Topical Cyclosporine Inhibits Mast Cell-Mediated Conjunctivitis

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Purpose. Allergic conjunctivitis is a common condition characterized by itching, hyperemia, and chemosis. The disorder usually is triggered by airborne allergens such as pollens and molds. Studies on the pathogenesis of allergic conjunctivitis suggest that the mast cell is critical for the development of the condition.1,2 Allergens dissolve in the tear film and bind to the immunoglobulin E attached to mast cells. This leads to a type I hypersensitivity reaction induced by mast cell degranulation and a release of inflammatory mediators, including histamine, platelet-activating factor, eosinophil chemotactic factors, and prostaglandins. Treatment for this disorder includes antihistamines, vasoconstrictors, corticosteroids, and mast cell stabilizing agents; however, many patients are intolerant of or resistant to these therapies.

Recently, cyclosporine has been shown not only to inhibit the release of mast cell mediators, such as histamine, but to suppress mast cell-leukocyte cytokine cascades.3,4 The purpose of this study was to investigate the effect of topical cyclosporine A (CsA) on an animal model of allergic conjunctivitis induced by compound 48/80. Compound 48/80 is a condensation product of formaldehyde with paramethoxyphenylethylamine that reliably induces the release of chemical mediators in the mast cell granules. Clinically, conjunctival erythema, chemosis, and mucous discharge develop from 15 to 60 minutes after the instillation of compound 48/80, and these clinical findings consistently correlate with histologic findings of conjunctival infiltration with neutrophils, macrophages, lymphocytes, and eosinophils.5

METHODS

Animals

Female C57BL/6 mice, 6 to 8 weeks of age, were obtained from Charles River (Raleigh Durham, NC) and...
TABLE I. Infiltrating Cells, Mast Cells, and Goblet Cells in the Conjunctivea

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Goblet Cells</th>
<th>Mast Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PBS (n = 10)</td>
<td>158.4 ± 24.5</td>
<td>2.2 ± 0.6</td>
<td>9.4 ± 4.6</td>
<td>4.6 ± 1.3</td>
</tr>
<tr>
<td>CsA 0.05% (n = 10)</td>
<td>34.2 ± 5.2</td>
<td>0.2 ± 0.1</td>
<td>14.4 ± 4.9</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>CsA 0.2% (n = 10)</td>
<td>55.8 ± 20.7</td>
<td>0.6 ± 0.3</td>
<td>11.0 ± 2.7</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>CsA 0.4% (n = 8)</td>
<td>29.2 ± 11.8</td>
<td>0.5 ± 0.3</td>
<td>22.0 ± 2.4</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>Pred (n = 10)</td>
<td>18.4 ± 3.0</td>
<td>0.2 ± 0.1</td>
<td>11.2 ± 2.2</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS (n = 3)</td>
<td>60.7 ± 15.3</td>
<td>1.2 ± 0.7</td>
<td>9.5 ± 2.6</td>
<td>8.5 ± 3.0</td>
</tr>
<tr>
<td>CsA 0.05% (n = 3)</td>
<td>13.3 ± 3.9</td>
<td>0.2 ± 0.2</td>
<td>24.5 ± 4.9</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>CsA 0.2% (n = 3)</td>
<td>6.2 ± 0.7</td>
<td>0.2 ± 0.2</td>
<td>37.8 ± 14.9</td>
<td>5.8 ± 1.4</td>
</tr>
<tr>
<td>CsA 0.4% (n = 2)</td>
<td>11.0 ± 0.5</td>
<td>0.2 ± 0.2</td>
<td>22.2 ± 1.8</td>
<td>3.2 ± 1.8</td>
</tr>
<tr>
<td>Pred (n = 3)</td>
<td>6.8 ± 1.3</td>
<td>0.05 ± 0</td>
<td>21.0 ± 6.0</td>
<td>3.5 ± 0.6</td>
</tr>
</tbody>
</table>

PBS = phosphate-buffered saline; CsA = cyclosporine A; Pred = prednisolone acetate 1%.
*a Data are mean ± standard error.

were kept under standard pathogen-free conditions. All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of Allergic Conjunctivitis**

Mice were anesthetized with methoxylurane inhalation (Metofane; Pitman–Moore, Mundelein, IL). Allergic conjunctivitis was induced by the instillation of 2 mg of compound 48/80 (Sigma Chemical, St. Louis, MO) dissolved in 5 µl of phosphate-buffered saline (PBS) into both eyes as previously detailed.6

**Treatment Protocol**

In two separate experiments (n = 48 for experiment 1, and n = 14 for experiment 2), animals were treated every 6 hours with topical CsA oil–water emulsion

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**FIGURE 1.** Number of infiltrating neutrophils. (A) Experiment 1. Compared to phosphate-buffered saline (PBS)-treated controls (n = 10), there was a significant decrease in infiltrating neutrophils into the conjunctiva of animals treated with topical cyclosporine A (CsA) 0.05% (n = 10), CsA 0.2% (n = 10), CsA 0.4% (n = 8), or prednisolone acetate (Pred) (n = 10). *P < 0.0001. (B) Experiment 2. Again, compared to PBS-treated controls (n = 3), there was a significant decrease in infiltrating neutrophils into the conjunctiva of animals treated with topical CsA 0.05% (n = 3), CsA 0.2% (n = 3), CsA 0.4% (n = 2), or pred (n = 3). *P = 0.0006; **P = 0.0015; ***P = 0.0023. Bars = standard error.
(0.05%, 0.2%, or 0.4%; Allergan, Irvine, CA), prednisolone acetate (pred) 1% (pred forte; Allergan), or PBS; each animal received drops 0, 6, 12, and 18 hours after compound 48/80 administration.

**Histologic Analysis**

Mice were killed 24 hours after compound 48/80 administration. The lower lid tarsal and bulbar conjunctiva attached to the right eye were removed and fixed with glutaraldehyde 4% for 30 minutes and then transferred into 10% buffered formalin for 24 hours. The conjunctiva and the globe were embedded in methacrylate, and 4-μm vertical sections through the pupillary–optic nerve head plane were stained with hematoxylin and eosin for neutrophils and eosinophils, periodic acid–Schiff for goblet cells, and toluidine blue.
for mast cells. The number of infiltrating neutrophils, eosinophils, preserved goblet cells, and undegranulated or partially degranulated mast cells were counted by a masked observer.

The lower lid tarsal and bulbar conjunctiva attached to the left eyes from animals treated with topical CsA, pred, or PBS were removed, embedded in optimum cutting temperature (OCT) compound (Miles Laboratory, Naperville, IL), and immediately snap frozen in a dry ice and methylbutane bath. Six-micrometer vertical (pupillary-optic) frozen sections were prepared on lysozyme-coated glass slides, and immunohistochemical staining was performed using an avidin–biotin–complex technique. Primary antibodies included mouse monoclonal antibodies against Lyt2 (CD8+ T lymphocytes), L3T4 (CD4+ T lymphocytes) (Becton Dickinson, Mountain View, CA), and M1/70.15 (macrophages) (Sera Laboratory, Westbury, NY). Rat immunoglobulin G (Sigma Chemical, St. Louis, MO) was used as the control primary antibody, and biotin-conjugated goat anti-rat immunoglobulin G (American Qualex, La Mirada, CA) was used as the secondary antibody. Avidin–biotin–peroxidase complex (Vector Laboratory, Burlingame, CA) was applied, and sections were developed in diaminobenzenedine. The number of infiltrating lymphocytes and macrophages per histologic section were counted by a masked observer.

**Statistical Analysis**

To control for multiple comparisons, the number of infiltrating neutrophils, eosinophils, preserved goblet cells, and undegranulated mast cells in the conjunctiva of animals treated with the three doses of topical CsA, pred, or PBS were compared using analysis of variance and the Fisher's protected least significant difference test. The number of infiltrating lymphocytes and macrophages between animals treated with topical CsA 0.4% or PBS were compared using an unpaired t-test. The null hypothesis that there was no difference in the number of cells between groups was rejected at a $P < 0.05$.

**RESULTS**

The numbers of infiltrating neutrophils and eosinophils, goblet cells, and undegranulated mast cells are listed in Table 1. Figure 1 shows the effect of topical CsA therapy on neutrophil infiltration into the conjunctiva. In the first experiment, compared to PBS-

**FIGURE 4.** Number of preserved goblet cells. (A) Experiment 1. Compared to phosphate-buffered saline (PBS)-treated controls ($n = 10$), there was an increase in preserved goblet cells in the conjunctiva of animals treated with topical cyclosporine A (CsA) 0.05% ($n = 10$), CsA 0.2% ($n = 10$), CsA 0.4% ($n = 8$), or prednisolone acetate (Pred) ($n = 10$). $*P = 0.0240$. (B) Experiment 2. Again, compared to PBS-treated controls ($n = 3$), there was an increase in preserved goblet cells in the conjunctiva of animals treated with topical CsA 0.05% ($n = 3$), CsA 0.2% ($n = 3$), CsA 0.4% ($n = 2$), or pred ($n = 3$). $*P = 0.034$. Bars = standard error.
treated animals, treatment with topical CsA at all three doses or with pred significantly reduced the number of infiltrating neutrophils into the conjunctiva 24 hours after compound 48/80 instillation ($P < 0.0001$ for each treatment group) (Fig. 1A). There was no statistically significant difference in the number of infiltrating neutrophils between animals treated with any dose of topical CsA and animals treated with pred. In addition, there was no statistically significant difference in the number of infiltrating neutrophils among the three doses of topical CsA. These results were confirmed when the experiment was repeated (Fig. 1B). Again, treatment with all three doses of topical CsA or with pred significantly reduced the number of infiltrating neutrophils. Figure 2 shows representative histologic sections of the conjunctiva from an animal treated with PBS (Fig. 2A) and with CsA 0.4% (Fig. 2B). There is not only less neutrophil infiltration in the CsA-treated eye but also less chemosis and better preservation of the normal conjunctival architecture.

Similar treatment effects were found on infiltrating eosinophils (Fig. 3). In the first experiment, treatment with all three doses of topical CsA and with pred significantly reduced the number of infiltrating eosinophils into the conjunctiva ($P = 0.001$ for each treatment group) (Fig. 3A). Again, no statistical differences were found between the treatment effect of the three doses of topical CsA compared with each other or with pred. These results were confirmed when the experiment was repeated (Fig. 3B), although, because of the small numbers of animals, only the decrease in infiltrating eosinophils in the pred-treated group reached statistical significance ($P = 0.038$).

Figure 4 shows the number of goblet cells identified in the conjunctiva. In the first experiment, there was a trend toward increased preservation of goblet cells in animals treated with topical CsA or pred (Fig. 4A). The greatest number of preserved goblet cells was found in animals treated with the highest dose of topical CsA (0.4%). The increase in goblet cells compared to PBS-treated animals was statistically significant only for the CsA 0.4% group ($P = 0.02$). In the second experiment, there was again a trend toward increased goblet cell preservation in animals treated with topical CsA or pred (Fig. 4B). The increase in goblet cells compared to PBS-treated animals reached statistical significance only for the topical CsA (0.2%) group ($P = 0.034$).

The number of undegranulated or partially degranulated mast cells remaining in the conjunctiva...
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FIGURE 6. Number of infiltrating lymphocytes. Compared to phosphate-buffered saline (PBS)-treated controls (n = 4), there was a significant decrease in the number of infiltrating lymphocytes in the conjunctiva of animals treated with topical cyclosporine A (CsA) 0.05% (n = 3), CsA 0.2% (n = 5), CsA 0.4% (n = 4), or prednisolone acetate (Pred) (n = 4). *P = 0.0033; **P = 0.0091; ***P = 0.0192; ****P = 0.03. Bars = standard error.

FIGURE 7. Number of infiltrating macrophages. Compared to phosphate-buffered saline (PBS)-treated controls (n = 5), there was a small decrease in the number of infiltrating macrophages in the conjunctiva of animals treated with the higher doses of topical cyclosporine A (CsA): CsA 0.2% (n = 3) and CsA 0.4% (n = 4). There was no difference in infiltrating macrophages in animals treated with CsA 0.05% (n = 2) or prednisolone acetate (Pred) (n = 4). *P = 0.0448. Bars = standard error.

decrease, *P = 0.0033; **P = 0.0091; ***P = 0.0192; ****P = 0.03.

Bars = standard error.

were counted to ensure an equal degranulating effect of compound 48/80 in each group (Fig. 5). In the first experiment, there was no difference in mast cells counted in the conjunctiva of animals treated with PBS, topical CsA, or pred (Fig. 5A). These findings were confirmed in the second experiment; no statistically significant differences in mast cells were found in any group (Fig. 5B).

Immunohistochemical staining of the conjunctiva showed a statistically significant decrease in the number of infiltrating lymphocytes in animals treated with topical CsA or pred when compared to controls (Fig. 6). There also appeared to be a decrease in the number of infiltrating macrophages in animals treated with the higher doses of topical CsA (0.2% and 0.4%) compared to pred-treated animals and PBS-treated controls (Fig. 7), although the differences were not statistically significant.

DISCUSSION

Our data show that the topical application of cyclosporine effectively inhibits a mast cell-mediated model of allergic conjunctivitis. Cyclosporine reduced the number of neutrophils, eosinophils, and lymphocytes infiltrating the conjunctiva 24 hours after compound 48/80 instillation. There was also a trend toward decreased infiltration of macrophages in CsA-treated animals, although the results did not reach statistical significance. Furthermore, CsA appeared to improve the preservation of goblet cells, consistent with the finding of decreased inflammation.

We also assessed the number of undegranulated and partially degranulated mast cells in each section of conjunctiva to assure an equal effect of compound 48/80. Compound 48/80 has been shown to induce a predictable degranulation response of mast cells. Maximal degranulation occurs within the first hour of a single topical dose of compound 48/80, leading to full degranulation in approximately 31% of mast cells.8 In our study, there was no difference in the number of undegranulated or partially degranulated mast cells in any of the treatment groups or controls, suggesting an equal degranulating effect of compound 48/80.

Although there was not a well-defined dose-response relationship between the concentration of CsA used and the number of infiltrating cells, this may be explained by the efficacy of even the lowest concentration of the drug used in this study (0.05%). Future
studies will investigate the therapeutic effect of lower doses of CsA. Of note, there was no statistical difference between the effects of CsA and prednisolone acetate on mast cell-mediated conjunctivitis at all three doses. Because topical corticosteroids remain the mainstay of therapy, it is important for any drug tested for allergic ocular disease to have an effect similar to corticosteroids.

However, although corticosteroids are used frequently for the treatment of chronic allergic disorders, many patients are resistant or intolerant to corticosteroid therapy. Topical corticosteroid therapy has been associated with cataract formation, increased intraocular pressure, and delayed wound healing. Therefore, other immunosuppressive agents, such as CsA, have been tried for the treatment of severe allergic conditions. Cyclosporine A has been used to treat patients with a number of allergic diseases, including asthma.\(^9\)

Topical CsA also has been used successfully to treat patients with vernal keratoconjunctivitis, an allergic disease characterized by the thickening and formation of giant papillae on the upper tarsal conjunctiva.\(^10,11\)

Recently, the mechanisms explaining how CsA acts to inhibit allergic disease have been elucidated. Although many of the immediate mast cell-mediated effects in allergic reactions are thought to result from the actions of mediators such as histamine, data suggest that cytokines produced by mast cells and infiltrating lymphocytes play an important pathogenic role.\(^12-17\)

Recently, CsA has been shown to inhibit mast cell-mediated cytokine production and to have a therapeutic effect on allergic disease. Hatfield and Roehm\(^18\) showed that cyclosporine inhibited murine mast cell cytokine production. Specifically, CsA completely inhibited IL-2, IL-3, IL-4, and granulocyte-macrophage colony stimulating factor secreted by all cell lines tested. Wershil and colleagues\(^4\) similarly showed that CsA blocked mouse mast cell TNF-\(\alpha\) production in vitro, and they demonstrated that CsA inhibited mast cell-dependent inflammation in vivo. The authors also suggested that CsA can inhibit the responsiveness of target cells to the produced cytokines. Additional data show that CsA interferes with the degranulation of basophils, another key inflammatory cell in the pathogenesis of allergy.\(^19\)

Cyclosporine is an immunosuppressive agent with predominant inhibitory effects against T lymphocytes by blocking early activation genes\(^20\) specifically related to cytokines.\(^21\) It is not surprising, therefore, that CsA interferes with both mast cell- and lymphocyte-mediated cytokine production and has an inhibitory effect on the development of allergic disease. Topical application of CsA results in good levels in ocular tissues,\(^22,23\) sparing many of the adverse systemic effects of the medication, which include hypertension and renal disease.\(^24-28\)

Given the fact that allergic ocular disease can threaten vision and be corticosteroid resistant, topical CsA may have an important role in the treatment of allergy-associated eye disease in humans.

**Key Words**

allergy, conjunctivitis, cyclosporine, inflammation, mast cell

**References**

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