and left corneal scars without neovascularization, and one animal developed a severe keratitis with neovascularization. No ulceration was observed in the immunized animals.
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Corneal Inoculation with HSV Strain mP

Immunized animals did not develop signs of encephalitis following corneal challenge with HSV-1 strain mP, \( 3 \times 10^5 \) PFU, (Fig. 1). All control mice (unimmunized mice challenged corneally with mP, \( 3 \times 10^5 \) PFU) developed encephalitis by Day 7 (Fig. 1) and all were dead by Day 9. Five of six immunized mice challenged in the cornea with mP, \( 3 \times 10^5 \) PFU, developed mild to moderate stromal keratitis which improved after Day 7 (Table 1). Unimmunized mice developed severe stromal keratitis during the first 7 days after corneal challenge, but because of the fatal encephalitis the clinical course of the keratitis could not be followed longer than 7–9 days after corneal challenge.

Four of nine immunized animals (44%) challenged in the cornea with HSV-1 mP, \( 5 \times 10^3 \) PFU, developed stromal keratitis with a maximum mean score of 0.4 at Day 14. Eight out of nine animals totally recovered from keratitis and none developed encephalitis (Table 1). In contrast, nine of eleven (82%) unimmunized mice developed severe keratitis with a maximum mean score of 3.4 at Day 21. The keratitis did not resolve in any of these animals. Eight mice developed encephalitis and four of these died (Table 1).

Corneal Inoculation with HSV-1 Strain KOS

None of the immunized or unimmunized animals developed clinical signs of encephalitis after corneal challenge with HSV-1 strain KOS, \( 5 \times 10^5 \) PFU (Table 1).

Two of five (40%) immunized animals developed mild stromal keratitis at Day 4 after corneal inoculation. This keratitis in the immunized mice totally resolved by Day 7. Four nonimmunized animals (80%) developed severe keratitis which did not resolve by Day 21.

Corneal Inoculation with HSV-1 Strain F

None of the immunized or unimmunized animals developed signs of encephalitis following corneal challenge with HSV-1 strain F, \( 5 \times 10^4 \) PFU (Table 1). Immunized animals did not develop stromal keratitis. Four of five unimmunized mice developed severe stromal keratitis.

Histopathology Studies

Neutrophils and mononuclear cells were rarely observed in the corneal stroma of immunized mice 11 days after corneal inoculation with HSV-1 strain MP; a small number of mononuclear cells were routinely present in the peripheral cornea. Corneas from unimmunized mice contained vast numbers of neutrophils and very rare mononuclear cells; significant neovascularization of the corneal stroma was observable by Day 11. At Day 21 after corneal inoculation, corneas from immunized mice appeared completely normal. The corneas from unimmunized mice showed stromal infiltration by neutrophils and mononuclear cells and the tissue became highly neovascularized.

Neutralizing Antibodies to HSV-1

Immunized mice had serum neutralizing antibodies for HSV-1 at a titer of 1:50.
at Day 21 following the first subcutaneous inoculation of HSV-1. Unimmunized mice had no detectable neutralizing antibodies.

DISCUSSION

Subcutaneous immunization with HSV-1 strain MP, which lacks the major viral glycoprotein C, protected A/J mice from keratitis and encephalitis induced by corneal inoculation with strains MP, mP, F, and KOS of the virus. These results indicate that glycoprotein C does not provide antigens essential for priming for immunologic protection from destructive HSK. Furthermore, these experiments demonstrated that the MP strain is much less virulent compared to the mP strain after corneal inoculation. Only 40% of unimmunized A/J mice inoculated corneally with the MP strain (3 x 10^5 PFU) developed fatal encephalitis compared with 100% of unimmunized animals inoculated with the same amount of mP strain HSV-1.

Previous studies by Lopez (7) have shown that A/J mice are extremely susceptible to HSV-1 strain 2931, with 50% of the animals developing fatal encephalitis after an intraperitoneal inoculation with 10^1.33 PFU of the virus. Other studies using live and inactivated preparations of HSV-1 in mice have shown that subcutaneous immunization with the virus protects against subsequent challenge by either the intraperitoneal or corneal route. Tullo and coworkers (2) have shown that previous infection in the contralateral ear pinna with a virulent HSV-1 strain (SC16) protected outbred Swiss mice from keratitis and death following corneal inoculation. Previously infected Swiss mice also developed a much lower incidence of latency in the trigeminal ganglion compared to controls following corneal challenge (8). Long et al. (9) have shown that BALB/c mice immunized with a subunit vaccine consisting of purified glycoprotein D from HSV-1 are protected from a lethal encephalitis after intraperitoneal challenge with either HSV-1 or HSV 2. Mice immunized with glycoprotein D developed antibodies which were capable of neutralizing both HSV-1 and HSV-2 in vitro (9). Berman et al. (10) recently used a similar preparation of purified glycoprotein D from HSV-1 to protect guinea pigs from HSV-2 genital infection. Another subunit HSV-1 vaccine "antigenoid" AcNFU(S-)-MRC (11), which contains all of the envelope glycoproteins, has been shown to protect mice from primary lid disease and latent trigeminal ganglion infection following subcutaneous immunization with the vaccine and corneal challenge with HSV-1 strain SC16 (12).

Our histopathological studies of corneas from immunized A/J mice indicated that acute inflammatory cells (neutrophils and monocytes) were not present in the corneas following HSV-1 inoculation. In contrast, unimmunized animals had large numbers of these cells in the cornea following HSV-1 challenge. This result suggests that the cellular inflammatory response to the virus in the cornea dramatically depends upon whether or not the inoculated cornea is in an animal previously immunized systemically with HSV: and the results strongly suggest that prior immunization results in, among other things, limitation of the corneal disease perhaps through limitation of the production of neutrophil or macrophage chemotactic factors. Further immunopathologic studies in progress in our laboratory are designed to investigate the relevance of these cells and of mononuclear
cell subsets in corneal pathology development and in corneal pathology prevention.

At present, we do not know whether the immunity to HSV-1 caused by subcutaneous inoculation with an avirulent strain of the virus is mediated by cellular or humoral elements of the immune system. Immunized A/J mice in our study had an increased titer of HSV-1 neutralizing antibodies at the time of corneal inoculation. Neutralizing antibodies were not detectable in unimmunized A/J mice. It is possible that neutralizing antibodies could have inhibited the spread of HSV-1 within the cornea and brain of the immunized animals, thus limiting corneal disease and encephalitis in these animals. Davis et al. (13) have shown that passive transfer of rabbit hyperimmune anti-HSV-1 serum to Balb/C mice protects against acute encephalitis and death following corneal inoculation with HSV-1. McKendall et al. (14) have also shown that the passive transfer of hyperimmune anti-HSV-1 serum to mice protects against encephalitis following footpad challenge.

Both neutralizing and non-neutralizing antibodies directed against HSV-1 may also mediate the lysis of infected host cells via antibody-dependent cellular cytotoxicity (ADCC) (15–17). Other studies have indicated that T cells (18–21), natural killer (NK) cells (22, 23), B-cells (24, 25), and macrophages (26, 27) are important in limiting the spread of HSV-1 at the site of inoculation and within the CNS. Schrier et al. (28) demonstrated that T cells obtained from draining lymph nodes of A/J mice 4 days following subcutaneous inoculation of HSV-1 (KOS strain) were capable of transferring immunity to naive A/J mice and protected against fatal encephalitis following intraperitoneal challenge with HSV-1 (MGH-10 strain). Further studies are necessary in our system to determine which cellular and humoral factors are responsible for the protection from herpes simplex keratitis observed when mice are first exposed subcutaneously to the MP strain of HSV-1.

REFERENCES

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