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 (Sjögren and non-Sjögren).

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 Sjögren syndrome and 20 with non–Sjögren 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 Sjögren 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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KERATOCONJUNCTIVITIS sicca (KCS), or dry eye syndrome, is characterized by chronic dryness of the cornea and conjunctiva.[1] Patients with KCS typically show symptoms of ocular discomfort ranging from irritation to severe pain. Redness, burning, itching, foreign body sensation, contact lens intolerance, photophobia, and blurred vision can occur.[2]

Although KCS can arise from various types of diseases, common to all is the involvement of immune-mediated or inflammatory-mediated pathways.[3] Immunopathologic studies of the lacrimal gland in patients with Sjögren syndrome show progressive lymphocytic infiltration, primarily consisting of CD4+ T and B cells.[4,5] This infiltration is believed to be responsible for the destruction of normal secretory function.[6] Lymphocytic infiltration of the lacrimal gland has also been described in patients with non-Sjögren KCS.[7,8] Although the immunopathologic analysis of the lacrimal gland has received considerable attention, less work has been done on pathological changes occurring in the ocular surface. The chronic dryness of the ocular surface in Sjögren syndrome has been attributed to deterioration of lacrimal gland function with decreased tear production.[9,10] However, in Sjögren syndrome, conjunctival epithelial and stromal T-cell infiltration (predominantly CD3+ and CD4+ T lymphocytes) has also been shown to occur along with drying of the ocular surface.[9,11]

Supporting a role for an immunopathogenesis of KCS are the reports of activated lymphocytes as demonstrated by expression of lymphocyte activation markers such as HLA-DR (MHC class II) and ICAM-1 (intercellular adhesion molecule-1) in the conjunctiva of patients with Sjögren syndrome.[12,13] To date, there is little information on the effect of modulating these molecules in the conjunctiva of patients with Sjögren and non–Sjögren syndrome.

SUBJECTS AND METHODS

SUBJECTS

Conjunctival biopsy specimens from 32 patients were examined; 13 patients were treated with 0.05% CsA, 6 with 0.1% CsA, and 13 with vehicle alone. This subject group was randomly chosen from a double-masked, vehicle-controlled clinical study designed by Allergan, Inc, Irvine, Calif, to investigate the efficacy and safety of topical CsA in the treatment of moderate to severe KCS.[21] The study was conducted in compliance with Good Clinical Practices, investigational site institutional review board regulations, sponsor and investigator obligations, informed consent regulations, and the Declaration of Helsinki. Potential patients signed a prescreening informed consent form and a second written informed consent form prior to actual enrollment.[21] The protocol for this study is described briefly here. Adult patients of either sex were eligible for participation if they had a diagnosis of moderate to severe KCS at initial examination as defined by the following criteria: (1) Schirmer test results (without anesthesia) less than or equal to 5 mm/5 min in at least 1 eye (if Schirmer test results without anesthesia equaled 0 mm/5 min, then Schirmer test results with nasal stimulation had to be >3 mm/5 min in the same eye); (2) sum of corneal and interpalpebral conjunctival staining greater than or equal to +5 in the same eye where corneal staining was greater than or equal to +2; (3) a baseline Ocular Surface Disease Index[22] score of 0.1 with no more than 3 responses of "not applicable"; and (4) a score greater than or equal to 3 on the Subjective Facial Expression Scale.[21] Signs and symptoms must have been present despite conventional management.

Patients were excluded from the study if they had participated in an earlier clinical trial with CsA ophthalmic emulsion or had used systemic or topical ophthalmic CsA within 90 days prior to the study. Other exclusion criteria were the presence or history of any systemic or ocular disorder or condition (including ocular surgery, trauma, and disease); current or recent use of topical ophthalmic or systemic medications that could affect a dry eye condition; known hypersensitivity to any component of the drug or procedural medications such as stains or anesthetics; required contact lens wear during the study; recent (within 1 month) or anticipated use of temporary punctal plugs during the study; permanent occlusion of lacrimal puncta within 3 months of the study; or if the patients were pregnant, lactating, or planning a pregnancy. Patients were also excluded if they appeared to have end-stage lacrimal gland disease (Schirmer reading with nasal stimulation <3 mm/5 min) or if their KCS was secondary to the destruction of conjunctival goblet cells or scarring.

A retrospective diagnosis of Sjögren syndrome was used with modified criteria reported by Vitali et al[23] to ensure that a consistent definition of Sjögren syndrome was assigned to the patients enrolled. Diagnosis included presence of at least one of the following autoantibodies in sera: antinuclear antibody (ANA), rheumatoid factor (RF), and Sjögren syndrome autoantibodies class SS-A (Ro) and class SS-B (La). In addition, oral and ocular symptoms were used to classify patients with Sjögren syndrome.

Patients instilled 1 drop of 0.05% or 0.1% CsA ophthalmic emulsions or vehicle of CsA ophthalmic emulsion twice daily in each eye for 6 months; once on waking in the morning and once at bedtime. Patients were allowed to use assigned artificial tears (REFRESH Lubricant Eye Drops; Allergan Inc) as needed up to month 4.

Full-thickness conjunctival biopsy specimens of a standard size (2-3 mm) were removed from the "worse" eye by surgeons following standard procedure. The worse eye was defined as the eye with the worse Schirmer tear test value (without anesthesia) and the worse sum of corneal and interpalpebral conjunctival staining. If both eyes were comparable, then the right eye was used. At the baseline visit, the conjunctival biopsy specimen was obtained from the inferonasal quadrant close to midline. At the 6-month visit, the sample was removed from the same eye but from the inferotemporal quadrant, also close to midline.

TISSUE PROCESSING FOR IMMUNOHISTOCHEMICAL ANALYSIS

After removal, the baseline biopsy specimens were immediately frozen in OCT embedding compound (Tissue-Tek; Miles Laboratories, Elkhart, Ind) in a cryomold (Miles Laboratories) and stored at –80°C until patient-matched

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.[14] Topical CsA has been used as treatment of ocular conditions such as vernal keratoconjunctivitis,[15] corneal transplants,[16] corneal ulcers,[17] and herpetic stromal keratitis.[18] 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).[19,20] 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.[21] 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

6-month biopsy specimens were obtained and similarly frozen. Six-micrometer sections were taken from each block, mounted on gelatin-coated slides, and processed for immunohistochemical analysis. Sectioning of tissue blocks and immunohistochemical experiments were performed as pairs of biopsies, pretreatment and posttreatment, to minimize differences due to experimental conditions.

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 on the Nikon microscope. 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.

(CD11a and HLA-DR) to further understand the underlying mechanism of CsA treatment.

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.

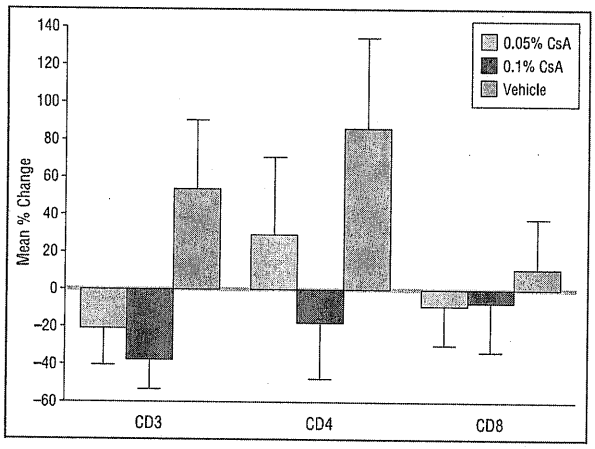

Figure 1. *Percent change for cells positive for the lymphocytic markers CD3, CD4, and CD8 in the overall patient population. Values presented are mean percent change±SE from baseline at month 6. CsA indicates cyclosporine.*

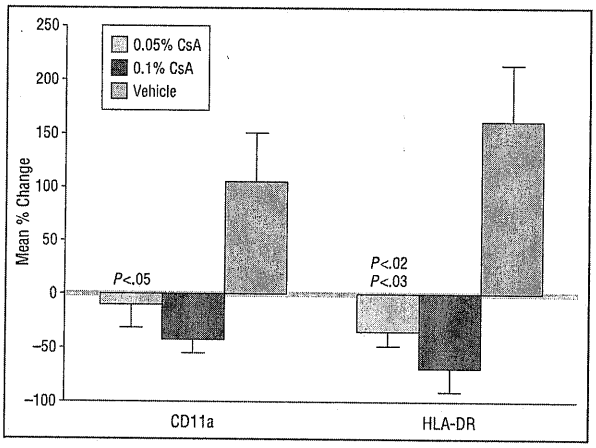

Figure 3. *Percent change for cells positive for the lymphocyte activation markers CD11a and HLA-DR in the overall patient population. Values presented are mean percent change±SE from baseline at month 6. The P values are relative to pairwise comparisons (P<.05) and within-group differences (P<.03). CsA indicates cyclosporine.*

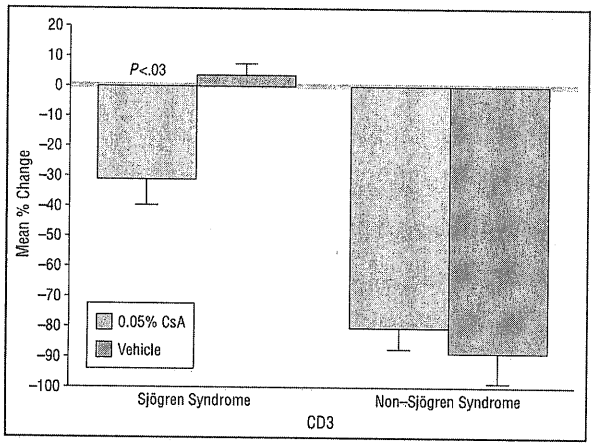

Figure 2. *Percent change for CD3-positive cells from the Sjögren syndrome and non–Sjögren syndrome subpopulations. Values presented are mean percent change±SE from baseline at month 6. The P value is relative to pairwise comparisons from 1-way analysis of variance. CsA indicates cyclosporine.*

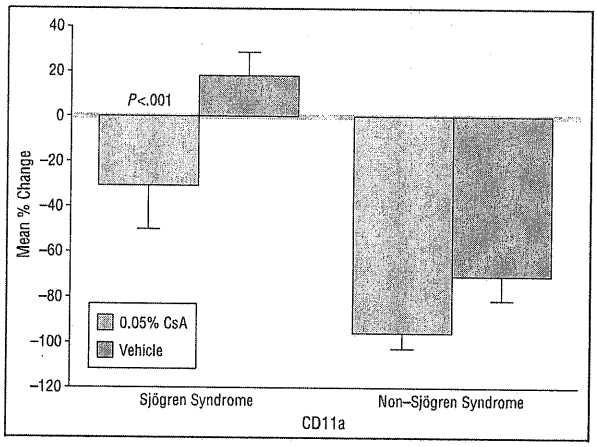

Figure 4. *Percent change for CD11a-positive cells from the Sjögren syndrome and non–Sjögren syndrome subsets. Values presented are mean percent change±SE from baseline at month 6. The P value is relative to pairwise comparisons from 1-way analysis of variance. CsA indicates cyclosporine.*

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 post-treatment 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.

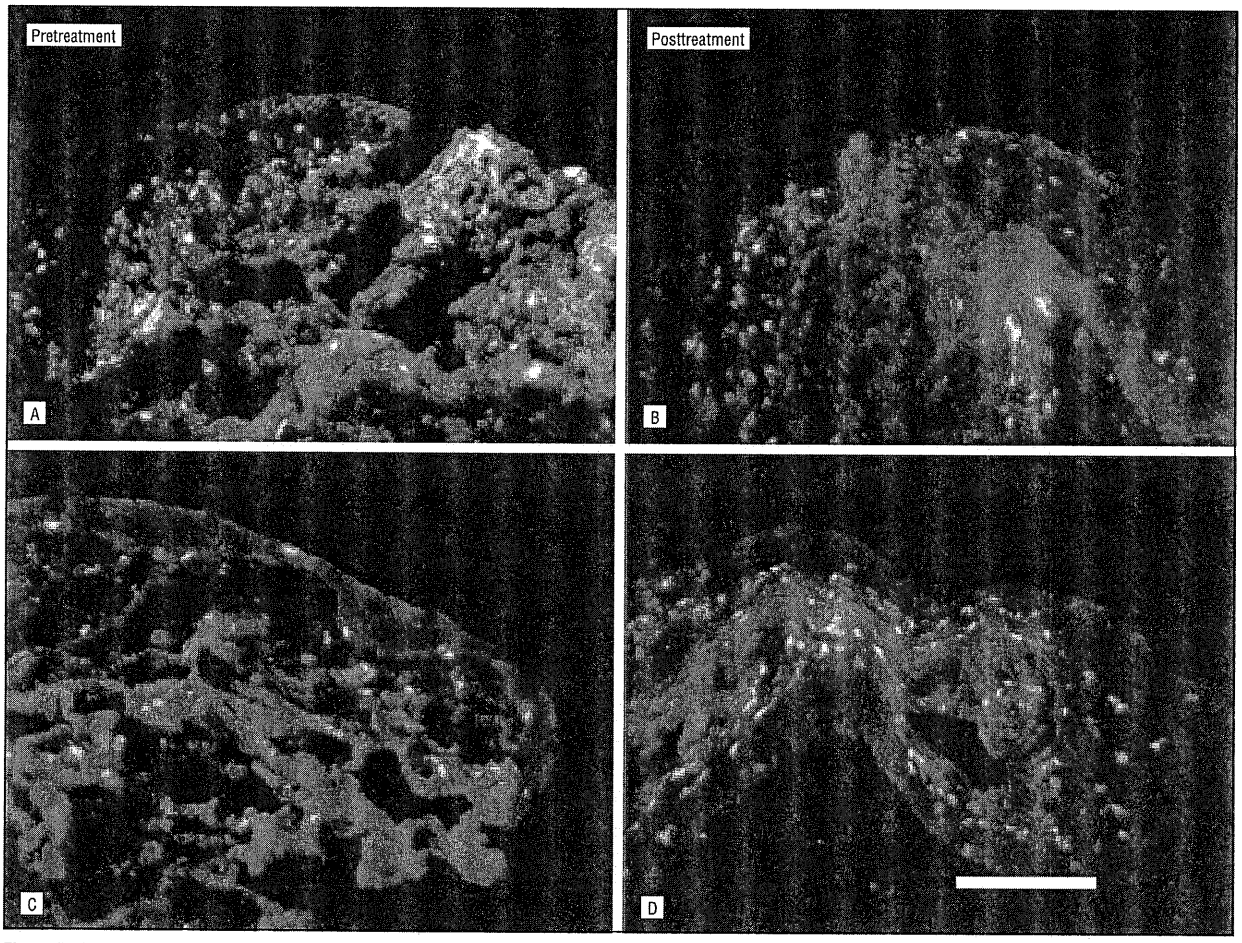

Figure 5. *Immunofluorescence micrographs demonstrating cells positive for the lymphocyte activation marker CD11a in conjunctival biopsy specimens of patients with non–Sjögren keratoconjunctivitis sicca pretreatment and posttreatment with (A and B) 0.05% cyclosporine and (C and D) vehicle. The number of positive cells within epithelium and substantia propria in the cyclosporine-treated group decreased, while the number in the vehicle-treated biopsy sample increased (bar=25 µm).*

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.[24-26]

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[27-29] with only 1 attempt to look at the effect of the treatment at a cellular level.[30] Power et al[30] 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

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