Resolution of HSV Corneal Infection in the Absence of Delayed-Type Hypersensitivity

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The role of delayed-type hypersensitivity (DTH) in the resolution of herpes simplex virus type 1 (HSV-1) ocular infection was examined. Infection of Balb/c mice on the scarified cornea with HSV-1 resulted in sensitization for DTH. This response, demonstrable by swelling of the ear following inoculation with ultraviolet-irradiated virus, was optimal 7 days postinfection. The reaction was immunologically specific and characterized histologically by a predominately mononuclear cell infiltrate. DTH responsiveness could be completely abrogated if the mice were inoculated intravenously with an attenuated strain of HSV-1 7 days before corneal infection. DTH-unresponsive mice were, nevertheless, resistant to corneal challenge with sublethal or lethal doses of HSV-1. Resistance was accompanied by a >30-fold reduction in infectious virus in the eye 24 hr post challenge. A cellular infiltrate characteristic of a DTH response was not observed within the cornea during virus clearance. Tolerance was restricted to DTH, as antibodies to HSV antigens could be readily demonstrated 6–7 days after intravenous virus immunization. These antibodies may have contributed to the resistance observed. The results establish that neither a systemic nor local DTH response is required by the host to resist HSV-1 ocular infection. Invest Ophthalmol Vis Sci 26:1509–1515, 1985

Recently, it has been shown that inoculation of attenuated HSV particles intravenously (i.v.) will render mice DTH unresponsive.14,15 The unresponsiveness was restricted to DTH as other immune parameters appeared normal. DTH tolerance was immunologically specific, and appeared to be mediated at least in part by virus-specific suppressor T cells.15,16

In the present report, we analyze the role DTH plays in controlling HSV ocular infection in a mouse model. In this model, virus applied to the scarified cornea replicates in ocular tissue, then spreads to the trigeminal ganglion and subsequently to the brain where a fatal encephalitis may ensue. We show that a classical “tuberculin” DTH response is induced following HSV-1 infection of the cornea. What effect abrogation of this response had on host recovery from ocular HSV-1 infection was investigated. It was found that an intact DTH response was not essential for virus clearance from ocular tissue.

Materials and Methods

Animals

Female Balb/c mice were obtained from Cumberland View Farms, Cumberland, Tennessee. The use of animals in this investigation conformed to the ARVO Resolution on the Use of Animals in Research.

Viruses

HSV-1 strains KOS, 35, RE, and ts LG4 were used. ts LG4 is a temperature-sensitive mutant of KOS17 and
was a gift provided by Dr. Rozanne M. Sandri-Goldin. RE strain was kindly provided by Dr. Ysolina Centi-fanto-Fitzgerald. Vaccinia virus was used for specificity studies. All virus stocks were grown in Vero cells maintained on Dulbecco’s medium supplemented with 5% newborn calf serum, 7.5% sodium bicarbonate and antibiotics. When gross cytopathic effects were evident, the infected cells were scraped into the medium and centrifuged to collect the cells. Infected cell pellets were washed one time in 10 ml serum-free RPMI-1640. The cells were resuspended in serum-free RPMI-1640 medium, usually at the equivalent of 1 ml medium per cells from a 32-oz culture flask, and frozen and thawed three times. Cell debris was removed from the virus preparations by centrifugation for 30 min at 2000 rpm in a Beckman TJ-6 centrifuge. The supernatant was aliquoted and stored at -70°C. Virus infectivity recorded as plaque-forming units (PFU) per ml was determined using Vero monolayers and a 48-hour plaque assay.

Induction and Elicitation of DTH

Balb/c mice were sensitized via corneal or subcutaneous inoculation of HSV-1 strain KOS or RE. For the former route, mice were anesthetized with 0.1–0.2 ml of 1:10 dilution of sodium pentobarbital (50 mg per ml stock solution), and the right eye was scarified by three twists of a 2-mm corneal trephine. A 3–10 μl-volume of the desired virus concentration was dropped onto the corneal surface and massaged into the eye with the eyelids. For subcutaneous inoculation, 0.1 ml of virus was distributed among the rear footpads and the base of the tail.

DTH responsiveness was determined using the ear swelling assay. The HSV-1 test antigen was the KOS strain diluted 1:4 in serum-free RPMI-1640 medium. The virus preparation was exposed to ultra-violet (UV) irradiation for 10 min. This reduced the infectivity of the preparation from 10⁵ PFU/10 μl to ≤10⁵ PFU/10 μl. To test for DTH responsiveness at the desired time after sensitization, 10 μl of virus antigen was inoculated into the dorsal side of the mouse right ear using a 50 μl syringe and a 30-gauge needle. The left ear (control) received 10 μl RPMI-1640 with 1.0% newborn calf serum. Ear swelling was measured (usually 24 h post-infection) using a Mitutoyo 7326 micrometer (Schleisinger Tools; Brooklyn, NY). Results are expressed as ear swelling of right (antigen) ear minus ear swelling of left (control) ear in units of 10⁻⁴ inches. Data were analyzed using Student’s t-test.

Induction of DTH Tolerance

Specific DTH tolerance was induced by intravenous inoculation (0.1 ml) of avirulent HSV-1 strains ts LG4 or 35. Neither strain at high dose (10⁹ PFU/ml) produced any signs of overt disease when given intravenously.

Measurement of Neutralizing Antibody

Equal volumes of plasma diluted as desired were added to tubes containing 0.2 ml of a HSV-1 suspension (3 × 10⁵ PFU/ml). These virus-plasma mixtures were incubated for 30 min at 37°C, then 0.1 ml aliquots were placed in 25 cm² plastic flasks (done in duplicate) containing Vero monolayers. Controls received virus only. After adsorption for 1 hr at room temperature, the monolayers were overlaid with 4 ml of 0.5% methylcellulose solution and incubated for 2 days at 37°C in a 5% CO₂ atmosphere. Plaques were then counted after staining with 0.1% crystal violet. The neutralizing titer was taken as that dilution of plasma that reduced the mean PFU by 50% in comparison with the controls.

Enzyme-linked Immunosorbent Assay (ELISA)

The plasma of virus-immunized mice was assayed for antibodies capable of binding to HSV-infected Vero cells but not uninfected cells. The protocol has been previously described.

Assay of Tissues for HSV-1

Eye globes, trigeminal ganglia and brains were dissected out of animals and homogenized in Ten Broeck homogenizer (Bello, Vineland, NH) containing minimal essential medium with 5% newborn calf serum to give a 10% suspension. The tissues were then frozen and thawed three times and centrifuged at 1000 × g for 10 min at 4°C. The supernatants were then assayed for virus content by plaque titration on monolayers of Vero cells.

Histological Procedures

Corneas were dissected free from enucleated eyes and fixed by immersion in 10% buffered neutral formalin for 24 hr. Likewise, following micrometry, the external ears (pinna) were removed and immersed in the same fixative. Following fixation, the tissues were washed overnight, dehydrated through a graded series of alcohols to 95%, and embedded in glycol methacrylate (Polysciences, Inc.; Warrington, PA). Sections were cut at 2 μm and stained with Lee’s stain (1% basic fuchsin, 1% methylene blue). Two or three tissue samples were examined per time point.

Results

Characterization of the DTH Response Following Ocular HSV Infection

We initially investigated whether Balb/c mice infected on the scarified cornea with 10⁶–10⁷ PFU/ml...
KOS strain would develop an inflammatory response when challenged in the ear with UV-irradiated virus 5 days later. Preliminary studies indicated that at 24–48 hr post challenge, ear swelling in the test ear was substantially greater than that of the control ear. A series of experiments was then conducted to characterize the response. To analyze the immunological specificity of the ear swelling response an unrelated antigen, vaccinia virus, was prepared in a manner identical to that of HSV-1, i.e. grown on Vero cells using Dulbecco’s medium supplemented with 5% newborn calf serum. It was found that mice infected ocularly with HSV-1 produced a significantly stronger DTH response ($P < 0.02$) to UV-irradiated homologous antigen challenge than to vaccinia virus antigen inoculation (Table 1). Conversely, the UV-irradiated vaccinia antigen elicited a significantly greater response ($P < 0.002$) in vaccinia-sensitized hosts than in HSV-1 infected mice. Thus, the ear swelling response was specific for the sensitizing antigen.

The kinetics of DTH induction were determined. Mice infected on the cornea on day 0 were ear tested 3, 5, 7, or 25 days later. Figure 1 shows that a modest response could be detected in animals challenged just 3 days after sensitization. Optimal responsiveness occurred at day 7 and sensitization persisted at least 25 days. In additional studies, the kinetics of DTH elicitation was examined. Ear swelling was optimal 24–48 hours after challenge. The histology of the ear reaction in ocularly sensitized mice at 48 hr is shown in Figure 2. A mononuclear cell infiltrate, similar to that of mice immunized subcutaneously, and typical of that seen in DTH reactions to HSV-1 antigen was observed. Obvious mononuclear cell infiltrates were not seen in the HSV-1 antigen challenged control (unimmunized) mice.

### Table 1. Immunological specificity of the ear swelling response

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>HSV-1</th>
<th>Vaccinia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>29.6 ± 5.2$^+$</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>10.0 ± 4.7</td>
<td>19.3 ± 2.7</td>
</tr>
<tr>
<td>None</td>
<td>2.3 ± 1.9</td>
<td>6.7 ± 3.3</td>
</tr>
</tbody>
</table>

*$^+$ Mice were immunized on the cornea with $0.5 - 1 \times 10^7$ PFU/ml of HSV-1 (KOS) or vaccinia on day 0 and challenged on day + 6 with UV-irradiated HSV-1 or vaccinia antigen. Ear swelling was measured 24 hr after challenge.

**Effect of DTH Tolerance on Recovery from HSV-1 Ocular Infection**

Preliminary studies showed that mice sensitized subcutaneously and positive for DTH and antibody will resist HSV corneal infection. We then proceeded...
to determine what effect DTH tolerance induction had on host capacity to resist ocular HSV-1 infection. It was observed that 7 days after sublethal KOS corneal infection, none of the 6 DTH-unresponsive mice exhibited ocular pathology (Table 4, Experiment #1). The cornea and pupil were normal in appearance and remained so thereafter. In marked contrast, mice challenged on the cornea with HSV-1, but otherwise untreated, showed obvious ocular pathology 7 days post-infection. The infected eye of each mouse was swollen shut. Gross inspection revealed corneal opacity, no visible pupil, and an accompanying heavy extraocular exudate.

In a second experiment, mice were infected ocularly with varying doses of strain RE, a stromal disease inducing virus. The DTH tolerant mice proved to be resistant even to the \(10^6\) PFU challenge dose (Table 4, Experiment #2).

In a third experiment, the resistance of animals pretreated with virus i.v. to a lethal dose of KOS was assessed. It was found that all DTH unresponsive mice had a normal appearing eye 7 days post challenge, and all remained healthy (Table 4, Experiment 3). Conversely, the control mice experienced severe ocular pathology which progressed to encephalitis. By 8 days postinfection, all controls were moribund. These results established that systemic DTH responsiveness is not essential for recovery from ocular HSV-1 infection.

**Virus Titers in Tissues of DTH-Unresponsive Mice**

The absence of overt ocular or neurological disease in the DTH-unresponsive mice was striking. We determined whether virus replication and spread was inhibited. Mice pretreated 7 days before with LG4 i.v. (\(3 \times 10^8\) PFU/ml) and untreated controls were infected on the scarified cornea with KOS (\(1 \times 10^7\) PFU/ml). At intervals thereafter, eyes, trigeminal ganglia, and brains were removed and examined for infectious virus content. The eyes of control mice at 24 hr postinfection

**Table 2. Effect of intravenous HSV-1 pretreatment on development of DTH sensitization following corneal infection**

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>Tolerogen i.v.</th>
<th>Corneal infection</th>
<th>Ear swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>KOS ((10^5))</td>
<td>30.0 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>35 ((1 \times 10^5))</td>
<td>KOS ((10^5))</td>
<td>5.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>LG4 ((7 \times 10^6))</td>
<td>KOS ((10^5))</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>RE ((10^6))</td>
<td>30.8 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>LG4 ((7 \times 10^6))</td>
<td>RE ((10^6))</td>
<td>3.3 ± 3.3</td>
</tr>
</tbody>
</table>

*See text for details.

†Mean of 3 mice per group.
had a high titer of HSV-1, and by 48 hr virus had spread to the trigeminal ganglion and brain where subsequently high levels of virus were synthesized (Table 5). In the eyes of i.v. sensitized mice, only very low virus titers were observed during the first 4 days, and none was found in samples taken 6, 8 or 10 days after infection (data for latter time points not shown). At no time during the 10-day sampling period was virus detected in trigeminal ganglia or brain tissue. In a second experiment, the virus titer at 24 hr was >30-fold lower than that of the control. These results indicated that within 24 hr after infection, virus replication in the eyes of DTH-tolerized mice was markedly inhibited and spread of virus to nervous tissue was prevented.

Absence of Corneal Cellular Infiltrate in DTH Tolerant Mice

Although a systemic DTH response was absent, it was still possible that a mononuclear cell infiltrate was occurring in the cornea and contributing to virus clearance. Therefore, corneas from tolerant mice were removed at 7 hr and 24 hr postinfection and examined for cellular infiltrates. Histological examination of the limbus and the area surrounding the virus infection site revealed some infiltration of polymorphonuclear neutrophils. However, no classical mononuclear infiltrate was observed at either time period in any of six eyes examined.

HSV-1 Antibody Titers in DTH Tolerant Mice

It has been reported that mice given HSV i.v. and rendered DTH tolerant are not tolerized at the level of the humoral immune response. 14,15 We tested the plasma of mice 6–7 days after virus inoculation i.v. for neutralizing antibody to HSV. Nine of 11 samples analyzed were positive, with titers ranging from 1:4 to 1:64. Preparations negative in the neutralization assay were positive when tested for their capacity to bind to virus-infected cells in the ELISA. It seems likely that the plasma antibodies contributed to the rapid clearance of virus from the eye, presumably via collaboration with accessory cells.

Discussion

Although generation of DTH to HSV-1 has been demonstrated in several species including humans, 22 guinea pigs, 20 and mice, 23 the role DTH plays in controlling virus infection in vivo has not been established. In this study we first showed that infection of the mouse cornea with HSV-1 would result in sensitization for DTH. In fact the kinetics, intensity, and histology of the response were essentially identical to that described for mice infected subcutaneously. 23 Furthermore, the DTH sensitization could be completely blocked by pretreatment of mice i.v. with attenuated HSV-1.

We have found that mice which were documented to be DTH-tolerant were nevertheless completely resistant to ocular challenge with a lethal dose of HSV-1. Virus titers in the eyes of such animals were reduced by 97–99% within 24 hr postinfection when compared with controls. Moreover, spread of virus into the central nervous system was inhibited. Histologically, studies

Table 3. Specificity of DTH tolerance induced by intravenous HSV-1 inoculation*

<table>
<thead>
<tr>
<th>HSV-1 tolerogen</th>
<th>Sensitizing virus</th>
<th>DTH test antigen</th>
<th>Ear swelling†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 i.v.</td>
<td>HSV-1 s.c.</td>
<td>UV-HSV-1</td>
<td>27.5 ± 2.4</td>
</tr>
<tr>
<td>None</td>
<td>HSV-1 s.c.</td>
<td>UV-HSV-1</td>
<td>46.8 ± 8.1</td>
</tr>
<tr>
<td>HSV-1 i.v.</td>
<td>Vaccinia s.c.</td>
<td>UV-Vaccinia</td>
<td>56.8 ± 7.4</td>
</tr>
<tr>
<td>None</td>
<td>Vaccinia s.c.</td>
<td>UV-Vaccinia</td>
<td>53.7 ± 1.9</td>
</tr>
</tbody>
</table>

* Mice were tolerized with 7 X 10⁸ PFU/ml strain LG4 given i.v. on day 0. Six weeks later the animals were immunized subcutaneously with 1 X 10⁸ PFU/ml HSV-1 strain RE or 4.5 X 10⁷ PFU/ml vaccinia and tested for DTH 7 days later.
† Ear swelling (10⁻⁴ in) ± SEM. Mean of 4 mice per group.

Table 4. Effect of HSV-1 challenge in cornea of mice which are DTH tolerant*

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>Infecting HSV-1 strain</th>
<th>Challenge inoculum (PFU/eye)</th>
<th>DTH tolerant</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KOS</td>
<td>10⁹</td>
<td>0/6</td>
<td>2/3</td>
</tr>
<tr>
<td>2</td>
<td>RE</td>
<td>10⁸</td>
<td>0/10</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁷</td>
<td>0/10</td>
<td>5/8</td>
</tr>
<tr>
<td>3</td>
<td>KOS</td>
<td>10⁴</td>
<td>0/9</td>
<td>8/8</td>
</tr>
</tbody>
</table>

* Mice were tolerized with HSV-1 LG4 intravenously as described in Table 2. Seven days later tolerized mice and their normal counterpart were challenged on the scarified cornea with HSV-1. The incidence of ocular pathology was that observed 7–8 days after challenge.

Table 5. HSV-1 titers in tissues of mice challenged on the cornea 7 days after virus inoculation i.v.

<table>
<thead>
<tr>
<th>Hours post challenge</th>
<th>Host status</th>
<th>Virus titer in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eye</td>
<td>Trigeminal ganglion</td>
</tr>
<tr>
<td>24</td>
<td>HSV i.v.</td>
<td>40*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.5 X 10⁴</td>
</tr>
<tr>
<td>48</td>
<td>HSV i.v.</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8.2 X 10⁴</td>
</tr>
<tr>
<td>96</td>
<td>HSV i.v.</td>
<td>90*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4.2 X 10⁴</td>
</tr>
</tbody>
</table>

* Values represent mean PFU/ml of tissue samples from 2 mice per group per time period.
of the cornea at the time of virus clearance revealed no mononuclear cell infiltrate. Therefore, we conclude that neither systemic nor local DTH is required for host resistance against ocular HSV-1 infection.

Clearly, i.v. inoculation induced a protective response as well as DTH tolerance. What mechanism(s) might be responsible for stopping HSV infection? It was found that neutralizing and/or infected cell-specific antibodies were present in the plasma of all DTH-tolerant mice examined at the site of ocular challenge. Since trephining elicited neovascularization in the cornea, such antibody could reach the site of infection and account for the rapid reduction of virus in the eye. This explanation is supported by earlier studies which showed that passive transfer of antibody promoted recovery from ocular HSV-1 infection. In addition to neutralizing extracellular virus, antibody might exert its protective effect via antibody-dependent cellular cytotoxicity with infiltrating polymorphonuclear neutrophils or some other cell type functioning as effector cells. Also, recent studies have indicated that antibody binding to virus-infected mouse trigeminal ganglion cells can suppress production of infectious HSV progeny.

In addition to antibody, antigen-responsive T cells have been reported to be present in the draining lymph nodes of DTH-tolerant mice. At least some of these cells have been shown to possess specific cytotoxic activity following in vitro culture. Although no obvious mononuclear infiltrate was seen in the cornea at the time of rapid virus clearance, T cells may have contributed to host resistance, perhaps via their cytotoxic action and/or by release of gamma interferon. An investigation of these possibilities is in progress.

Metcalf et al. studied the inflammatory response in HSV corneal infection in athymic nude and heterozygous Balb/c mice. They found that necrotizing keratitis developed only in those hosts which had an intact T cell response. Thus, it could be argued that in our model, acute ocular inflammation and opacity were absent because DTH was absent. This argument, however, does not account for the rapid drop in virus titer in ocular tissue. Thus, our results reflect immunity and not simply virus replication without inflammation.

It should be emphasized that our observations do not rule out the possibility that DTH may be important in limiting HSV infection under different experimental conditions, i.e., in mice lacking antibody, or following infection of other tissues. In this regard Nash et al. initially reported that immune lymph node T cell transfer of antiviral immunity was correlated with transfer of DTH sensitization. Then it was discovered that enhanced virus clearance also occurred in DTH tolerant mice. However, in a subsequent study, adoptive transfer of lymph node cells from DTH-tolerant donors did not enhance clearance of HSV-1 although transfer of cells from mice able to generate DTH did so. Thus, the role of DTH in controlling subcutaneous infection remains unclear.

Continued experimentation using the ocular disease model should provide greater insight into how HSV infections are controlled in vivo. The results of our study do suggest that by appropriate manipulation of the immune response, it will be possible to minimize hypersensitivity responses potentially detrimental to corneal tissue while at the same time inducing protective immunity.

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

References