Analysis of Topical Cyclosporine Treatment of Patients With Dry Eye Syndrome

Effect on Conjunctival Lymphocytes

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Objective: To study the effect of topical cyclosporine on lymphocyte activation within the conjunctiva of patients with moderate to severe dry eye syndrome (Sjogren and non-Sjogren).

Methods: Biopsy specimens were obtained at baseline and after 6 months of cyclosporine treatment from eyes of 32 patients with moderate to severe dry eye syndrome; 19 were cyclosporine treated (0.05% cyclosporine, n=13; 0.1% cyclosporine, n=6) and 13 were vehicle treated. Within this group there were 12 with Sjogren syndrome and 20 with non–Sjogren syndrome. Biopsy tissue was analyzed using immunohistochemical localization of binding of monoclonal antibodies to lymphocytic markers CD3, CD4, and CD8 as well as lymphocyte activation markers CD11a and HLA-DR.

Results: In cyclosporine-treated eyes, biopsy results of conjunctivae showed decreases in the number of cells positive for CD3, CD4, and CD8, while in vehicle-treated eyes, results showed increases in these markers, although these differences were not statistically significant. Following treatment with 0.05% cyclosporine, there was a significant decrease in the number of cells expressing the lymphocyte activation markers CD11a (P<.05) and HLA-DR (P<.05), indicating less activation of lymphocytes as compared with vehicle treatment. Within the Sjogren patient subgroup, those treated with 0.05% cyclosporine also showed a significant decrease in the number of cells positive for CD11a (P<.001) as well as CD3 (P<.03), indicating a reduction in number of activated lymphocytes.

Conclusion: Treatment of dry eye syndrome with topical cyclosporine significantly reduced the numbers of activated lymphocytes within the conjunctiva.


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Currently, administration of artificial tears is the most common therapy available for lubricating a dry ocular surface. This palliative treatment gives only temporary and incomplete symptomatic relief and does not address the cause of the symptoms, which may include immune-mediated inflammation of the ocular surface. Evidence of inflammatory processes in the pathogenesis of KCS led to the development of cyclosporine (CsA) as a first attempt to treat this condition therapeutically. Cyclosporine is an immunosuppressive agent commonly used systemically to treat inflammatory diseases such as psoriasis or rheumatoid arthritis or to prevent organ transplant rejection. Topical CsA has been used as treatment of ocular conditions such as vernal keratoconjunctivitis, corneal ulcers, and herpetic stromal keratitis. The effect of this drug on inflammatory diseases is due to its ability to inhibit T-cell–mediated inflammation by preventing the activation of T cells (by antigen-presenting cells or cytokines). Activated T cells are responsible for the production of inflammatory substances such as cytokines, which lead to further tissue damage and, in turn, to the activation of more T cells and the production of even more inflammatory substances.

Clinical trials with this drug have shown improvement in various objective measures of KCS such as corneal staining and Schirmer test values. To attempt to find tissue correlates in these patients, conjunctival biopsy specimens from patients with Sjögren and non-Sjögren KCS treated with CsA or vehicle were evaluated immunohistochemically for the presence of activated T cells (CD3+ [Pan-T cell], CD4+ [T helper cell], and CD8+ [cytotoxic T cell]) and lymphocyte-activation markers.
Within this group, there were 12 Sjögren and 20 non-Sjögren patients (range, 28.8-84.2 years), including 27 women and 5 men. The mean±SD age of our subjects was 59.0±13.5 years for Sjögren patients.

RESULTS

PATIENT POPULATION

The mean±SD age of our subjects was 59.0±13.5 years (range, 28.8-84.2 years), including 27 women and 5 men. Within this group, there were 12 Sjögren and 20 non-Sjögren patients.

LYMPHOCYTIC MARKERS

In general, there was a decrease from baseline in the number of cells positive for CD3, CD4, and CD8 following treatment with either concentration of CsA. The only exception was that there was a mean increase from baseline in the CD4-positive T helper cell population following 0.05% CsA treatment. In comparison, all cells positive for the lymphocytic markers increased from baseline following vehicle treatment.

Figure 1 shows the percent change from baseline for cells expressing the lymphocytic markers (CD3, CD4, and CD8) after 6 months of treatment for the overall patient population. Note that there was a reduction from baseline in the number of CD3-positive cells in the CsA-treated groups, while there was an increase from baseline in the vehicle-treated group. There was also an increase from baseline in the numbers of CD4-positive cells in the vehicle group, with a smaller increase in the 0.05% CsA group and a slight decrease in the 0.1% CsA group.

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical staining for lymphocytic markers as well as lymphocyte activation markers was conducted using monoclonal antibodies to CD3 (PharMingen, San Diego, Calif), CD4 (Becton-Dickinson, San Jose, Calif), CD8 (Becton-Dickinson, San Jose), CD11a (PharMingen, San Diego), and HLA-DR (PharMingen). Cryostat sections were fixed in cold acetone (−20°C) for 3 minutes and air dried at room temperature for 30 to 45 minutes. They were then rinsed in 3 changes of phosphate-buffered saline (PBS) and incubated in PBS with 1% bovine serum albumin (BSA) (Sigma Chemical Co, St Louis, Mo) for 10 minutes. Sections were incubated for 1 hour at room temperature in primary antibodies at concentrations derived empirically: CD3, 1.0 µg/mL; CD4, 5.0 µg/mL; CD8, 2.5 µg/mL; CD11a, 10.0 µg/mL; and HLA-DR, 1.0 µg/mL. Sections were rinsed in PBS alone, followed by 10 minutes in PBS with 1% BSA before incubation for 1 hour at room temperature in the secondary antibody, fluorescein isothiocyanate–conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson Immunoresearch, West Grove, Pa) at a dilution of 1:50. Sections were then rinsed in PBS, mounted in Vectashield (Vector Labs, Burlingame, Calif), cover-slipped, and viewed under a microscope (Eclipse E800; Nikon, Melville, NY) interfaced with a digital camera (Spot Digital Camera; Diagnostic Instruments Inc, Micro Video Instruments, Avon, Mass). Secondary antibody controls omitting the primary antibody for all biopsy specimens for each immunohistochemical analysis were run. Three separate images were acquired for each antibody and biopsy specimen under a ×20 objective using a Spot acquisition program (Diagnostic Instruments Inc). The first field selected for imaging was the field with the highest number of positive cells, followed by images to the left and right of that area. In this manner the entire biopsy area was usually captured.

COUNTING PROCEDURE

Measurement of the entire area of epithelium and stroma (substantia propria) was achieved by tracing the area using the lasso tool under the Adobe Photoshop computer program (Adobe Systems Inc, San Jose, Calif). The total data area, measured in pixels, was acquired through the “Image: Histogram” command in Photoshop. Two independent counts were recorded for cells positive for each antibody within the traced area. Cells per unit area of pixels were adjusted to real unit area or cells per millimeter squared of real tissue area, based on 28.346 pixels per centimeter in Photoshop and the fact that 1 mm equals 67.8 cm equals 1922 pixels at ×20 magnification in Photoshop. Data were recorded as cells per millimeter squared for all markers, and statistical analysis was based on these measurements.

STATISTICAL METHODS

Baseline characteristics were tabulated and summarized by treatment groups. Overall differences among treatment groups were tested using a 2-way analysis of variance (ANOVA) for continuous variables and the Fisher exact test for categorical variables.

Percent changes in the number of cells expressing lymphocytic and/or lymphocyte activation markers were summarized using descriptive statistics (ie, sample size, mean, SD, minimum, maximum, and median). A 1-way ANOVA with main effect for treatment was used to test for differences in percent change from baseline and ratios among treatment groups by visit. If the test for among-group differences in main effect was significant, then all 3 pairwise comparisons were made. Within-group changes from baseline were analyzed by the paired t test method.

The same analysis was performed on Sjögren and non-Sjögren subpopulations, excluding the 0.1% CsA treatment group in which there was only 1 patient in the Sjögren subset.
The CD8-positive cells exhibited the same pattern as CD3-positive cells but with less of a decrease from baseline following CsA and less of an increase from baseline following vehicle treatment. However, the change from baseline in the number of T lymphocytes (CD3+, CD4+, and CD8+) did not reach statistical significance, either among or within treatment groups (Figure 1).

Within the Sjögren subgroup, 0.5% CsA treatment resulted in significantly greater (P < .03) decreases in CD3-positive cells than did vehicle. The CD3-positive cells decreased from baseline in all treatment groups among the non-Sjögren subgroup. However, this decrease was not statistically significant in either group (Figure 2).

LYMPHOCYTE-ACTIVATION MARKERS

In general, there was a decrease from baseline in the number of cells positive for lymphocyte activation markers CD11a and HLA-DR following CsA treatment compared with an increase from baseline in these cells following vehicle treatment for the overall patient population.

Statistical analysis revealed a significant among-group difference in change from baseline for cells expressing CD11a (P = .04) and HLA-DR (P = .02) for the overall patient population. Pairwise comparisons showed significant reductions with 0.05% CsA treatment compared with treatment with vehicle in cells positive for both markers CD11a (P = .05) and HLA-DR (P = .016) (Figure 3). Furthermore, a comparison within individual treatment groups, comparing pretreatment to posttreatment results, revealed a statistically significant decrease from baseline for HLA-DR in the 0.05% CsA group (P = .03) (Figure 3).

Within the Sjögren subgroup treated with 0.5% CsA, there were significantly greater (P < .001) decreases in cells positive for CD11a than in vehicle. There was a decrease from baseline in both treatment groups (CsA and vehicle) among the non-Sjögren subgroup (Figure 4). This decrease did not reach statistical significance.
Figure 5 and Figure 6 show a representative set of immunofluorescence micrographs for cells positive for the markers CD11a and HLA-DR from the non-Sjögren subgroup treated with 0.05% CsA or vehicle. Figure 7 shows immunofluorescence micrographs for cells positive for the markers CD3 and CD11a from patients with Sjögren KCS treated with 0.05% CsA.

**COMMENT**

In the present study, immunohistochemical analysis was used to evaluate changes in the presence of cells positive for lymphocytic and lymphocyte activation markers in conjunctival biopsy specimens of patients with moderate to severe KCS, following treatment with 0.05% CsA, 0.1% CsA, or vehicle. We found that CsA treatment reduced the number of activated T lymphocytes within the ocular surface of patients with and without Sjögren syndrome. After 6 months of treatment with 0.05% CsA, statistically significant decreases were seen in cells positive for CD11a and HLA-DR compared with those in vehicle for the overall patient population. Within the Sjögren patient subgroup treated with 0.05% CsA, there were also significantly greater decreases than with vehicle in the number of cells positive for CD3 and CD11a.

These findings provide additional evidence that inflammation plays a role in the pathogenesis of KCS and suggests that modulating the underlying immune response may prove more efficacious in the treatment of KCS than the frequent use of artificial tears. Topical CsA has been successfully used for the treatment of canine dry eye for many years. Studies in the canine KCS model have demonstrated that CsA decreases the conjunctival and lacrimal gland lymphocytic infiltrates.

However, there have been only a limited number of reports on the use of topical CsA in the treatment of dry eye syndrome in humans with only 1 attempt to look at the effect of the treatment at a cellular level. Power et al reported a significant reduction in CD4-positive T lymphocytes in both the conjunctival epithelium and the substantia propria of patients with secondary Sjögren syndrome compared with non–dry eye controls following treatment with CsA. The present study also demonstrated a significant decrease in CD3-positive cells after 6 months of 0.05% CsA treatment in patients with Sjögren syndrome.

Furthermore, the number of cells positive for CD11a and HLA-DR, which are lymphocyte activation markers, decreased significantly in patient populations treated with CsA. HLA-DR is a class II major histocompatibility...
complex antigen that is expressed in inflamed regions and serves as a ligand for the T-cell receptor. CD4+ T lymphocytes are activated through a signal from HLA-DR molecules of antigen-presenting cells. Immunopathologic studies show evidence of immune activation of the conjunctival epithelium in Sjögren syndrome. Compared with control eyes, a significantly greater percentage of conjunctival epithelial cells from patients with Sjögren syndrome express the HLA-DR molecule. Hingorani et al report a decrease in HLA-DR expression on cells in the substantia propria of patients with atopic keratoconjunctivitis following 3 months of treatment with CsA. In confirmation of these findings, the data presented here demonstrate a reduction in the number of cells positive for the lymphocyte activation marker HLA-DR after 6 months of 0.05% CsA treatment.

CD11a/LFA-1 (lymphocyte function–associated antigen) is associated with adhesion of lymphocytes, macrophages, and granulocytes and is a ligand of intercellular adhesion molecule-1 (ICAM-1), which supports the binding of lymphocytes to antigen-presenting cells. CD11a is up-regulated during activation of human lymphocytes and, with its ligand ICAM-1, plays an important role in cell-to-cell interactions and cell migration of

Figure 6. Immunofluorescence micrographs demonstrating cells positive for HLA-DR in conjunctival biopsy specimens of patients with non–Sjögren keratoconjunctivitis sicca pretreatment and posttreatment with (A and B) 0.05% cyclosporine and (C and D) vehicle. A decrease in the number of positive cells within epithelium and substantia propria in the 0.05% cyclosporine-treated group is apparent compared with an increase in number in the vehicle-treated biopsy sample. E and F, Example of a negative control for a vehicle biopsy in which the primary antibody was omitted. Bar=25 µm (A–C).
lymphocytes into the surrounding tissue such as the conjunctival epithelium and substantia propria. Cyclosporine has been shown to regulate immune-based inflammation within epithelial tissues by inhibiting ICAM-1 production. Our data support these results, showing reduced immune activation by means of a decrease in the number of cells positive for CD11a after a 6-month course of 0.05% topical CsA treatment.

Part of the beneficial effect of CsA might be due to the reduction in T-cell activation as illustrated by a decrease of cells positive for HLA-DR. By preventing the migration of new lymphocytes into the conjunctiva, as suggested by the reduction in CD11a-positive cells, CsA may help to reduce the inflammatory process. The fact that the data show a reduction in positive cells mainly for the lymphocyte activation markers CD11a and HLA-DR suggests that CsA is promoting lymphocytes to a more quiescent status rather than eliminating present lymphocytes. This might explain why the change from baseline in the number of T lymphocytes (CD3+, CD4+, and CD8+) did not reach statistical significance for the overall patient population. However, another contributing factor may be the small patient number and high variability within each treatment group.

These results provide further evidence that topical use of CsA may have a local immunoregulatory effect on inflammation in the conjunctiva of patients with dry eye syndrome. This effect is evident in the reduction of the number of cells positive for lymphocyte activation markers. In preventing the activation of T cells in the conjunctiva, topical administration of CsA may interrupt an ongoing immune reaction. Even though the reduction in Pan-T cells (CD3) and CD11a seems to be larger in patients with Sjogren syndrome, our data on the lymphocyte activation markers, especially HLA-DR, provide evidence that CsA treatment is providing benefits for both types of dry eye syndrome. The larger effect in patients with Sjogren syndrome may be due to the fact that there are greater numbers of lymphocytic infiltrates in patients with this disease.

In conclusion, this study demonstrates a reduction in activated lymphocytes with topical CsA use in patients with moderate to severe KCS. Consistent with these findings, clinical symptoms of KCS also appear to improve with the use of CsA in the overall patient population of the multicenter study conducted by Allergan Inc. This suggests that CsA treatment may help to reduce the pathophysiological factors contributing to the development of KCS.

Figure 7. Immunofluorescence micrographs demonstrating cells positive for (A and B) CD3 and (C and D) CD11a in conjunctival biopsy specimens of patients with Sjogren keratoconjunctivitis sicca pretreatment and posttreatment with 0.05% cyclosporine. Note the decrease in number of positive cells within the epithelium and substantia propria in the posttreatment biopsy specimens (bar=25 µm).
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