Cell-mediated immunity in herpes corneal stromal disease

Rose Marie Nagy, Rosemary C. McFall, and Theodore W. Sery

Regional draining lymph nodes (RDLN) from rabbits with herpes virus disciform keratitis (defined as corneal edema or corneal edema with concomitant epithelial lesions) were tested for general as well as specific immune capacity. Although general RDLN immune capacity did not seem to be impaired, specific reactivity to herpes antigens was found only in those rabbits which presented with disciform keratitis and concomitant epithelial lesions; specific reactivity to herpes antigens was not found in those animals which presented with disciform edema alone. Additionally, the unilateral nature of the RDLN immune response during ocular infections of unequal severity was demonstrated.

Key words: herpes virus, disciform keratitis, disciform edema, cell-mediated immunity, regional draining lymph nodes, T-cell, lymphocyte transformation

The etiology of endothelial dysfunction associated with corneal edema during herpes simplex virus (HSV) disciform keratitis remains uncertain. Although viral invasion of the endothelium, cell-mediated immunity (CMI), and humoral immunity have been suggested as causative mechanisms for disciform edema, it is generally accepted that immune factors are probably most important in disciform edema of HSV stromal disease.

The HSV disciform keratitis model described by Sery and associates is unique in that it permits the separation of discrete disciform edema from the more progressive stages of corneal stromal disease. The purpose of the present study was to evaluate the cell-mediated immune response of the regional draining lymph nodes (RDLN) from animals undergoing experimental disciform keratitis (defined as corneal edema, or corneal edema with concomitant epithelial lesions). The immune response of RDLN was measured via the lymphocyte transformation assay. The mitogen phytohemagglutinin (PHA) was used to measure general T-cell immune status, while HSV antigens were used to monitor specific immune capacity.

Materials and methods

Virus propagation. Rabbit corneal fibroblast cell cultures (RC-1) were grown in Minimum Essential Medium (Eagle) supplemented with 10% heat-inactivated fetal calf serum. When confluent, the monolayer cultures were washed three times with phosphate-buffered saline (pH 7.2) and then infected with the H-4 strain of HSV at a multiplicity of 4 to 7 virus particles per cell. After incubation at 34°C for 40 hr, infected cell lysates were prepared by rapid freezing and thawing of the infected RC-1 cultures to release intracellular virus. The supernatants were collected and cellular debris removed by centrifugation at 500 x g at 4°C for 5 min.
Table 1. Mode of antigen presentation

<table>
<thead>
<tr>
<th>Days post-inoculation</th>
<th>No. of rabbits tested</th>
<th>Response to antigen preparations*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PV</td>
<td>S</td>
</tr>
<tr>
<td>5-7 (E)</td>
<td>4</td>
<td>10.4 ± 1.4</td>
</tr>
<tr>
<td>18-20 (L)</td>
<td>4</td>
<td>10.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>PV: S p &lt; 0.02†</td>
<td>S: C p &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>PV: S NS</td>
<td>S: C NS</td>
</tr>
</tbody>
</table>

*Mean stimulation index ± SD of response to particulate (PV), soluble (S), and combination (C) HSV antigens. For each rabbit, lymph nodes from right and left side were removed, pooled, and tested.
† p values were obtained by Student’s t-test with p > 0.05 considered nonsignificant (NS).

Animal inoculation and lymph node removal.
Normal gray chinchilla rabbits weighing 2.0 to 2.5 kg were used in this study. Prior to corneal inoculation, the ICL were titered and then diluted in Medium 199 to a virus concentration of 10,000 TCID<sub>50</sub>/ml. Corneas were anesthetized by topical administration of 0.5% tetracaine hydrochloride, and both eyes of all test rabbits were given an intrastromal injection of 200 TCID<sub>50</sub> of H-4 virus in 0.02 ml. An exception to this protocol was utilized for the testing of the mode of HSV antigen presentation in the lymphocyte transformation assay. Four times the amount of virus (i.e., 800 to 1,000 TCID<sub>50</sub>) was injected intrastromally to ensure that all rabbits within this test group would present with corneal epithelial dendritic lesions, stromal edema, and subsequent stromal necrosis.

During clinically observable disciform keratitis (defined as corneal edema or corneal edema with concomitant epithelial lesions), test animals were sacrificed with an overdose of sodium pentobarbital. Regional draining preauricular and cervical lymph nodes were removed and single cell suspensions prepared for immunologic evaluation via the lymphocyte transformation assay. When individual rabbits presented with similar ocular lesions (OD vs. OS), RDLN were removed, pooled, and tested; when ocular lesions were dissimilar (OD vs. OS), lymph nodes draining the respective eyes were removed and tested unilaterally.

RDLN from individual non-HSV infected rabbits were removed, pooled, and tested. This group served as a control. In addition, RDLN from infected rabbits with similar ocular lesions (OD vs. OS) were removed and tested unilaterally. These rabbits served as a second control group.

HSV antigen preparation. After initial low-speed centrifugation, ICL were centrifuged at 30,000 X g at 4° C for two additional 5 min intervals. Virus was concentrated by centrifugation at 82,500 X g at 4° C for 1 hr in a Beckman L265B ultracentrifuge. The pellet containing semipurified virus was designated antigen PV and the supernatant containing soluble virus protein antigen S. ICL containing both virus and soluble virus proteins (i.e., nonultracentrifuged supernatant) was designated antigen C. Control antigens consisted of noninfected cell lysates prepared by rapid freezing and thawing of noninfected BC-1 cultures and removal of cellular debris by centrifugation at 500 g at 4° C for 5 min. Prior to use in the lymphocyte transformation assay, both the control and HSV antigen preparations were irradiated with ultraviolet light.

Lymphocyte transformation assay. A modification of the method of Williams<sup>10</sup> for measuring tritiated thymidine incorporation into mitogen- and antigen-stimulated lymphocytes was used in this study.

Preauricular and cervical lymph nodes of sacrificed rabbits were removed and immediately placed in RPMI 1640 medium (Grand Island Biological Co.) containing, per milliliter, 200 IU penicillin, 200 IU streptomycin, and 5 μg Fungizone. Single cell suspensions were made by mincing the lymph nodes with fine scissors; then the lymphocyte preparations were washed twice in RPMI 1640 medium and resuspended to a final concentration of 40 x 10<sup>6</sup> viable cells/ml as determined by dye exclusion. Aliquots of 0.025 ml were added to each well of a Falcon Plastics Micro Test II tissue culture plate containing an equal volume of RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum and, per milliliter, 100 IU penicillin, 100 IU streptomycin, and 2.5 μg Fungizone. The mitogen phytohemagglutinin (PHA) (PHA 15, Burroughs Wellcome Co.), as well as HSV and control antigens, were added in 0.025 ml aliquots to each of four replicate wells, and the plates were incubated at 37° C in a 95% air–5% CO<sub>2</sub> humidified atmosphere. After a 72 hr incubation period, 0.025 ml

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Table II. Response of RDLN lymphocytes during HSV corneal stromal disease of unequal severity

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Side</th>
<th>PHA</th>
<th>PV</th>
<th>S</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>34.0 (97.9)</td>
<td>2.1 (6.1)</td>
<td>1.9 (5.4)</td>
<td>1.2 (3.3)</td>
<td>2.9 (5.9)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25.0 (124.7)</td>
<td>11.5 (50.6)</td>
<td>22.1 (97.6)</td>
<td>6.6 (28.9)</td>
<td>4.4 (18.4)</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>10.1 (76.3)</td>
<td>3.1 (23.1)</td>
<td>5.1 (38.7)</td>
<td>2.5 (19.3)</td>
<td>7.6 (28.9)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.4 (92.1)</td>
<td>11.0 (43.3)</td>
<td>21.6 (85.1)</td>
<td>11.5 (45.1)</td>
<td>3.9 (12.4)</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>46.1 (83.2)</td>
<td>2.3 (4.2)</td>
<td>3.6 (6.5)</td>
<td>4.1 (7.4)</td>
<td>1.8 (5.6)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.0 (87.6)</td>
<td>7.4 (18.4)</td>
<td>11.8 (29.6)</td>
<td>13.9 (34.9)</td>
<td>2.5 (6.7)</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>29.4 (61.1)</td>
<td>2.3 (4.8)</td>
<td>6.5 (13.6)</td>
<td>7.3 (15.3)</td>
<td>2.0 (5.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22.6 (85.6)</td>
<td>10.9 (31.8)</td>
<td>12.6 (36.7)</td>
<td>11.5 (33.3)</td>
<td>2.9 (7.8)</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>1</td>
<td>23.9 ± 15.0</td>
<td>7.9 ± 15.3</td>
<td>3.6 ± 1.9</td>
<td>3.8 ± 2.6</td>
<td>0.3 ± 2.6</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>2</td>
<td>27.3 ± 5.7</td>
<td>10.2 ± 1.9</td>
<td>17.0 ± 5.6</td>
<td>10.9 ± 3.1</td>
<td>3.4 ± 0.9</td>
</tr>
</tbody>
</table>

*p values:
- Stimulation index: NS
- Thymidine incorporation (cpm): p < 0.001

Table II continued...

Results

Particulate, soluble, and combination HSV antigens in deep stromal corneal disease with dendritic lesions. By day 5, all rabbits in this test group showed similar bilateral corneal lesions with evidence of stromal edema. Four animals within the test group were sacrificed early (E), days 3 thru 7, while corneal edema and concurrent epithelial dendritic lesions were evident. The remaining four animals were sacrificed later (L), days 18 thru 20, when edema and dendritic keratitis had subsided but mild stromal necrosis was still evident. RDLN were removed from each rabbit and the mode of HSV antigen presentation in the lymphocyte transformation assay was evaluated.

HSV antigen S (soluble HSV proteins) elicited the greatest lymphoproliferative response in the early (E) test group (Table I). The response to antigen S differed significantly from the other antigens.

Student's t-test. The percent variability of each replicate culture was usually less than 20%. Generally, the range of variability was between 10% and 15%.

Results

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Table III. Response of RDLN lymphocytes during HSV corneal stromal disease of equal severity

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Side</th>
<th>Response to mitogen and antigen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>V</td>
<td>OD</td>
<td>40.5 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>33.7 ± 3.0</td>
</tr>
<tr>
<td>VI</td>
<td>OD</td>
<td>51.1 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>57.9 ± 4.4</td>
</tr>
<tr>
<td>VII</td>
<td>OD</td>
<td>72.1 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>72.4 ± 6.5</td>
</tr>
</tbody>
</table>

*Incorporation of ³H-thymidine (cpm × 10³, mean ± SD) in response to optimal concentrations of PHA and combination (C) HSV antigens. For each animal, comparison of ³H-thymidine incorporation by unilaterally tested RDLN lymphocytes, all values were not significantly different (OD vs. OS) at p > 0.05 by Student's t-test.

significantly when compared to either HSV antigen PV (particulate semipurified virus) or HSV antigen C (combination of particulate virus and soluble HSV proteins). In contrast, no significant difference in the lymphoproliferative response to the three antigen preparations was evident in the later stages (L) of stromal disease.

Unilateral response of regional draining lymph nodes. The data in Table II indicate the unilateral nature of the immune response of RDLN from animals which presented with HSV ocular disease of differing severity. Four test rabbits presented with deep stromal edema with a few punctate dendritic lesions in one eye (side 1), while the contralateral eye (side 2) presented with deep stromal edema and multiple small dendritic lesions. Examination of the unilaterally harvested preauricular and cervical lymph nodes draining the respective eyes revealed size variation; nodes from side 1 were relatively normal in size and appearance, while the contralateral nodes, side 2, were hyperplastic and slightly hemorrhagic. No significant difference in the lymphoproliferative response to PHA was evident between contralateral nodes, whereas the response to specific HSV antigens was significantly different. RDLN lymphocytes from side 1 showed relatively low levels of response to specific HSV antigens when compared to lymphocytes from the contralateral nodes (side 2), which showed an augmented lymphoproliferative response to HSV antigens.

Table III compares the immune response of RDLN lymphocytes from rabbits with bilaterally similar HSV ocular lesions. The three test rabbits in this control group presented with deep stromal edema and punctate dendritic lesions. RDLN were harvested and tested unilaterally; no significant difference in the lymphoproliferative response to either PHA or HSV was evident between contralateral nodes.

Disciform keratitis as a function of clinical disease. The 40 animals included in this study were grouped according to the severity of clinical disease. Group A consisted of 14 animals which presented with only disciform edema; group B consisted of 10 animals with disciform edema and small dendritic and punctate epithelial lesions; group C consisted of five animals with disciform edema and epithelial lesions involving approximately 25% of the cornea; and group D consisted of 11 noninfected control animals. The lymphoproliferative responses of RDLN lymphocytes from rabbits in this study are shown in Table IV. As is evident from the data, the responses to PHA were quite variable in all groups tested (as indicated by the large standard deviations). In addition to the unique immunologic capacity of each test animal, this variability may reflect shifts in PHA responsive cells within regional draining lymph nodes during the course of clinical disease (unpublished data). As is also evident from the data presented, augmentation of the RDLN lymphoproliferative response to specific HSV antigens correlated with the degree of HSV epithelial infection during disciform keratitis. The increase in the levels of HSV antigen responsiveness as clinical disease became more severe is strikingly apparent in Fig. 1. T-cell responsiveness to specific HSV antigens during disciform edema (group A) was near control antigen level; however, as epithelial involvement became more severe (groups B and C), the mean SI and cpm ³H-thymidine incorporation increased accordingly. Differences in SI and cpm ³H-thymidine...
Table IV. Disciform keratitis as a function of clinical disease

<table>
<thead>
<tr>
<th>Animal group†</th>
<th>PHA (15.9 ± 10.3)</th>
<th>HSV (3.2 ± 0.2)</th>
<th>Control (10.5 ± 4.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 14)</td>
<td>(152 ± 84.5)</td>
<td>(10.8 ± 4.1)</td>
<td></td>
</tr>
<tr>
<td>B (n = 10)</td>
<td>28.0 ± 15.2</td>
<td>30.0 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>C (n = 5)</td>
<td>(202 ± 77.6)</td>
<td>(29.5 ± 27.5)</td>
<td>(11.3 ± 4.6)</td>
</tr>
<tr>
<td>D (n = 11)</td>
<td>(93.9 ± 17.9)</td>
<td>(82.0 ± 31.5)</td>
<td>(4.6 ± 2.6)</td>
</tr>
</tbody>
</table>

\*Mean stimulation index ± SD and mean \(^{3}H\)-thymidine incorporation (cpm × 10^3 ± SD) in response to PHA, combination HSV antigen, or control antigen.
†Disciform edema only (A); disciform keratitis with slight infection (B); disciform keratitis with moderate infection (C); and noninfected controls (D).
\(t\) Student's t-test statistical analysis with \(p > 0.05\) considered nonsignificant (NS).

...incorporation between groups A and B were significant at \(p < 0.01\) and \(p < 0.05\), respectively; differences between groups A and C were significant at \(p < 0.001\) for both SI and thymidine incorporation.

Interestingly, the responses to control antigen were significantly higher in the HSV-infected groups A and B as compared to the infected group C or to the control (animal) group D. These results may indicate that the RDLN lymphocytes from groups A and B were in the lymphoproliferative stage after initial contact with HSV antigen delivered from the infected cornea. In contrast, RDLN lymphocytes from group C probably passed the stage of lymphoproliferation following initial HSV antigen contact; thus the level of control antigen responsiveness was equal to that of control group D.

Discussion

The mode of antigen presentation in the lymphocyte transformation assay seems most important when testing RDLN harvested during early HSV deep stromal ocular disease. The augmented lymphoproliferative response to soluble structural and nonstructural HSV proteins indicated the early escape of soluble protein during primary HSV infection of the cornea. Whether this augmented response involves a specific subset of lymphocytes within the RDLN requires further investigation. The lymphoproliferative responses to the three antigen preparations were more variable in the late stages of stromal disease. No statistical difference between the modes of antigen presentation was evident, although the combination of whole virion and soluble HSV proteins (group C) elicited the highest mean SI. This may indicate that antigen C is the lymphocyte transformation assay antigen of choice when testing RDLN of late stromal disease.

The unilateral nature of the lymphoproliferative RDLN response to specific HSV antigens during ocular disease of limited but unequal severity was demonstrated. These findings suggest early entrapment of viral antigen by RDLN with stimulation and sensitization of localized lymphocytes. In contrast, the lymphoproliferative potential of PHA-responsive T-cells was not significantly different between the contralateral nodes from animals with ocular disease of unequal severity. These findings suggest that the augmented response to specific HSV antigens by lymphocytes from nodes draining the more severely infected eye was not a function of an enhanced proliferative potential of this PHA responsive lymphocyte population but was probably due to the quantity of herpes antigen available for stimulation and sen-
Fig. 1. PHA (■) and HSV (□) lymphoproliferative response of RDLN from rabbits with disciform edema only (A); disciform edema and small dendritic lesions (B); and disciform edema and large epithelial lesions (C). The increase in the levels of HSV antigen responsiveness as clinical disease became more severe is strikingly apparent in the histogram.

sitzation of RDLN lymphocytes. Localization and antigen dose–dependency of the immune response in RLDN has been described for various antigens and other routes of antigen administration.12–14

Although previous investigations have suggested the role of CMI in the pathogenesis of disciform edema,4, 16–19 we were unable to demonstrate RDLN lymphoproliferative response to specific HSV antigens in animals with disciform edema only. Significant levels of response to the T-cell mitogen PHA suggests that the lack of lymphocyte reactivity to HSV antigen was not due to impairment or suppression of this T-cell population. In contrast, lymphoproliferation to specific HSV antigens was demonstrated in animals with disciform edema and concomitant epithelial lesions; the levels of response correlated with the severity of epithelial involvement. The tendency toward higher levels of CMI to specific HSV antigens during epithelial disease has been previously reported.13

The higher levels of 3H-thymidine incorporation by RDLN lymphocytes tested with control antigens suggest that local lymph node stimulation (possibly by low quantities of viral antigen) had occurred in animals with disciform edema. This finding, in conjunction with two previous investigations from this laboratory,17, 19 suggests that the etiology of disciform edema may indeed be of immune origin. The apparent contradiction between this suggestion and our inability (in the present study) to demonstrate specific HSV induced lymphoproliferation via the lymphoblast transformation assay supports the concept of dissociation of different measurements of in vitro correlates of CMI.20 We are presently investigating other correlates of CMI to determine their association with herpes simplex disciform edema.

We thank Antonio Ortiz for technical assistance and Ruth Grevious for secretarial assistance.

REFERENCES
12. Landy M and Baker PF: Cytodynamics of the distinctive immune response produced in regional

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