Superiority of Antibody Versus Delayed Hypersensitivity in Clearance of HSV-1 From Eye

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The contribution that antibody and delayed type hypersensitivity (DTH) make in promoting HSV-1 clearance from the infected cornea was investigated. Balb/c mice were immunized intravenously or subcutaneously with an attenuated strain of HSV-1 to generate hosts which were antibody-producing DTH-tolerant or antibody-producing DTH-responsive. Anti-mu serum treated mice were likewise sensitized intravenously or subcutaneously to obtain hosts which were antibody depressed-DTH tolerant or antibody-depressed DTH-responsive. Eight days after sensitization, these four sensitized groups and unsensitized controls were infected on scarified corneas with a stromal keratitis inducing strain of HSV-1, and the extent of virus replication was determined 1, 3, and 7 days later. Very different results were obtained depending upon the host’s immune status. Virus proliferated extensively (>3-4 logs) in the eyes of nonimmune mice and antibody-depressed DTH-tolerant hosts during the first 3 days after infection. In striking contrast, HSV-1 could not be detected even 24 hr post challenge in antibody-producing DTH-tolerant mice. In fact, such mice cleared virus from the eye as efficiently as immunologically intact hosts. However, in mice with the reverse immune status, ie antibody-depressed DTH-responsive, virus growth was clearly evident (>2-3 logs) during days 1-3, and only thereafter did complete clearance occur. These results indicate that in the sensitized host antibody is both independent of and significantly more effective than DTH in promoting HSV-1 eradication from the infected eye. Invest Ophthalmol Vis Sci 28:565-570, 1987

Herpes simplex virus type 1 (HSV-1) infection is known to induce both cellular and humoral immune responses. Studies conducted in mice have indicated that transfer of immune T cells 1-6 or antibody 7-10 could set in motion events resulting in clearance of virus from infected tissues. However, the relative contribution of these two arms of the immune response in accomplishing this task is unclear. T cells are multi-functional and could exert their protective effect in a number of ways. The known possibilities include mediation of delayed type hypersensitivity (DTH), 11 lysis of virus infected cells, 12 and inhibition of virus replication by production of gamma interferon. 13,14 In addition, helper T cells can collaborate with B cells to produce antibody. 15

It is also not known how antibodies mediate recovery, but previous work has indicated that for optimal protection, the immunoglobulins must be passively transferred into recipients with a functionally intact immune system. 7,8,16 This latter observation implies that antibody collaborates with host cells, or their products, to eradicate HSV. It is important to identify the mechanism(s) most efficient at promoting rapid and complete virus clearance. Firstly, the inflammatory process with its attendant tissue damage would be minimized. 17-19 Secondly, prompt HSV clearance might prevent the establishment of the latent state, avoiding the prospect of subsequent virus reactivation with potential clinical disease. 20

Recently Nash et al 21 reported that inoculation of attenuated HSV particles intravenously would render mice unresponsive in DTH tests, although antibody production was not impaired. Mice so immunized were highly resistant to subsequent subcutaneous HSV challenge. We subsequently showed that Balb/c mice inoculated intravenously with HSV-1 were resistant to corneal challenge with sublethal or lethal doses of virus although tolerant for DTH. 22 In the present study, the capacity to selectively destroy host B cells via anti-mu serum treatment has been exploited in order to investigate the mechanisms of resistance in DTH tolerant hosts. In particular, the relative contributions of DTH and antibody in promoting HSV clearance from ocular tissue was evaluated. It was found that immune clearance was greater than 1000-fold more effective in mice which were producing antibody but were DTH tolerant, than in hosts which were DTH responsive but made little or no antibody.
Materials and Methods

Animals

Four-week-old female Balb/c mice were obtained from Cumberland View Farms, Cumberland, TN as were late term pregnant Balb/c mice. The latter served as the source for infant mice. The use of animals in this investigation conformed to the ARVO Resolution on the Use of Animals in Research.

Viruses

HSV-1 strains tsLG4 and RE were used. tsLG4 is an avirulent temperature-sensitive mutant of KOS. RE strain was isolated from a patient with stromal keratitis and will induce this disease in rabbits and mice. Both virus stocks were grown and titrated on Vero cells as previously described.

HSV Inoculations

Humoral immunity and specific DTH tolerance were simultaneously induced by a single intravenous inoculation of 0.1 ml containing $5 \times 10^7$ PFU tsLG4. Subcutaneous immunization was carried out by distributing the above inoculum among the rear footpads, tail base, and back of the neck. For corneal infection, the mice were anesthetized with 0.2 ml of a 1:10 dilution of sodium pentobarbital (50 mg per ml stock solution), and each eye was scarified by three twists of a corneal trephine. A 3µl volume containing $3 \times 10^6$ PFU RE strain was dropped onto the corneal surface and massaged into the eye with the eyelids.

Elicitation of DTH

DTH responsiveness was assessed using the ear swelling test as previously described.

Assay of Ocular Tissue For HSV-1

Eye globes were excised and placed in 1.0 ml RPMI-1640 medium with antibiotics and no serum. Preparations were frozen at $-70^\circ$C, then thawed and homogenized in Potter-Elvehjem (Wheaton Scientific, Millville, NJ) tissue grinders fitted with Teflon-coated stainless steel grinders. The homogenates were frozen and thawed a total of three times and centrifuged at 1000 xg for 10 min at 4$^\circ$C. The supernatants were then titered for infectious virus on Vero monolayers in a 48-hr plaque assay.

Anti-IgM Suppression

Newborn mice were inoculated with anti-mu serum within 24 hr of birth. 50 µl of undiluted antiserum were given intraperitoneally on Mondays and Wednesdays and 100 µl on Fridays for the duration of the experiment. The anti-mu serum was prepared in the rabbit or the goat. The former was a gift from Dr. James Rohrer and the latter was obtained from Miles Laboratories, Inc., Elkhart, Indiana (Lot G420). The effectiveness of this treatment was shown by the finding that antibody titers to HSV were decreased by at least 100-fold in comparison to the titers obtained in untreated controls.

Enzyme-linked Immunosorbent Assay (ELISA)

The serum of virus immunized mice was assayed for antibodies capable of binding to HSV-infected Vero cells but not uninfected cells. The ELISA protocol and reagents used have been previously described.

Results

Protocols Used to Develop Hosts With Defective Antibody Production and/or DTH Responsiveness to HSV Antigen

In this study we evaluated the relative contribution of antibody and DTH individually and collectively in clearing HSV-1 from ocular tissue. Accordingly, protocols which led to the development of mice with suppressed humoral or/and cellular immune responses to HSV antigens were used. To obtain antibody-depressed DTH-responsive hosts, 5-week-old mice pretreated from birth with anti-mu serum were immunized once subcutaneously with tsLG4, an attenuated strain of KOS. Antibody-producing DTH-tolerant mice were generated by the same inoculum given intravenously. Intravenous inoculation of tsLG4 into anti-mu serum treated hosts yielded mice which were suppressed with respect to both their humoral and DTH immune responses. Finally, to serve as the intact immune control, antibody-producing DTH-responsive, additional mice of the same age were immunized subcutaneously with the tsLG4 strain.

Eight days after sensitization all four immunized groups were infected with virulent HSV-1 on the corneas. Eyes were collected 1, 3, and 7 days later and individually titered for infectious virus content. At the same time the eyes were excised, a serum sample from each mouse was collected for HSV antibody testing. DTH responsiveness was assessed by inoculating the ear pinna with ultraviolet irradiated HSV antigen 24 hr before tissue collection and measuring ear swelling just before sacrifice. Using this protocol, it was possible to evaluate the immune status of each animal in each of the four sensitized groups.

Evaluation of Host Immune Status

The immune status of the individual mice within a given test group proved to be very similar. Therefore, the data were pooled. The results for each group are
summarized in Table 1. Mice immunized subcutaneously with HSV-1 made a good antibody response. All eight mice had a titer of at least 1:10,000 as assessed by the enzyme-linked immunosorbent assay (ELISA) using virus-infected cells as targets. These immunologically intact animals also made a strong ear swelling response measured 24 hr after virus antigen challenge.

Mice immunized intravenously with virus also made a good antibody response, but the DTH response was not significantly different from that of the unsensitized controls (6.3 ± 1.2). This is in accord with previous studies showing that intravenous inoculation of attenuated HSV-1 produces hosts which were making antibody to the virus although DTH tolerant.21 22

As expected animals treated with anti-mu from birth made little or no antibody following subcutaneous immunization but DTH responsiveness was unimpaired. Finally, mice given anti-mu and subsequently immunized intravenously were strongly depressed with respect to both antibody production and DTH responsiveness. This analysis established that all animals in each group had the immune status desired, and that this immune status was not altered following ocular HSV challenge.

Table 1. Evaluation of antibody and DTH status in normal and anti-mu serum treated mice following subcutaneous (SC) or intravenous (IV) immunization with attenuated HSV-1

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Route of HSV immunization</th>
<th>Number of mice with antibody titer of</th>
<th>Ear swelling*</th>
<th>Immune status</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>SC</td>
<td>&lt;10^2</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>None</td>
<td>IV</td>
<td>—</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Anti-mu</td>
<td>SC</td>
<td>6</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Anti-mu</td>
<td>IV</td>
<td>4</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>

* Mean ear swelling (10^-4 in) ±SEM. Number of mice tested is shown in parentheses. AB^+ = antibody-producing; DTH^+ = DTH-responsive; AB^0 = antibody-depressed; DTH^T = DTH-tolerant.

groups, two important observations were made. First, it is evident that antibody-producing DTH-tolerant mice were just as efficient in clearing virus as the antibody-producing DTH-responsive hosts. Second, antibody-producing DTH-tolerant animals were markedly superior to antibody-depressed DTH-responsive mice in their ability to eradicate HSV from the eye. It should be noted that the latter were inefficient rather than totally ineffective. Some virus suppression was apparent at day 3 when compared with the unimmunized control, and at day 7 virus was no longer detected. As expected, virus readily proliferated in mice which were depressed in both their humoral and cellular immune response (Day +7 not studied).

**Correlation of Virus Clearance With Ocular Pathology**

At 7 days post infection, the eyes were examined for ocular pathology. The results are summarized in Table

**Effect of Immune Status on Virus Clearance From the Eye**

As noted above, 8 days after immunization the four different groups as well as unimmunized mice were infected on both scarified corneas with 3 × 10^6 PFU of the HSV-1 stromal keratitis inducing strain RE. The virus titers in the eyes of the different immune groups and control on days 1, 3, and 7 postinfection are shown in Figure 1. Nonsensitized controls had substantial virus titers (>10^8 PFU) per eye during the first 3 days of the postinfection period. However, by day 7 the virus titer had dropped approximately 2 logs, presumably reflecting the de novo developing immune response. In contrast, antibody-producing DTH-responsive hosts promptly cleared HSV-1 from the eye. Comparing the results among the immunologically altered and control
production and/or DTH expression were constructed.

Thus, by using anti-mu treated or untreated normal mice, and giving the antigen intravenously or subcutaneously, panels of mice defected for antibody responsiveness to the mitogen lipopolysaccharide, and responding to the mitogen lipopolysaccharide, and of immunoglobulin-bearing cells, abrogate B-cell responsiveness to the mitogen lipopolysaccharide, and strongly depress antibody formation. Anti-mu treatment also is selective in that development of DTH, a T cell-mediated defense mechanism, is not significantly impaired. Thus, by using anti-mu treated or normal mice, and giving the antigen intravenously or subcutaneously, panels of mice defected for antibody production and/or DTH expression were constructed.

We investigated how these selective immune defects would affect the kinetics of virus clearance from the eye.

For this study, every animal was examined immunologically and shown to have the immune status desired. The results are summarized as follows: intravenous HSV immunization yielded antibody-producing DTH-tolerant hosts which could completely clear a 3 × 10^6 PFU virus challenge dose less than 24 hr after infection. Indeed virus eradication was as efficient as that seen in subcutaneous sensitized hosts which were antibody-producing DTH-responsive. However, HSV-1 infection of immunized but B cell suppressed animals was not followed by rapid virus clearance. Instead, at 3 days postinfection a virus titer of >2 logs was seen in subcutaneous immunized, DTH-responsive hosts and 5 logs of infectious HSV were detected in intravenous sensitized, DTH-tolerant mice. The latter observation would indicate that intravenous sensitization did not induce an antibody-independent defense mechanism, such as immune interferon or cytotoxic lymphocytes, which was solely responsible for rapid virus eradication.

Our results provide strong support for the hypothesis that to rapidly clear HSV from the immunized host participation of B cells is essential. The mechanism responsible for rapid clearance is not known. Previous passive antibody transfer studies have suggested that in addition to neutralizing extracellular virus antibody-mediated lysis of virus-infected cells is important. Immune T cells are also probably required. The importance of these latter cells has been indicated by prior observations that antibody per se was not an efficient protector in immunosuppressed hosts. How immune T cells contribute to rapid virus clearance in the antibody producing host is not known, but our results indicate that it is not via mediation of DTH.

Unlike the already immunized host, antibody made following primary infection does not appear to play a dominating role in virus elimination. Kapoor et al concluded that the kinetics of virus clearance from the ear pinna of B cell-suppressed unimmunized mice was the same as that of normal hosts. Schrier et al found that protection from acute HSV infection could be transferred with immune T cells but not serum from donors immunized 4 days previously. In addition to their slow appearance, the initial antibodies produced following primary infection are not as efficient at neutralizing virus or lysing virus-infected cells as those generated upon maturation of the immune response. These characteristics of the initial humoral immune response could explain why it may be less efficient at stopping primary HSV infection.

There is increasing evidence that the host immune response is an integral component in HSV-induced

| Table 2. Ocular pathology 7 days post HSV-1 corneal infection |
|-------------------|-------------------|-------------------|-------------------|
| **Immune status** | **Number of murine eyes with score** |
|                   | 0-+              | ++ +++++          | ++++              |
| Nonsensitized     | —                | —                | 8                |
| AB+ DTH*          | 4                | 2                | —                |
| AB+ DTH*          | 6                | —                | —                |
| AB+ DTH*          | 3                | 3                | —                |

* Abbreviations are defined in Table 1.
† Ocular pathology scoring: cornea normal or slightly cloudy (0-+); cornea partially or completely opaque (++-+++); cornea completely opaque with necrosis (+++).
stromal keratitis. T-lymphocytes have been implicated because stromal keratitis does not develop in athymic mice, but will occur in such hosts following adoptive transfer of these cells. In the rabbit ocular model, it has been suggested that antibody acting in concert with polymorphonuclear leukocytes causes an immunopathological reaction. In the present study, there was a significant incidence of clinically apparent corneal opacity in mice with intact DTH responsiveness. Conversely, all corneas of antibody-producing DTH-tolerant hosts were clear at 7 days postinfection. Our results are consistent with the concept that T cells can mediate virus clearance from the eye but in the absence of antibody, the process is inefficient, and can be accompanied by immunopathological sequelae. On the other hand, antibody mediates virus clearance very efficiently without development of ocular opacity. If, however, antibody and immune T cells are both present at the time of virus infection, some ocular pathology may occur despite rapid virus clearance.

Additional studies are needed to identify precisely how antibody promotes rapid virus clearance, and also to determine whether there are conditions in which B cells or their products mediate adverse immune reactions in the eye. Ultimately, sufficient information should be collected to permit the design of strategies suitable for optimizing protective and minimizing immunopathological responses.

Key words: herpes simplex virus, delayed-type hypersensitivity, corneal herpetic infection, antibody

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