Flow Cytometric Analysis of Inflammatory Markers in KCS: 6-Month Treatment with Topical Cyclosporin A

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PURPOSE. Immune-based inflammation has been observed as a common mechanism of keratoconjunctivitis sicca (KCS). In KCS-affected eyes, upregulated expression of HLA DR and various immune- or apoptosis-related markers by conjunctival epithelial cells has been demonstrated in an earlier study, by a technique of flow cytometry in impression cytology (IC) specimens. The purpose of this study was to monitor the effects of topical cyclosporin A on the expression of these markers throughout a 6-month period of treatment.

METHODS. Patients with moderate to severe KCS included in a large European multicenter clinical trial (Cyclosporin Dry Eye Study, Allergan, Irvine, CA) underwent collection of IC specimens at baseline, month 3, and month 6. For 6 months, they randomly received 0.05% or 0.1% cyclosporin A or vehicle. Specimens were processed and analyzed in a masked manner by flow cytometry, using monoclonal antibodies directed to HLA DR, CD40, CD40 ligand, Fas, and the apoptotic marker APO2.7. Percentages of positive cells were calculated and levels of expression quantified after conversion into standardized units of fluorescence.

RESULTS. One hundred fifty-eight patients had at least two IC specimens available for flow cytometry analysis. HLA DR expression, both in percentage of positive cells and level of expression, was highly significantly reduced after 0.05% and 0.1% cyclosporin A treatment at months 3 and 6 compared with baseline values, whereas vehicle did not induce any change in HLA DR expression over time. The 0.05% and 0.1% cyclosporin emulsions were significantly more effective than the vehicle in reducing HLA DR at months 3 and 6 (0.05%), and at month 6 (0.1%). CD40 expression was significantly reduced at month 3 and partially at month 6, compared with baseline, with no reduction in patients who received the vehicle. CD40 ligand expression also decreased at months 3 and 6 in patients taking both concentrations of cyclosporin A. APO2.7 expression was significantly increased in all three groups, whereas percentage of Fas-positive cells decreased only in patients treated with 0.05% cyclosporin A at months 3 and 6.

CONCLUSIONS. Flow cytometry provided an objective technique to monitor the effects of topical cyclosporin A on immune- and apoptosis-related markers in the conjunctival epithelium of patients with KCS enrolled in a large multicenter trial. Topical cyclosporin A strikingly reduced HLA DR and to a lesser extent, other inflammatory and apoptotic markers, whereas the vehicle, used as a control tear substitute, had almost no effect. This study confirms that cyclosporin A may be efficacious in reducing conjunctival inflammation in moderate to severe KCS and is consistent with clinical results in this indication. (Invest Ophtalmol Vis Sci. 2001;42:90-95)

There is growing evidence that inflammation plays a key role in the development of keratoconjunctivitis sicca (KCS). The presence of inflammatory cell infiltrates in the lacrimal glands and conjunctiva, upregulation of immune-related antigens and inflammatory cytokines at the level of conjunctival epithelium, impaired apoptotic regulation in lacrimal acini, and alterations in membrane trafficking of acinar cells are common features of KCS in association with squamous metaplasia and loss of goblet cells.¹⁻⁵ Moreover, these ocular surface abnormalities have been demonstrated not only in the autoimmune form of KCS, Sjögren’s syndrome, but also in nonautoimmune KCS, as a consequence of severe tear deficiency, absence of trophic factors, neural deregulation, and repeated abrasion of the corneal surface by the lids.⁶⁻⁹

Treatments of the most severe forms of KCS with steroids,¹⁰ and topical cyclosporin A (CsA) have therefore been successfully attempted, first in Sjögren’s syndrome animal models of mouse,¹¹ and dog,¹²,¹³ and then in human clinical trials.¹⁴⁻¹⁷ Topical CsA has been shown in animal models to be effective in reducing lymphocytic infiltration of the lacrimal glands and conjunctiva, both in the epithelium and substantia propria, and in reversing the abnormal apoptotic imbalance of the lacrimal glands in KCS (i.e., decreased lymphocyte apoptosis and increased epithelial apoptosis).¹⁵ CsA thus appears to be an efficacious drug capable of reducing inflammatory infiltrates, modulating immune reactions and apoptotic pathways, and even increasing tear fluid secretion.¹⁶,¹⁷

Large phase 2 and 3 clinical trials with topical emulsions of CsA have been undertaken in patients with moderate to severe KCS,¹⁶,¹⁷ and have shown significant efficacy of topical CsA in this indication. Recently therefore, a large European multicenter clinical trial has been conducted in a similar complementary way to evaluate efficacy and safety of topical CsA in the treatment of moderate to severe KCS. As a part of this clinical study, impression cytology (IC) specimens were taken in a large series of patients as a tertiary complementary test, to investigate the expression of immune-related markers by the conjunctival epithelium, confirm the presence of inflammation in KCS, and monitor these markers throughout a 6-month treatment with CsA. We used a previously validated method of flow cytometry in IC specimens to quantify the percentage of conjunctival cells expressing various inflammation- and apoptosis-related antigens, HLA DR class II antigens, CD40, CD40 ligand, Fas, and the apoptotic marker APO2.7, and to objectively measure their levels of expression. Results at baseline, before the randomized treatment, were recently published and showed highest levels of HLA DR expression and to a lesser extent of the other markers in the conjunctival epithelium in KCS, both in Sjogren’s and non-Sjogren’s syndrome eyes.²¹ We hereby present the effects of these markers of two concentrations of CsA topical emulsions and of their vehicle, using flow cytometry in IC specimens repeatedly obtained during a 6-month treatment.
MATERIALS AND METHODS

Study Design

Study design and flow cytometric methods have been previously described in detail. Briefly, a multicenter, double-masked, randomized, vehicle-controlled, parallel-group study of the safety and efficacy of cyclosporin A 0.05% and 0.1% ophthalmic emulsions used twice daily in patients with moderate to severe KCS was designed by Allergan Inc., Irvine, CA, (Protocol 192371-501-03). After a 2-week run-in phase during which patients received only un_preserved tear substitute (Re-Fresh; Allergan), patients who fulfilled inclusion criteria entered the masked treatment phase. From day 0 to month 6, patients were allowed to use the tear substitute daily as needed, in addition to the masked treatment (at month 4, patients were instructed to use the tear substitute less than eight times a day, if possible). Inclusion criteria excluded the use of topical or systemic cyclosporin A within 90 days before the screening visit or of topical ophthalmic steroids in the prior 3 weeks; contact lens wear or use of any other topical treatments during the study; acute ocular infection, severe blepharitis, or non-KCS inflammation, including atopic keratoconjunctivitis, recurrent herpetic keratitis within the prior 6 months; anterior segment surgery or trauma within the prior 12 months, KCS secondary to the destruction of conjunctival goblet cells (as with vitamin A deficiency); or scarring (such as that with cicatricial pannus, alkali burns, Stevens-Johnson syndrome, trachoma, or irradiation); and ocular rosacea currently treated with systemic tetracycline.

At selected centers, as part of the whole study, IC specimens were collected at day 0, from the worse eye, defined as the one showing the highest degree of corneal staining, or the lowest Schirmer’s test when both eyes had the same corneal staining scores. If the two criteria were equal in both eyes, the right eye was chosen for IC. The same eye was used throughout the study. Patients providing samples for this study were recruited in 28 centers from four countries in Europe. Immediately after collection, specimens were shipped to the Department of Immunohematology, Ambroise Paré Hospital, Boulogne, France, for processing and analyses in a centralized procedure.

The laboratory work for this study as well as the study protocol were conducted in compliance with the Ethics Committee (CCPPRB) at the Ambroise Paré Hospital and the relevant ethics committees in each of the participating countries. Additional specific written informed consent was obtained to collect repeated conjunctival impressions. This study was conducted in compliance with the Declaration of Helsinki, South Africa amendment, 1996.

Experimental Procedures

IC specimens were obtained with patients under topical anesthesia (0.04% oxybuprocaine), using 0.20-μm polyethersulfone filters (Supor; Gelman Sciences, Ann Arbor, MI), applied on the superior and superotemporal bulbar conjunctiva, according to previously published procedures. Specimens were collected at least 15 minutes after instillation of the last staining eye drop (i.e., fluorescein and lissamine green), to avoid any interference with immunofluorescence (IF) analysis. After collection, membranes were immediately dipped into tubes containing 1.5 ml of cold phosphate-buffered saline (PBS) with fixative (0.05% paraformaldehyde, prepared monthly and sent regularly from the central laboratory to the centers). Tubes were to be kept at or below 4°C before impression collection and sent within 2 days to the Department of Immunohematology, Ambroise Paré Hospital, in cold_conditioned containers. Cells were extracted by gentle agitation for 30 minutes and centrifuged (1600 rpm, 5 minutes). The cells were then counted in a Malassez cell before processing for flow cytometry, according to previously validated methods.

Five different monoclonal antibodies and two corresponding negative controls were used in this study. Mouse IgG1 anti-HLA DR α chain (clone TAL.1B5, 50 μg/ml, Dako, Copenhagen, Denmark), mouse IgG1 anti-CD40 (clone MAB89, 1 mg/ml, Immunotech, Marseille, France), and mouse IgG1 anti-CD40 ligand (clone TRAP1, 1 mg/ml, Immunotech) were used in indirect IF procedures. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins were used as the secondary antibody (Dako) and nonimmune mouse IgG1 (Dako) as a negative isotopic control. FITC-conjugated mouse IgG1 anti-human Fas/CD95 (clone UB2, 1 mg/ml, Immunotech) and phycoerythrin (PE)-conjugated mouse IgG1 anti-human Apo 2.7 (clone UB2, 1 mg/ml, Immunotech), as an apoptosis marker, were used in a direct IF technique. The FITC-PE-conjugated nonimmune mouse IgG1 (Immunotech) was used as a negative isotopic control for the direct IF procedure. Antibodies were used in a 1:50 dilution in PBS containing 1% bovine serum albumin. After 30 minutes of incubation, cells were washed in PBS by a 5-minute centrifugation and, for indirect IF procedures, were reacted with the secondary anti-mouse immunoglobulins in a 1:50 dilution, for 30 minutes. After incubation, cells were centrifuged in PBS (1600 rpm, 5 minutes), resuspended in 100 μl of PBS, and analyzed on a flow cytometer (FACScan; Becton Dickinson) according to previously validated methods. The same flow cytometer was used during the study.

All specimens were analyzed in a masked manner. For each marker, at least 1,000 cells were analyzed, and specimens with fewer than 10,000 cells were therefore discarded. The percentages of positive cells were obtained from logarithmic cytograms of mean fluorescence intensities, by comparison with the negative isotopic control. Fluorescence intensities were further quantified by using calibrated fluorescent spheres to translate the mean fluorescence of each sample into standardized arbitrary fluorescence units (AUF). A calibration curve was therefore established during each flow cytometric procedure by using four different beads (Immunobrite; Coulter, Hialeah, FL) with standardized fluorescence intensities. The actual AUF value was obtained by subtracting the isotopic negative control from the total AUF calculated for each marker.

Statistical Analyses

For both the percentage of positive cells and the AUF analyses, a nonparametric method was used because of the high variability of the data. A Kruskal-Wallis test was used to compare differences in change from baseline among treatment groups. If the test for among-group differences was significant (P < 0.05), then all three pairwise comparisons were performed using a Wilcoxon rank sum test. Within-group changes from baseline were analyzed by the Wilcoxon signed-rank test. Statistical analysis software (SAS, ver. 6.12 for UNIX; SAS Institute, Cary, NC) was used for computation and analysis.

RESULTS

At baseline, 169 patients, aged 18 to 86 years (mean, 57.1) and composed of 86.4% females and 41% with Sjögren’s syndromes, yielded valid specimens. Of these 169 samples, 134 to 158, according to the marker tested (Figs. 1 through 5), allowed at least two analyses throughout the study (intent-to-treat analyses). No difference was found among the three groups in mean age, sex ratio, and coexisting medical conditions or systemic medications. Percentages of patients with and without Sjögren’s syndrome did not differ in the three groups, nor did clinical data at baseline, for any of the criteria investigated in this multicenter trial. Figures 1 through 5 summarize mean ± SE obtained at each time point, with statistical analyses of mean between- and within-group differences. At baseline, the three groups were fully comparable with no statistical difference for any marker or criterion.

The percentage of HLA DR-positive conjunctival cells significantly decreased in the two cyclosporin A groups (Fig. 1), compared with baseline, both at month 3 and month 6, decreasing from 61.67% ± 29.54% (mean ± SD) at baseline to 39.03% ± 31.56% (P < 0.001) and 39.45% ± 33.06% (P < 0.001) at months 3 and 6, respectively, for patients treated with 0.05% CsA, and from 57.53% ± 31.73% to 41.75% ±
33.57% (P = 0.004) and 38.59% (P = 0.001) for patients taking 0.1% CsA. Simultaneously, patients receiving vehicle did not show any significant change from baseline (mean differences 2.59% and 6.68% respectively at months 3 and 6, nonsignificant). Moreover, a significant difference was found between 0.05% CsA and vehicle at months 3 (P = 0.006) and 6 (P = 0.028) between 0.05% and 0.1% CsA at month 3 (P = 0.013) and between 0.1% CsA and vehicle (P = 0.011) at month 6.

Levels of expression of HLA DR confirmed the striking decrease of this marker with the CsA treatment. Mean expression of HLA DR decreased from 101,004 ± 117,356 AUF (mean ± SD) at baseline to 41,557 ± 44,842 AUF (P < 0.001) and 39,748 ± 38,492 AUF (P < 0.001) at months 3 and 6, respectively, for patients receiving 0.05% CsA, and from 124,883 ± 188,883 AUF to 61,949 ± 72,717 AUF (non-significant) and 38,492 ± 39,054 AUF (P < 0.001) for 0.1% CsA. Patients receiving vehicle did not show any significant difference from baseline (mean changes, −13,140 AUF and −16,387 AUF, respectively, at months 3 and 6, nonsignificant). Again, a significant difference was found between 0.05% CsA and vehicle at months 3 (P = 0.009) and 6 (P = 0.022) between 0.05% and 0.1% CsA at month 3 (P = 0.013) and between 0.1% CsA and vehicle (P = 0.011) at month 6.

The expression of the other markers also showed significant changes from baseline in the CsA-treated groups, whereas the vehicle had no or poor effect. Percentage of CD40-positive cells (Fig. 2) significantly decreased in those patients treated with 0.05% CsA at month 3 (P = 0.003) and in the 0.1% CsA group at months 3 (P < 0.001) and 6 (P < 0.001). The levels of CD40 expression significantly decreased at month 3 with 0.05% CsA treatment (P = 0.049). Treatment with vehicle slightly decreased CD40 expression but did not change significantly either the percentages of positive cells and levels of expression.

Percentages of cells positive to CD40 ligand (Fig. 3) decreased in all three groups, and differences from baseline reached significance, with 0.05% CsA treatment at month 6 (P = 0.047), 0.1% CsA at months 3 and 6 (P = 0.001 and P < 0.001, respectively) and vehicle at months 3 and 6 (P = 0.020 and P < 0.001, respectively). However, CD40 ligand expression remained low in all groups. Mean percentages of cells expressing this marker ranged from 7% to 10% at baseline, 0.4% to 5% at month 3, and 1.5% to 2.9% at month 6. Levels of expression of CD40 ligand also remained low but significantly decreased only in the CsA groups, whereas levels of expression in the vehicle group did not significantly decrease. Differences were thus significant at months 3 and 6 for patients treated with 0.05% CsA (P = 0.007 and P = 0.008, respectively) and 0.1% CsA (P < 0.001 and P = 0.044, respectively).

The apoptotic marker APO2.7 (Fig. 4) paradoxically showed an increase of expression in the three groups in percentages of positive cells, at the two time points, excepted for vehicle at month 3, but in levels of expression a difference was
only found in patients treated with 0.05% CsA at months 3 (P < 0.001) and 6 (P = 0.001), and with 0.1% CsA at month 3 (P = 0.043). Fas (Fig. 5) showed only a significant decrease of percentage of positive cells in the 0.05% CsA group at months 3 (P < 0.046) and 6 (P = 0.001), whereas levels of expression did not significantly change in any group.

For all markers, additional statistical analyses in stratified groups of patients with and without Sjögren's syndrome were performed, to detect eventual differences in efficacy of CsA treatment in either subgroup. At baseline, 41% of patients were found to have Sjögren's syndrome according to clinical and biologic criteria given in detail in our previous study at baseline. Although the number of patients decreased in the stratified analyses, similar results and tendencies were found in the two subpopulations of Sjögren and non-Sjögren's syndrome, which did not statistically differ from each other at any time and for any marker, as well as from the overall population (data not shown).

**DISCUSSION**

The present study clearly demonstrates that topical cyclosporin A is efficient in reducing the expression of inflammatory markers by conjunctival epithelial cells. As we previously reported, highly elevated levels of expression of HLA DR class II antigens, CD40, and CD40 ligand were found in KCS-affected eyes compared with normal eyes. HLA DR expression was significantly reduced in the two CsA groups after 3 months of treatment, compared with baseline values. A striking decrease in both percentage of class II-expressing epithelial cells and levels of expression was thus found, although levels reached at month 6 remained above mean values obtained with similar techniques in normal eyes. However, treatment with 0.05% CsA seemed to be more effective than that with 0.1% CsA, in that the latter concentration showed significant superiority compared with the vehicle only at month 6 and was significantly less efficient than 0.05% CsA at month 3. HLA DR expression seemed to stabilize between months 3 and 6 in the 0.05% CsA group, whereas it continued to decrease in the 0.1% CsA group between the two time points, reaching levels comparable to those of the 0.05% CsA group at month 6.

In our previous study using this technique of flow cytometry, the gating process on the flow cytometer clearly showed a dense homogenous population of conjunctival cells that was mainly composed of epithelial cells with rare smaller inflammatory cells. Although interferences between expression of HLA DR by the two populations could not be totally excluded, it is therefore most unlikely that the eventual decrease of inflammatory cells alone could influence levels of HLA DR expression measured in the whole cell population. Nevertheless, the lipid vehicle, despite potential therapeutic effects as a viscous tear substitute, showed no effect on HLA DR expression. Although decreasing slightly, neither percentage of HLA DR expressing cells nor objectively quantified levels of expression significantly changed.

**FIGURE 3.** Changes in CD40 ligand expression by conjunctival cells in percentage of positive cells (A) and in AUF (B). Error bars: SE.
HLA DR is a major immune-related marker normally expressed by immunocompetent cells, which has been shown to be upregulated in epithelial cells in cases of autoimmune and inflammatory disorders. In KCS, conjunctival and lacrimal acinar cells overexpress this marker at very high levels, especially, but not only, in Sjögren’s syndrome.\(^1\)\(^2\)\(^5\) In this study similar results were obtained for eyes with and without Sjögren’s syndrome, both at baseline and with CsA treatment, which confirms that class II antigen expression is not a specific consequence of an autoimmune disease. HLA DR expression by conjunctival cells is dependent on various cytokines, such as interferon (IFN)-\(\gamma\) or tumor necrosis factor (TNF)-\(\alpha\).\(^3\)-\(^24\) IFN-\(\gamma\) induces class II antigen expression together with Fas and CD40 overexpression and apoptosis in conjunctival epithelial cells in a dose- and time-dependent manner.\(^20\)-\(^22\) Many inflammatory cytokines are involved in ocular surface diseases, and IFN-\(\gamma\) is therefore a good candidate for some of the major mechanisms found in ocular structures in KCS, especially inflammation and apoptosis.\(^9\)-\(^13\) It remains to be determined whether conjunctival epithelial cells acquire antigen-presenting properties when they normally express class II antigens, as do corneal epithelial\(^25\) and lacrimal acinar cells.\(^5\) Nevertheless, class II-expressing epithelial cells after stimulation by inflammatory cytokines could participate in immune reactions and/or recruitment of inflammatory cells.\(^3\)

The other markers we tested also showed significant changes with CsA treatment. CD40 and CD40 ligand significantly decreased in the CsA groups, whereas the vehicle had no effect for CD40. CD40 belongs to the TNF receptor family and is involved in regulation of immune response and apoptosis.\(^26\)-\(^2\) It is normally expressed by conjunctival epithelial cells and has been shown to be upregulated during inflammatory processes of the conjunctiva, including dry eye.\(^28\) In the present clinical trial on moderate to severe dry eye syndrome, baseline levels were significantly higher in KCS eyes than in normal ones, and in eyes with Sjögren’s syndrome compared with those without. Moreover, both CD40 and CD40 ligand expressions were found to be significantly correlated with HLA DR levels, thus confirming their involvement in the inflammatory process in the ocular surface.\(^21\)

In contrast, Fas, although previously found at higher levels in dry eyes than in normal ones,\(^21\) seemed to be poorly influenced by CsA treatment in this study, in that a percentage of positive cells significantly decreased only in the 0.05% CsA group at months 3 and 6. This could be explained by the wide SDs and the low levels of expressions obtained by using direct immunofluorescence procedures, compared with indirect ones that amplify the detection process. However, possible persistence in the conjunctival epithelium of Fas-positive lymphocytes that would be inactivated by topical CsA could not be definitively eliminated.

More paradoxical were the findings of increased APO2.7 expression, both in percentages of positive cells and in levels of staining. Treatment with CsA at both concentrations significantly increased APO2.7 at months 3 and 6, and even the vehicle increased the percentage of positive cells. According to previous work performed in conjunctival cells with this apoptotic marker\(^29\) and the description of apoptotic epithelial cells in lacrimal glands of KCS models,\(^21\) we would have expected a greater level of expression of APO2.7 at baseline and a lower number of apoptotic cells after CsA treatment. At baseline we did not find any difference between normal and KCS-affected eyes, in contrast with a study previously conducted in a patient group with less severe dry eye.\(^20\) A toxic effect of the lipid vehicle cannot be eliminated that may explain these results. In addition, the accurate significance of APO2.7 cannot be ascertained, because differences between TUNEL- and APO2.7-positive apoptotic cells may exist. However, it could also be hypothesized that the increase of the early marker of apoptosis APO2.7\(^21\) after CsA treatment may indicate a form of regulation of a very severely impaired epithelium. In severe KCS, and especially in Sjögren’s syndrome, conjunctival epithelium has been shown to exhibit increased numbers of S-phase cells with a loss of normal epithelial differentiation.\(^3\) The epithelium may therefore become hyperplastic under permanent cytokine stimulation and after decreased cell maturation. Increased APO2.7 expression observed with CsA, and to a lesser extent with the vehicle, could thus in such severely affected epithelial cells reflect an initial step of normalization of epithelial differentiation by elimination of the hyperplastic epithelial layers. Further long-term monitoring of this marker with CsA treatment as well as with various tear substitutes could thus provide additional interesting information.

Nevertheless, CsA appeared to be an effective drug in reducing inflammatory markers in conjunctival cells, thus confirming results in animal models of KCS.\(^1\)\(^13\)-\(^18\)\(^1\)\(^9\) A clinical study conducted with a similar design in the treatment of KCS with CsA also showed a significant decrease of infiltrating HLA DR-positive cells with CsA in conjunctival biopsy specimens, both in the epithelium and subconjunctiva.\(^29\) CsA has also been shown to be efficient in atopic keratoconjunctivitis in reducing HLA DR-positive infiltrates, which confirms these results.\(^50\) CsA could act as an anti-inflammatory, immunosuppressive drug and possibly a modulator of apoptosis.\(^30\)-\(^13\) CsA has been
shown to inhibit phosphatase and more specifically calcineurin, but also to stimulate substance P release and therefore has an effect on neurotransmitter regulation, with positive properties for tear fluid secretion as a consequence.19

Although obtained by indirect techniques designed to evaluate expression of biological markers by conjunctival cells, our results strongly support the role of CsA as a potent regulator of the ocular surface in KCS. Clinical studies have been performed with topical CsA in KCS eyes either in former14,15 or currently used16,17 ophthalmic formulations. Clinical improvement, especially on corneal staining as a main outcome for subjective and objective measures with 0.05% and 0.1% CsA ophthalmic emulsions. Results of these large multicenter placebo-controlled clinical trials in KCS are providing important information, both for the pathogenesis of this very complex disease and for therapeutic issues, until now limited to the repeated instillation of poorly effective tear substitutes.

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