Conjunctival T-Cell Subpopulations in Sjögren's and Non-Sjögren's Patients with Dry Eye

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Purpose. To examine the conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca (SS-KCS) and non-Sjögren's keratoconjunctivitis sicca (NS-KCS) for evidence of immune-based inflammation.

Methods. Conjunctival biopsy specimens were obtained from 15 patients with SS-KCS and 15 with NS-KCS. Immunohistochemistry was performed on frozen sections to characterize and quantify T-cell subtypes (CD3, CD4, and CD8) and markers of immune activation (major histocompatibility complex [MHC] class II: HLA-DR, HLA-DQ) and inflammation (intercellular adhesion molecule [ICAM]-1). The numbers of cells positive for each marker were counted by two masked observers and averaged.

Results. Conjunctival biopsy specimens from patients with SS-KCS or NS-KCS revealed lymphocytic infiltration and increased immunoreactivity for the markers of inflammation and immune activation. The extent of cellular immunoreactivity did not differ significantly between SS-KCS and NS-KCS tissue samples.

Conclusions. The authors' findings indicate that patients with SS-KCS or NS-KCS have conjunctival inflammation manifested by inflammatory cell infiltrates and upregulation of expression in markers of immune activation. Clinical symptoms of KCS may be more dependent on T-cell activation and resultant inflammation than previously believed. In addition to tear substitutes, anti-inflammatory therapeutics should be investigated for the treatment of KCS. (*Invest Ophthalmol Vis Sci.* 2002;43: 2609–2614)

Keratoconjunctivitis sicca (KCS), dry eye syndrome, is characterized by ocular discomfort, ocular surface damage, and abnormal tear film quantity and/or quality. The syndrome can be painful and debilitating. It is currently believed that KCS is caused by disease of the lacrimal functional unit, which comprises the ocular surface, the main and accessory lacrimal glands, and the interconnecting innervation.[1] In individuals with KCS, there are detrimental alterations in tear composition and decreases in tear production that lead to symptoms of ocular irritation. Some individuals have KCS secondary to systemic autoimmune diseases such as Sjögren's syndrome (SS-KCS), a disorder characterized by altered lacrimal and salivary gland function and one of the leading causes of KCS.[2] In addition other systemic autoimmune syndromes such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) may include KCS as pathologic feature.[2] There have been no large epidemiologic studies to determine exactly what proportion of patients with KCS actually have Sjögren's syndrome.

One of the diagnostic criteria for Sjögren's syndrome is histologic evidence of significant lymphocytic infiltration of the exocrine glands.[2,3] This is most commonly assessed by incisional biopsy of the minor salivary glands. These infiltrating lymphocytes are predominantly $CD4^+$ T cells and B cells, which contribute to the dysfunction and eventual destruction of the exocrine glands (salivary, lacrimal) by initiating an inflammatory response.[4] Initiation of the immune response is multifactorial but is primarily due to synthesis and secretion of proinflammatory cytokines, which results in upregulation of a series of proinflammatory mediators, including cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1; causes T-cell homing; and facilitates immune activation. Thus, the pathogenic process that leads to alterations in glandular function involves not only lymphocytic infiltration, but also the secretion of proinflammatory cytokines and the presentation of autoantigens.[5] However, the actual mechanism of glandular dysfunction in Sjögren's syndrome and other forms of KCS remains unclear.

Current dogma proposes that the ocular pathogenesis of SS-KCS and non-Sjögren's KCS (NS-KCS) are entirely different, and as a result, the therapies differ between the conditions.[1] Treatment of severe dry eye in SS-KCS may require topical corticosteroids.[6] In contrast, patients with NS-KCS typically use ocular lubricating drops or ointments. If patients with SS-KCS or NS-KCS have similar ocular immune activation and inflammation, current therapies may have to be adjusted and more effective treatments established. Inadequate treatment can result in ocular surface damage, and compromised visual acuity can result from irregular astigmatism, corneal scarring, or perforation. Our hypothesis therefore was that ocular immune activation and inflammation are important in the pathogenesis of KCS and warrant more widespread use of immunomodulatory therapy. To investigate this, we examined conjunctival biopsy specimens of patients with SS-KCS or NS-KCS to determine the presence of immune-based inflammation as a measure of disease status and as a target for therapeutic intervention.

Materials and Methods

Subjects and Biopsy

Thirty patients ranging in age from 36 to 80 years (mean 59; 6 men 24 women) with moderate to severe dry eye were enrolled in a prospective, randomized, masked clinical trial of topical cyclosporine for the treatment of dry eye. Twenty-six subjects were white, two were African American, and two were Hispanic. Baseline evaluations included a complete eye examination and conjunctival biopsy and are

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the basis for this study. Eligible patients had moderate to severe KCS with a Schirmer test result of less than or equal to 8 mm/5 minutes without anesthesia or a Schirmer test result of less than or equal to 10 mm/5 minutes with anesthesia, with concomitant corneal or conjunctival staining. Patients were classified as having SS-KCS if they met at least four of the following criteria: ocular symptoms, ocular signs, oral symptoms, oral signs (sialography, scintigraphy, unstimulated saliva production, or minor salivary gland biopsy), or serology (AntiNuclear Antibody, Sjögren's syndrome A [SSA], Sjögren's syndrome B [SSB], or Rheumatoid Factor). Patients who satisfied the criteria for dry eye (KCS with Schirmer results within the described limits) but not for Sjögren's syndrome were classified as having NS-KCS. Fifteen patients were classified as having SS-KCS and 15 as having NS-KCS. The patients with NS-KCS did not have any associated condition. The protocol was approved by the National Eye Institute's institutional review board, and the research was conducted according to the tenets of the Declaration of Helsinki. Written, informed consent was obtained from all patients before they were enrolled in the study.

Topical anesthetic (Ophthaine; Bristol-Myers Squibb, Princeton, NJ) and subconjunctival injection of 2% lidocaine with epinephrine were administered, and incisional biopsy was performed, with specimens of approximately 2 × 3 mm removed from the inferonasal bulbar conjunctiva. Because there are topographical differences in the conjunctiva, this area was chosen to maintain consistency among the study groups. The tissue was then bisected, with one portion fixed in 4% paraformaldehyde for histology and the other portion embedded in optimal cutting temperature (OCT) compound and fresh frozen with liquid nitrogen for immunohistochemistry.

Immunohistochemistry and Antibodies

Colorimetric Immunostaining. OCT-embedded conjunctival specimens were cryosectioned at 5 μm and collected onto multiwell glass slides (Shandon, Pittsburgh, PA) that were pretreated with an adhesive reagent (Vectabond; Vector Laboratories, Burlingame, CA). Frozen sections were postfixed in 4°C acetone for 3 minutes. Slides were air dried at room temperature for 30 minutes and rinsed in phosphate-buffered saline (PBS) and treated with 0.6% hydrogen peroxide for 10 minutes. Sections were then blocked with 4% normal goat serum in PBS for 60 minutes at room temperature, rinsed in PBS, and incubated with primary antibody in a humidified chamber at 4°C overnight. Subsequently, sections were incubated with the biotinylated species-specific secondary antibody (Oncogene, Cambridge, MA, and Vector Laboratories) for 60 minutes at room temperature. Elite avidin-biotin conjugated system (Vector Laboratories) was optimized for each antibody and applied to the tissue for antigen localization. The time required for color development varied from 3 to 5 minutes, depending on the type of antibody. Negative control experiments were performed for each antibody with 1% to 2% normal sera from the same species.

Six primary antibodies were used as immunohistochemical markers. The following conditions were used: 1.0 μg/mL CD3 (PharMingen, San Diego, CA), 5.0 μg/mL CD4 (BD Bioscience, Franklin Lakes, NJ), 2.5 μg/mL CD8 (BD Bioscience), 1.0 μg/mL HLA-DR (PharMingen), 1.0 μg/mL HLA-DQ (PharMingen), and 1.0 μg/mL ICAM-1 (BioSource, Camarillo, CA). B-cells (CD20, 2 μg/mL; BD Bioscience) were evaluated at baseline in a subset of patients.

Double-Immunofluorescence Labeling. Double-immunofluorescence labeling was performed, with specific monoclonal antihuman CD3 and CD4 antibodies used to identify CD3 and CD4 double-positive cells in a subset of patients with dry eye. The primary antibodies for CD3 and CD4 were conjugated with fluorescein (FITC) or biotin (both from eBioscience, San Diego, CA), respectively. Frozen sections were incubated with primary antibody mixture (20 μg/mL for CD3 and 10 μg/mL for CD4 antibodies) at room temperature for 2 hours. The secondary antibody for detecting the biotinylated antibody was rhodamine red-X-conjugated with streptavidin (Jackson ImmunoResearach Laboratory, Inc., West Grove, PA) and used at a 1:50 dilution. The distribution of CD3 and CD4 immunoreactivity was examined by fluorescence microscope (Eclipse E800; Nikon, Inc., Melville, NY) and the images were captured with a digital camera (SPOT RT; Diagnostic Instruments, Inc., Sterling Heights, MI). Because of the size limit of biopsy samples, we were not able to conduct double staining to identify $CD8^+$ T cells.

Quantitative Analysis

Quantitative analysis of the immunohistochemical antibody binding was performed on noncounterstained sections to maximize the signal contrast. Alternating tissue sections were counterstained with hematoxylin to assess morphology and to confirm the localization of the staining. Color micrographs were taken of each section, with a photomicroscope and a ×20 objective. The biopsy images were traced on a computer screen with an image-analysis software package (Image Pro Plus, ver. 4.0; Media Cybernetics, Silver Spring, MD), and the total area of the biopsy section was determined. The numbers of cells positive for immunohistochemical staining against each antibody were counted by two masked observers. Scores were averaged, divided by the area of the sample, and expressed as cells per square millimeter. If the scores between the two observers differed by more than 30%, a third masked observer scored the image and the two closest scores were averaged.

Statistical Evaluation

The mean cell densities for each inflammatory cell marker of NS-KCS were compared with those of SS-KCS by a standard ANOVA. In addition, a MANOVA was performed to compare NS-KCS and SS-KCS cell densities simultaneously. Cell densities were transformed with a natural logarithm to obtain a symmetric distribution.

Results

Progressive lymphocytic infiltration primarily comprising T cells in the lacrimal gland and conjunctiva is known to be characteristic of the ocular surface histopathology in KCS. To further determine and compare the subpopulations of T lymphocytes in the ocular tissues of patients with KCS with the systemic autoimmune disease (SS-KCS) as well as those of patients without the systemic autoimmune disease (NS-KCS), immunohistochemical staining was performed on conjunctival specimens obtained from them by incisional biopsy. Large numbers of infiltrating lymphocytes were found in both SS-KCS and NS-KCS specimens. The cellular infiltrates were examined for T cells (CD3; Fig. 1A), including the subsets of CD4 (Fig. 1B) and CD8 T cells (Fig. 2). The results showed that most of the infiltrating cells were positive for CD3 (a marker definitive for T cells). On sequential sections from the same tissue block, the same area was stained for $CD4^+$ or $CD8^+$ cells. Double immunofluorescent staining confirmed that a large population of $CD3^+$ cells were also $CD4^+$, indicating that they were $CD4^+$ T cells (Fig. 3). In addition, a small number of B cells were found in conjunctival tissue in both SS-KCS and NS-KCS (data not shown).

To investigate the activation status of these infiltrating lymphocytes in the conjunctiva, the immunoreactivity of major histocompatibility complex (MHC) class II antigen HLA-DR and its essential comediator for antigen presentation and immune activation, HLA-DQ, were subsequently evaluated for the same tissue blocks from patients with SS-KCS or NS-KCS. The conjunctiva of both groups had large numbers of cells expressing HLA-DR (Figs. 1E, 4A, 4B). HLA-DQ was also detected in a substantial number of cells (Fig. 4C). These two markers were specifically chosen to evaluate immune activation. It is known that HLA-DQ and HLA-DR can be expressed by professional antigen-presenting cells (APCs) including T cells and B cells. In the current study, in the conjunctival tissue of SS-KCS and NS-KCS, HLA-DR and HLA-DQ were not only expressed by

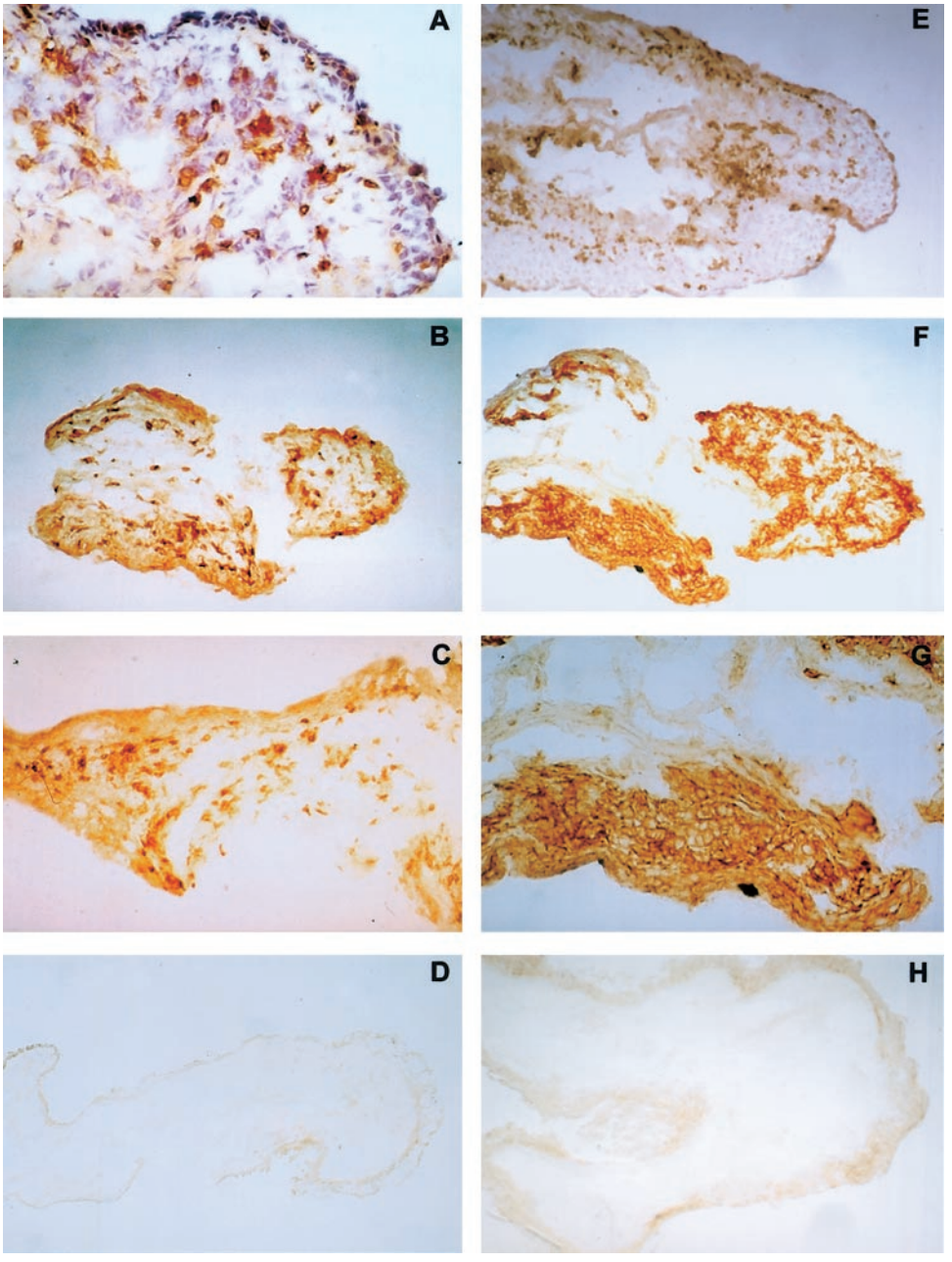

FIGURE 1. Representative micrographs of conjunctival biopsy specimens from patients with KCS. Sections were stained for $CD3^+$, $CD4^+$, HLA-DR, and ICAM-1. (**A**) $CD3^+$ immunoreactivity was detected primarily in the substantia propria and to a lesser extent in the subepithelium. Specific membrane staining confirmed the surface expression of $CD3^+$. (**B**) $CD4^+$ cells were apparent in the substantia propria, with most cells accumulating anteriorly. (**C**) A few migrating $CD4^+$ T lymphocytes in the epithelium were evident at a higher magnification. (**D**) No significant immunoreactivity for $CD4^+$ antibody was detected in the conjunctival specimen incubated with correspondent IgG (as a negative control). (**E**) Cells immunopositive for HLA-DR were in both the substantia propria and epithelium. (Strong membrane staining in the epithelium is shown in Fig. 4A at a higher magnification). (**F**) ICAM-1 expression was exhibited on both infiltrating lymphocytes and conjunctival epithelial cells. (**G**) Specific ICAM-1 membrane staining on most but not all epithelial cells was evident at a higher magnification. (**H**) No significant ICAM-1 immunoreactivity was detected in the conjunctival specimen incubated with correspondent IgG (as a negative control). Magnification: (**A**, **G**) ×40; (**B**, **D**–**F**, **H**) ×20; (**C**) ×63.

lymphocytes (Fig. 1E), but also by conjunctival epithelial cells (as shown in Fig. 4). For this reason, the number of cells positive for HLA-DR or HLA-DQ appeared greater than the number of $CD3^+$ T cells (Fig. 5). More important, MHC II molecules expressed on nonprofessional APCs, such as conjunctival epithelial cells, are indicative of an active role of resident epithelial cells on the ocular surface in the pathogenesis of dry eye disease.

Further, the expression of ICAM-1, an adhesion molecule that plays an important role in the amplification of inflammation by facilitating lymphocyte homing, was also examined. ICAM-1 immunoreactivity was detected on the vascular endothelial cells, infiltrating lymphocytes in the substantia propria and in residential epithelial cells of the conjunctival tissue in SS-KCS and NS-KCS (Fig. 1F). In most of the conjunctival biopsy specimens tested in the present study, ICAM-1 positivity was detected in some but not all epithelial cells in the conjunctiva. In some cases (both SS-KCS and NS-KCS), the basal epithelium appeared to exhibit relatively stronger immunoreactivity for ICAM-1 than the superficial epithelium. This may be due to the physical location of the basal epithelium, which is the first line attacked by migrating lymphocytes extravasated from blood vessels in the substantia propria of the conjunctiva.

Overall, the conjunctiva of patients with SS-KCS or NS-KCS demonstrates increased immunoreactivity for the markers for immune activation and inflammation (Fig. 5). The number of T cells positive for subtypes $CD4^+$ or $CD8^+$, as well as the number of cells expressing immune activation markers HLA-DR and HLA-DQ, were not significantly different between SS-KCS and NS-KCS tissue; all probabilities exceeded 0.10 (Fig. 5). Strong expression of ICAM-1 was evident in the conjunctival tissue in both SS-KCS and NS-KCS, and the number of positively labeled cells did not significantly differ ($P > 0.10$) between the groups (Fig. 5). In addition, a simultaneous comparison (MANOVA) resulted in a probability of 0.36, indicating no difference between groups.

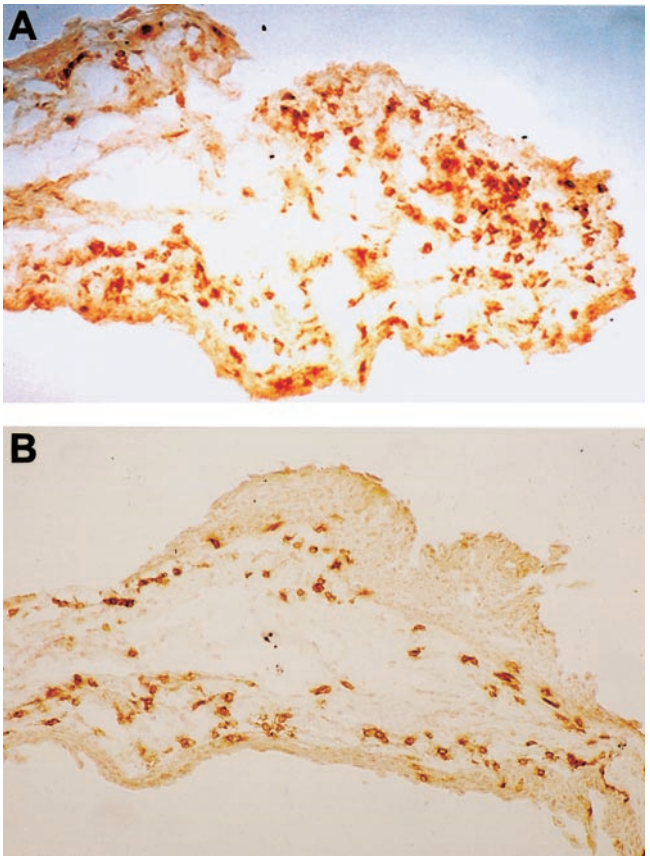

FIGURE 2. $CD8^+$ expression was comparable in the conjunctival biopsy specimens of patients with KCS, with (**A**) and without (**B**) Sjögren's syndrome. In this example, the number of $CD8^+$ cells in the section in (**A**) was 95 and in that shown in (**B**) was 89. The $CD8^+$ cells were located in the substantia propria, with most accumulating anteriorly. Small follicles positive for $CD8^+$ were often present in SS-KCS specimens (**A**). Magnification, ×20.

DISCUSSION

Dry eye is a condition affecting millions of people around the world. With medical advances, people are living longer, and the number of people with KCS is increasing. Currently, it is thought that only patients with KCS who have systemic conditions such as Sjögren's syndrome benefit from topical anti-inflammatory treatment. In our study, similar to patients with SS-KCS, patients with NS-KCS had an immune-based inflammation and therefore such patients may benefit from a treatment that alleviates symptoms through reduction of this underlying pathologic process.

The two diseases have differ causes: SS-KCS is caused by systemic autoimmunity, with very little environmental induction of the disease processes, whereas NS-KCS is without systemic autoimmunity and typically is caused by chronic ocular surface irritation. Past studies have differentiated SS-KCS from NS-KCS by several criteria, including the presence of exocrine gland lymphocytic follicular infiltrates.[7–9] More recent evidence suggests that in SS-KCS the formation of lymphocytic foci is a late-stage event subsequent to salivary and lacrimal glandular dysfunction.[10,11] In addition, we have shown that glandular dysfunction can occur in noninfiltrated portions of accessory lacrimal glands in biopsy sections of dogs with dry eye.[12] Together these findings support the notion that follicle formation, although indicative of systemic autoimmunity and disease progression, does not preclude a common pathophysiology for aspects of SS-KCS and NS-KCS.

Glandular dysfunction appears to be related to both the homing of lymphocytes to the main and accessory lacrimal glands and the activation status of the cells at the time of diapedesis from conjunctival blood vessels into the substantia propria.[1] Our current findings demonstrate that patients with SS-KCS or NS- KCS have large numbers of infiltrating T cells, demonstrated by their positivity for the pan T-cell antibody CD3. To further define the subpopulation of immune cells within the biopsy, antibodies against CD4 and CD8 were used. Immunoreactivity to both CD4 and CD8 was detected. Double staining was performed to verify that CD4 was expressed on T cells. The results illustrate that a large number of $CD3^+$ cells were indeed $CD4^+$ T cells (CD3 and CD4 double positive). The presence of activated $CD4^+$ T cells (as demonstrated by HLA-DR and HLA-DQ immunoreactivity) in the conjunctival tissue of both SS-KCS and NS-KCS may cause ocular surface dysfunction, due to their secretion of proinflammatory cytokines.[13] The significance of the current findings is that the conjunctival cellular infiltrates in both SS-KCS and NS-KCS are made up of similar amounts of $CD4^+$ and $CD8^+$ T cells (Fig. 5), indicating a common cellular basis of ocular surface inflammation.

Our results also demonstrate that both SS-KCS and NS-KCS exhibit conjunctival ICAM-1 immunoreactivity. Increased expression of lymphocyte function-associated antigen (LFA)-1, the ligand for ICAM-1, was also detected (data not shown). ICAM-1 is a cell surface adhesion molecule that facilitates entry of lymphocytes into the site of inflammation. Vascular endothelial ICAM-1 binding to LFA-1 on the lymphocytes promotes peripheral lymphocyte extravasation and homing to the target tissue.[14] ICAM-1 expression on infiltrating lymphocytes can be a secondary signaling molecule for potential antigen presentation and exacerbates the immune-mediated inflammatory response.[15] The resident epithelial cells' expression of ICAM-1 may provide a direct contact between epithelial cells and infiltrating lymphocytes, resulting in epithelial cell damage, such as apoptosis.[12] Finally, in both conditions HLA-DR and HLA-DQ are expressed and upregulated in the infiltrating lymphocytes and conjunctival epithelial cells allowing for T cell–epithelial cell (potential APCs[16,17]) interactions that lead to immune activation. The finding that multiple markers for immune-based inflammation exist in both medical conditions further supports a common pathophysiology.

Ocular irritation, such as that caused by inadequate tears, may lead to chronic inflammation as measured by inflammatory infiltrates in the target tissue. However, the present study demonstrates not only the presence of immune cells, but also the immunoreactivity for MHC class II antigens on the surface of both lymphocytic infiltrates and resident conjunctival epithelial cells. It is still not certain that nonprofessional APCs can present activating antigens. The expression of class II antigen, however, is most likely not merely the result of irritation. It is a rather strong indication of immune reactivity. Therefore, we believe that the commonality in the pathophysiology of SS-KCS and NS-KCS is immune-based inflammation.

Conjecture surrounds the cause of KCS. One theory focuses on an autoimmune response to the local presentation of self-antigen in both SS-KCS and NS-KCS. In the normal individual, the inappropriate immune response is suppressed through the anti-inflammatory environment created by circulating androgens. A decrease in the levels of circulating androgens below a certain threshold facilitates an environment in which the initiation of KCS can proceed.[18,19] The processes of aging, especially menopause, causes a decrease in the presence of these protective hormones, thereby compromising the normal anti-inflammatory umbrella.[18,20] This leaves the lacrimal functional unit susceptible to a second event (e.g., viral infection, chronic low humidity, wind, or allergy), which repeatedly triggers the

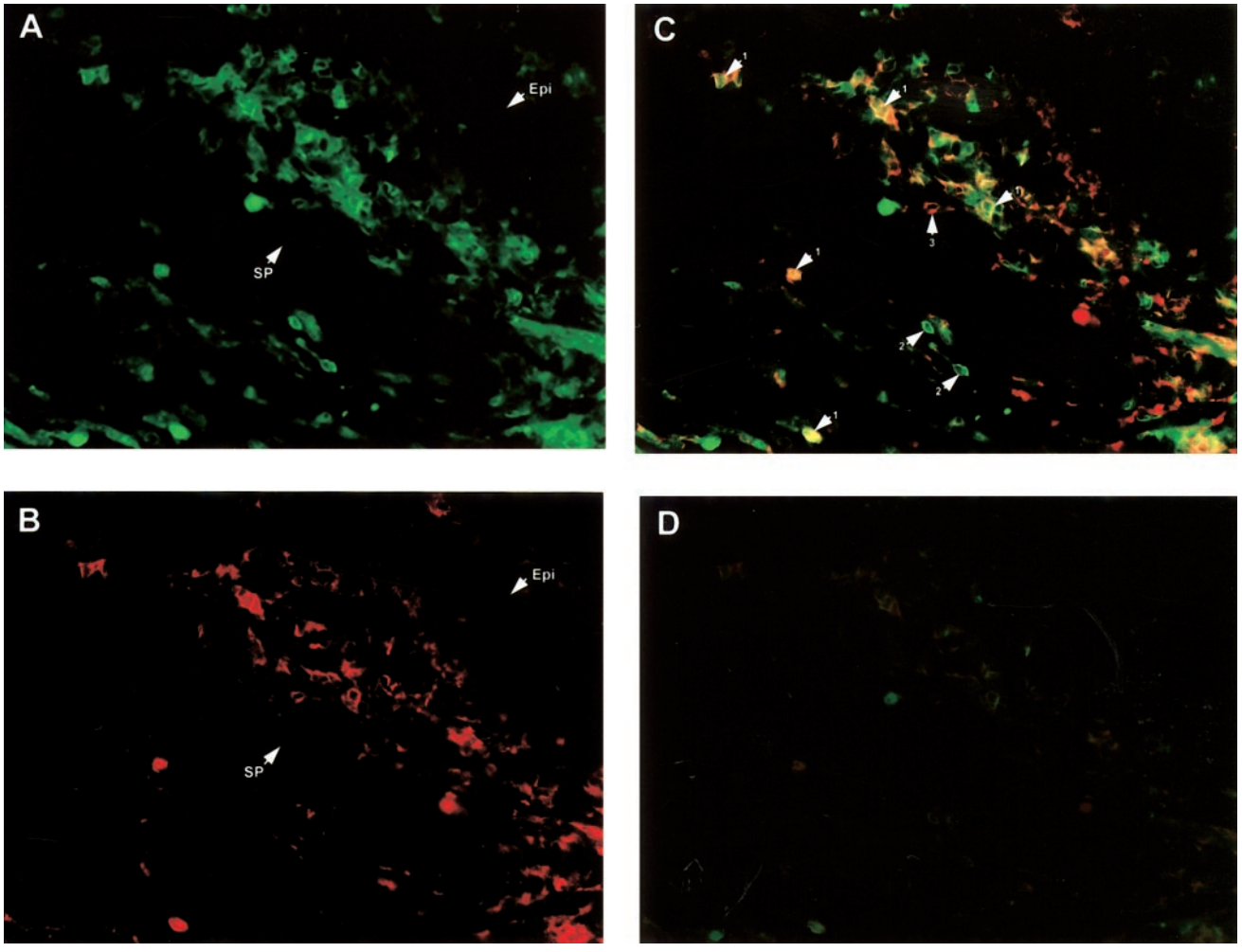

FIGURE 3. Colocalization of CD3 and CD4 immunoreactivity in the conjunctival tissue of a patient with SS-KCS. (**A**) $CD3^+$ cells (FITC *green* fluorescence) were detected primarily in the area of the substantia propria (SP) approaching the conjunctival epithelium (Epi). (**B**) A similar staining pattern of $CD4^+$ cells was observed (rhodamine *red* fluorescence). (**C**) The superimposed images of (**A**) and (**B**) revealed CD3 and CD4 double-positive cells (*arrow 1*, *yellow-orange*) in the same biopsy section. A few $CD3^+$ cells (*arrow 2*, *green*) and $CD4^+$ cells (*arrow 3*, *red*) were also detected. (**D**) No significant positive staining was detected in a serial section of the same patient that was incubated in the staining solution without primary antibodies (the negative control).

lacrimal reflex (the functional unit) and initiates neurogenic inflammation within the main and accessory lacrimal glands.[1,21] Inflammation of the secreting lacrimal acinar epithelial cells could provide an initial signal for activation of vigilant trafficking T cells. The result of this activation is further recruitment and activation of mature T cells and the amplification of the inflammatory processes, as seen in patients with KCS.

In the present study, the conjunctiva of patients with KCS, with and without Sjögren's syndrome, exhibited infiltrating lymphocytes and evidence of markers of immune activation and inflammation. This suggests that the factors that impair lacrimal gland secretion in both SS-KCS and NS-KCS may have a common pathophysiology. Further, lubrication-based treatment with artificial tears, ointments, and sustained-release pel-

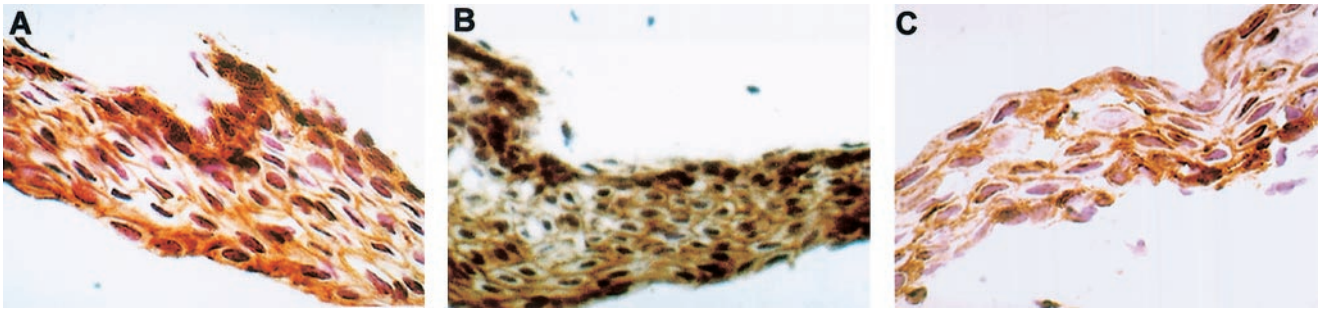

FIGURE 4. Expression of HLA-DR (**A**, **B**) and HLA-DQ (**C**) in the conjunctival epithelium of patients with SS-KCS (**A**) or NS-KCS (**B**, **C**). The pattern and number of $HLA\text{-}DR^+$ cells in the SS-KCS tissue section (**A**) was similar to that in the NS-KCS section (**B**). Specific membrane staining was detected in all three cases. Magnification, ×100.

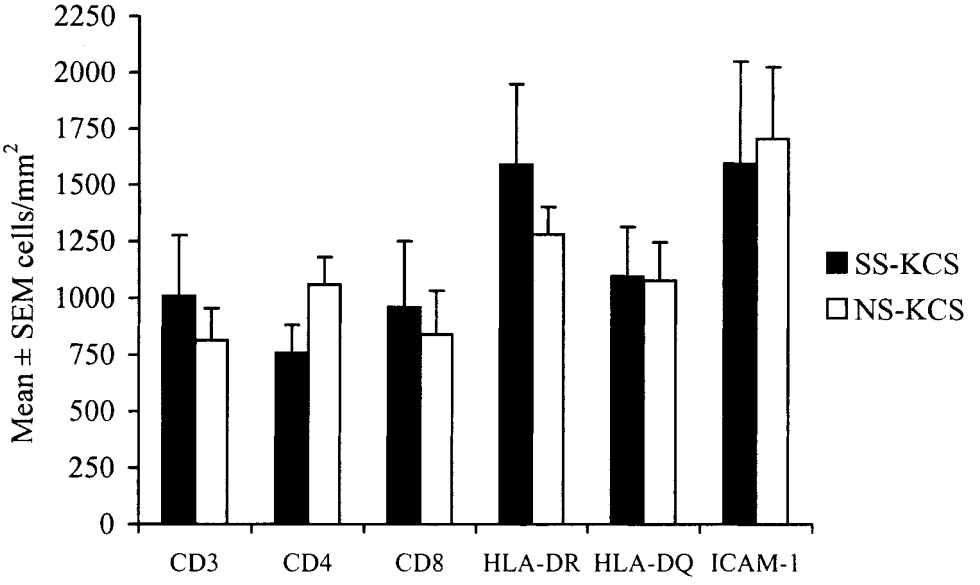

FIGURE 5. Comparison of the presence of cellular markers of inflammation and immune activation between patients with SS-KCS (n = 12–13) and those with NS-KCS (n = 12–15).

lets are solely palliative and may have limited effectiveness in the treatment of NS-KCS. Patients with either SS-KCS or NS-KCS may benefit from a similar therapeutic regimen directed against inflammation, despite their different origins. Based on the findings in this baseline study, a study is underway examining alterations in inflammatory cell populations after ocular application of cyclosporine, an immunomodulatory agent, in patients with SS-KCS or NS-KCS.

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