

The Ocular Surface of Glaucoma Patients Treated over the Long Term Expresses Inflammatory Markers Related to Both T-Helper 1 and T-Helper 2 Pathways

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Purpose: To investigate the expression of CCR5 and CCR4, two chemokine receptors, as markers of the T helper (Th) 1 and Th2 pathways, respectively, and class II antigen HLA-DR as a hallmark of inflammation on conjunctival cells obtained from patients receiving long-term glaucoma treatment.

Design: Case-control study.

Participants: A total of 18 normal subjects and 70 glaucoma patients treated with topical antiglaucoma drugs for more than 1 year: 14 receiving a β -blocker as monotherapy, 38 treated with a prostaglandin analog alone (19 with latanoprost, 6 with travoprost, 13 with bimatoprost), and 18 receiving multiple treatments.

Methods: Impression cytologic specimens (ICSs) were obtained from 1 eye of the patients and processed for flow cytometry. Conjunctival cells were extracted and incubated with monoclonal antibodies against CCR4, CCR5, HLA-DR, or their specific controls to measure, in a masked manner, the percentages of conjunctival cells positive for the 3 markers.

Main Outcome Measures: HLA-DR and chemokine receptors (CCR4 and CCR5) in ICSs.

Results: Compared with all other groups, HLA-DR expression was raised significantly in the multitreatment group, whereas all monotherapies showed slight and nonsignificant increases. Both CCR4 and CCR5 were increased significantly in all 5 glaucoma groups compared with normal subjects, with no between-group differences.

Conclusions: This study demonstrates the overexpression of 2 chemokine receptors in the conjunctival epithelium of glaucoma patients treated over the long term. These results show the simultaneous overexpression of CCR4 and CCR5, suggesting that the chronic use of topical treatments may stimulate both the Th1 and Th2 systems simultaneously. These results also suggest that inflammatory mechanisms combining allergy with toxicity are at work and illustrate the complexity of inflammatory reactions occurring in the ocular surface of glaucoma patients. *Ophthalmology* 2008;115:109–115 © 2008 by the American Academy of Ophthalmology.

Long-term use of ocular drugs, as in glaucoma patients who are treated for decades after they are diagnosed, frequently causes tear film and conjunctival involvement, sometimes resulting in sight-threatening ocular surface disorders (OSDs).^{1–3} At a subclinical level, chronic use of eyedrops may induce ocular surface changes identified by the abnormal production of interleukins and inflammatory markers^{4,5} and may have an influence on further outcomes in subsequent glaucoma surgery.⁶ Ocular surface impairment in

glaucoma, therefore, has become the focus of great interest and debate because of its importance and complexity, because it may involve the active compound, the preservative, or both, and it may combine allergic and toxic mechanisms in conjunctiva, lacrimal film, and possibly the cornea.

Most previous studies conducted ex vivo or in vitro have focused on the role of the preservative, benzalkonium chloride (BAC), on ocular surface cells,^{7–9} but little is known on the effects of new generation eyedrops on conjunctiva,

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especially prostaglandin analogs. Indeed, the 3 prostaglandin analogs—latanoprost, travoprost, and bimatoprost—are the most powerful and efficient agents for controlling intraocular pressure among all current ocular hypotensive medications.^{10,11} Latanoprost has been shown to induce squamous metaplasia,¹² to stimulate HLA-DR overexpression at the conjunctival surface,^{13,14} and to cause significant changes in the metalloproteinase and tissue inhibitor balance.¹⁵ To the authors' knowledge, the only comparative clinical study conducted with the 3 prostaglandin analogs showed that central corneal mechanical sensitivity was reduced significantly by all treatments, correlating with significant changes of Schirmer and tear break-up time tests, with no significant differences between the 3 drug preparations.¹⁶ Recently, the authors' group comparatively studied the proinflammatory and toxicity profiles of the 3 prostaglandin analogs *in vitro*.^{17,18} They showed that none seemed to induce direct stimulation of the inflammatory pathways involving adhesion molecules or class II antigens. Their toxicity was mild and seemed to be related primarily to the concentration of their common preservative, BAC.¹⁷ Latanoprost and travoprost were even found to be responsible for significant protective effects against BAC toxicity, when compared with the corresponding concentration of BAC alone, by effects possibly related to antioxidant properties.¹⁸

Because the authors' group also developed the technique of flow cytometry in impression cytology specimens (ICSs), which can demonstrate the overexpression of inflammatory markers and cytokines in various ocular surface diseases and glaucoma patients treated over the long term,^{4,13} they further wished to focus on the effects of prostaglandin analogs on conjunctival epithelium. In a previous study, they demonstrated that prolonged treatment with latanoprost eyedrops induced only mild inflammation, even lower than that caused by preserved β -blockers, confirming this good *in vitro* safety profile.¹³ In a more recent study also conducted in ICSs using a similar technique, they further investigated markers associated with the T helper (Th) 1 and Th2 profiles of inflammation in OSDs, using the expression of CCR5 and CCR4, respectively,¹⁹ 2 markers already demonstrated to reflect the 2 T-helper pathways.^{20–22} In this

study, vernal keratoconjunctivitis (VKC) was characterized by a high CCR4 and low CCR5 profile, whereas keratoconjunctivitis sicca had a low CCR4 and high CCR5 profile, consistent with what is assumed to be Th2 and Th1 involvement, respectively.^{23,24} Interestingly, the authors also studied a group of glaucoma patients treated over the long term and found that their conjunctiva expressed high levels of both CCR4 and CCR5, suggesting the simultaneous involvement of Th1 and Th2 pathways under the stimulation of topical drugs, as also was found using different techniques in other OSDs, such as atopic keratoconjunctivitis (AKC).²⁵ To the authors' knowledge, no other research has focused on the Th1 and Th2 profile in glaucoma patients, and little information is known on the conjunctival effects of the currently available prostaglandin analogs. The authors therefore undertook this complementary ICSs study to improve their knowledge of the inflammatory pathways involved in glaucoma patients treated over the long term (i.e., Th1, Th2, or both) and to look for possible differences in the inflammatory profiles of the 3 prostaglandin analogs.

Patients and Methods

Patients

A series of 70 patients with chronic primary open-angle glaucoma using the conjunctival impression cytologic technique was compared with a group of normal eyes, using the technique previously developed in the authors' laboratory (Table 1).¹⁹ Glaucoma patients had been treated for at least 1 year with the same treatment when examined. Patients evidencing allergic phenomena or with symptoms or signs of poor tolerance to their eye drops at presentation were excluded: repeated stinging or ocular discomfort on and between instillations, pronounced conjunctival hyperemia, tear break-up time less than 8 seconds, or positive corneal fluorescein staining. Glaucoma patients were divided into 5 groups: 14 treated with preservative-containing timolol, 19 treated with latanoprost eyedrops (Xalatan, Pfizer, New York, NY), 6 treated with travoprost (Travatan, Alcon, Fort Worth, TX), 13 treated with bimatoprost (Lumigan, Allergan, Irvine, CA), and 18 receiving at least 2 topical treatments, including the latter 2 families, carbonic anhydrase inhibitors, or both or α -2 agonists. Eighteen normal

Table 1. Demographic Characteristics of Normal Subjects and the 5 Groups of Glaucoma Patients

	Normal Subjects	Monotherapy*				Multitreatments [†]
		Preserved Timolol	Latanoprost (Xalatan)	Travoprost (Travatan)	Bimatoprost (Lumigan)	
No.	18	14	19	6	13	18
Age (yrs)						
mean \pm SD	46.4 \pm 19.5	49.8 \pm 9.1	59.5 \pm 11.2	60.8 \pm 13.4	54.8 \pm 9.0	59.9 \pm 16.8
Range	17–69	45–69	46–80	47–79	50–69	21–80
Male-to-female ratio	8:10	8:6	9:10	3:3	6:7	10:8
Total duration of treatment (yrs)						
mean \pm SD	NA	5.8 \pm 2.6	4.9 \pm 1.3	4.7 \pm 2.0	5.9 \pm 1.8	6.8 \pm 2.5
Range	NA	2–8	2–7	2–3	2–3	3–9

ANOVA = analysis of variance; NA = not applicable; SD = standard deviation.

No between-group statistical differences for age or duration of treatments (ANOVA test).

*Glaucoma patients receiving only 1 topical drug.

[†]Patients receiving at least 2 topical drugs.

subjects who had no history of ocular disease or clinical ophthalmic abnormality, assessed after slit-lamp examination, and who had not received any topical treatment for at least 3 months, also were investigated after approval by the Ethics Committee of Dijon University, France. Concerning the patients treated for glaucoma over the long term, the Ethics Committee of Saint Antoine Hospital/Paris 6 University had stated that the exploration of OSDs, including investigations on chronically used topical treatments using impression cytologic collection, did not require specific approval. Nevertheless, all patients received specific explanations on impression cytologic principles and the study aims and gave informed consent for the procedure and subsequent use of conjunctival specimens.

Impression Cytologic Specimens

Only 1 eye was examined to avoid any bias related to immune between-eye dependency. In case of bilateral treatment, the right eye was selected for impression cytologic analyses. As previously described,^{4,13,19} specimens were collected in the superior bulbar conjunctiva using 0.20- μ m polyether sulfone filters (Supor Membranes; Gelman Sciences, Ann Arbor, MI) after instillation of 1 drop of 0.04% oxybuprocaine. Two pieces of filters, measuring 13 \times 6.5 mm, were applied gently on the conjunctiva in 2 neighboring areas covered by the upper eyelid to collect a homogeneous population of superficial conjunctival cells. Given that this procedure was validated previously, specimens were obtained at least 15 minutes after the use of fluorescein eye drops so as to avoid any interference with the immunofluorescence analyses. The membranes immediately were fixed in cold phosphate buffered saline (pH, 7.4; 4 $^{\circ}$ C) containing 0.05% paraformaldehyde. Conjunctival cells were extracted from their support by mechanical agitation and then centrifuged at 2000 rpm for 5 minutes before immunofluorescence procedures and flow cytometry.

Immunofluorescence Procedures and Flow Cytometry Processing

The immunoeexpression of HLA-DR, CCR4, and CCR5 by conjunctival epithelial cells was evaluated using flow cytometry. An indirect immunofluorescence procedure was used with purified anti-HLA-DR (α chain, clone TAL.1B5) from DakoCytomation (Glostrup, Denmark). Anti-HLA-DR antibody was used at a 1:25 dilution and was incubated with conjunctival cells for 30 minutes in the dark at room temperature before being counterstained with a 1:25 dilution of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin (DakoCytomation) for 30 minutes. Direct immunofluorescence procedures were used for the chemokine receptors, phycoerythrin-conjugated anti-CCR4 (mouse immuno-

globulin (Ig)G1, clone 1G1) from DakoCytomation and fluorescein isothiocyanate-conjugated anti-CCR5 (mouse IgG2a, clone 2D7) from Pharmingen BD Biosciences (San Diego, CA), at dilutions of 1:50 and 1:25, respectively, for 30 minutes. The corresponding isotypic negative controls were used for the 3 markers: mouse IgG1 (Immunotech, Marseilles, France) for HLA-DR, mouse IgG1-phycoerythrin for CCR4, and mouse IgG2a-phycoerythrin for CCR5 (Pharmingen). Cells were processed on a flow cytometer EPICS XL (Beckman Coulter, Miami, FL). As previously described,^{13,19} cells were gated on a side scatter versus forward scatter histogram in logarithmic and linear modes, respectively, so as to discriminate epithelial and lymphocyte populations. Because previous research conducted in a similar way in various ocular surface diseases, including a subgroup of glaucoma patients,¹⁹ showed that CD45-positive cells bearing CCR4 or CCR5 were not increased significantly in superficial cells collected in ICS, this marker was not tested again in the present study and analyses were performed only on the epithelial cell population easily recognizable on the biparametric scales of the flow cytometer. For each antibody, at least 2000 conjunctival cells were analyzed. The results were expressed in percentages of cells gated on the epithelial cell population found positive for each marker compared with its corresponding isotypic negative control. All flow cytometric analyses were performed in a masked manner for patient characteristics. Statistical comparisons were performed using an analysis of variance and the nonparametric Mann-Whitney *U* test to compare the immune marker levels between patient groups.

Results

The mean percentage of HLA-DR-positive conjunctival cells in the multitreatment group was at the highest level (mean \pm standard error, 67.1 \pm 5.1%), significantly higher than in the control (22.1 \pm 4.6%), latanoprost (35.2 \pm 6.5%), travoprost (29.5 \pm 6.2%), and bimatoprost (23.8 \pm 5.1%) groups (P <0.001 compared with the control and 3 prostaglandin groups; Fig 1). Although β -blockers showed a lower percentage of HLA-DR-positive cells (47.5 \pm 8.6%) compared with the multitreatment group, the difference was not significant, and they were associated with a higher percentage than normal subjects and the bimatoprost group (P <0.02 for these 2 groups). There was no significant difference among the 3 prostaglandin monotherapy groups, although a tendency toward lower levels was found with travoprost and bimatoprost, possibly reflecting their lower benzalkonium chloride preservative content.¹⁷

The results of CCR4-positive cells (Fig 2) showed a significant difference between the control group (3.5 \pm 0.6%) and the other 5 glaucomatous groups: 15.9 \pm 4.3% for the β -blocker group,

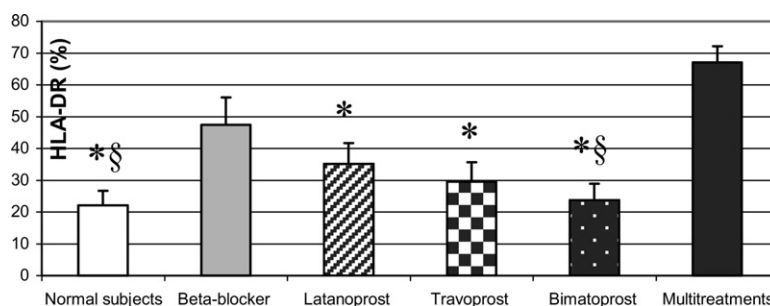


Figure 1. Bar graph showing the percentage of HLA-DR-positive cells from flow cytometry analyses in impression cytologic specimens (mean \pm standard error) in normal eyes and in the 5 glaucoma groups. * P <0.001 compared with multitreatments. § P <0.02 compared with β -blocker group.

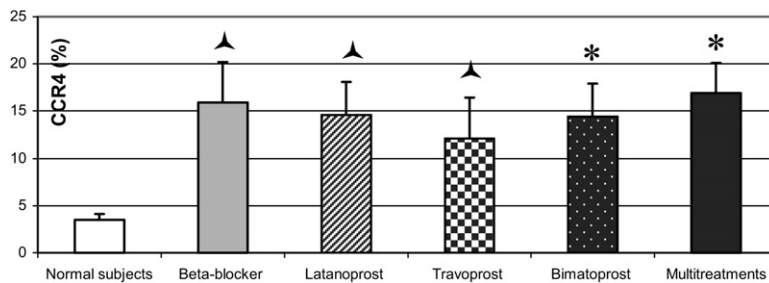


Figure 2. Bar graph showing the percentage of CCR4-positive cells in the conjunctival epithelium. * $P < 0.001$ compared with normal subjects. ▲ $P < 0.005$ compared with normal subjects.

14.6±3.5% for latanoprost, and 12.1±4.3% for the travoprost group ($P < 0.005$ for these 3 groups); 14.4±3.5% for bimatoprost and 16.9±3.2% for the multitreatment group ($P < 0.001$ for the last 2 groups). The 5 glaucoma groups showed no significant differences for CCR4.

These 5 groups also demonstrated higher percentages of CCR5-positive cells (Fig 3) than normal eyes: 25.7±6.4% for the β -blocker group, 29.9±5.3% for the latanoprost group, 36.3±4.7% for the travoprost group, 25.5±8.0% for the bimatoprost group, and 33.9±3.7% for the multitreatment group ($P < 0.002$ for all glaucomatous groups compared with control eyes; 2.4±0.6%). Among all the glaucomatous groups, no significant difference could be found. Of the entire glaucoma population, only 3 patients were found to have negative results for both CCR4 and CCR5, 1 in each of the β -blocker, latanoprost, and multitherapy groups. Most patients expressed only 1 marker; of 20 patients with high levels of both CCR4 and CCR5, 9 were evenly distributed among all monotherapy groups and 11 were in the multitherapy group.

Discussion

Flow cytometry in impression cytologic analysis is a reliable technique for assessing inflammatory changes in ocular surface cells. It is minimally invasive and can be performed routinely for OSDs and evaluations of topical drug effects on the eye surface, because the technique of cell collection is the same as that used in standard impression cytologic analysis. The authors' group has developed this technique and has found a wide variety of applications, such as examining inflammation in dry eye²⁶ and monitoring the effectiveness of topical cyclosporine A in large multicenter studies²⁷ or the effects of antiglaucoma drugs over the long term.^{4,13} The authors showed that glaucoma patients, although clinically asymptomatic, exhibit significant overexpression of HLA-DR class II antigens, intercellular adhe-

sion molecule 1, and interleukin (IL)-6, IL-8, and IL-10 in epithelium. Preserved drugs and multiple treatments reliably showed higher levels of inflammatory markers or cytokines, which was consistent with what is most likely the influence of the preservative BAC, a well-known toxic compound that causes proapoptotic effects even at a low concentration in a time- and dose-dependent manner.^{17,28} Interestingly, in an ex vivo and in vitro study investigating latanoprost eye drops, the only prostaglandin available at that time, the authors found that proinflammatory and proapoptotic effects were lower than expected with regard to the concentration in benzalkonium contained in the commercial preparation, and these effects were at even lower levels than those found with β -blockers.¹³ The authors confirmed these findings with a series of in vitro studies comparing the effects of the 3 prostaglandin analogs commercially available, showing mild toxic effects proportional to the solution's concentration in benzalkonium, but lower than those induced by the preservative solution alone at the same concentration. This suggests protective properties on the part of the lipid compound resulting from the chemical combination of the lipid and the lipophilic quaternary ammonium or from a possible antioxidative effect.^{17,18}

Although the toxicity of BAC in cell cultures has been well established,^{13,17,28} long-term use of this toxic compound for periods of several years or decades, even at low concentrations, may induce different effects in tissue. This is particularly true when BAC treatment is associated with other intercurrent OSDs or in cases of multiple treatments, as required in more than half the glaucoma population.²⁹ Experimental models or biopsies of conjunctiva have confirmed that topical treatments exert effects on an inflammatory mode rather than a toxic mode, inasmuch as the currently available tools can discriminate the 2 reactions.

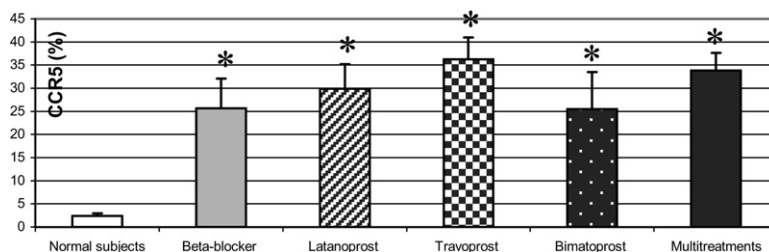


Figure 3. Bar graph showing the percentage of CCR5-positive cells in the conjunctival epithelium. * $P < 0.002$ compared with normal subjects.

Conjunctival biopsies have shown inflammatory infiltrates directly related to duration and number of treatments.⁵⁻⁷ More recently, even with modern therapies, tissue changes have been observed over the long term.¹⁵ It is most likely that the toxicity exerted at the conjunctival level after instillation of BAC results in enhanced desquamation of superficial layers, further compensated by an increased turnover of epithelial replacement and associated with inflammatory infiltrates. It remains to be determined whether (1) these cells are stimulated directly by the low doses of toxic compounds administered over the long term; (2) they result from the chronic injury of the epithelial cells; and (3) they are the consequence of the damage to the lacrimal film, either at the lipidic layer level, given that quaternary ammoniums are potent detergents, or secondary to the destruction of goblet cells and impairment of soluble mucins. Indeed, BAC has been shown to penetrate conjunctival tissues deeply and to accumulate as long as 1 week after a 1-drop instillation,³⁰ and goblet cell destruction is known to be very rapid after instillation of BAC-containing solutions,^{31,32} which the authors also confirmed as occurring after long-term use of glaucoma drugs.¹³ However, because immune cell infiltrates or HLA-DR overexpression are non-specific indicators of inflammation, the accurate mechanisms of these inflammatory changes remain to be elucidated: are they allergy driven, toxicity dependent, or both?

Recently, the authors adapted the flow cytometry technique in ICS by using new markers designed to attempt to discriminate between Th1 and Th2 pathways, the 2 major inflammatory systems involving T cell-dependent reactions. The Th2 pathway is especially implicated in allergic diseases with secretion of IgE, whereas Th1 plays a part in the immune response to infections and is the key effector of type IV delayed hypersensitivity. CCR4 and CCR5 have been widely demonstrated as associated with Th2 and Th1 immune reactions, respectively.²⁰⁻²² Although the methodology chosen in our ICS studies did not allow direct analyses of the cytokines produced by T cells, but rather focused on epithelial involvement, it is most likely that CCR4 and CCR5 reflect the same inflammatory pathways in the ocular surface as in other systems; the authors' first study also clearly supported this hypothesis.¹⁹ Indeed, allergic ocular surface diseases have been the most thoroughly studied with regard to Th1 and Th2 balance.³³⁻³⁵ Secretion of IL-4, a Th2 cytokine, was reported in the tears and conjunctiva of patients with VKC and AKC. The conjunctiva of AKC and VKC patients mainly expressed Th2-type cytokines and was found to be infiltrated by Th2-type lymphocytes. Conversely, keratoconjunctivitis sicca involves Th1-related cytokines, such as IFN- γ , the principal product of Th1 lymphocytes, with little or no IL-4 mRNA.^{36,37} This activation of the Th1 system also was confirmed by salivary gland biopsies from Sjögren syndrome patients,³⁸ and an experimental model of dry eye syndrome showed high production of IL-1 β and tumor necrosis factor α in animal tears.³⁹ However, although Th1 and Th2 systems usually are considered mutually antagonistic, some OSDs such as AKC seem to have a mixed mechanism and rely on both pathways.²⁵

The inflammatory pathway involved in OSDs can be

determined directly from the cytokines released by T cells in tears or from conjunctival biopsies, but these techniques remain complex or not fully reliable. Another approach indirectly detects chemokine receptors involved in the recruitment of the leukocytes and is associated with either system. CCR4 and CCR5 thus are expressed by cells of the Th2 and Th1 pathways, respectively.^{21,22} Abu El-Asrar et al⁴⁰ showed that these receptors are not expressed or are expressed only slightly at the normal eye surface in conjunctival biopsies, whereas CCR4 and CCR5 were observed at the level of inflammatory cells in VKC. Using more sensitive techniques, CCR5 was found to be overexpressed by epithelial cells of the conjunctiva in Sjögren syndrome.²⁴

In the authors' first study investigating Th1 and Th2 balance using flow cytometry in ICS,¹⁹ they also found abnormally high levels of CCR4 in epithelial cells in patients with VKC and in a population of glaucoma patients treated over the long term, but not in normal eyes or in dry eye syndrome where this marker was restricted to CD45-bearing inflammatory cells. Conversely, CCR5 was found to be overexpressed only in keratoconjunctivitis sicca as well as in most glaucoma patients. The coexpression of CCR4 and CCR5 in glaucoma motivated the current study, with particular attention focused on the effects of prostaglandin analogs and a comparison between the 3 commercially available eyedrops. The authors' results first confirmed that the conjunctiva of glaucomatous patients treated over the long term was highly inflammatory, especially in patients receiving more than 1 medication, but with lower inflammatory profiles when prostaglandin analogs were used. No major differences were found between the 3 compounds. Moreover, glaucoma treatments were confirmed to activate not only CCR4, and therefore most likely are associated with the Th2 pathway, suggesting allergic reactions, but also CCR5, the marker associated with Th1. Consequently, the Th1 and Th2 pathways seemed to be implicated simultaneously in this iatrogenic ocular pathologic setting, resembling what has been found with other techniques in AKC patients. Only 3 eyes had negative results for both markers, whereas 20 eyes simultaneously overexpressed CCR4 and CCR5, including 11 of the 18 multitreated eyes, thus suggesting mixed mechanisms in iatrogenic inflammation of the ocular surface. The other eyes had a prominent expression of 1 of the 2 markers, with no correlation with a specific treatment or a clinical condition. This illustrates the complexity of the mechanisms occurring in the ocular surface in glaucoma, for example, in allergic or toxic reactions, direct stimulation of inflammatory cells, impairment of the lacrimal film, or destruction of epithelial cells and their further stimulation. In addition, it shows that the active compound, the preservative, or multiple combinations of different drugs may intervene in this reactive process. Interestingly, no major difference was found between the β -blockers and the 3 prostaglandin analogs, thus confirming their favorable inflammatory profile, provided they are used alone in monotherapy. Although not significant, a tendency toward slight differences was found only with HLA-DR expression, parallel to the concentrations of BAC contained in the eyedrops; that is, the higher the concentration, the higher the HLA-DR expression, similar to the apoptotic and

toxic effects found in vitro.¹⁷ No apparent relationship could be established with the known frequency of hyperemia, which therefore seems to correspond to a different mechanism, likely not caused by inflammatory processes.⁴¹

Although the toxicity of the preservative BAC has been shown clearly, which may account for the large majority of inflammatory and degenerative changes in the ocular surface of glaucomatous patients, there are still complex mechanisms to understand and discriminate. Tolerance of eye-drops usually is satisfactory in prospective clinical trials investigating a single drug in selected patients. However, the question of tolerance should be investigated differently in real-life conditions: when treatments are used for decades, when they are used in multiple and complex combinations or are associated with intercurrent ocular surface disorders, very frequent use in elderly patients that increases with age, tear film impairment, and accumulation of aggressions to the ocular surface.

References

- Schwab IR, Linberg JV, Gioia VM, et al. Foreshortening of the inferior conjunctival fornix associated with chronic glaucoma medications. *Ophthalmology* 1992;99:197–202.
- Broadway D, Grierson I, Hitchings R. Adverse effects of topical antiglaucomatous medications on the conjunctiva. *Br J Ophthalmol* 1993;77:590–6.
- Pisella PJ, Pouliquen P, Baudouin C. Prevalence of ocular symptoms and signs with preserved and preservative free glaucoma medication. *Br J Ophthalmol* 2002;86:418–23.
- Baudouin C, Hamard P, Liang H, et al. Conjunctival epithelial cell expression of interleukins and inflammatory markers in glaucoma patients treated over the long term. *Ophthalmology* 2004;111:2186–92.
- Baudouin C, Pisella PJ, Fillacier K, et al. Ocular surface inflammatory changes induced by topical antiglaucoma drugs: human and animal studies. *Ophthalmology* 1999;106:556–63.
- Broadway DC, Grierson I, O'Brien C, Hitchings RA. Adverse effects of topical antiglaucomatous medication. II. The outcome of filtration surgery. *Arch Ophthalmol* 1994;112:1446–54.
- Mietz H, Niesen U, Krieglstein GK. The effect of preservatives and antiglaucomatous medication on the histopathology of the conjunctiva. *Graefes Arch Clin Exp Ophthalmol* 1994; 232:561–5.
- de Jong C, Stolwijk T, Kuppens E, et al. Topical timolol with and without benzalkonium chloride: epithelial permeability and autofluorescence of the cornea in glaucoma. *Graefes Arch Clin Exp Ophthalmol* 1994;232:221–4.
- Dogan AL, Orhan M, Soylemezoglu F, et al. Effects of topical antiglaucoma drugs on apoptosis rates of conjunctival epithelial cells in glaucoma patients. *Clin Experiment Ophthalmol* 2004;32:62–6.
- Parrish RK, Palmberg P, Sheu WP, XLT Study Group. A comparison of latanoprost, bimatoprost, and travoprost in patients with elevated intraocular pressure: a 12-week, randomized, masked-evaluator multicenter study. *Am J Ophthalmol* 2003;135:688–703.
- Orzalesi N, Rossetti L, Bottoli A, Fogagnolo P. Comparison of the effects of latanoprost, travoprost, and bimatoprost on circadian intraocular pressure in patients with glaucoma or ocular hypertension. *Ophthalmology* 2006;113:239–46.
- Hong S, Lee CS, Seo KY, et al. Effects of topical antiglaucoma application on conjunctival impression cytology specimens. *Am J Ophthalmol* 2006;142:185–6.
- Pisella PJ, Debbasch C, Hamard P, et al. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. *Invest Ophthalmol Vis Sci* 2004;45:1360–8.
- Guglielminetti E, Barabino S, Monaco M, et al. HLA-DR expression in conjunctival cells after latanoprost. *J Ocul Pharmacol Ther* 2002;18:1–9.
- Ito T, Ohguro H, Mamiya K, et al. Effects of antiglaucoma drops on MMP and TIMP balance in conjunctival and subconjunctival tissue. *Invest Ophthalmol Vis Sci* 2006;47:823–30.
- Kozobolis VP, Detorakis ET, Maskaleris G, et al. Corneal sensitivity changes following the instillation of latanoprost, bimatoprost, and travoprost eyedrops. *Am J Ophthalmol* 2005; 139:742–3.
- Guenoun JM, Baudouin C, Rat P, et al. In vitro study of inflammatory potential and toxicity profile of latanoprost, travoprost, and bimatoprost in conjunctiva-derived epithelial cells. *Invest Ophthalmol Vis Sci* 2005;46:2444–50.
- Guenoun JM, Baudouin C, Rat P, et al. In vitro comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. *Invest Ophthalmol Vis Sci* 2005;46:4594–9.
- Baudouin C, Liang H, Bremond-Gignac D, et al. CCR 4 and CCR 5 expression in conjunctival specimens as differential markers of T(H)1/T(H)2 in ocular surface disorders. *J Allergy Clin Immunol* 2005;116:614–9.
- Bonecchi R, Bianchi G, Bordignon PP, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998;187:129–34.
- Annunziato F, Galli G, Cosmi L, et al. Molecules associated with human Th1 or Th2 cells. *Eur Cytokine Netw* 1998; 9(suppl):12–6.
- Loetscher P, Ugucioni M, Bordoli L, et al. CCR5 is characteristic of Th1 lymphocytes [letter]. *Nature* 1998;391:344–5.
- Leonardi A. Vernal keratoconjunctivitis: pathogenesis and treatment. *Prog Retin Eye Res* 2002;21:319–39.
- Gulati A, Sacchetti M, Bonini S, Dana R. Chemokine receptor CCR5 expression in conjunctival epithelium of patients with dry eye syndrome. *Arch Ophthalmol* 2006;124:710–6.
- Calder VL, Jolly G, Hingorani M, et al. Cytokine production and mRNA expression by conjunctival T-cell lines in chronic allergic eye disease. *Clin Exp Allergy* 1999;29:1214–22.
- Brignole F, Pisella PJ, Goldschild M, et al. Flow cytometric analysis of inflammatory markers in conjunctival epithelial cells of patients with dry eyes. *Invest Ophthalmol Vis Sci* 2000;41:1356–63.
- Brignole F, Pisella PJ, De Saint Jean M, et al. Flow cytometric analysis of inflammatory markers in KCS: 6-month treatment with topical cyclosporine A. *Invest Ophthalmol Vis Sci* 2001; 42:90–5.
- Debbasch C, Pisella PJ, De Saint Jean M, et al. Mitochondrial activity and glutathione injury in apoptosis induced by unpreserved and preserved beta-blockers on Chang conjunctival cells. *Invest Ophthalmol Vis Sci* 2001;42:2525–33.
- Feiner L, Piltz-Seymour JR. Collaborative Initial Glaucoma Treatment Study: a summary of results to date. *Curr Opin Ophthalmol* 2003;14:106–11.
- Champeau EJ, Edelhofer HF. Effect of ophthalmic preservatives on the ocular surface: conjunctival and corneal uptake and distribution of benzalkonium chloride and chlorhexidine digluconate. In: Holly FJ, Lamberts DW, MacKeen DL, Esquivel ED, eds. *The Preocular Tear Film in Health, Disease*

- and Contact Lens Wear. Lubbock, TX: Dry Eye Institute; 1986:292–302.
31. Rolando M, Brezzo V, Giordano G, et al. The effect of different benzalkonium chloride concentrations on human normal ocular surface: a controlled prospective impression cytology study. In: van Bijsterweld OP, Lemp MA, Spinelli D, eds. *The Lacrimal System*. Amsterdam: Kagler & Ghedini; 1991:89–91.
 32. Herreras JM, Pastor JC, Calonge M, Asensio VM. Ocular surface alteration after long-term treatment with an antiglaucomatous drug. *Ophthalmology* 1992;99:1082–8.
 33. Uchio E, Ono SY, Ikezawa Z, Ohno S. Tear levels of interferon-gamma, interleukin (IL) –2, IL-4 and IL-5 in patients with vernal keratoconjunctivitis, atopic keratoconjunctivitis and allergic conjunctivitis. *Clin Exp Allergy* 2000;30: 103–9.
 34. Leonardi A, DeFranchis G, Zancanaro F, et al. Identification of local Th2 and Th0 lymphocytes in vernal conjunctivitis by cytokine flow cytometry. *Invest Ophthalmol Vis Sci* 1999;40: 3036–40.
 35. Metz DP, Hingorani M, Calder VL, et al. T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol* 1997; 100:817–24.
 36. Ajjan RA, McIntosh RS, Waterman EA, et al. Analysis of the T-cell receptor Valpha repertoire and cytokine gene expression in Sjogren’s syndrome. *Br J Rheumatol* 1998;37:179–85.
 37. Willeke P, Schotte H, Schluter B, et al. Interleukin 1beta and tumour necrosis factor alpha secreting cells are increased in the peripheral blood of patients with primary Sjogren’s syndrome. *Ann Rheum Dis* 2003;62:359–62.
 38. Kolkowski EC, Reth P, Pelusa F, et al. Th1 predominance and perforin expression in minor salivary glands from patients with primary Sjogren’s syndrome. *J Autoimmun* 1999;13: 155–62.
 39. Luo L, Li DQ, Doshi A, et al. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. *Invest Ophthalmol Vis Sci* 2004;45:4293–301.
 40. Abu El-Asrar AM, Struyf S, Al-Mosallam AA, et al. Expression of chemokine receptors in vernal keratoconjunctivitis. *Br J Ophthalmol* 2001;85:1357–61.
 41. Leal BC, Medeiros FA, Medeiros FW, et al. Conjunctival hyperemia associated with bimatoprost use: a histopathologic study. *Am J Ophthalmol* 2004;138:310–3.