Effects of Cyclosporine A on Clinical and Immunological Parameters in Herpes Simplex Keratitis

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The immunosuppressive effects of cyclosporine A (CyA) on the clinical and antiviral immune responses were examined in experimental herpes simplex virus (HSV) keratitis in the rabbit in order to clarify the role that immune lymphocytes play in herpetic stromal disease. Cyclosporine A was administered intramuscularly to rabbits daily starting from the time of corneal infection with HSV until day 14 postinfection. Control HSV-infected rabbits received daily injections of the solvent vehicle alone. HSV-infected rabbits receiving CyA treatment showed more severe and persistent stromal keratitis, and a greater incidence and duration of virus recovery from the cornea. Suppression of cellular immune responses to T cell mitogens, B cell mitogens (anti-rabbit immunoglobulins), and HSV antigens were observed in the CyA treatment group. These results show that in CyA-treated HSV-infected rabbits the antiviral immune responses are inhibited. Acute viral infections with cytopathic viruses such as HSV may therefore be more dramatic, suggesting that CyA may facilitate the potentiation of HSV infections ordinarily suppressed by immune cells. Invest Ophthalmol Vis Sci 28:1170-1180, 1987

Cyclosporine A (CyA) is a potent immunosuppressive agent derived from active fungal metabolites and is being used as an anti-rejection drug in organ transplants and in treating certain autoimmune diseases. CyA acts preferentially on T lymphocytes and is believed to prevent early activation processes such as acquisition of receptors for interleukins, as well as interfering with macrophage-activating properties of T cells.1-3

In ocular herpesvirus infections, the importance of the host immune response has been clinically documented.4-6 Experimentally induced herpes simplex virus (HSV) infection in animals has increased our understanding of the pathogenesis and has confirmed the importance of the immune system.7-14 The presence of herpes virions, viral antigens, antiviral antibody, complement, polymorphonuclear leukocytes, and mononuclear cells has been described during primary HSV keratitis in the rabbit.15-20 However, the function of these cells in the immunopathogenesis of stromal keratitis has not been clearly established. Although delayed-type hypersensitivity mechanisms have been implicated in recovery from systemic herpes virus infections,21 it remains unclear whether T lymphocytes play a protective or immunopathologic role in determining the severity of both primary and recurrent ocular infections.

The mode of action of CyA in viral infections is not completely understood. Immune suppression allows infectious agents to thrive unchecked, and CyA-treated patients continue to get viral infections.22 However, CyA is an important immunological reagent that may play a critical role in dissecting various complex immunological phenomena. The immunosuppressive effect of CyA on T cell-mediated antiviral immune responses has recently been reported.23 The results show that in CyA-treated mice primary and secondary antiviral T cell responses are strongly inhibited, resulting in more acute viral infections with cytopathic viruses. In contrast, immunopathological T cell-mediated disease caused by noncytopathic viruses such as lymphocytic choriomeningitis virus is less severe.23

In order to elucidate the role that T lymphocytes play in herpetic stromal disease, we studied the effects of CyA on the course of experimental HSV keratitis in the rabbit.24 Parameters investigated were the effects of CyA on: (1) the clinical disease; (2) virus re-
covery from the cornea; (3) histopathology of corneal infiltrates; (4) immunological parameters of T lymphocyte and B lymphocyte function; and (5) the immune response to HSV antigens.

Materials and Methods

Animals

New Zealand white male rabbits (Elkhorn Rabbitry, Watsonville, CA) weighing approximately 2 kg were used. All corneas were clear by biomicroscopic examination prior to use. Animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Virus and Ocular Infection

Fifty-four rabbits (108 eyes) were topically infected with HSV-1 on the abraded cornea with $3 \times 10^5$ plaque-forming units (PFU) per eye as previously described, except that the HSV-1 strain HF virus was propagated in SIRC rabbit corneal cells (RCC) (Staats Institut Rabbit Cornea, American Type Culture Collection, Rockville, MD) with normal rabbit serum instead of fetal bovine serum in the culture medium.

CyA and Solvent

CyA batch 84060 (Sandoz, Ltd., Basel, Switzerland) was dissolved in Miglyol 812 (Dynamit Nobel, Kay-Fries Chemical, Mont Vale, NJ). CyA was prepared by dissolving 1 g in 6 ml of Miglyol to which 0.4 ml of absolute ethanol was added, warming to 37°C until dissolved, then adjusting the volume to 10 ml with Miglyol for a final concentration of 100 mg/ml. Solvent control consisted of Miglyol and ethanol prepared as described above.

Treatment

Rabbits received daily intramuscular injections beginning at the time of infection and continuing until day 14 postinfection. Twenty-six rabbits received 25 mg CyA per kilogram of body weight, and 28 rabbits received an equal volume (0.25 ml/kg) of solvent only. All rabbits received tetracycline (Sumycin® syrup, E. R. Squibb & Sons, Inc., Princeton, NJ) administered daily in drinking water (maximum daily dose 375 mg) beginning 3 days prior to HSV infection through day 14 postinfection, as a prophylactic against secondary bacterial infection during immunosuppression.

Clinical Evaluation

Eyes were examined on days 3, 7, 9, 14 and 21 postinfection and scored for epithelial and stromal keratitis as previously described. Anterior chamber inflammation was evaluated by the following criteria: 0 = no inflammation, 1+ = slight injection of iris vessels, 2+ = moderate injection of iris vessels, 3+ = severe injection of iris vessels with or without anterior chamber infiltrate, and 4+ = severe injection of iris vessels with hypopyon.

Virus Isolation

Corneal swabs were taken from left eyes on days 3 and 9 postinfection and inoculated and examined as previously described. In some cases an aliquot of the swabbed material was used directly in an antibody overlay assay for PFU as described by Hampar et al.

Tissue Preparation

Three to seven animals from each treatment group were sacrificed on days 3, 7, 14, and 21 postinfection. In the solvent-treated group, seven animals were sacrificed at each of these time points except four animals which were taken at day 21. In the CyA-treated group, three animals were sacrificed at day 3, seven animals at day 7, and six animals at day 14. Right eyes were removed and placed in formalin for histologic examinations. Kidneys from day 14 were also processed for histologic evaluation for nephrotoxicity. Peripheral blood (PBL), spleen (SPL), and preauricular lymph nodes (PALN) were taken at time of sacrifice. SPL and PALN were dissected free of fat, minced, passed through a #40 mesh screen, and washed three times in RPMI medium without serum. Heparinized blood was centrifuged anduffy coat was obtained and further purified on Ficoll-Isopaque cushions (Pharmacia, Piscatway, NJ). Lymphocytes obtained at interface were washed three times as above. Cell suspensions appropriate to the various assays were prepared in lymphocyte culture medium (RPMI-LC) consisting of RPMI 1640 medium supplemented with fetal bovine serum (10%), sodium pyruvate, MEM vitamins, MEM nonessential amino acids, MEM essential amino acids, penicillin (10 U/ml), and streptomycin (100 µg/ml), modified from that described by Metzger et al.

T Cell Mitogen Response Assays

PBL, SPL, and PALN cells were suspended in RPMI-LC and dispersed in 0.2 ml volumes containing $2 \times 10^5$ lymphoid cells per well of a 96-well microwell plate (Falcon Labware 3072, Oxnard, CA). Stock mitogen solutions were added to give the following final concentrations: concanavalin A (Con A)
10, 5, 1, 0.5 and 0.2 μg/ml (Miles-Yeda cat. 79-003, lot 165, Rehovot, Israel), phytohemagglutinin (PHA-P) 5 μg/ml (Difco cat. 3110-56, lot 685049) and Protein A 200 μg/ml (Sigma P6650, lot 91f-6882, St. Louis, MO). Concentrations used were optimal as determined by titration assays. Cultures were incubated at 37°C in a 5% CO₂ atmosphere for a total of 3 days. One μCi of ³H-thymidine (Amersham, cat. no. TRA.310, 2 Ci/mmol, Arlington Heights, IL) was added per well 18 hr prior to termination of the cultures. Harvesting was done on glass fiber paper with a Mash II cell harvester (MA Bioproducts, Walkersville, MD). Discs containing harvested cells were placed in plastic vials containing 5 ml PCS (Amersham-Searle) liquid scintillation cocktail and counted in a Beckman LS7500 scintillation counter.

Response to Anti-Rabbit Immunoglobulins

PBL, SPL, and PALN were prepared and plated as described above except that stock solutions of anti-rabbit immunoglobulins were used instead of mitogens. Anti-rabbit IgG (Cappel Laboratories, cat. no. 0212-0081, Lot 19316, Cochranville, PA) was added to give final concentrations of 50 μg and 5 μg protein per ml. Anti-rabbit IgM (Cappel cat. no. 0212-0201, Lot 18438) was added to give final concentrations of 200 μg and 50 μg protein per ml. The higher concentration used was optimal as determined by titration assays. Cultures were incubated for a total of 2 days, labelled and harvested as described above. The shorter incubation time was selected because additional incubation time did not increase the response, as has been indicated by other investigators studying rabbit B cells.²⁸

Response to HSV Antigens

Cultures were prepared, labelled, and harvested as described above, except that 20 μl of virus or control antigen was added per well, and cultures were incubated for a total of 4 days. All antigens were ultraviolet (UV)-inactivated for 40 min at a distance of 4.5 cm from a G15T8 germicidal lamp (General Electric, Bridgeport, CT). No infectious virus could be recovered after UV inactivation. Antigens consisted of cell lysates prepared from cells infected with HSV-1, strain HF, (HSV antigens), or uninfected cell lysates (control antigens). Lysates contained 1% fetal bovine serum, were clarified by centrifugation at 3000 g prior to use. and were prepared from both RCC and rabbit kidney cells (RKC)-13 (American Type Culture Collection, Rockville, MD). RCC-derived HSV antigen had 2.7 × 10⁷ PFU/ml prior to inactivation, and 20 μl of undiluted, 1:5, 1:15, 1:30, 1:60, and 1:120 dilutions were used. RKC-derived HSV antigen had 2.2 × 10⁶ PFU/ml prior to inactivation, and 20 μl of undiluted, 1:5, 1:15, 1:30, and 1:60 dilutions were used. Uninfected cell lysates were used at the same dilutions as stated above.

Results

Survival

Increased mortality was noted in the CyA-treated animals, with 10 of 26 animals dying between days 6 and 14 after treatment. Eight of these animals exhibited no obvious symptoms except rapid weight loss and mild lethargy, and died quickly after the symptoms became evident. Two of these eight animals had no gross findings on necropsy, except that the intestinal tract was entirely empty. Two other CyA-treated animals died due to secondary pulmonary infections, with cultures revealing Bordetella bronchiseptica from one animal and a Pseudomonas species from the second. In the solvent-treated control animals, two of twenty-eight died from secondary pulmonary infections. An increase in pulmonary disease, most frequently associated with isolation of Bordetella bronchiseptica, has been noted in HSV-infected rabbits as compared with uninfected controls (RH Meyers-Elliott et al, unpublished results). Animals that had died were not included in the experimental data results except for the clinical response evaluations made before death.

Clinical Response

The percentage of eyes involved in each group is shown in Figure 1 and the mean score of positive eyes is shown in Figure 2. Animals in the CyA-treated group showed a lower incidence but slightly longer duration of epithelial disease than solvent-treated animals, but no difference in severity was noted (Fig. 2). Stromal disease was much more severe and its incidence was slightly greater in CyA-treated animals than in solvent-treated, with onset and duration equal in both groups through day 14 postinfection (Figs. 1 and 2). Corneal vascularization followed a parallel course in both groups, with slightly higher incidence in the CyA-treated group (Fig. 1). The most marked difference between the two groups was the increased incidence of iritis in the CyA-treated group (75% of eyes at day 14) compared with the solvent-treated group (0% at day 14). Conjunctivitis was more severe and more persistent in the CyA-treated group than in the control group.

Virus Isolation

Virus recovery in traditional flask culture was equal in both groups at day 3 postinfection (Table 1),
Fig. 1. Clinical response during the course of HSV keratitis. Percentage of positive eyes after HSV infection for each of the clinical parameters shown for CyA-treated and solvent-treated HSV corneal infected rabbits.

Fig. 2. Clinical response during the course of HSV keratitis. Mean score ± SE of positive eyes after HSV infection for each of the clinical parameters shown for CyA-treated and solvent-treated HSV corneal infected rabbits.
Table 1. Presence of infectious HSV in rabbit corneas

<table>
<thead>
<tr>
<th>Day post infection</th>
<th>Eyes positive* (Eyes positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment group</td>
</tr>
<tr>
<td>Day 3</td>
<td>Solvent</td>
</tr>
<tr>
<td>Day 3</td>
<td>CyA</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.006†</td>
</tr>
<tr>
<td>Day 9</td>
<td>Solvent</td>
</tr>
<tr>
<td>Day 9</td>
<td>CyA</td>
</tr>
</tbody>
</table>

* Each positive value represents isolation of virus from one rabbit. Virus recovery from corneal swabs by plaque method. † Probability value obtained by student t-test analysis comparing CyA with solvent control groups. A value less than 0.05 is considered significant. NS = not significant.

although flasks inoculated with material from the CyA group showed cytopathological effect characteristic of HSV at least 1 day earlier than those flasks receiving material from the control group. At day 9 postinfection, no virus was recovered from the control group, but 66% of cultures (8 of 12) from the CyA group were positive. Virus recovery was also assayed directly by the plaque assay method. At day 3 postinfection, material from CyA-treated animals showed slightly higher PFU recovery (22.14 ± 5.40 × 10^3 PFU/ml) than controls (12.06 ± 3.27 × 10^3 PFU/ml). At day 9 only cultures from the CyA group were positive in the plaque assay.

Histology

Examination of hematoxylin-eosin (H & E) stained sections revealed that the onset of cellular infiltration in the CyA-treated group was somewhat delayed as compared with the control group. At day 3, eyes from the CyA group demonstrated epithelial damage and edema, stromal edema, and enlargement of the limbus vessels, but no cellular infiltrates were evident. Similar findings were noted in control group eyes, and in addition polymorphonuclear cells (PMN) were seen in the epithelium, at the limbus, in the corneal stroma, and in the trabecular meshwork iris angle area in some instances, although the degree of infiltration varied. By day 7 postinfection, PMN were noted in the epithelium and mixed PMN and mononuclear cells at the limbus in corneas from both groups. Stromal infiltrates in the CyA group at day 7 were dense and consisted entirely of PMN, while infiltrates in the control group were moderate, with both PMN and mononuclear cells. Vascularization of the cornea was more evident in the CyA group than in the control. The most dramatic difference between the two groups at day 7 was that seen in anterior chamber cellular infiltrates. Control eyes showed little or no infiltrates, with PMN seen only in the trabecular meshwork near the base of the ciliary body in three of seven eyes (Fig. 3), while six or seven CyA eyes showed anterior chamber cellular infiltrates (Fig. 4). Four of these showed extensive PMN and moderate mononuclear cell infiltration of the iris, ciliary body, and endothelium, as well as free cells in the anterior chamber. One eye showed PMN infiltrates posterior to the iris, at the most anterior area of the retina, as well as near the lens capsule. By day 14, this difference in anterior chamber involvement was still evident, with one of six control eyes showing a few mononuclear cells only in the endothelium and ciliary body, and free in the anterior chamber, while four of six CyA eyes still showed moderate PMN and light mononuclear cell infiltrates in the iris, ciliary body and endothelium, and free cells in the anterior chamber. At day 14, stromal involvement in both groups consisted of mixed PMN and mononuclear cell infiltrates, although the density of PMN was greater in the CyA eyes, and there was some stromal scarring in both groups. At day 14 epithelial involvement in both groups was resolving, with fewer than half of the eyes showing epithelial defects, usually at the periphery of an underlying stromal lesion, and a few PMN seen within the epithelium in some cases. Residual vascularization was slightly greater in the CyA group, with some enlargement of the limbus vessels still evident. Limbal infiltrates were reduced, with mononuclear cells predominating in both groups, although fewer cells were seen in the CyA eyes. At day 21 there were no survivors in the CyA group. The four control group eyes showed complete resolution of epithelial involvement; only one eye showed a few infiltrating mononuclear cells in the stroma and three showed slight mononuclear cell infiltrates at the limbus.

Total Cell Recovery

The total cell recovery from the organs assayed did not decline appreciably at time intervals postinfection, which indicated that adequate numbers of lymphocytes were present at all times. We did not examine any population shifts that may have been occurring. Recovery of lymphoid cells from PALN of CyA-treated HSV-infected rabbits was similar to that from HSV-infected solvent-treated animals, approximately 100 × 10^6 lymphocytes per pair of PALN. However, the PALN from the CyA-treated animals were often smaller and more necrotic in appearance. By day 14, the cell yields had decreased in both groups to a mean of 58.4 ± 20.17 in the solvent treated and 10.9 ± 2.96 × 10^6 in the CyA-treated groups. Cell yields from the spleen were usually com-
parable, with group means ranging from 60.4 to 96.2 \( \times 10^6 \) throughout the time course of the experiment. PBL recovery from approximately 45 ml of heparinized blood did not vary appreciably in either treatment group, remaining between 11 and 22 \( \times 10^6 \) lymphocytes. Despite apparent differences in the group means in the PALN and SPL cell recoveries, no significance was found with the student t-test because of the relatively small sample size and normal variability within groups.
Table 2. Effect of CyA on rabbit lymphocyte responses to T cell mitogens during the course of HSV keratitis*  

<table>
<thead>
<tr>
<th>Cell source†</th>
<th>Mitogen‡</th>
<th>Intercept§</th>
<th>Slope¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CyA</td>
<td>Solvent</td>
<td>CyA</td>
</tr>
<tr>
<td>Spleen</td>
<td>Con A</td>
<td>29,020</td>
<td>154,993</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td>449</td>
<td>18,084</td>
</tr>
<tr>
<td></td>
<td>Prot A</td>
<td>929</td>
<td>19,577</td>
</tr>
<tr>
<td>PBL</td>
<td>Con A</td>
<td>12,827</td>
<td>163,203</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td>814</td>
<td>19,181</td>
</tr>
<tr>
<td></td>
<td>Prot A</td>
<td>-5</td>
<td>23,087</td>
</tr>
<tr>
<td>PALN</td>
<td>Con A</td>
<td>403,679</td>
<td>227,784</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td>24,871</td>
<td>23,162</td>
</tr>
<tr>
<td></td>
<td>Prot A</td>
<td>9,702</td>
<td>24,865</td>
</tr>
</tbody>
</table>

* Regression analysis of 3H-thymidine uptake data expressed as counts per minute (cpm) corrected for unsupplemented culture cpm. Data from solvent-treated group compared with data from CyA-treated group during the course of HSV keratitis representing days 3, 7, 14, and 21 postinfection.
† Intercept is the point at which the regression line crosses the y axis (cpm).
‡ Slope is the rate of change from intercept value over time (x axis = days postinfection).
§ Solvent-treatment intercept differs significantly (P < 0.01) from CyA-treatment intercept.

Statistical Analysis of Data

Linear regression analysis (SAS Institute, Inc., Cary, NC, data programs) of test counts per minute (cpm) values corrected for background unsupplemented cpm was used for statistical evaluation. Examination of the plots revealed that the data appeared linear over time. Thus the method of analysis best answers the question essential to this experiment, ie, whether there is a difference over time in the response of CyA-treated animals compared with the response of solvent-treated control animals. Although it is not the most traditional method of presenting mitogen response data, in this case it is the most effective because it allows all the data for a given mitogen to be used in the analysis and thereby increases the statistical power. Use of regression analysis also avoids the multiple comparison problem of performing several t-tests at the different time points. Regression analysis data is presented in table form in most instances. Probability values of 0.05 and lower were considered to represent significant differences.

Table 3. Effect of CyA on rabbit lymphocyte responses to B cell mitogens during the course of HSV keratitis*  

<table>
<thead>
<tr>
<th>Cell source†</th>
<th>Mitogen‡</th>
<th>Intercept§</th>
<th>Slope¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CyA</td>
<td>Solvent</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>Anti-IgG</td>
<td>14,868</td>
<td>95,414</td>
</tr>
<tr>
<td></td>
<td>Anti-IgM</td>
<td>5,184</td>
<td>53,896</td>
</tr>
<tr>
<td>PBL</td>
<td>Anti-IgG</td>
<td>2,725</td>
<td>69,076</td>
</tr>
<tr>
<td></td>
<td>Anti-IgM</td>
<td>585</td>
<td>28,237</td>
</tr>
<tr>
<td>PALN</td>
<td>Anti-IgG</td>
<td>63,852</td>
<td>51,039</td>
</tr>
<tr>
<td></td>
<td>Anti-IgM</td>
<td>44,763</td>
<td>33,592</td>
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</table>

* Regression analysis of 3H-thymidine uptake data expressed as counts per minute (cpm) corrected for unsupplemented culture cpm. Data from solvent treated group is compared with data from CyA-treated group during the course of HSV keratitis representing days 3, 7, 14, and 21 postinfection.
† Spleen, peripheral blood lymphocytes (PBL), preauricular lymph node (PALN).
‡ Anti-rabbit IgG 50 μg/ml, anti-rabbit IgM 200 μg/ml.
§ Intercept is the point at which the regression line crosses the y axis (cpm). Differ-ences in slope were negligible, except for Protein A responses which declined in the control group with time. PALN responses to Con A were strong in both groups, but with lower significance values. Differences in slope were negligible, except for Protein A responses which declined in the control group with time. PALN responses to Con A were strong in both groups. Responses were significantly higher in the CyA-treated group, but declined more rapidly with time than in the control group. PALN responses to Protein A were significantly greater in the control group, with both groups declining at the same rate over time. No difference in PALN response to PHA was noted, with response remaining steady throughout the experiment. In summary, SPL and PBL responses to T mitogens were markedly reduced in CyA-treated animals, while PALN responses were reduced to a much lesser extent.

Response to Anti-Rabbit Immunoglobulins

Lymphocyte response to anti-rabbit IgG and anti-rabbit IgM for the SPL, PALN, and PBL are shown in Table 3. SPL response to both anti-rabbit IgG and anti-rabbit IgM was significantly lower in CyA-
Table 4. Effect of CyA on proliferative responses of rabbit lymphocytes to HSV antigens on day 14 postinfection*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rabbit no.</th>
<th>CyA-treatment response to HSV stimulation $^\dagger$</th>
<th>Solvent-treatment response to HSV stimulation $^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm ± SE</td>
<td>SI $^\ddagger$</td>
</tr>
<tr>
<td>Spleen</td>
<td>1</td>
<td>482 ± 62</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>235 ± 17</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>607 ± 29</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1,236 ± 252</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>164 ± 28</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>338 ± 47</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td>510 ± 159$^\dagger$</td>
<td>0.51 ± 0.08$^\dagger$</td>
</tr>
<tr>
<td>PALN</td>
<td>1</td>
<td>2,344 ± 406</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,379 ± 145</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>254 ± 101</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td>1,533 ± 478</td>
<td>1.54 ± 0.45</td>
</tr>
</tbody>
</table>

*3H-thymidine incorporation in the presence of rabbit corneal cell-derived HSV antigens (1:30).
†Counts per minute (cpm) ± standard error for triplicate cultures for each individual rabbit.
‡Stimulation index (SI) = cpm in presence of antigen divided by cpm in the presence of medium only.
§ND = not determined.
$^*$P < 0.05, possibly significant differences.

Response to HSV Antigens

Individual responses to RCC-derived HSV antigen are shown in Table 4. Regression analysis of results for all HSV antigens tested show that the response to HSV antigens increased with time after infection in the control group but did not increase appreciably in the CyA-treated animals. PBL response to anti-IgG and anti-IgM was significantly lower in the CyA-treated animals. PBL response to anti-IgG did not decline during the course of infections in either group. PALN responses to anti-IgG and anti-IgM were strong in both groups, with no statistically significant difference between the two. Both groups demonstrated a significant decline in response during the course of infection. In summary, SPL responses to both anti-IgG and anti-IgM were markedly reduced, and PBL response to anti-IgG and anti-IgM was reduced significantly. The PALN responses of the CyA-treated group to both anti-IgG and anti-IgM were not significantly reduced at any times.

Discussion

CyA treatment of animals with primary HSV keratitis resulted in more severe and persistent ocular disease than that seen in solvent-treated control animals with primary HSV keratitis. We found that systemic CyA potentiated stromal disease, but we did not see any evidence of CyA increasing the severity of epithelial disease. This is in contrast to results of a study on prophylactic topical CyA therapy in an experimental...
rabbit model of immunogenic herpetic keratitis, which involved systemic presensitization of rabbits with HSV prior to corneal intrastromal challenge with live HSV. In that study, the severity of stromal disease was found to be decreased in eyes treated with combined cyclosporine-trifluridine, and CyA was found to increase the severity of epithelial disease when antiviral coverage was not provided.29

The high incidence of mortality in the CyA-treated animals was unexpected, since other investigators have used CyA in rabbits at comparable doses without mortality.30,31 Virus-infected animals may be especially sensitive to the cytotoxic effects of CyA (J. F. Borel, personal communication). We observed symptoms of severe gastrointestinal cytotoxicity similar to those previously reported in rabbits,33 as well as symptoms and histopathologic nephrotoxicity consistent with renal toxicity. Cyclosporine has been reported to impair natural resistance of mice and to increase the susceptibility to a fatal HSV type 2 infection.35 An otherwise mild vesicular stomatitis virus infection in mice, which are not a natural host for this virus, became a fatal infection when these animals were given CyA.34 Increased mortality may be due to CyA depressing cellular immunity, impairing viral clearance by acting on macrophage processing,35 or by inhibiting interferon production.36,37 It is clear that more work is needed in understanding the action of CyA in viral infections.

Cyclosporine A has a number of well-described immunosuppressive effects, including the inhibition of T cell proliferation and lymphokine production.3 The data presented here confirm and extend other studies on the inhibition by CyA of T lymphocyte responses and also the effects of CyA on B lymphocytes.38,39 We found T cell function as measured by responses to T cell mitogens was significantly reduced (P < 0.01) in CyA-treated HSV-infected animals compared with solvent-treated HSV-infected animals, but was not eliminated entirely. At clinically useable doses of CyA, there may be little effect on these responses. Responses to viral antigens were also reduced in CyA-treated animals compared with solvent-treated animals, with P < 0.05 significance at days 7 and 14 postinfection for SPL cells, and observed differences for PALN that were not statistically significant.

An unexpected finding was the marked reduction in response to anti-rabbit immunoglobulins, a response used to measure B cell activity in the rabbit.28 Rabbit T cells may bear surface immunoglobulins40,41 and might therefore be affected by this assay. Marked reduction in responses in the CyA group to anti-rabbit immunoglobulins were noted for SPL and PBL but not for PALN. The lack of a mitogen that specifically activates rabbit B cells without prior sensitization prevented any further clarification of this response, since reagents cited by other investigators42 were not available. Rabbit lymphocytes do not respond well to LPS, and reagents such as Protein A, which stimulates B cells in other species, have been shown to be T mitogens in the rabbit.43 Studies conducted by Lindsey et al40 indicate that CyA administered at the same dose and by the same method used in our study entirely abrogated the primary antibody response to human serum albumin in rabbits, and that significant levels of antigen remained present during the 20-day assay period. Since the secondary immune response was not affected by CyA, it was concluded that CyA at this dose, but not at lower doses, probably acted on the T helper cells required to initiate the T and B cell collaboration required for primary antibody response in the rabbit.30 The continued presence of antigen noted in their study would seem to correlate well with the extended recovery of virus noted in our study, and supports the findings that the reduction of response to anti-rabbit immunoglobulins and viral antigens may be related to effects on T helper function. Nevertheless, a strong possibility remains that rabbits may have a CyA-sensitive B cell population such as that described in the mouse system by Kunkl and Klaus.44 Oh et al45 reported that CyA treatment of immune rabbits which had recovered from an intravitreally-induced herpetic uveitis suppressed induction of a secondary herpetic uveitis following intravitreal challenge with UV-inactivated HSV antigens. We cannot compare results from that study with ours, since this is a secondary immunogenic uveitis model, but our results would argue against the use of CyA for the prevention and treatment of immune-mediated herpetic uveitis.

In summary, CyA is an effective immunosuppressive agent in the experimental HSV keratitis model, but further studies are required to identify the cell populations affected by CyA treatment as well as the in vivo immune responses of CyA-treated rabbits against HSV antigens. Attempts to try to reduce the high mortality rate in the rabbit undergoing CyA treatment during herpetic keratitis are required. If these objectives can be attained, an effective tool will be available to clarify the complex cellular interactions seen in primary HSV keratitis. Our study has shown that CyA-sensitive cells are involved in limiting virus replication, and that CyA treatment results in a more severe disease course. Additional studies will help clarify the roles of specific subpopulations of
T or B cells which control aspects of immune or immunopathologic responses in HSV keratitis.

Key words: herpes simplex virus, herpetic keratitis, cyclosporine A, immunosuppression, rabbit, cellular immune response, T cells, B cells

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