Induction of cell-mediated immunity in herpes simplex virus keratitis

Kinetics of lymphocyte transformation and the effect of antiviral antibody

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The in vitro lymphoproliferative responses to herpes simplex virus (HSV) antigens were followed in rabbits with herpesvirus infection of the cornea. The proliferative cellular responses occurred early in infection and were demonstrated by lymphoid cells from the local lymph nodes at day 5, the peripheral blood at day 11, and the spleen after day 14. The presence of autologous serum antibodies suppressed lymphoproliferative responses of the lymph node lymphocytes to HSV antigens early in infection at day 5, the time at which antibody production is first noted. Peripheral blood and spleen cells were not appreciably influenced at early time periods. However, at 7 months after infection, the presence of autologous serum antibodies stimulated spleen lymphocytes from animals with recurrent disease. These results indicate that antiviral antibodies can affect the cellular immune response in herpesvirus infections by modulating the lymphoproliferative response to herpes antigens.

Key words: cell-mediated immunity, herpes simplex keratitis, lymphocytes, antibody

Recent evidence indicates that cell-mediated immunity (CMI) is an important defense mechanism in viral infections. The fact that recurrent herpes infections are common in the presence of antibody lends support for the primary role of CMI in modifying both the frequency and severity of recurrent infections. Herpesvirus primary infection is usually a self-limited disease. Persistence of the virus is lifelong, but disease is inapparent except when localized reactivation occurs. Reactivated infections can manifest themselves by the reappearance of infectious virus at the site of the original infection. Both the humoral and cellular immune responses have been documented in the acute as well as recurrent infections. However, data on the sequential evaluation of the immune response over the course of herpesvirus disease are lacking. Consequently, there has been no clear resolution of the question whether an immunologic defect in a cellular or humoral immune response is responsible for the increased incidence of severity for a recurrent infection or, conversely, whether heightened immune response is necessary for resistance to recurrent infection.

In this report we studied the pathogenesis and host-virus relationship to herpes simplex...
virus (HSV) infection of the rabbit cornea. The clinical disease in this model parallels the sequence of disease in human herpesvirus keratitis. We studied the sequential immunologic events transpiring in acute herpesvirus keratitis, recovery from keratitis, and recurrent infection in a limited number of animals by determining the lymphoproliferative response to HSV antigens and the development of antiviral antibody as well as its effects on the lymphoproliferative response.

Materials and methods

**Animals.** New Zealand male rabbits, 2.0 to 2.5 kg, were used for this study. Preinfection serum samples were obtained from arterial bleedings.

**Virus.** HSV type 1, HF strain, was propagated in Statens Serum Institut rabbit cornea cells (RCC) (American Type Culture Collection, Rockville, Md.). RCC were grown in Earle’s balanced salt solution supplemented with 0.17% lactalbumin hydrolysate, 0.05% yeast extract, penicillin (250 μg/ml), streptomycin (250 μg/ml), and 10% fetal calf serum.

**Infection.** Ocular infections were induced by instilling 3 × 10^4 pfu of virus in a volume of 0.05 ml onto both corneas abraded with sterile Q-Tips. Three to six animals were sacrificed at each time point assayed, i.e., days 5, 7, 9, 11, and 14 as well as 7 months after infection. All clinical evaluations were carried out by gross inspection and biomicroscopic examination. Each eye was examined daily, and corneas were stained with fluorescein to observe the epithelial lesions. The presence of conjunctival inflammation, extent and intensity of epithelial disease, and extent and intensity of stromal disease were scored separately and graded on a scale from 0 to 4 as modified from Smolin and Okumoto.8

**Virus isolation.** Specimens for virus isolation were obtained by very gently rolling a sterile, dry cotton-tipped applicator over the cornea, inferior fornix, and conjunctival surfaces. The corneal swabs were inoculated in bottles with monolayers of rabbit kidney cells and examined for cytopathic effects for up to 2 weeks.

**Virus antigens.** Antigens were prepared from HSV type 1, HF strain, propagated in primary rabbit kidney (PRK) cells in order to avoid detection of hypersensitivity responses to the cell line and serum component used in the initial infection. PRK cells were grown in Eagle’s minimal essential medium (MEM) (Flow Labs, Rockville, Md.) supplemented with 10% NCTC-135 medium, penicillin (250 μg/ml), streptomycin (250 μg/ml), neomycin (250 μg/ml), mycostatin (500 μg/ml), and 10% normal rabbit serum. A modification of the method of Rosenthal et al.9 was employed to obtain virus antigen preparations. Confluent PRK monolayers in 32 oz bottles were infected with virus for 1 hr at 37°C in a humidified 5% CO₂ atmosphere. The medium was then replaced, and the cultures incubated for 24 hr. After this time, the culture medium was discarded, monolayers were washed twice with 50 ml of sterile phosphate-buffered saline (PBS), and a final volume of 2 ml of sterile PBS was added. The monolayers were frozen and thawed three times to liberate intracellular virus. The cell-PBS suspension was then centrifuged at 3000 × g for 15 min to remove the debris. The resultant supernatant was used as HSV antigen. Stock antigen preparations were then inactivated by exposure to ultraviolet irradiation (General Electric Germicidal lamp, G 15t8, 15-watt) until no infectious virus (usually after 5 min at a distance of 10 cm) was detectable by plaque formation on RCC. The titer of the virus before inactivation was 3 × 10^8 pfu/ml. Control antigen was prepared similarly but without infection of the cells. Both antigens were stored at −70°C until use. The antigens were used at a 1:10 dilution, which was determined to be optimal for in vitro tests with cells from hyperimmune animals.

**Cell suspensions.** Animals were sacrificed under chlorpromazine (Thorazine; SmithKline Corp., Philadelphia, Pa.) sedation (12.5 mg/kg) by exsanguination via cardiac puncture. Twenty milliliter aliquots of blood were retained for serum, the remainder being heparinized at 100 U of heparin per milliliter of blood. Spleen and preauricular (draining) lymph nodes were removed and stored in RPMI 1640 (Microbiological Associates, Bethesda, Md.) without serum in an ice bath until the cell suspensions were prepared. Peripheral blood lymphocytes (PBL) were separated on Ficoll-Hypaque. The blood was centrifuged, the buffy coat was removed, and 0.4 ml was layered on 0.4 ml of Ficoll-Hypaque solution cushions in a Fisher microcentrifuge tube and centrifuged 4 min at 1500 × g at room temperature.10 Lymphocytes at the interface were recovered and washed three times with RPMI medium without serum. Spleens were minced, suspended in RPMI, filtered through cotton gauze, and centrifuged at

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Fig. 1. Relationship between cell-mediated immune response to HSV antigens expressed as SI (••••) and the clinical severity of HSV keratitis (○○○○) as plotted as a function of time after infection. Each data point represents the mean of results from three or more animals.

Cell pellets were briefly treated with distilled water to lyse red cells, restored immediately to isotonicity with RPMI, and washed two times with RPMI. Lymph nodes were dissected free of fat and connective tissue, pressed through a No. 20 mesh sieve, and washed three times with RPMI. Each cell type was resuspended in RPMI without serum at 1 X 10^6 cells/ml. Viability was greater than 98% in all cases.

**Lymphocyte transformation assay.** A 1 ml volume of cell suspension was cultured in triplicate in a 16 mm well of a Linbro FB-16-24-TC plate (Linbro Chemical Co., New Haven, Conn.) with 0.1 ml of ultraviolet-inactivated HSV or control antigen. After mixing, 0.1 ml of autologous heat-inactivated preinfection or postinfection serum was added. Cultures were incubated for 5 days at 37° C in a 5% CO_2 atmosphere. For the final 6 hr of culture, 1.0 μCi of [3H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each culture. The cultures were harvested with an automatic MASH cell harvester (Microbiological Associates) which had been modified to accommodate the larger plates. Cell deposited on glass fiber paper discs were washed three times with PBS, once with 5% trichloroacetic acid, and finally with 95% ethanol. The discs were transferred to scintillation vials, and 20 ml of flour containing 20% Beckman Biosolve 3 were added. Samples were counted 20 min in a Packard Tri Carb scintillation counter.

The average counts per minute (cpm) of triplicate samples were determined and the results were expressed as stimulation index (SI): cpm incorporated in the presence of HSV antigens divided by the cpm incorporated in the presence of uninfected control cells. An SI was determined for each dilution of antigen tested and the maximum SI was found to be a 1:10 dilution of antigen when tested with lymphocytes from HSV-hyperimmune rabbits. To show that the lymphoid preparations were capable of being stimulated in vitro and to assess the effect of virus infection in lymphocyte responsivity, phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Mich.) was added to triplicate cultures at a concentration of 5 μg/culture.

**Viral neutralization assay.** A modification of the microneutralization test as developed by Pauls and Dowdle was employed. Serial twofold dilutions of heat-inactivated (56° C, 30 min) serum were tested. Monolayers of BCC in Linbro FB-16-24-TC dishes were overlaid with a mixture of 1 ml of serum dilution and 1 ml of virus suspension containing 1 x 10^4 pfu that had been reincubated for 1 hr at 37° C. The plates were examined 24 hr later for cytopathic effect. The viral antibody titer was determined as the reciprocal of that dilution of serum that would neutralize 50% of the virus inoculated, in this case 1 x 10^4 pfu.

**Results**

**Disease course.** All corneas infected with the HF strain of HSV developed an initial episode of punctate and dendritic keratitis, which progressed to a geographic ulcer. Mild to moderately severe conjunctivitis was noted early in the course of infection. By day 7 after infection, conjunctivitis was clearing, and epithelial lesions were regressing at the time of appearance of stromal edema and disciform keratitis. Stromal infiltrates were seen by days 9 and 10. The stromal edema
and infiltrates cleared in most eyes by day 21. In a small number of eyes the corneal lesions remained severe for an extended period but finally cleared, leaving a scar. The HF strain is not encephalitogenic, and no animal deaths due to infection were noted.

**Viral isolation.** Positive cultures were formed uniformly through days 3 and 4 after infection and some cases up to day 7 but in greatly decreased titers. The two surviving animals studied at 7 months after infection had positive cultures at this time.

**Kinetics of development of lymphoproliferative response and clinical disease.** The ability of HSV infection of the rabbit cornea to sensitize lymphocytes from the local draining lymph node, PBL, and spleen was determined by measurement of the lymphocyte transformation responses of the cells to HSV antigens as a function of time after infection. All animals developed lymphocyte responses to HSV antigens during the acute infection. Lymphocytes from the preauricular lymph node, when tested in the presence of autologous preinfection serum, responded maximally to HSV antigens at days 5 and 7 after infection. We have noted low levels of virus-specific lymphocyte transformation with lymph node cells at 3 days after infection (unpublished observations). PBL capable of responding to HSV antigens were not seen until day 11 after infection when lymphocytes were assayed in the presence of autologous preinfection serum (Fig. 1). At day 14, the response had returned to normal levels. The spleen cells in the presence of preinfection serum responded weakly at days 5 and 7, with a small response to viral antigens at day 14 (Fig. 1). At 7 months after infection, spleen cells exhibited good responses to viral antigens in the presence of preinfection serum.

The data in Fig. 1 show that the severity of disease does not correlate with the SI which

Fig. 3. Temporal relationship of the development of humoral immune response in herpes keratitis. Each triangle represents data from three or more rabbits. Antibody to HSV was quantitated by the microplaque-neutralization technique, and the titer was expressed as the reciprocal of the highest serum dilution that reduced the number of plaques by 50%.

reflects the cellular sensitivity to HSV antigens. The SI of the local draining lymph node was highest with severe epithelial disease and lowest with stromal edema and infiltration. The spleen cell response was low during the initial infection and became positive after the infection began to clear. The peripheral blood response to HSV antigens was greatest several days following the onset of disciform keratitis, roughly paralleling the appearance of stromal infiltrates. There was no correlation between the clinical state of disease in the animals at day 14 (i.e., subsiding vs. severe) and the in vitro cellular response to HSV antigens by the animals in either group.

Antibody modulation of the lymphoproliferative response. The importance of antiviral antibody in modulating the lymphoproliferative immune response was suggested by our studies (unpublished) showing the depressive effects of hyperimmune anti-HSV serum on the lymphoproliferative response of rabbit lymphocytes sensitized to HSV antigens. In order to further study the effect of antiviral antibody, lymphocyte transformation assays were performed in the presence of postimmunization serum obtained at the same time as the lymphocyte populations. Both the preimmunization and postimmunization sera were employed at a final concentration of 10%. Addition of autologous postinfection serum to virus-sensitized lymphocytes from the draining preauricular lymph node at days 5 and 7 after infection drastically reduced the lymphoproliferative responses (Fig. 2, right). This time coincided with the appearance of viral neutralizing antibody (Fig. 3) and complement-dependent cytotoxic antibody, both of which appeared at this time and persisted throughout the course of the disease. PBL obtained from animals at 7 months after infection showed approximately a 20-fold increase in SI in the presence of autologous postinfection serum vs. a sevenfold increase in the presence of autologous preinfection serum (Fig. 2). The spleen cells also showed an augmented response, approximately a 60-fold increase when tested in postinfection serum vs. a 10-fold increase in the preinfection serum (Fig. 2).

Table I represents the lymphoproliferative and clinical responses for two animals studied at 7 months after infection. The only apparent difference was the absence of highly sensitized PBL in the animal without active disease; this may merely reflect a lack of in vivo stimulus to maintain an active circulating population in the blood, since spleen cells were clearly capable of responding. Blood and spleen cells in this animal responded bet-
Table I. HSV antigen lymphoproliferative responses of lymphocytes from rabbits with recurrent and nonrecurrent infection

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>State of disease 7 months after infection</th>
<th>Source of lymphocytes</th>
<th>Responses to HSV antigens (SI)(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>With autologous preinfection serum</td>
</tr>
<tr>
<td>603</td>
<td>Corneas clear, no apparent disease</td>
<td>Blood</td>
<td>0.95 ± 0.02(^{c})</td>
</tr>
<tr>
<td>612</td>
<td>Corneas opaque, recurrent episodes of conjunctivitis</td>
<td>Spleen</td>
<td>10.52 ± 3.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymph node</td>
<td>9.06 ± 1.85</td>
</tr>
</tbody>
</table>

*Few rabbits received intraocular infection with HSV. Data reported on two surviving animals. Cells and serum were collected at 7 months after infection.
*S I: cpm of HSV antigen-stimulated cultures divided by cpm of control antigen-stimulated cultures.
*Mean ± S.E.M. of triplicate cultures.

Table II. PHA response of lymphocytes from normal-uninfected and HSV-infected rabbits at various times after infection\(^{a}\)

<table>
<thead>
<tr>
<th></th>
<th>PHA response (SI)(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>HSV infected:</td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>1.78 ± 0.94(^{c})</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.78(^{d})</td>
</tr>
<tr>
<td>Day 9</td>
<td>0.37</td>
</tr>
<tr>
<td>Day 11</td>
<td>4.00 ± 3.49</td>
</tr>
<tr>
<td>Day 14</td>
<td>3.56 ± 0.41</td>
</tr>
<tr>
<td>7 months</td>
<td>65.94 ± 8.94</td>
</tr>
<tr>
<td>Normals</td>
<td>10.10 ± 1.86</td>
</tr>
</tbody>
</table>

\(^{a}\)Rabbits received corneal infection with HSV. Cells and serum were collected at indicated times after infection, and the cells were assessed for lymphoproliferative responses to PHA (5 \(\mu\)g/culture) in autologous serum.

\(^{b}\)For calculation see footnote to Table I.

\(^{c}\)Mean ± S.E.M. of triplicate cultures on three or more rabbits per experimental time point.

\(^{d}\)S.E.M. for days 7 and 9 were not calculated because of insufficient data.

In the presence of autologous postinfection serum, but the degree of augmentation was substantially less than in the animal with active disease. It is interesting to note that in both animals, the draining lymph nodes contained cells capable of responding well to viral antigens in the presence of preinfection serum. When autologous postinfection serum was added, this response was reduced but not entirely eliminated.

**Kinetics of development of the humoral immune response.** In order to compare CMI with the humoral immune response to virus, the sera of infected rabbits were tested individually for the presence of serum neutralizing antibodies at time intervals after infection (Fig. 3). Neutralizing antibodies developed in all infected animals and reached measurable titers at day 5, achieving maximum titer from days 11 to 21 after infection.

**Lymphoproliferative responses to mitogens.** To determine the effect of HSV infection on lymphocyte function, the in vitro lymphocyte responses not related to specific viral antigens, i.e., the mitogenic response to PHA, was assessed. The PHA- and HSV-induced proliferative responses were measured at the same time with the same cells and culture conditions to minimize the variability of these assays and difficulty in com-
paring data from different experiments. The responses of lymphocytes from peripheral blood, spleen, and draining lymph node from normal, uninfected, and HSV-infected rabbits to PHA is shown in Table II. The PHA response was suppressed on all experimental days studied from day 5 through day 14 after infection. At 7 months after infection, a strong response to PHA was demonstrated by all cell populations.

Discussion

These studies were undertaken to demonstrate the kinetics of the immune response after HSV ocular infection. The results clearly indicate that HSV infection of the rabbit eye induces cell-mediated immune responses, measured as in vitro lymphoproliferation in the regional draining lymph node, spleen, and peripheral blood. The lymph node cell responses are greater and of a different time course, suggesting antigen escape into the systemic circulation. The significant stimulation in the draining lymph nodes presumably reflects lymphocyte proliferation associated with the primary immune response to a high local antigen concentration.

Lymphoproliferative cell-mediated immune responses to HSV antigens are noted early in HSV-1 and HSV-2 experimental infections. We observed lymphocyte stimulation to HSV antigens by lymph node cells occurring within 3 days after acute ocular infection and reaching a peak at day 5. The proliferative response of the PBL coincides with the severe stromal responses. We did not note a significant spleen cell response during the acute infection in our study. The spleen cell proliferative responses were noted only after recovery. Perhaps ocular infection does not lead to significant stimulation of the spleen at early time periods. Jacobs et al. reported a significant positive lymphocyte transformation response in the local draining node of rabbits immunized with HSV in adjuvant at day 10. Lesser responses were noted in the spleen and peripheral blood, and no responses were found in the distal lymph nodes. A late onset of responsiveness to HSV was shown by PBL from guinea pigs infected by the footpad route in studies by Scriba. The stimulation increased over several weeks and reached a maximum at 63 to 70 days after infection. The difference in time course in these experimental models of acute infection vs. immunization may reflect the persistence of HSV or HSV antigens in boosting the immune response. The site of primary infection would influence the responses of the lymphoid population studied and might represent the effect of local antigen depot of an increased antigen concentration.

Infectious virus titers peaked on days 1 and 2, coinciding with the onset of corneal epithelial lesions. At later times during stromal disease at day 5, viral antigens were found to be localized in corneal keratocytes, with immunohistochemical electron microscopy techniques (unpublished results). The percentage of positive virus cultures declined rapidly after the fourth day of infection. Culture results following herpetic keratitis vary with the strain of HSV-1 studied. Employing the Rodanu strain of HSV-1, Laibson and Kibrick recovered virus from the cornea through approximately the first week after infection. Infectious virus of the PH strain was maximal at 24 hr after corneal infection, was present for 4 to 5 days, and then declined, so that no infectious virus was recoverable from the cornea later than the tenth day. Nesburn et al. found positive cultures through day 5 in rabbit herpes keratitis induced with the Mac Krae strain of HSV-1. The appearance of serum antibody paralleled corneal stromal disease. Using another strain of HSV-1, Sawicki et al. found similar results in HSV corneal infection of rabbits. Antibody was found in corneal extracts on day 14, a time when corneal virus had been undetectable for 2 days. In the study of Sawicki et al. virus titers were not always correlated with the onset of clinical signs, and appearance of local antibody did not correlate with elimination of HSV from the cornea or conjunctiva. We have found a refractory period of about 30 days after initial infection when the eye is resistant to subsequent infection. This can probably be explained by the presence of
local antiviral neutralizing antibody. Interestingly, at this time abortive reinfection does not lead to significant increases in lymphocyte stimulations (unpublished observations).

No correlation has been reported between the antibody titers and SI for different experimental animal models of HSV infection.12, 14, 16, 21 However, it appears from our data that autologous antiviral antibodies may modulate lymphoproliferative responses to HSV. Other investigators using soluble protein antigens have found that antibodies increase or impair the antigen-induced stimulation of lymphocytes.20, 22–24 In the case of herpesvirus, Rosenberg and Notkins21 and Gerber and Lucas25 did not find that antibody altered lymphocyte transformation responses. However, using a system of cell-bound HSV, Rosenberg and Notkins21 later reported that hyperimmune antiviral antibody, when used in high concentrations, markedly depressed lymphocyte stimulation. Scriba16 showed that addition of hyperimmune anti-HSV antibodies could reduce stimulation of PBL by HSV. Our finding that greater depressive effects of autologous serum on the HSV-induced proliferative response of lymph node cells could probably correspond to the in vivo phenomenon of antibody-mediated immune suppression. It also could possibly reflect the greater amounts of HSV antigen in the draining node and possibly the greater proportion of T lymphocytes in the rabbit lymph node than in spleen and peripheral blood populations. In animals with recurrent clinical disease at 7 months after infection, autologous antibody enhanced the spleen cell stimulation to HSV. The enhancement of the proliferative response may be due to antigen aggregation by antibody similar to the findings of Oppenheim29 using nonviral antigens. Infectious herpesvirus-antibody complexes have been shown to stimulate blastogenesis of immune lymphocytes35, 26 and may well explain the antibody effect. It is possible that anti-HSV antibody may affect the lymphoproliferative responses to HSV by removing recognition and binding sites and thus modulating or capping virus antigens, as has been reported for measles virus infections.27 The antibody suppression of lymphocyte transformation may act as a blocking mechanism akin to immunologic enhancement28 observed in tumors. However, we cannot discount the possibility that any direct interaction between antibody and antigen might serve as a binding function that would make antigen less accessible to the cell for its proliferative response. More critical work is needed to establish the extent to which antiviral serum blocking and enhancing factors detected in vitro play a role in vivo.

During the acute HSV infection, all lymphoid populations were found to have a reduced ability to respond to the T lymphocyte mitogen PHA. Kelsey et al.29 reported that splenic lymphocytes from murine cytomegalovirus-infected mice had suppressed responses to PHA and lipopolysaccharide during acute and recovery phases of infection, yet the animals demonstrated a specific lymphocyte response to cytomegalovirus-infected cells. It was suggested that the virus-induced immunosuppression may result from the regulation of the immune response of the host rather than a direct immunosuppressive effect of the virus. It is possible that HSV may impair lymphocyte function by direct infection. Infectious virus suppression of the ability of lymphocytes to respond to PHA or viral antigens has been reported for HSV by Willems et al.30 and Olson.31 Wallen et al.32 examined the in vitro lymphocyte response to general mitogens in the owl monkeys with HSV-induced malignant lymphoma. A loss of response to mitogens, particularly PHA, was noted during the development of the neoplasm. This loss of response to general mitogens was suggested to be due to the activation of a subpopulation of T cells which suppressed the mitogenic response. Although the presence of infectious HSV is possible in our assay, it is doubtful because we (unpublished observations) as well as Rosenberg and Notkins21 have found that infectious virus was about 50% less effective in stimulating lymphocytes than ultraviolet-inactivated virus. No cytopathic effects of our viral antigen were found after testing on cell cultures.
In summary, the results demonstrate that following corneal infection with HSV, the development of virus-specific immunologic memory, measured by lymphocyte transformation, develops in the local draining lymph node. Peripheral blood and spleen develop immune response although at a considerably smaller magnitude than the draining lymph node. The effects of antibody on modulating CMI to HSV antigens is suggested by our results. However, further experiments need to be done to show whether the addition of unbound antigen would reverse the antibody effects. Antigenic modulation is of interest clinically. Antigenic modulation is a mechanism whereby virus may persist by evading immunologic recognition and thereby persist in the host in a state of maintained latency.

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