Delayed Hypersensitivity to Herpes Simplex Virus: Murine Model

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Cell-mediated immunity has been shown to be clinically important in recovery from herpes simplex virus (HSV) infections. To investigate the role of delayed hypersensitivity (DH) in immunity and protection against HSV, we developed a murine model using the ear-swelling assay. Mice were infected subcutaneously with HSV-1 and ear-challenged, and the swelling was quantified. Significant ear swelling was detected by 3 to 4 days postinfection and peaked at 6 days. The kinetics of development of ear swelling were typical of DH: maximal swelling occurred 24 h post challenge and was diminished by 48 h, and the cellular infiltrate was predominantly mononuclear. Four-hour swelling, indicative of antibody-mediated, immediate-type hypersensitivity, was not detected until 15 days post immunization. The DH response was virus specific and could be transferred to normal recipients with lymph node T cells, but not with B cells or immune serum. This system will provide a useful model for evaluating the protective role of DH in HSV infection and for studying the specificity and interaction of T cells which mediate the response.

Exposure to a complex antigen, such as a virus, stimulates a variety of immune responses involving various subpopulations of cells. The relative effectiveness of each type of response in protecting the host from infections is dependent on the nature of the particular virus. With the increasing ability to assay separately for different immune mechanisms, it has become possible to examine which responses are triggered and which play a protective role in infection with a given pathogen.

We chose to study the delayed hypersensitivity (DH) response to herpes simplex virus (HSV) to examine the role of this response in protection from virus infection. Little is known specifically about DH to HSV, although the importance of cell-mediated immunity in host defense against HSV is well established. For example, individuals with depressed cell-mediated immunity due to immunosuppressive drugs or immunodeficiency disease often develop HSV infections, especially if they were previously susceptible to recrudescent infections (1, 13, 15, 23, 24). In general, these individuals maintain high levels of neutralizing antibody to HSV which do not seem to afford a protective effect. Similar findings have been made in experimental animals. Transfer of HSV-immune T cells prevents death in HSV-infected adult thymectomized mice (7), normal mice (3), nude mice (16), and mice treated with anti-T lymphocyte serum (18). Drugs and treatments which suppress cell-mediated immunity can increase reactivation of latent virus (19). Also, HSV-specific cytotoxic T cells (Tc) are induced in HSV-infected mice, although this generally requires manipulation of the host with drugs to eliminate suppressor cells (10, 20). Thus, although macrophages (22), natural killer cells (2), and antibody (16) have also been shown to play roles, it seems clear that T cells are important and perhaps obligatory for immune protection against HSV infections. In this report we describe the basic parameters of induction and elicitation of DH to HSV in mice. This basic system will be used in future experiments to investigate the role of DH effector cells in protection against primary and recrudescent HSV infections.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from the American Medical Center, Lakewood, Colo.

Virus. HSV-1 KOS 5 is a temperature sensitive mutant which replicates normally at 34°C but produces few if any infectious particles at higher temperatures. HSV-2 186 is a wild-type strain. Both virus strains were grown in rabbit skin cells and 3T6 cells (of BALB/c origin), using Eagle modified minimal essential medium supplemented with 1% fetal calf serum, 1% glutamine, 0.1 mM arginine, 100 U of penicillin per ml, and 1 µg of streptomycin per ml. Polyoma virus was grown in 3T6 cells.

To grow HSV, we infected cell layers three-fourths confluent for 1.5 h at 0.1 PFU/cell. Forty-eight hours later, when cells showed pathology, they were scraped.
into the media and centrifuged at 100 × g. Infected cells were suspended in serum-free media at a concentration of 2 × 10^7 cells per ml and stored at −70°C.

For use, virus preparations were thawed and sonicated for two 30-s pulses with a Branson well sonicator at setting 9. The cell debris was removed by centrifugation at 10,000 × g, and the titer of the supernatant fluid were determined by plaque assay; it was then diluted and used for immunization. For use in ear challenge, the virus, as prepared above, was UV inactivated for 10 min. This procedure, which reduced PFU by 4 logs but did not alter the ability of the preparation to elicit an immune response, altered the nonspecific toxicity of the elicitation preparation in unimmunized animals.

**Induction and elicitation of DH.** Mice were injected subcutaneously in four sites on the back (over each limb). Total dosage was 4 × 10^6 PFU/mouse in 0.4 ml, except in dose-response experiments. Six days after immunization, mice were challenged by injection of 10 μl containing 10^5 or 10^6 PFU of UV-inactivated virus into the dorsal side of each ear with a 27-gauge needle on a 1-ml tuberculin syringe. Ear swelling was measured 4 and 24 h after challenge with a Mitutoyo engineering micrometer. Experimental results are expressed either as units of ear swelling, in which case the response of unimmunized controls is shown, or as delta (Δ) ear swelling, in which the response of unimmunized controls is subtracted from the values obtained for the immunized groups.

As mentioned above, virus stocks were grown in both rabbit skin cells and 3T6. This procedure was used so that virus grown in one cell type could be used for immunization and the other could be used for challenge, thereby insuring that the immunity measured was to viral antigens and not to cellular components.

**Transfer of DH.** For transfer experiments, mice were immunized as above and the draining lymph nodes (inguinal, cervical, and brachial) were removed 4 days later. Single-cell suspensions were made by gently pushing the nodes through a 60-mesh wire screen. Cells were washed twice in Earle balanced salt solution (Colorado Serum Co., Denver, Colo.), and 50 × 10^6 lymph node cells (LNC) were injected intravenously in a volume of 0.5 ml. Recipient mice were ear challenged within 1 h of transfer, and ear swelling was measured after 24 h.

**Antiserum.** Anti-brain-associated θ serum (anti-BA-θ) was prepared in rabbits according to Golub (5). Polyvalent rabbit anti-mouse immunoglobulin serum (anti-Mlg) was prepared as previously described (25). Before use, the sera were heat inactivated at 56°C and then adsorbed. Anti-BA-θ was adsorbed with normal mouse bone marrow and erythrocytes; anti-Mlg was adsorbed with normal mouse thymocytes and erythrocytes. The cytotoxicity and specificity of both antisera have been described previously (14). Lymph node cells (10^8 per ml) were suspended in 1:10 diluted antiserum, incubated for 1 h on ice, and washed with balanced salt solution. The cells were then suspended in 1:6 diluted guinea pig serum (10^6 cells/ml) and incubated for 5 min on ice followed by 40 min at 37°C. After washing, the cells were suspended to 10^9 ml in balanced salt solution, and 0.5 ml was injected IV into each recipient.

**Nylon-wool fractionation.** Enrichment of lymph node T cells by passage through nylon wool was done using the method of Julius et al. (8). Nonadherent cells were washed, suspended at 10^9/ml, and 0.5 ml was injected IV into each recipient.

**RESULTS**

**Dose-response for immunization and challenge.** To determine the optimal HSV dose for sensitization, groups of mice were immunized with a range of virus concentrations and ear challenged 6 days later. The results in Fig. 1 show that for immunization doses of 4 × 10^5 to 4 × 10^6 PFU/mouse, the response was statistically on a plateau. When the immunization dose was lower than 4 × 10^5, the response dropped rapidly. A total of 4 × 10^3 PFU was used for immunization in all succeeding experiments.

The optimal challenge dose was determined by varying the number of PFU injected into the ears of mice immunized 6 days previously with 4 × 10^6 PFU of HSV. Table 1 shows a dose-dependent increase in the ear swelling response to 10^6 PFU per ear, the maximum dose tested. In all subsequent experiments, 10^5 to 10^6 PFU per ear was used to elicit the DH response.

Histology of the mouse ears was also examined (data not shown). Ears from HSV-infected and control mice, 24 h after ear challenge, were fixed in buffered Formalin, sectioned, stained with hematoxylin-eosin, and examined under oil emersion at ×100. Ears from the HSV-immune mice showed a uniform mononuclear cell infiltration characteristic of DH responses. In con-
TABLE 1. Dose-response for ear challenge with HSV-1

<table>
<thead>
<tr>
<th>HSV-1 challenge dose (PFU/ear)</th>
<th>Ear swelling ± SE (immunized)</th>
<th>Ear swelling ± SE (unimmunized control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>101.9 ± 2.9</td>
<td>9.2 ± 3.4</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>70.9 ± 1.6</td>
<td>1.5 ± 2.4</td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>32.2 ± 2.2</td>
<td>6.1 ± 3.4</td>
</tr>
<tr>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>12.2 ± 1.1</td>
<td>3.7 ± 3.3</td>
</tr>
</tbody>
</table>

a Mice were immunized with 4 × 10<sup>6</sup> PFU of HSV-1 on day 0 and ear challenged with increasing doses of (UV-inactivated) HSV 6 days later. Ear swelling was measured 24 h after challenge.

Ear swelling was measured in units of 10<sup>-4</sup> in (2.54 × 10<sup>-4</sup> cm).

trast, ears from control mice had a very localized infiltrate which consisted almost entirely of polymorphonuclear cells.

Development of DH to HSV. To determine the kinetics of development of DH to HSV, mice were immunized on day 0, and individual groups were challenged on days 4, 6, 9, 12, and 15. Ear measurements were made at 4 and 24 h after challenge. Figure 2 demonstrates that the 24-h ear-swelling response was significant by day 4 (and on day 3, data not shown), peaked on day 6, and remained high at least through day 15. The 4-h ear-swelling response was not significant until 15 days post immunization. Additional studies (data not shown) indicate that HSV-immunized mice showed significant DH 3 months post immunization. Thus, immunity to HSV, as measured by DH, was long-lasting despite the fact that the temperature-sensitive mutant virus used to immunize would not be expected to replicate in the host.

Specificity of the DH response in mice immunized with HSV-1. To examine the specificity of the DH response, HSV-1-immunized mice were ear challenged with HSV-1, HSV-2, and polyoma virus. As can be seen in Fig. 3, mice immunized with HSV-1 showed no response to polyoma, a totally unrelated virus. However, HSV-1-immunized mice did show a significant ear-swelling response to HSV-2 challenge. HSV-1 and HSV-2 have been shown to have proteins in common (21).

Transfer of DH to HSV and characterization of the effector cells. To study the cell type involved in DH to HSV, immune LNC were fractionated or antisera and complement treated and transferred to naive recipients, which were then ear challenged. Donors of immune LNC were immunized 4 days before transfer. Figure 4 shows that immune LNC treated with either normal rabbit serum or anti-Mlg plus complement were able to transfer the DH response to HSV. However, anti-BA-Ø treatment of immune LNC destroyed their ability to transfer immunity. Immune serum taken from the LNC donors was able to transfer the immune response. These results indicate that immune T cells, but not B cells or their products, were able to transfer DH to naive recipients.

To demonstrate that the DH response to HSV is T cell mediated, as well as T cell dependent, immune LNC were fractionated on a nylon-wool column, and nonadherent cells were injected into normal recipients. Figure 4 shows that nylon-wool-enriched T cells transferred DH to HSV. Additional experiments (data not shown) were carried out to see whether the DH response to HSV could be induced in nude mice. No response was observed with nude mice although their litter mates displayed significant DH to HSV.

DISCUSSION

The experiments presented in this report describe a classical DH response to herpes simplex virus in mice. A nonreplicating form of the virus (HSV-1 KOS 5) was used to characterize DH in primary, acute infection without the toxic effects of disease. However, additional experiments have shown that immunization with wild-type virus (HSV-1 KOS) produces similar results. A strong response to HSV was rapidly induced (by 3 to 4 days post immunization) and was measu-
able 24 hr, but not 4 hr, after ear challenge. Although the antibody-mediated (4-h) response was not apparent during the 6-day interval used in the assay, it was significant at 15 days. The DH response was shown to be mediated by T cells since anti-MIg-plus-complement-treated or nylon-wool nonadherent immune LNC could transfer DH to HSV, although anti-BA-0-treated cells or immune serum could not transfer immunity.

Examination of the specificity of the response demonstrated that DH to HSV-1 was virus specific (no response to polyoma virus was seen in HSV-1-immunized mice), but not type specific, since mice immunized with HSV-1 responded significantly to challenge with HSV-2. The reciprocal experiment showed similar results, i.e., mice immunized with HSV-2 responded strongly to challenge with HSV-1 (data not shown). This reactivity probably reflects the considerable number of determinants shared by HSV-1 and HSV-2. These strains share 50% DNA homology (9), antibody to several of the glycoproteins of HSV-1 and HSV-2 cross-react (6, 21), and peptide mapping has recently shown similar amino acid sequences in a corresponding HSV-1 and HSV-2 glycoprotein (G. Cohen, personal communication). However, in spite of the biochemical similarities between HSV-1 and HSV-2 and in contrast to antibody and DH cross-reactivity it has been reported that HSV-specific Tc are able to distinguish between HSV-1- and HSV-2-infected target cells (20). It is not known whether this difference in specificity is due to differences in virus preparations or strains or in vivo versus in vitro assays or whether the T-cell-mediating DH and antibodies recognize type-common determinants, whereas Tc detect unshared determinants on HSV-1 and HSV-2. Currently, little is known concerning which viral determinants are recognized in the DH response. Since HSV is a complex virus, express-

![Graph](image1.png)

**FIG. 3.** Antigen specificity of response induced with HSV-1. Mice were immunized with 4 x 10⁶ PFU of HSV-1 on day 0 and challenged on day 6 with 10⁵ PFU of (UV-inactivated) HSV-1, HSV-2, or polyoma virus. Ear swelling was measured 24 hr after challenge.

![Graph](image2.png)

**FIG. 4.** Ability of different cell populations to transfer DH to HSV. Mice were immunized with 4 x 10⁶ PFU of HSV-1, and the draining lymph nodes were removed 4 days later. In experiment I, the immune LNC were treated with normal rabbit serum (NRS) plus complement (C'), anti-MIg plus complement, or anti-BA-0 + C' and transferred into normal recipients. A 0.4-ml amount of immune serum was also transferred. In experiment II, the immune LNC were either passed over a nylon-wool column and the nonadherent fraction was collected, or they were left unfractionated and injected into normal recipients. In both experiments, recipients were ear challenged with 10⁴ (UV-inactivated) PFU of HSV within 1 hr of transfer. Ear swelling was measured 24 hr after challenge.
ing approximately 10 virus-specific proteins on the surface of infected cells (4), it is possible that immune recognition involves multiple proteins. On the other hand, one or two membrane proteins may induce DH. Studies of specificity of DH to other viruses, such as influenza and reovirus, give mixed results. Although DH to reovirus is serotype specific (26), DH to influenza virus appears to be highly cross-reactive among serotypes. Liew et al. (12), immunizing with a detergent extract of type A influenza, and Leung and Ada (11), infecting with type A influenza virus, both report that DH is cross-reactive for all A strain influenza.

Protection against HSV infections (either latent or primary) has long been considered to be primarily dependent on cellular mechanisms, although there is evidence that macrophages and natural killer cells may be important, especially in primary infections (2, 22). Until recently, T cell subpopulations have not been examined separately. One report (7) showed that transfer of HSV-immune cells that shared K/D determinants of the major histocompatibility complex with the recipient strain gave transient protection from HSV infection, whereas immune cells sharing I region determinants with the recipient transferred long-term protection. H-2-incompatible cells gave no protection. Other reports (20) have shown that Tc to HSV are K/D restricted. Attempts to induce Tc to HSV have resulted in low levels of response, and development of significant Tc activity requires cyclophosphamide pretreatment of extended in vitro culture of the Tc before the assay (10, 20). This difficulty in detecting Tc to HSV brings into question their in vivo significance in immunopotentialization. In contrast to the difficulty found in demonstrating Tc to HSV, DH was readily induced. Recently, Nash et al. (17), also describing a DH response to HSV, found that transfer of HSV-immune T cells into normal recipients was able to reduce the local virus titer when virus was injected, indicating that T cells are capable of acting against HSV in vivo. The model system described here will be used to examine cell-mediated immunity to HSV, as measured by DH and to determine whether there is a correlation between the responsive state and protection against acute infection or establishment of latency by HSV.

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LITERATURE CITED


