Suppression of Secondary Herpes Simplex Uveitis by Cyclosporine

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The authors studied the effect of an immunosuppressive agent, cyclosporine (CyA), on experimental secondary herpes simplex (HS) uveitis. Secondary HS uveitis was induced in a rabbit eye that had recovered from primary HS uveitis by challenging it with an intravitreal injection of herpes simplex virus (HSV) antigen. Daily intramuscular injections of CyA (25 mg/kg body weight) for 7 days prior to the intravitreal challenge with HSV antigen significantly suppressed the induction of secondary HS uveitis, but daily injections of CyA after the challenge with HSV antigen was ineffective. Intravitreal injections of CyA (5 mg) 7 days and 3 days prior to the HSV challenge were less effective, but the combined treatment with seven daily intramuscular CyA and two intravitreal CyA injections prior to the HSV challenge was most effective in the prevention of the uveitis. The daily intramuscular treatment with CyA resulted in a marked reduction of cell-mediated immunity while leaving the level of circulating HSV specific antibody high. No reactivation of latent HSV was detected in trigeminal and superior cervical ganglia of CyA-treated rabbits.


Our previous studies indicated that secondary herpes simplex (HS) uveitis is mediated by immunologic mechanisms\(^\text{1}\) and that the interaction of herpes simplex virus (HSV) antigen, HSV-sensitized lymphocytes, and HSV-specific antibody is essential in the pathogenesis of the disease.\(^\text{2}\) However, cyclophosphamide, a known immunosuppressant, failed in our previous study to modify the secondary HS uveitis, and this failure was thought to be due in part to its ineffectiveness in modifying the host’s immune system.\(^\text{3}\)

Cyclosporine (CyA) is a new fungal cyclic polypeptide which has been shown to be a potent immunosuppressive agent. It can be distinguished from conventional immune suppressants, such as cyclophosphamide, because of its selective inhibitory effect on T-cells,\(^\text{4}\) and because it causes minimum side effects at therapeutic dosages in laboratory animals.\(^\text{5,6}\) Furthermore, Borel et al\(^\text{5}\) and Thomson et al\(^\text{7}\) each reported the suppressive effects of CyA on secondary immune responses in vivo. We therefore investigated the effects of CyA on clinical, immunologic, and virologic aspects of secondary HS uveitis in rabbits.

Materials and Methods

Experimental Animals

New Zealand white male rabbits weighing approximately 2 kg each were used in the study. Before the experiment, none of them had any ocular anomalies or antibody against HSV. The investigations utilizing these rabbits, as described in this article, conform to the ARVO Resolution on the Use of Animals in Research.

Virus

Shealey strain of type 1 HSV was grown in primary cultures of rabbit kidney cells maintained in medium 199 with 5% rabbit serum and antibiotics. It had a titer of \(10^8\) plaque forming units (PFU) per milliliter.

Herpes Simplex Virus Antigen

The Shealey strain of type 1 HSV, prepared as above, was partially purified by a differential centrifugation as described previously.\(^\text{8}\) The partially purified HSV was inactivated by ultraviolet (UV) light in a 35-mm tissue culture plate with 1 ml volume; the virus suspension was exposed to a G 15T8 germicidal lamp (Sylvania Electric Products, Inc.; Salem, MA)

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at a distance of 10 cm for 10 min with occasional agitations. No infectious HSV could be recovered from UV-inactivated HSV antigen after inoculation into monolayer cultures of Vero cells.

Rabbit Kidney Antigen

Primary rabbit kidney cells were maintained in medium 199 with 5% rabbit serum and antibiotics for 5 days at 37°C, and the culture medium was collected. It then was subjected to the differential centrifugation, the pellet was resuspended in PBS and was UV-irradiated in the same manner as for the preparation of HSV antigen. Rabbit kidney antigen was used as a control antigen in the transformation assay for lymph node cells by HSV antigen, since primary rabbit kidney cell culture was used for cultivation of HSV and for the preparation of HSV antigen.

Cyclosporine (CyA)

CyA was kindly provided by Dr. J. F. Borel (Sandoz Ltd.; Basel, Switzerland). It was dissolved in Miglyol 810 (Dynamit Nobel AG, Germany), a semisynthetic neutral oil, and sterilized by filtering through a milipore filter.

Virus Isolation

The conjunctival swabs were shaken vigorously in the medium consisting of Eagle's minimum essential medium (MEM) with 5% fetal calf serum and antibiotics, and the medium was placed in Vero cell culture tubes. Ocular tissue and ganglia were homogenized with a mortar and pestle, and the supernatant fluid was inoculated into Vero cell tubes. The tubes were incubated at 36°C and examined daily for 14 days for cytopathic effects (CPE). The virus isolates were confirmed as HSV by neutralization test with HSV-1 specific antiserum.

Neutralizing Antibody Titration

As in our previous work, we mixed 0.5 ml of serial two-fold dilutions of unheated serum with 0.5 ml of HSV (Shealey strain) containing about 100 PFU. After keeping this mixture at room temperature for 1 hr, we inoculated 1 ml of the mixture into tubes of Vero cells and incubated the tubes at 36°C for 7 days. The titer of neutralizing antibody was the reciprocal of the highest dilution of the serum that inhibited HSV-induced CPE completely.

Transformation Assay of Lymph Node (LN) Cells

The incorporation of (³H) thymidine into acid-insoluble material was used to measure the degree of LN cell transformation by HSV antigen. One-tenth milliliter of HSV antigen (1:2 dilution) or rabbit kidney antigen (1:2 dilution) was added to each of the duplicate LN cell cultures (10⁶ cells/tube) and the cultures were incubated at 36°C for 72 hr in an atmosphere of 5% CO₂ and 95% air. As determined by the dye-exclusion method, 95–97% of LN cells were viable. The culture medium for LN cells was nine parts RPMI 1640 medium and one part of fetal calf serum fortified with antibiotics. One μCi of (³H) thymidine was added to each culture for the final 4-hr incubation. The cells then were washed once with 3 ml of PBS, twice with 3 ml of 5% trichloroacetic acid, and once with 3 ml of absolute methanol. The resulting precipitates were dissolved at 50°C for 30 min in 0.2 ml NCS (Amersham-Searle Corp.; Arlington Heights, IL), and 3 ml of Ready-Solve HP scintillation fluid (Beckman Instruments; Palo Alto, CA) was added. Samples were counted in a Beckman LS 100C liquid scintillation spectrophotometer. Results are expressed as the ratio (stimulation ratio) of cpm of (³H) thymidine incorporated in the presence of HSV antigen divided by cpm incorporated in the presence of rabbit kidney antigen.

Induction of Secondary Herpes Simplex (HS) Uveitis

As in our previous studies, secondary HS uveitis was produced in rabbits as follows. Both eyes of normal rabbits received an intravitreal injection of live HSV suspension containing about 10⁴ PFU. Primary HS uveitis developed within 2 days and healed completely in 30 days. On day 31, both eyes were challenged with an intravitreal injection of HSV antigen to produce secondary HS uveitis.

Evaluation of Uveitis

Eyes were examined by slit-lamp biomicroscopy before the intravitreal inoculation and daily thereafter by a person who had no knowledge of the CyA treatment schedule. The uveal inflammation was scored according to the criteria of Hogan et al with some modification; flare and cells in the anterior chamber were each graded from 0 (no reaction) to 3 (maximum).
Fig. 1. Effect of intramuscular injections of CyA on the induction of secondary herpes simplex uveitis (postchallenge treatment). Daily intramuscular injections of CyA (25 mg per kg body weight) were given immediately after the challenge with HSV antigen. Control animals (no CyA) received daily intramuscular injections of Miglyol 810, a semisynthetic neutral oil, which was used as a solvent for CyA. Numbers indicate number of eyes with reaction/number of eyes tested.

Results

Effects of Systemic CyA on the Induction of Secondary Herpes Simplex Uveitis

Systemic CyA treatment after challenge with HSV antigen: Ten rabbits whose eyes recovered from primary HS uveitis were challenged with intravitreal injection of HSV antigen into both eyes to produce secondary HS uveitis. Immediately they were divided into two groups of five rabbits each, a test group and a control group; the rabbits in the test group received an intramuscular injection of CyA, 25 mg/kg body weight daily, while the control rabbits received a daily intramuscular injection of Miglyol 810 (a solvent for CyA). Eyes were checked for flare and cells in the anterior chamber with a slit lamp each day for 8 consecutive days.

As shown in Figure 1, there was no significant difference between CyA-treated group and control group in the induction of secondary HS uveitis (P > 0.1).

Systemic CyA treatment prior to challenge with HSV antigen: Rabbits whose eyes recovered from primary HS uveitis were divided into two groups, a test group (15 eyes) and a control group (13 eyes). Then rabbits in the test group received an intramuscular injection of CyA, 25 mg/kg body weight daily, for 7 days, while the control rabbits received an intramuscular injection of Miglyol 810 daily, for 7 days. Upon completion of seven daily CyA or Miglyol injections, all the eyes of both groups were challenged intravitreally with HSV antigen to produce uveitis.

In control animals, flare appeared in all eyes within 24 hr after the challenge and persisted throughout the experimental period. Mean values of flare in the control rabbits were 1.6 on day 1–3 (Fig. 2). On the other hand, although flare was observed in many eyes of the CyA-tested rabbits on days 1 and 2, it was very mild, and no flare was observed in nine of 15 eyes on day 3. Mean values of flare in the CyA-treated group were 0.8 on day 1 and 0.6 on days 2 and 3; thus, they were significantly less than those of the controls (P < 0.05).

Cells were observed in the anterior chambers of all the eyes of control rabbits on days 2 and 3, while cells were present in only four of 15 eyes of the CyA-treated group on the same time period. The degree of cell infiltration into the anterior chamber was milder in the eyes of CyA-treated rabbits compared to that in the control group; mean values of only 0.3, 0.3 and 0.5 were obtained on days 1–3, respectively, in CyA-treated rabbits, while mean values of 1.4, 1.8 and 1.9 were noted in the control (P < 0.01) (Fig. 2).

The eyes of rabbits treated with CyA, however, became fully responsive to HSV antigen 2 weeks after the cessation of daily CyA injections.

Effect of Systemic CyA on Immune Responses and Virus Shedding in the Rabbit which Recovered from Primary Herpes Simplex Uveitis

Fourteen rabbits that had recovered from primary HS uveitis were divided into two groups of seven rabbits each, a test group and a control group, as in the preceding experiment. Blood was collected from all the rabbits to obtain a pre-CyA treatment titer of neutralizing antibody. Then rabbits in the test group received an intramuscular injection of CyA, 25 mg/kg body weight daily, for 7 days, while the control rabbits received an intramuscular injection of Miglyol 810 daily, for 7 days. To investigate whether daily CyA or Miglyol injections induce shedding of HSV
to tear, cultures of conjunctival swabs for HSV isolation also were made daily. Upon completion of seven daily CyA or Miglyol injections, blood was collected again for posttreatment antibody titers. Animals were killed and the eyes and both trigeminal and superior cervical ganglia were dissected out for virus isolation. Cervical lymph nodes were also obtained for lymphocyte transformation assay. Vitreous humor was collected for neutralizing antibody assay.

In the control rabbits, a high degree of lymphocyte transformation was observed following stimulation with phytohemagglutinin (mean stimulation index [SI] of 23.9) as well as with HSV antigen (mean SI of 16.1), and no significant changes in neutralizing antibody titers were noted between those obtained before and after the injection of Miglyol (Table 1). On the other hand, the CyA treatment resulted in a marked depression of lymphocyte transformation with both phytohemagglutinin (SI = 2.8) and HSV antigen (SI = 3.0). However, CyA treatment had no significant effect on neutralizing antibody titers in blood or vitreous humor (Table 1).

No infectious HSV could be isolated from conjunctival swabs or from tissue homogenates of eyes. Tissue homogenates of trigeminal or superior cervical ganglia of either CyA-treated or control rabbits also failed to yield virus.

**Effect of Local, Systemic or Combination of Local and Systemic CyA on the Induction of Secondary Herpes Simplex Uveitis**

Two groups of seven rabbits each which had recovered from primary herpetic uveitis were subjected to the following treatments: One group received an intramuscular injection of CyA (25 mg/kg body weight) daily, for 7 days. Rabbits in the other group received one intramuscular injection of Miglyol (solvent for CyA) daily, for 7 days. In addition, the right eyes of all the rabbits in both groups were injected intravitreally with 5 mg of CyA in a volume of 0.05 ml on days 0 and 4, and the left eyes of all the rabbits were injected intravitreally with 0.05 ml of Miglyol in the same manner. On day 8, both eyes of all the rabbits in both groups were challenged with the intravitreal injection of HSV antigen, and the eyes were examined daily with a slit lamp for anterior chamber reactions.

As shown in Figure 3, both flare and cells in the eyes which were pretreated with intravitreal injections of CyA appeared to be less severe, and to occur less frequently than in the eyes pretreated with intravitreal injections of Miglyol. However, the differences are not significant (P > 0.05). On the other hand, the combined treatment with intravitreal and intramuscular injections of CyA completely suppressed secondary herpetic uveitis in all rabbits, showing that this treatment is far more effective than either intramuscular or intravitreal injections of CyA alone (P < 0.01). No toxic reaction was noted in the eye following intravitreal injections of CyA.

**Discussion**

Recent studies have shown that CyA was an effective immunosuppressive agent capable of inhibiting a variety of primary immune responses in vivo as well as in vitro, and that S-antigen-induced au-
Table 1. Immune responses in CyA-treated rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Rabbit no.</th>
<th>Stimulation index</th>
<th>Serum antibody</th>
<th>Antibody in vitreous tumor</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PHA</td>
<td>HSV</td>
<td>Pretreatment</td>
</tr>
<tr>
<td>CyA</td>
<td>1</td>
<td>0.9</td>
<td>2.0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.0</td>
<td>0.8</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
<td>4.1</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>1.7</td>
<td>2.6</td>
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</tr>
<tr>
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<td>5.3</td>
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<td>80</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8.3</td>
<td>2.9</td>
<td>80</td>
</tr>
<tr>
<td>Miglyol 810 (Control)</td>
<td>1</td>
<td>15.8</td>
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<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.0</td>
<td>8.0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.8</td>
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<td>35.6</td>
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</table>

Our rabbit model of secondary HS uveitis has the following characteristics for an immune-mediated disease: (1) secondary uveitis developed immediately after intraocular injection of either live HSV or HSV antigen; (2) following intraocular injection of live HSV, no infectious virus could be isolated from the eye with secondary uveitis at any time suggesting noninfectious nature of the disease; and (3) secondary uveitis could be induced by inactivated HSV but not by antigenically unrelated virus, eg, inactivated vaccinia virus indicating HSV-specific nature of the reaction. Utilizing this rabbit model, we have shown that pretreatment of the rabbit with CyA effectively suppressed the induction of secondary HS uveitis. The combined treatment with local and systemic administrations of CyA was most effective. The suppression of the uveitis by pretreatment of systemic CyA was closely associated with the depressed cell-mediated immunity, namely low stimulation index.
of lymphocytes against phytohemagglutinin as well as HSV antigen, while circulating HSV specific antibody was not affected by the CyA treatment. The suppressive effect of CyA on the secondary HS uveitis, however, appears to be dependent on its continuous administration. The eyes of rabbits treated with CyA became fully reactive to HSV antigen 2 weeks after the cessation of daily CyA administration. Others also have shown that skin allografts are retained by CyA-treated animals only as long as the drug is administered.14-16

It is interesting that secondary HS uveitis was significantly suppressed in this animal model only if the animal was treated with CyA prior to the challenge with HSV antigen. A similar observation was made by Borel et al5 who reported a suppressive effect of CyA on secondary responses to oxazolone contact sensitivity only when the animal was treated with CyA prior to the antigen challenge. We do not yet know the mechanisms through which pretreatment of these animals with CyA suppresses the induction of secondary HS uveitis. There is general agreement that many in vivo effects of CyA stem from this modification of T-cell function. However, the mechanism of its action on secondary immune responses is still unknown. Our present study shows that the suppression of secondary HS uveitis is associated with the depletion of cell-mediated immune responses. Hess et al17 demonstrated that the inhibition of secondary mixed lymphocyte response by CyA appears to be mediated in part by the direct impairment of T-lymphocyte stimulating growth factors. There is also in vitro evidence that both antigen-specific and antigen-nonspecific suppressor T-cells can be generated in the presence of CyA.18,19 Therefore it may be possible that the suppressor T-cells generated in vivo by CyA may create a tolerance mechanism in vivo. Another possible mechanism may be that the memory cells are inhibited by CyA as suggested by Borel et al who observed the depression of the secondary responses by CyA in oxazolone hypersensitivity.5 It remains to be determined whether such effects of CyA indeed play any role in the suppression of secondary HS uveitis in CyA-treated rabbits.

Although intravitreal injection of CyA alone had no significant suppressive effect on secondary HS uveitis, it appears to exert an additive effect on systemic CyA, since the combined treatment of intravitreal and intramuscular CyA was much more effective than any of these routes alone in the suppression of the uveitis. The additive effect of intravitreal CyA need not be due to a specific effect on the antigen induction of T-lymphocytes. It has been noted that CyA in amounts as small as 10^{-5} \text{ mol/L}^{-1} damaged resting human lymphocytes in vitro20 and inhibited the production of T-cell growth factors in cultures of murine spleen cells.21 Such nonspecific local lymphocytotoxic effects of CyA might have played a role in the local administration of highly concentrated CyA solution (5 mg in our study) and could be responsible for the results obtained. The suppressive effects of local CyA on corneal allograft responses22 and herpetic disciform keratitis23 also have been reported by others, yet its mechanisms are unknown.

Patients treated with immunosuppressive drugs are prone to recurrent HSV infection, and it also has been shown in experimental animals that reactivation of latent HSV infections could be induced readily by treatment with immunosuppressive agents.24 However, in the present study no reactivation of latent HSV was noted in any rabbits treated with CyA, even when they were immunosuppressed. Because of its low toxicity in vivo, and because it causes no reactivation of latent HSV infection, CyA appears to be a promising immunosuppressive drug for the prevention and treatment of immune-mediated HS uveitis and other related diseases.

**Key words:** secondary uveitis, herpes simplex virus, cyclosporine, immunosuppression, rabbit

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### References