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Conjunctival Epithelial Cell Expression of Interleukins and Inflammatory Markers in Glaucoma Patients Treated over the Long Term

Christophe Baudouin, MD, PhD,[1,2] *Pascale Hamard, MD, PhD,*[1,2] *Hong Liang, MD,*[2]
Catherine Creuzot-Garcher, MD, PhD,[3] *Larry Bensoussan, MD,*[1] *Françoise Brignole, MD, PhD*[2,4]

Purpose: To compare the conjunctival epithelial cell expressions of inflammatory cytokines in normal subjects and in glaucoma patients treated over the long term.

Design: Case–control study.

Participants: A total of 69 glaucoma patients treated over the long term and 15 normal subjects with no ocular abnormality or topical treatment.

Methods: Amongst the 69 glaucoma patients, 27 were treated with preserved β-blockers, 24 with unpreserved 0.5% timolol, and the other 18 patients with an association of ≥2 preserved drugs. All patients were treated for more than 1 year with the same treatment, with no significant differences between groups for mean ages and durations of treatment at the time of the study. Impression cytology specimens were taken and processed for immunofluorescence techniques. Conjunctival cell expressions of HLA DR, as a standard for inflammatory level, and the interleukins IL-6, IL-8, and IL-10 were obtained and quantified using flow cytometry.

Main Outcome Measures: Immune markers and proinflammatory cytokines in impression cytology specimens.

Results: We found a significantly increased expression of all immunoinflammatory markers and mediators in the conjunctival epithelium of glaucoma patients compared with normal eyes. Human leukocyte antigen DR was significantly higher in the 2 groups receiving preserved drugs than in the unpreserved timolol group. The 3 interleukins were similarly overexpressed in all glaucoma groups, with no significant between-groups differences except for the expression level of IL-8, which was significantly higher in the multitreatment group than in the preservative-free one.

Conclusions: The present study confirms the increased expression of immunoinflammatory markers by the conjunctival epithelium of glaucoma patients treated over the long term. The development of nontoxic preservatives or preservative-free solutions is therefore of great interest. *Ophthalmology 2004;111:2186–2192 © 2004 by the American Academy of Ophthalmology.*

There is now growing evidence from experimental and clinical studies that the long-term use of antiglaucoma drugs may induce ocular surface changes, causing ocular discomfort upon instillation,[1] tear film instability,[2] conjunctival inflammation,[3] subconjunctival fibrosis,[4] conjunctival epithelium apoptosis,[5] corneal surface impairment,[1,6] and potential risk for failure of further glaucoma surgery.[7,8] Subclinical inflammation has also been described with significant infiltration of the conjunctival epithelium and substantia propria by inflammatory cells,[3,9,10] as has conjunctival epithelial cell expression of inflammatory markers,[11,12] in patients receiving antiglaucoma treatments for long periods. However, the respective roles of the active compound and the preservative in inducing toxic and/or

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[1] Departments of Ophthalmology, Quinze-Vingts National Ophthalmology Hospital and Ambroise Paré Hospital, APHP, Paris-Ouest School of Medicine, University of Versailles, Paris, France.

[2] INSERM U598, Cordeliers Biomedical Institute, Paris, France.

[3] Department of Ophthalmology, University Hospital of Dijon, Burgundi University, Dijon, France.

[4] Department of Toxicology, Faculty of Biological and Pharmacological Sciences, University Paris 5 René Descartes, Paris, France.

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Correspondence to Christophe Baudouin, MD, PhD, Quinze-Vingts National Ophthalmology Hospital, 28 rue de Charenton, 75012 Paris, France. E-mail: baudouin@quinze-vingts.fr.

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Table 1. Demographic Characteristics of Normal Subjects and the 3 Groups of Glaucoma Patients

	Preservative-Free Timolol	Preserved Timolol	Multitreatments	Normal Subjects
n (eyes)	24	27	18	15
Age (yrs)				
Mean ± SD	62.7 ± 15.5	60.0 ± 7.5	56.6 ± 16.9	49.3 ± 20.7
Range	45–84	46–72	28–79	37–66
Gender				
Male	10 (42%)	12 (44%)	10 (55%)	7 (46%)
Female	14 (58%)	13 (56%)	8 (45%)	8 (54%)
Type of treatment	0.5% timolol	0.5% timolol	0.5% timolol (n = 18)* Prostaglandin (n = 11)* CAI (n = 9)* α-2 agonist (n = 5)*	NA
Total duration of treatment (yrs)				
Mean ± SD	4.7 ± 2.5	4.6 ± 2.8	5.9 ± 6.6	NA
Range	2–7	1–10	2–24	NA

CAI = carbonic anhydrase inhibitor; NA = not applicable; SD = standard deviation.
No statistical difference between each group for age and duration of treatment (Mann–Whitney *U* test).
*Eleven patients had 2 medications; 7 received 3 different drugs.

proinflammatory effects of antiglaucoma ophthalmic solutions are still being debated. The most frequently used preservative, benzalkonium chloride, has widely demonstrated its toxic effects in laboratory,[13,14] experimental,[9,15,16] and clinical studies.[1,17,18] As a quaternary ammonium, this compound is much more than an excipient and has been shown to cause directly or has shown some evidence of causing tear film instability,[2] loss of goblet cells,[17,19] conjunctival squamous metaplasia and apoptosis,[5,20] disruption of the corneal epithelium barrier,[21] severe loss of endothelial cells if accidentally introduced in the anterior chamber,[22] and even blood–aqueous barrier disruption in the early phase of pseudophakia,[23] leading to the new concept of pseudophakic preservative maculopathy.[24]

In a previous series of reports on the ocular surface, our group developed new objective and reliable techniques for exploring the conjunctival epithelium and assessing the inflammatory and apoptotic status of conjunctival cells by using flow cytometry in impression cytology specimens.[25–27] In a recent article,[12] we used this technique in patients who had received antiglaucoma drugs over an extended period and demonstrated for the first time in a case–control study that patients treated with preserved β-blockers exhibited high levels of HLA DR class II antigens and intercellular adhesion molecule (ICAM) 1, as well as a dramatic decrease in MUC-5AC–expressing cells, whereas eyes receiving unpreserved drugs showed almost normal patterns of inflammation and goblet cell density. Little is known, however, about the inflammatory pathways and mediators involved in ocular surface inflammation related to toxic side effects of ophthalmic drugs and preservatives. In the present case–control study, we used the flow cytometry technique to assess the expression of 3 major interleukins involved in inflammation and inflammatory cell recruitment: IL-6, IL-8, and IL-10. As the overexpression of these 3 interleukins has previously been described in different ocular surface diseases,[28–30] our aims were to validate the technique of flow cytometry in impression cytology for these intracellular markers, investigate interleukin expression by conjunctival epithelial cells in normal eyes and glaucoma patients, and compare their expression in different groups of patients receiving preserved or unpreserved drugs.

Patients and Methods

Patients

A total of 69 patients with glaucoma and 15 normal subjects were included in this case–control study conducted in compliance with the Declaration of Helsinki (Scotland amendment, 2000). All glaucoma patients had chronic primary open-angle glaucoma but no other ocular disease, as assessed after a complete ocular examination. They were treated for at least 1 year with the same treatment, 27 patients with preserved 0.5% timolol (containing 0.01% benzalkonium chloride as a preservative), 24 patients with unpreserved 0.5% timolol, and the other 18 patients with an association of ≥2 preserved drugs (Table 1). In the first 2 groups, no patient had received other antiglaucoma drugs before the ones investigated in this study or a concomitant treatment during the last 6 months before the time of conjunctival imprint collection. There was no significant difference for age and total treatment durations between all groups of glaucoma patients and normal subjects. The multitherapy group had a longer though not significantly different history of glaucoma treatment and served as a control group with multiple and long-term glaucoma treatments. In this group, treatments consisted of a preserved β-blocker in all eyes associated with a prostaglandin analog (11 eyes), a carbonic anhydrase inhibitor (9 eyes), or an α-2 agonist (5 eyes), 11 eyes receiving 2 drugs and 7 eyes 3 drugs. At the time of specimen collection, patients did not show any clinical evidence of intolerance to the administered drugs or ocular surface impairment. Patients with other diseases such as ocular or systemic inflammatory disorders or contact lens wear that might affect the conjunctival epithelium were excluded from the study. Fifteen normal subjects who had no history of ocular disease or clinical ophthalmic abnormality and who had not received any topical treatment for at least 6 months were also investigated, after approval by the ethics committee of

Dijon University, France. The ethics committee of University Paris 6 had previously indicated that the exploration of the ocular surface of glaucoma patients using impression cytology collection did not require specific approval. Nevertheless, all patients had received specific explanations of impression cytology and gave informed consent for the procedure.

Impression Cytology Specimens

One eye was randomly selected for impression cytology analyses. Specimens were collected in the superior bulbar conjunctiva using 0.20-μm polyether sulfone filters (13 mm in diameter; Supor Membranes, Gelman Sciences, Ann Arbor, MI), according to previously described techniques.[12,27] After instillation of 1 drop of 0.04% oxybuprocaine, 2 pieces of filters, each 13 × 6.5 mm, were applied to the superior and superotemporal bulbar conjunctiva without the exertion of any pressure. Specimens were collected at least 15 minutes after the use of fluorescein eyedrops, to avoid interference with immunofluorescence analyses. Membranes were immediately suspended and fixed in cold phosphate-buffered saline (PBS; pH 7.4, at 4° C) containing 0.05% paraformaldehyde. Conjunctival cells were further extracted by gentle agitation and then centrifuged at 1600 rpm for 5 minutes before processing for flow cytometry.

Immunofluorescence Procedure

Four antibodies and 3 isotypic negative controls were used in this study. To determine the inflammatory profile of impression cytology specimens, indirect immunofluorescence was performed with mouse immunoglobulin G1 (IgG1) anti–HLA DR α chain (clone TAL.1B5, 50 μg/ml, Dako SA, Copenhagen, Denmark) as the primary monoclonal antibody. The secondary antibody was fluorescein isothiocyanate–conjugated goat antimouse immunoglobulins (Dako). A nonimmune mouse IgG1 (Dako) was used as a negative isotypic control. For intracellular interleukin assessment, after a first step of cell permeabilization with 1% saponin for 5 minutes (Sigma Chemical Co., St. Louis, MO), phycoerythrin-conjugated anti–IL-6 mouse IgG2b, anti–IL-8 rat IgG2a, and anti–IL-10 rat IgG2a (BD Biosciences, San Diego, CA) were used in direct immunofluorescence procedures, with their respective mouse and rat isotypic negative controls (BD Biosciences). All antibodies were diluted in 1% bovine serum albumin–containing PBS to obtain a final dilution of 1:50, following the manufacturer's instructions. After 30 minutes of incubation with monoclonal antibodies, cell suspensions were washed in PBS, centrifuged for 5 minutes (1600 rpm), and resuspended in 500 μl of PBS before flow cytometry analysis. For HLA DR assessment, cells were additionally incubated for 30 minutes with the secondary antimouse immunoglobulins in a 1:50 dilution, centrifuged in PBS (1600 rpm, 5 minutes), and resuspended in 500 μl of PBS for flow cytometry processing, performed with an Epics-XL (Beckman Coulter, Miami, FL).

Flow Cytometry Analysis

Cells were analyzed from a 2-parameter histogram showing side scatter (cell size) versus forward scatter (cell granulometry) in logarithmic and linear modes, respectively. For each antibody, at least 1000 conjunctival cells were gated to give a logarithmic fluorescence histogram showing the number of cells as a function of fluorescence intensities. A cursor was first set at the highest fluorescence level obtained for each isotypic negative control, which determined the limit of background fluorescence and the threshold of positivity for the tested antibodies. Results were obtained in percentages of positive cells and in mean fluorescence intensities. Mean fluorescence intensity levels of HLA DR and the 3 interleukins tested were further quantified by the Quantitative Indirect Fluorescence Intensity (Dako) and Dakofluorosphere (Dako) methods, respectively, for indirect and direct immunofluorescence techniques. Calibration curves were then obtained, giving mean fluorescence intensities versus the number of molecules of bound antibody, thus defining arbitrary units of fluorescence (AUFs). The actual number of AUFs for a specific marker was then obtained by subtraction of that found for the corresponding isotypic negative control. All flow cytometric analyses were performed in a masked manner for treatment groups and patient characteristics. Statistical comparisons were performed using the nonparametric Mann–Whitney U test.[31]

Results

The mean percentage of HLA DR–positive conjunctival cells (Fig 1A) was higher in both the preserved and the multitreatment groups (46.4% and 63.4%, respectively) than in the control group (13.1%; $P<0.001$ for both groups) and the eyes receiving preservative-free timolol (19.3%; $P\leq0.03$ for both groups). No difference was found between the control and preservative-free groups. Although the mean percentage of HLA DR–positive cells was higher in the multitreatment group than in the preservative group, the difference did not reach significance. The mean levels of HLA DR expression given in AUFs showed similar results (Fig 1B), with values in the preservative treatment and multitreatment groups (47 784 and 77 406 AUFs, respectively) significantly higher than those in the control group (5119 AUFs; $P<0.01$ for both groups) and the eyes not treated with preservatives (29 480 AUFs; $P\leq0.03$ for both groups). There was also a significant difference between the control and preservative-free groups ($P = 0.035$).

The result of IL-6 expression in percentage of positive cells (Fig 2A) showed a significant difference between the control group and the other 3 groups ($P<0.001$), without a significant difference between the 3 glaucoma groups. Similarly, IL-6 intracellular expression levels assessed in AUFs (Fig 2B) showed a highly significant increase in the 3 glaucoma groups as compared with control normal eyes ($P<0.001$), with no significant difference between the 3 groups receiving antiglaucoma treatments, despite a slight nonsignificant increase in the multitreatment group.

The preservative-free, preservative-containing, and multitreatment groups also had IL-8 expression significantly higher than that of the control group (Fig 3), both in percentage of positive cells ($P<0.001$ for the 3 groups) and in levels of expression ($P<0.001$ for the 2 preservative-containing groups; $P = 0.01$ for the preservative-free group). Arbitrary unit of fluorescence results, however, showed higher levels in the multitreatment group than in the preservative-free one ($P = 0.03$).

The mean percentage of IL-10–positive conjunctival cells (Fig 4) was higher in the preservative and multitreatment groups than in the control group ($P\leq0.006$ for both comparisons), but not in the preservative-free group. Levels of intracellular expression similarly reached a significant difference between the 2 preservative-containing groups and normal eyes ($P<0.05$ for both groups).

Discussion

In previous studies on ocular surface immunopathology, some evidence has suggested that conjunctival epithelial cells may play an active role in ocular inflammation.[28–30] Conjunctival epithelial cells have therefore previously been

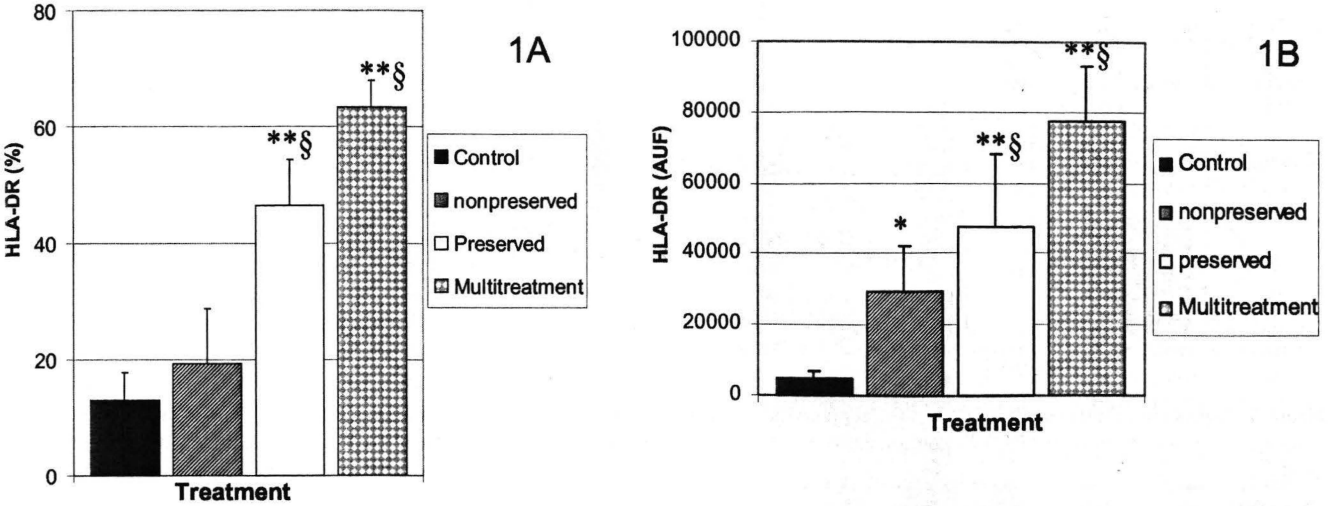

Figure 1. Results of flow cytometry in impression cytology specimens (means and standard errors). **A,** Percentage of HLA DR–positive cells in normal eyes and glaucomatous patients. §$P \leq 0.03$, compared with the preservative-free group. **$P < 0.001$, compared with controls. **B,** Level of expression, expressed in arbitrary units of fluorescence (AUF). §$P \leq 0.03$, compared with the preservative-free group. *$P = 0.035$, compared with controls. **$P < 0.01$, compared with controls.

shown to express immune-related markers[29,32] and to be a possible source for proinflammatory cytokines.[33,34] Normal conjunctival epithelial cells express mRNA for IL-1, IL-6, IL-8, tumor necrosis factor α (TNF-α), and regulated-on-activation normal T-cell expressed and secreted (RANTES) protein.[35,36] However, ICAM-1 and HLA DR are not expressed by normal conjunctival epithelial cells,[26,27,36] but are induced to be expressed at high levels on conjunctival epithelial cells in inflammatory conditions.[29,37] In the present study, we used a reliable and objective technique of flow cytometry in impression cytology to investigate the expression of 3 major interleukins by conjunctival cells. We also analyzed HLA DR expression as a hallmark for conjunctival inflammatory status and confirmed the overexpression of this immune marker in glaucoma patients receiving long-term treatments, consistent with a previous similarly conducted case–control study.[12] Our results also confirmed that preserved drugs, especially when ≥2 drugs are associated, induced significantly higher HLA DR expression than did unpreserved β-blockers. Human leukocyte antigen DR expression was slightly more elevated in the preservative-free group than in the nontreated eyes, which suggests a low level of subclinical inflammation induced by topical treatment, even though the active compound timolol was found in previous in vitro studies not or almost not to be toxic.[15] The mechanisms by which topical treatments may induce HLA DR expression are not fully determined, but it is likely that in the ocular surface proapoptotic and proinflammatory cytokines, including interferon γ and TNF-α, may stimulate common pathways.[26]

The conjunctival profile of interleukin expression, however, differed from those of HLA DR and ICAM-1.[12] Except for an increase in IL-8's level of expression (Fig 3B) in the preservative-containing groups as compared with the preservative-free group, IL-6, IL-8, and IL-10 were similarly overexpressed in all glaucoma groups, whatever the type of treatment received. This would suggest that interleukin expression by conjunctival cells is dependent upon immune mechanisms and pathways other than those influencing adhesion molecules and class II antigens.

As HLA DR, IL-6 is known to be secreted in response to TNF-α. Although IL-6 can be found in the normal eye, it is

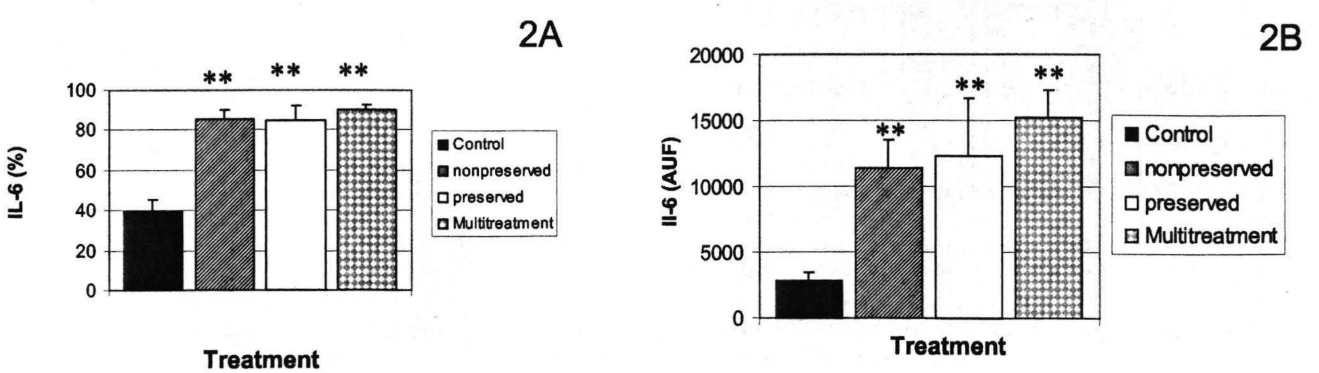

Figure 2. Expression of interleukin 6 (IL-6) by conjunctival cells. **A,** Percentage of positive cells. **B,** Levels of intracellular expression. AUF = arbitrary units of fluorescence. **$P < 0.001$, compared with controls.

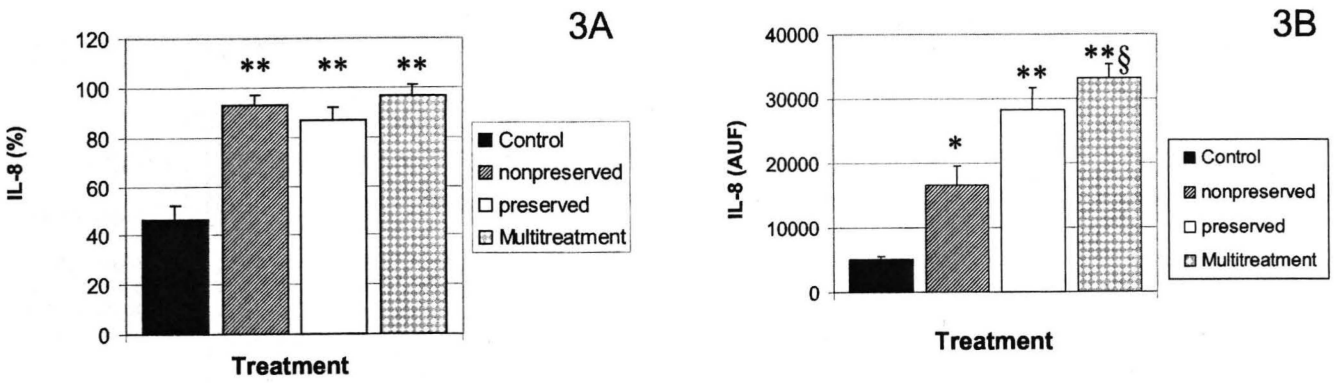

Figure 3. Expression of interleukin 8 (IL-8) in the conjunctival epithelium. **A,** Percentage of positive cells. **B,** Levels of intracellular expression. AUF = arbitrary units of fluorescence. *$P<0.01$, compared with controls. **$P<0.001$, compared with controls. §$P = 0.03$, compared with the preservative-free group.

generally considered to be an inflammatory cytokine. Jones et al[35] and Pflugfelder et al[38] observed a very high expression of mRNA encoding IL-6 and IL-8, as well as HLA DR, ICAM-1, TNF-α, IL-1α, IL-1β, and transforming growth factor β1, in the conjunctival epithelium of Sjögren's syndrome eyes as compared with normal eyes using the reverse transcriptase–polymerase chain reaction method in impression cytology. Human leukocyte antigen DR and IL-6 gene expressions were also found at higher levels in Sjögren's syndrome conjunctival cells than in normal conjunctiva.[37] Interleukin 6 and IL-8 have been shown to be present in normal tears[39] and to be expressed abundantly in pterygium epithelium.[40] In this latter study, mRNAs and proteins were stimulated by ultraviolet radiation in a time- and dose-dependent manner, thus showing the influence of external noninflammatory stimulations on these cytokines.[40] Other models of ocular surface diseases, such as corneal and limbal debridement or vernal keratoconjunctivitis, demonstrated an overexpression of proinflammatory cytokines and markers, including IL-6 or ICAM-1.[41,42]

In addition to IL-6, we found overexpression of 2 other major interleukins, IL-8 and IL-10. Interleukin 8 has powerful chemotactic effects on T lymphocytes and neutrophils and may lead to inflammatory reactions. Little is known about IL-8's role in ocular surface diseases, but significantly increased levels were found in the conjunctival epithelium of Sjögren's syndrome patients[36] and in chronic allergic disorders,[37,43,44] as compared with normal eyes. Interleukin 10, a cytokine of the Th2 profile, mainly acts as an inhibitory factor to restrain proinflammatory cytokines produced by inflammatory cells.[45] In ocular surface tissues, T cells found in atopic keratoconjunctivitis were shown to produce increased levels of IL-10 in comparison with those from vernal keratoconjunctivitis and giant papillary conjunctivitis groups.[46]

In our study, not only the expression of HLA DR and inflammatory cytokines (IL-6, IL-8) but also expression of the theoretically inhibitory factor IL-10 were higher in preserved and multitreatment groups than in the control group composed of normal untreated eyes. Interleukin expression could be hypothesized as a global reaction to toxic drugs administered for long periods of time to the ocular

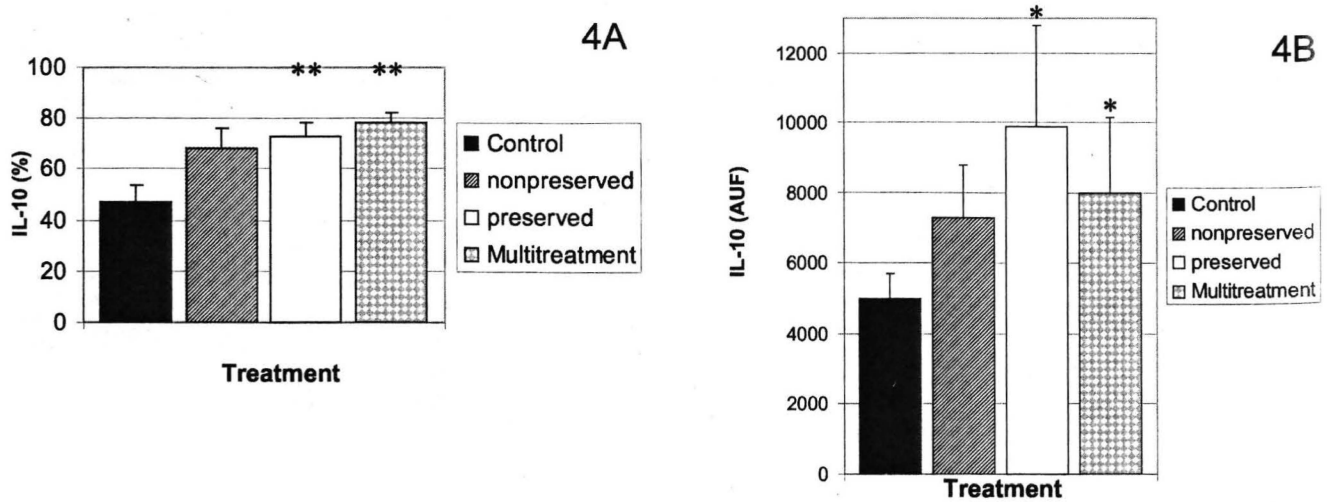

Figure 4. Expression of interleukin 10 (IL-10) in the conjunctival epithelium. **A,** Percentage of positive cells. **B,** Levels of intracellular expression. AUF = arbitrary units of fluorescence. *$P<0.05$, compared with normal eyes. **$P\leq0.006$, compared with normal eyes.

surface. However, the conjunctival profiles of HLA DR and the 3 interleukins seemed to differ, and further studies will be necessary better to understand the roles of immune markers and chemotactic, proinflammatory, or inhibitory cytokines in conjunctival immunopathology. Indeed, in vitro studies in conjunctival epithelial cells showed that release of proinflammatory cytokines may be stimulated by different mediators, as TNF-α and IL-1β are potent stimulators of IL-8, but not of ICAM-1 and HLA DR, whereas interferon γ has the inverse profile.[32,47] Histamine also stimulates the secretion of IL-6 and IL-8 by conjunctival epithelial cells in a dose- and time-dependent manner,[35] which confirms the role of the conjunctival epithelium in inflammatory and/or allergic disorders and illustrates the complex pathways leading to cytokine expression in the ocular surface.

An explanation for our results in glaucoma patients could therefore be that IL-6, IL-8 and IL-10 are highly sensitive to any proinflammatory stimulation, including the weakly toxic preservative-free β-blockers, whereas HLA DR and ICAM-1 may require higher levels of stimulation. Indeed, it has been shown that closed-eye tears contain high levels of IL-6 and IL-8 in comparison with open-eye tears,[48] which may demonstrate that these cytokines can be easily overexpressed in pathophysiological conditions. It is also possible, however, that these interleukins are released after inflammatory or toxic challenges in a soluble form in the tear flow and that the high levels of expression in the subcellular compartment do not reflect the actual amounts of cytokines present in tears and globally synthesized by conjunctival cells. Further studies combining cellular and tear concentrations would be of interest to try to discriminate between the different groups of glaucoma treatments, especially in differentiating preservative-free and preservative-containing topical drugs.

Nevertheless, subclinical inflammation may raise further serious ocular surface issues in glaucoma patients, especially at the time of filtering surgery.[9] Care should be taken to avoid preservatives in a long-term use as much as possible and to limit their concentration, as their toxicity is dose- and time-dependent,[15] or to develop new nontoxic preservatives[49] capable of reducing allergic reactions and improving ocular surface tolerance.

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