Flow Cytometric Analysis of Inflammatory Markers in Conjunctival Epithelial Cells of Patients with Dry Eyes

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PURPOSE. To investigate in impression cytology (IC) specimens the expression of inflammatory and apoptosis-related markers by conjunctival epithelial cells from patients with dry eye as a rationale for treatment with topical cyclosporine.

METHODS. Immunologic anomalies were identified at baseline, before treatment with the masked medication, in a homogeneous series of patients with dry eye syndrome, who were enrolled in a large European multicenter clinical trial (Cyclosporin A Dry Eye Study; Allergan, Irvine, CA). IC specimens were collected in 243 patients with moderate to severe keratoconjunctivitis sicca (KCS), with or without Sjögren’s syndrome (SS). Fifty normal subjects were separately examined to provide normal control values. Specimens were analyzed in a masked manner by flow cytometry, using antibodies directed to markers of the immune system and/or apoptotic pathway: HLA DR, CD40, CD40 ligand, Fas, and APO2.7. Levels of expression were quantified, and results were compared with those obtained in the 50 normal patients.

RESULTS. One hundred sixty-nine specimens were successfully interpreted at baseline, including 41% from patients with SS. A highly significant increase of HLA DR expression by conjunctival cells was found in KCS-affected eyes compared with normal eyes, which did not express this marker or did so very weakly. HLA DR expression in eyes with SS was significantly higher than in KCS-affected eyes without SS. Fas and APO2.7 were found at low levels in all normal and KCS-affected eyes. CD40 and CD40 ligand expressions were significantly increased in eyes with KCS compared with normal eyes. HLA DR, CD40 and Fas were found at significantly higher levels in the SS group than in the non-SS group.

CONCLUSIONS. Conjunctival cells from patients with dry eye with moderate to severe KCS, with or without SS, overexpress inflammatory and apoptosis-related markers. Whether inflammation is a primary phenomenon in KCS or is the consequence of repetitive abrasion of the ocular surface after tear deficiency remains to be determined. These data, nevertheless, support the use of immunomodulatory and/or anti-inflammatory drugs in the treatment of patients with KCS. (Invest Ophthalmol Vis Sci. 2000;41:1356–1363)

Dry eye disease or keratoconjunctivitis sicca (KCS) is one of the most frequently encountered categories ofocular morbidity in the United States, with as many as 4.3 million persons above age 65 having symptoms of dry eye often or all the time.1 The tear-deficient dry eye is characterized primarily by a deficiency in tear production and includes KCS associated with Sjögren’s syndrome (SS), an autoimmune disease of the lachrymal gland and ocular surface. The histopathologic changes of the lachrymal gland in patients with SS therefore consist of lymphocytic infiltration leading to atrophy and destruction of glandular function.2

Most cases of lachrymal gland insufficiency, however, cannot be attributed to this syndrome. Williamson et al.3 and Damato et al.4 also demonstrated the presence of lymphocytic infiltrates in the lachrymal glands of patients who had KCS but not SS and suggested that atrophy of the lachrymal gland that occurs with senescence represents a chronic, progressive inflammatory process. A mechanism for this inflammatory process in patients without SS recently has been proposed that describes alterations in membrane trafficking of acinar cells leading to the expression of major histocompatibility complex class (MHC) II molecules capable of triggering an autoimmune response.5 Even in non-SS-affected dry eyes, an inflammatory reaction has been demonstrated that could be hypothesized to result from chronic ocular surface dryness and degeneration after tear deficiency. A strong expression of class II antigens by conjunctival epithelial cells has thus been assessed in KCS by immunocytologic techniques, using impression cytology (IC)6–8 or brush cytology.9

Immune-based inflammation is a common feature of many ocular surface syndromes including dry eye disease. A strong relationship has also been proposed between inflammatory pathways and apoptosis, which directly affects epithelial turn-over10–11. Increased expression of apoptotic modulators in
ocular surface models of wound healing include the Fas (CD95) system (Fas and Fas ligand) and the CD40 system (CD40 and CD40 ligand), both as membrane-bound and soluble proteins.12–14 Fas and CD40 antigens are two membrane receptors that belong to the tumor necrosis factor (TNF)/nerve-growth factor receptor family and mediate apoptosis when bound respectively to Fas ligand and CD40 ligand (two members of the TNF family). Fas15 and CD4016 overexpressions were shown in conjunctival IC specimens from patients with KCS and various inflammatory ocular surface disorders. Demonstration of increased expression of immune markers and antigens of apoptotic pathways in patients with KCS, with and without SS, would therefore support the role of ocular surface inflammation in the pathogenesis of KCS and the use of immunomodulatory and/or anti-inflammatory drugs in its treatment.

The objective of this study was thus to assay inflammatory and apoptosis-related markers in conjunctival cells from patients with KCS enrolled in a large European multicenter trial on cyclosporine ophthalmic emulsion (Cyclosporin A; Allergan, Irvine, CA) in the treatment of moderate to severe KCS, using a flow cytometry technique previously validated in IC specimens.7–15 This study was undertaken to confirm the existence of inflammatory and apoptosis-related markers in ocular surface cells of patients with KCS and to determine whether treatment with cyclosporine could induce changes in these markers. IC was performed at baseline, 3, 6, and 12 months. The present study reports investigations on five markers involved in apoptosis and immune reactions that were previously shown to be overexpressed in ocular surface diseases.6–9,12–16 Expressions of Fas, CD40, CD40 ligand, the apoptotic marker APO2.7 and HLA DR class II antigen, as the main standard of inflammation, were therefore analyzed at baseline, before cyclosporine or vehicle treatment, to assess the baseline inflammatory status of the ocular surface in a large series of patients with moderate to severe KCS.

**MATERIALS AND METHODS**

**Study Design**

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 for up to 2 years in patients with moderate to severe KCS was designed by Allergan (protocol 192371-501-03). Qualified patients entered a 2-week run-in phase during which they were instructed to administer only ophthalmic lubricant (Refresh; Allergan) to each eye as needed daily (day -14 to day 0). The qualification, or baseline, examination (day 0) included questionnaires to quantify subjective assessments, a Schirmer’s tear test (with and without anesthesia), visual acuity, biomicroscopy, tear break-up time, corneal and interpalpebral conjunctival staining (lissamine green and sodium fluorescein), and intraocular pressure. Blood samples were obtained to test for SS-related autoantibodies before randomization. Patients who completed the run-in phase and still fulfilled the inclusion and exclusion criteria (Table 1) were qualified to enter the masked treatment phase at the qualification/baseline visit.

At selected centers, IC specimens were collected by the physician on the qualification-baseline visit (day 0). Specimens were obtained from the worse eye of patients who met the entry criteria and qualified for randomization and who had given specific consent for this procedure to be performed. The worse eye was defined as the one showing the highest degree of corneal staining, or the lowest Schirmer’s test result when both eyes had the same corneal scores. If the two criteria were equal in both eyes, the right eye was chosen for IC. The patients providing samples for this study were recruited from 29 of 35 centers involved in the European trial. The conjunctival samples were collected by the investigators from four countries throughout Europe and shipped to the Immunohematology Department, Ambroise Paré Hospital (Boulogne, France) for centralized analyses.

Baseline data presented in this report were analyzed by flow cytometry on samples gathered from 169 patients. To provide normal reference values for the tested markers, 100 control eyes from 50 normal subjects were also examined under similar procedures in independent investigations performed after ethics committee approval. Patients were assessed as normal according to history data, complete slit lamp examination, tear break-up time recording, fluorescein test, and lissamine green staining. Only patients with absolutely normal criteria and not having received eye drops for at least 2 months were collected for normal population analyses.

The laboratory work for this study and the study protocol were conducted in compliance with the Ethics Committee (CCPPPAB) at the Ambroise Paré Hospital and the relevant Ethics Committees in each of the participating countries. Written, informed consent was obtained before enrollment in the study, and patients were given the option to provide or not provide conjunctival impressions for this study. Specific additional consent for the impression procedure was obtained in all cases. This study was conducted in compliance with the Declaration of Helsinki, Somerset–West amendment, 1996.

**Sample Collection and Handling**

After the instillation of one drop of topical anesthetic (0.04% oxybuprocaine), two to three filters 13 × 6.5 mm in size...
(polyethersulfone filters, 0.20-µm pores, 13-mm in diameter; Supor; Gelman Sciences, MI) were applied to neighboring areas of the superior and superotemporal bulbar conjunctiva without exerting any pressure, 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 analyses. Care was taken to collect IC samples from nonexposed regions of the conjunctiva. Membranes were removed immediately after contact. Approximately 50% to 70% of the total surface of the filter was to be covered by cells. If not, investigators were instructed to collect a new specimen from an adjacent area. All membranes from each eye were immediately dipped into tubes containing 1.5 ml of cold phosphate-buffered saline (PBS, pH 7.4) with fixative (0.05% paraformaldehyde, prepared monthly and sent regularly from the central laboratory to the centers). Tubes were to be kept at 4°C before impression collection and sent within 2 days to the Department of Immunohematology, Ambroise Paré Hospital, in cold-conditioned containers. After reception by the central laboratory, cells were extracted by gentle agitation for 30 minutes and centrifuged (1600 rpm, 5 minutes). They were then counted in a Malassez cell before processing for flow cytometry. The conjunctival samples were processed up to a week after samples were collected. Because the samples were kept in fixative solution at 4°C, no major sample degradation was observed, as assessed elsewhere in repeated flow cytometric analyses over 4 weeks after collection (data not shown).

Antibodies and Immunofluorescence Procedures

Five sets of antibodies and two corresponding negative controls were used for assaying: CD95/Fas, the apoptotic marker APO2.7, class II antigen HLA DR, CD40, and CD40 ligand. One antibody was used for the direct immunofluorescence procedure for the following two labels: fluorescein isothiocyanate (FITC)-conjugated mouse IgG1 anti-human CD95 (clone UB2, 1 mg/ml; Immunotech, Marseilles, France), and phycoerythrin (PE)-conjugated mouse IgG1 anti-human APO2.7 (clone UB2, 1 mg/ml; Immunotech). APO2.7 is a mitochondrial protein reliably expressed by cells involved in the apoptotic pathway. The FITC/PE-conjugated nonimmune mouse IgG1 was used as a negative isotypic control for direct immunofluorescence procedure. Two sets of antibodies were successively used for the indirect immunofluorescence procedure. The primary antibodies were 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), and mouse IgG1 anti-CD40 ligand (clone TRAP1, 1 mg/ml; Immunotech). FITC-conjugated goat anti-mouse immunoglobulins was used as the secondary antibody for all the assays (Dako). The nonimmune mouse IgG1 was used as a negative isotypic control for the indirect immunofluorescence procedure (Dako).

Antibodies were used in a 1:50 dilution in 1% bovine serum albumin containing PBS. After 30 minutes of incubation, cell suspensions were washed in PBS by 5-minute centrifugation and, for indirect immunofluorescence procedures, reacted with the secondary anti-mouse immunoglobulins in a 1:50 dilution, for 30 minutes. At the end of incubations, cells were then centrifuged in PBS (1600 rpm, 5 minutes), resuspended in 100 µl/ml of PBS, and analyzed on a flow cytometer (FACScan; Becton Dickinson, Meylon, France), according to previously validated methods.

Flow Cytometry Processing

The linear plot giving granulometry versus cell size consistently revealed a single cell population (Fig. 1). Analytic gates were set around this population to exclude cellular debris and aggregates. The number of positive conjunctival cells was then obtained from logarithmic cytograms of mean fluorescence intensities. The superior level of fluorescence intensity obtained for the isotypic control antibody was considered as the limit of background fluorescence and the threshold of positivity for the tested antibodies. In each sample, at least 1000 cells were analyzed. All specimens were analyzed in a masked manner, in that the examiner did not know the clinical history of patients.

Data were further expressed as a quantimetric assessment of fluorescence intensities by using calibrated fluorospheres to translate the mean fluorescence of each sample into standardized arbitrary fluorescence units (AUFs), according to a previously published method. A calibration curve was established during each flow cytometric procedure by using four different beads (Immunobrite; Coulter, Hyaleh, FL) with standardized fluorescence intensities. This technique allowed quantification and objective comparisons between days and therefore controlled the reliability and quality of measurements. The real number of AUFs was obtained by subtraction of the isotypic negative control from the total AUFs calculated for each marker. The same flow cytometer was used during the study. On a weekly basis, the machine was tested for calibration by processing a set of calibrated beads used as controls.

Statistical comparisons were performed with the Mann-Whitney test and the Z correlation test, at a 0.05 level of significance (Statview IV for Windows; Abacus, Berkeley, CA).

RESULTS

Study Population and Specimen Characteristics

Flow cytometry was performed on 243 IC samples collected before randomization, which generated valid baseline data.
CD95/Fas (n) withdrawal (n) contamination by fluorescein before collection (n) ratio, or clinical data.

Two populations of patients with and without SS in age, sex nasal and temporal lissamine green staining reached 5.47 (of a maximal score of 5), and total mean Schirmer's test was 1.47 mm at 5 minutes, corneal

TABLE 3. Vitali et al.18 (Table 2). At baseline, in the overall population, according to the clinical and biological criteria reported by IC was therefore 10,000 to 685,000 cells per specimen that specimens were discarded. Range of cell numbers collected by however, because at least 1000 cells were required per analysis and a minimum of seven analyses (five markers and two controls) were performed, the number of cells in the sample was to be greater than 10,000, and poorer or nonhomogeneous specimens were discarded. Range of cell numbers collected by IC was therefore 10,000 to 685,000 cells per specimen that reached the level of quality required for reliable analyses.

For the 169 patients with valid baseline samples, the mean (range) age was 57.1 years (18–86 years) and 86.4% (146/169) were women. From these 169 patients, 41% (70/169) had SS, according to the clinical and biological criteria reported by Vitali et al.18 (Table 2). At baseline, in the overall population, mean Schirmer's test was 1.47 mm at 5 minutes, corneal staining score was 2.96 (of a maximal score of 5), and total nasal and temporal lissamine green staining reached 5.47 (of a maximal score of 10). No difference was found between the two populations of patients with and without SS in age, sex ratio, or clinical data.

TABLE 2. Criteria for Diagnosis of SS

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<td>One of the following three dry eye ocular symptoms: Daily persistent troublesome dry eyes for more than 3 months Recurrent sensation of sand or gravel in the eyes Uses of tear substitutes more than three times a day</td>
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<tr>
<td>Plus any one of the following three dry eye oral symptoms: Daily feeling of dry mouth for more than 3 months Recurrent or persistently swollen salivary glands as an adult Frequently need for liquid to aid in swallowing dry foods</td>
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<tr>
<td>Plus a positive ocular sign (Schirmer's test result #)</td>
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<tr>
<td>Plus any one of the following three dry eye oral symptoms: Recurrent or persistently swollen salivary glands as an adult Occasionally need for liquid to aid in swallowing dry foods</td>
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the two criteria). CD40 ligand was also found significantly more expressed in eyes with KCS than in normal ones \( (P = 0.007 \text{ in percentage of positive cells; } P = 0.02 \text{ in AUF}) \).

Data from the apoptosis-related marker APO2.7 were available from 168 patients, all found positive. The range of percentage of positive cells was 8% to 100% with a mean of 77% 
\( \pm 25\% \), and a mean level of expression of 6999 \( \pm 6087 \) AUF. No difference was found between SS and non-SS groups. Fas was also found on surface conjunctival cells in all specimens. The analysis of Fas expression showed that most cells were immunolabeled (95% \( \pm 9\% \) with minimal and maximal values respectively of 22% and 100%. Fas fluorescence levels ranged from 2,956 to 82,026 with a mean \( \pm \text{SD} \) of 15,760 \( \pm 10,224 \) AUF in the overall population. Results of Fas expression from the SS group showed significantly higher levels than in the non-SS group (17,565 \( \pm 11,037 \) AUF versus 14,038 \( \pm 9878 \) AUF, \( P = 0.01 \)). However, no significant difference could be found between normal subjects and patients with KCS for Fas and APO2.7.

Highly significant positive correlation was found between HLA DR and CD40, both in percentages of positive cells and in AUFs \( (R^2 = 0.37, P < 0.0001; \text{ and } R^2 = 0.31, P < 0.0001, \text{ respectively}) \), between HLA DR and CD40 ligand percentages and AUF \( (R^2 = 0.28, P = 0.0005; \text{ and } R^2 = 0.22, P = 0.005, \text{ respectively}) \), between HLA DR and Fas, only in AUF \( (R^2 = 0.36, P < 0.0001) \). CD40 percentages and AUF were also significantly correlated with CD40 ligand and Fas, but APO2.7 was not significantly correlated with any of the other markers.

**DISCUSSION**

The results from this analysis at baseline and comparisons with normal patients as controls confirm that the ocular surface of patients with moderate to severe KCS presents clear signs of inflammation and imbalance of apoptosis-related receptors. SS is an autoimmune disease involving not only the lacrimal glands but the whole ocular surface and causing chronic inflammation, with lymphocytic infiltrates and apoptosis of ocular epithelial cells. Even in non-SS dry eye, however, an inflammatory reaction has been demonstrated in KCS3–8 that could be hypothesized to result from chronic ocular surface dryness and epithelial cell degeneration. As suggested by Stern et al.,19 neural deregulation in the ocular surface and lacrimal glands may result from continuous secretion of proinflammatory cytokines and may exacerbate ocular surface damage by decreasing the tearing reflex. The role of apoptosis in ocular cells has also been shown in ocular surface disorders and in KCS; Fas and APO2.7 were found to be significantly correlated to the expression of inflammatory markers.15 In lacrimal glands of patients with SS, acinar cells are infiltrated by CD8\textsuperscript{+} lymphocytes that adhere to acinar cells and induce epithelial apoptosis by implying the Fas–Fas ligand pathway.20 Similarly, in dog models of SS, apoptotic acinar cells and lymphocytes with decreased levels of apoptosis can be observed within lacrimal glands. Lymphocyte infiltration results in complete atrophy of lacrimal glands that may be reversed by topical treatment with cyclosporine.21

High HLA DR expression by surface conjunctival epithelial cells was thus found in this large series of patients with moderate to severe dry eye. Almost all eyes showed high levels of HLA DR-positive epithelial cells. These results correlated well, however, with those previously reported using similar methods of flow cytometry in impression7,15 or brush9 cytolysis. Class II antigens HLA DR are membrane antigens expressed by immunocompetent cells, normally restricted to antigen-presenting cells. In inflammatory disorders, HLA DR expression
may be induced in epithelial cells. HLA DR has thus been reported to be overexpressed in the conjunctival epithelium in chronic conjunctivitis and in dry eyes.6–9,22

In the present study, similar results were observed in SS and non-SS eyes in patients with high levels of inflammatory and apoptotic markers, although HLA DR, CD40, and Fas were expressed at significantly higher levels in patients with SS. Tsubota et al.9 also found very high HLA DR expression in patients with SS but much lower levels in those with KCS without SS. The ocular surface in patients with dry eye without SS may in their study have been less severely injured than in the present work, in which all patients, whatever the underlying diagnosis, had significant KCS, assessed by very low Schirmer's test results and intense corneal staining. This could indicate that KCS per se may induce chronic inflammation in the ocular surface by the chronic injury to epithelial cells caused by tear deficiency, mechanical abrasion by the eyelid, and possibly the absence of trophic factors, such as epidermal growth factor or transforming growth factor beta.18,23 Conversely, it may be hypothesized that proinflammatory mediators chronically liberated on the ocular surface cause epithelial cells to degenerate over time, because various cytokines may stimulate apoptosis in many cell types. Such mediators also may cause degeneration by interfering with neural connections that regulate ocular surface homeostasis.19

Strong relationships between inflammation and apoptosis have therefore been demonstrated in various epithelial cells and especially on ocular surface cells. Fas is normally ex-
pressed by conjunctival epithelial cells, in which it is positively correlated with levels of HLA DR antigens, and stimulating anti-Fas antibodies causes epithelial cell apoptosis. Moreover, interferon-γ has been shown to increase expression of Fas and HLA DR by conjunctival cells and to induce apoptosis, by different metabolic pathways, including stimulation of the Fas system. In the present study, we confirmed the significant correlation between HLA DR and Fas in patients with dry eye, with or without SS. We did not find in this population, however, the overexpression of the apoptotic marker APO2.7, as previously demonstrated in ocular surface disorders. This could be related to the low levels of expression obtained when using direct immunofluorescence procedures, which reduce the possibility for slight differences to be raised to significance. We cannot exclude, however, the hypothesis that in the most severe cases of KCS, tissue differentiation may have been impaired, as shown by Jones et al. in SS, resulting in an increased number of epithelial layers and possibly a low level of apoptosis in the superficial layers.

CD40 and CD40 ligand were also found in ocular surface cells of patients with KCS, at upregulated levels in dry eyes compared with normal eyes, and at higher levels in eyes with than in eyes without SS. CD40 was also positively correlated with HLA DR, CD40 ligand and Fas expressions. CD40 upregulation may be observed in inflammatory conditions in various tissues and cell systems. CD40 has been shown to be involved in lymphocyte stimulation, chronic inflammation, and apoptosis. As HLA DR, its expression in epithelial cells is stimulated by various cytokines, such as interferon-γ or TNF-α, which could be synthesized in the ocular surface and lachrymal glands by infiltrating lymphocytes. CD40 has also been shown in other systems to interfere with the Fas–Fas ligand pathway. However, CD40–CD40 ligand interaction alone could not be sufficient to provide a mitogenic signal to T cells and should rather be implicated in amplifying the inflammatory reaction. Mechanisms of such overexpressions of inflammatory markers may therefore involve such cytokines as interferon-γ and TNF-α. These two cytokines have a synergistic effect on HLA DR expression by conjunctival epithelial cells. Interferon-γ also stimulates Fas expression, Fas-induced apoptosis and CD40 expression in a conjunctival cell line. Whether HLA DR–expressing conjunctival epithelial cells acquire antigen-presenting properties, as do corneal epithelial and lachrymal acinar cells, remains to be determined. However, it may be hypothesized that immunologically activated epithelial cells can be targeted by lymphocytes in cytotoxic reactions and/or that they participate in recruitment of inflammatory cells.

Nevertheless, overexpression of inflammation- and apoptosis-related antigens confirms that epithelial cells, even in nonautoimmune KCS, are directly involved in the inflammatory process. These data provide additional rationale for using cyclosporine ophthalmic emulsion in KCS, whatever its origin, to reduce both inflammatory and apoptotic involvement of ocular surface cells. Flow cytometry provided a valuable tool to reliably assess and quantify the level of inflammatory impairment of conjunctival epithelial cells, both at baseline, and throughout the study, to monitor the immunomodulatory effect of cyclosporine. Based on the present study, analyses of eyes receiving the masked treatments in this large European multicenter trial on Cyclosporin A Ophthalmic Emulsion, will further be presented and will provide major information concerning the effect of this drug on the ocular surface in moderate to severe KCS.

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References