New Tools for the Evaluation of Toxic Ocular Surface Changes in the Rat

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Purpose To assess the usefulness of noninvasive combined technologies used to observe ocular surface changes in toxicology studies.

Methods. Benzalkonium chloride (BAC) at 0.01, 0.1, 0.25, and 0.5% was applied to rat corneas for 11 days. The eye was evaluated macroscopically from day (D) 0 to D52. The cornea was examined with the slit lamp, a fluorescein test was performed, and a confocal microscope was used in vivo to calculate corneal thickness, score corneal epithelial and endothelial defects, and quantify corneal stromal inflammation and neovascularization. Conjunctival impression and brush cytology specimens were taken for labeling with MUC-5AC antibodies and sub-G1 peak analysis by flow cytometry, respectively. Histologic analyses were performed on D11.

Results. Although macroscopic and slit lamp examinations revealed signs of ocular irritation in the 0.25% and 0.5% BAC-treated eyes only, in vivo confocal microscopy revealed epithelial defects in the 0.01% and 0.1% BAC-treated corneas, and sub-G1 peak analyses showed increased apoptosis for all the BAC concentrations on D8 and D11. BAC at 0.25% and 0.5% induced increased corneal thickness, loss of goblet cells, reversible corneal inflammation, and persistent neovascularization.

Conclusions. Sub-G1 peak analysis of conjunctival brushings, in conjunction with in vivo confocal microscopy of the cornea and immunolabeling of conjunctival imprints, constitutes a noninvasive, reliable, and sensitive tool to evaluate toxic drug-induced ocular surface damage in rats, in addition to standard clinical assessments and at a wide range of concentrations, including the lowest ones. This study is consistent with the international strategy aimed at reducing the use of animals and refining animal toxicologic models. (Invest Ophthalmol Vis Sci. 2007;48:5473–5483) DOI:10.1167/iovs.06-0728

The ocular surface is a functional unit organized to maintain the integrity of the cornea, which is essential for visual function. Complex interactions exist between the three main components: the cornea, the conjunctiva, and the tear film. Any inflammatory or toxic reaction may disturb the homeostasis of the ocular surface and result in corneal opacification, consequently impairing vision. As topical ophthalmic preparations are often used for years or decades, especially in glaucoma or dry eye disease and may directly influence the ocular surface structures, they should be thoroughly tested before use to establish their potential to cause ocular surface alterations and disturbances, in particular corneal damage. The test currently used for the assessment of ocular irritancy is the Draize test in rabbit eyes, which is mainly based on the scoring of observed macroscopic changes in the rabbit cornea, conjunctiva, and iris. This test has been extensively criticized because of its subjectivity, intra- and interlaboratory variability, and overpredictiveness and because of ethical considerations. Many researchers have therefore tried to develop alternative in vitro or in vivo strategies. In vitro testing remains the principal alternative to animal experiments. Although it offers numerous advantages such as simplicity, low cost, and reproducibility, it does not reproduce the complexity of the pathophysiological mechanisms involved in the ocular defense system after exposure to a test compound. Noninvasive in vivo tests have therefore been developed to overcome these limitations. Toxic effects on the cornea have already been evaluated by pachymetry, tonometry, electrophysiology, corneal permeability, and in vivo confocal microscopy. Evaluating a single parameter even in an in vivo model may not precisely reflect the complexity of the mechanisms involved in chronic use of toxic compounds, especially when used at low concentrations. Few toxicological studies have focused on the assessment of the entire ocular surface system—the cornea, the conjunctiva, and the tear film—although toxicological processes involve not only the cornea but all components. Consequently, methods must be developed that better monitor the ocular surface as a complex pathophysiological system, allow the follow-up of ocular surface changes over time, avoid subjecting the animal to invasive procedures by using non- or minimally invasive techniques, and reduce the number of animals used.

Nonirritants are major components of ophthalmic preparations used to preserve the sterility of topical medications. Their ocular surface toxicity has been documented over the past six decades. As early as 1944, Swan demonstrated that benzalkonium chloride (BAC), the most commonly used preservative in topical ophthalmic medications, causes punctate disturbances to corneal epithelium at a concentration of 0.04%, and edema and cellular desquamation with corneal lesions at a concentration of 0.1%. Since then, a great number of reports have demonstrated the toxic and cytotoxic effects of BAC in laboratory, experimental, and clinical studies. BAC was found to induce or favor the infiltration of the conjunctival epithelium and stroma by inflammatory cells, as well as the expression of inflammatory markers by conjunctival epithelial cells. Ex vivo studies conducted on conjunctival
specimens from patients treated over the long term with preserved antiglaucoma drugs showed a significant subclinical inflammation associated with an alteration of the mucin goblet cell–associated system compared with normal eyes or patients treated with preservative-free drugs. In this study, we used the known toxic properties of BAC to develop an in vivo model of dose-dependent ocular surface toxicity and neovascularization. This model was induced by topically applying low to supertoxic doses of BAC to the ocular surface of rats. Because of the constant need to develop new techniques that could provide more reliable, objective, and quantifiable data as a function of morphologic and physiologic parameters, we propose herein a set of combined technologies and tools that can be used as a supplement for the classic Draize test–based toxicological studies or for helping to monitor neovascularization and inflammatory cell-associated events. New-generation in vivo confocal microscopy offers the advantage of providing histologic-like images. Its usefulness associated with the analysis of MUC-5AC on conjunctival impression cytology specimens, the sub-G1 peak analysis of conjunctival brushings, and slit lamp examination with the fluorescein test was assessed in a rat model of ocular surface toxicity.

The goals of our study were to compare histologic-like in vivo images generated with a new-generation in vivo confocal microscope to the classic ex vivo analysis of histologic slides and to address the question of providing better tools than the Draize test for ocular surface evaluation in animal models.

**Material and Methods**

**Animals and BAC Treatment**

A total of 60 male Lewis rats (24 weeks of age; Janvier, Le Genest St. Isle, France) were randomly assigned to five groups of 12 rats each. They weighed between 350 and 400 g and were free of clinically observable ocular surface disease. The animals were housed in cages at constant temperature (20 ± 1°C) and humidity (50% ± 5%). They were fed with chow and water ad libitum. For 11 days, they received different concentrations of BAC—0.01%, 0.1%, 0.25%, or 0.5% (Théa Laboratories, Clermont-Ferrand, France)—or 0.9% NaCl as the control, twice daily in the right eye. Three rats per group were killed on D11 for histology. The others were kept alive for the follow-up of ocular lesions after the treatment was stopped. Thirty rats were reserved for brush cytology sampling on day (D)8 and D11, with three new rats used on each analysis day. Conjunctival brushings being collected only once in each rat. All the experimental and animal care procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under the supervision of a health authority–accredited staff member for animal care and management.

**Ocular Surface Toxicity Evaluations**

The ocular surface was evaluated after intraperitoneal injection of a ketamine-xylazine solution (37.5 mg/kg ketamine; Merial, Lyon, France; and 2 mg/kg xylazine; Bayer, Puteaux, France) and to address the question of providing better tools than the Draize test for ocular surface evaluation in animal models.

Standard Draize test macroscopic criteria were completed by slit lamp evaluation and fluorescein test. At day 0, all rats had normal findings in physical and ophthalmic examinations. In animals under general anesthesia, the slit lamp (BQ 900; Haag-Streit, Bern, Switzerland) was used to examine corneal changes, separately evaluating corneal opacities, permeability barrier loss induced by epithelial defects, and scoring any corneal neovascularization. Corneal opacities were scored based on the Draize criteria by multiplying the degree of opacity by its area. The degree of opacity was scored as follows: 1, scattered or diffuse area, details of iris visible; 2, easily discernible translucent areas, details of iris slightly obscured; 3, opalescent areas, no details of iris visible, size of pupil barely discernible; and 4, opaque, iris not visible. The area of opacity was scored as follows: 1, one quarter (or less) but not zero; 2, greater than one quarter, but less than one half; 3, greater than one half, but less than three quarters; and 4, greater than three quarters, up to the entire area. The maximum total score possible was 16. The fluorescein test was performed as follows: 10 μL of a 0.5% fluorescein sodium solution (Fluorescène Faure 0.5%; Novartis France, Rueil-Malmaison, France) was instilled in each eye of the rats and then rinsed with 0.9% NaCl. The eyes were examined with cobalt blue light by two masked observers. Pictures were taken with a digital camera. The extent of corneal damage (width and intensity of fluorescein take-up area) was scored according to the following scale: 0, no staining; 0.5, slight punctate staining; 1, diffuse punctate staining; 2, diffuse staining covering less than one third of the cornea; 3, diffuse staining covering more than one third of the cornea; and 4, staining covering more than two thirds of the cornea. The neovascularization area was scored as follows: 1, one quarter (or less) but not zero; 2, between one quarter and one half; 3, between one half and three quarters; and 4, between three quarters and the entire surface of the cornea.

**In Vivo Confocal Microscopy**

A new laser scanning confocal microscope (Heidelberg Retina Tomograph [HRT] II/Rostock Cornea Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany) was used to examine corneas in vivo from D0 to D52. Because of the low responsiveness of 0.01% BAC–treated eyes in preliminary experiments, the effects of this dose during the treatment period were also examined 5 minutes after the evening instillation on D0, D2, D7, and D10 in addition to the classic confocal microscopic examination before the morning eye drop instillation on D3, D8, and D11. The first and the second examinations were assumed to model the immediate and the long-term chronic effects of BAC, respectively. The use of this microscope has been described previously. The laser source used is a diode laser with a wavelength of 670 nm. The objective of the microscope is an immersion lens (Olypus, Hamburg, Germany), magnification ×60, covered by a polymethyl methacrylate cap. Images consist of 384 × 384 pixels covering an area of 400 μm², with a transverse optical resolution of 4 μm (Heidelberg Engineering). Images were acquired in the automatic-gain mode. During the examination, the in vivo confocal microscope was manually focused through the cornea from the superficial epithelium to the endothelium, according to a technique adapted to imaging animal eyes. X-y position and the depth of the optical section were controlled manually. The depth of the optical section was automatically displayed and registered. The entire corneal surface was examined and more than 10 images were taken for each of the following structures of the cornea: superficial and basal epithelium, stroma, and endothelium. Images were then analyzed qualitatively and quantitatively in a masked manner using the software included in the tomography system (HRT II/RCM; Heidelberg Engineering GmbH).

The mean corneal thickness was calculated based on the depth difference between the most superficial and the deepest corneal structures.

We assigned severity scores to the epithelial corneal changes as follows: (−) normal; (+) slight: cell border loss/anisocytosis; (+++) mild: at least three of these parameters: cell border loss, cell metapla-
sia, marked anisocytosis, microcytosis, abnormal reflectivity pattern, epithelial erosion; and (+ + +) severe: denudation, ulceration.

The endothelium was also scored as follows: (−) normal; (+) slight: cell border loss, anisocytosis; (+ +) mild: partial visibility of cell structures; and (+ + +) severe: cell structures not visible.

The presence of brightly reflective material within the stroma has been thought to correspond to keratocyte activation/fragmentation, protein precipitation, and inflammatory cell infiltration. The presence and the persistence of white round dots resembling polymorphonuclear leukocytes and macrophages, however, was consistent with an inflammatory infiltrate. These cells were counted so as to evaluate inflammation severity, whereas the degree of neovascularization was evaluated by counting the number of neovascular nodes. For the quantification of inflammatory cells and neovascular nodes, a single identical 400-μm² counting area was defined for all images, and each object counted was marked once on the computer display. At the end, the density was automatically calculated using the tomograph-associated software. Based on 12 images, the means and standard deviations were calculated for each parameter.

**Impression Cytology Specimens and Immunofluorescence Labeling**

For the purpose of the study, a technique was adapted to the rat eye based on that developed in our laboratory for clinical investigations in humans. On D11, after general anesthesia, two pieces of filter, 3 × 6 mm in size (Gelman Supor, polyethersulfone filter, 0.2 μm pores; Pall Sciences, New York, NY) were applied to the bulbar conjunctiva of each animal in two adjacent locations, without exerting any pressure. The specimens were stored overnight in dry tubes at room temperature, fixed with 4% paraformaldehyde (PFA), permeabilized with 0.01%-diluted Triton X-100 (Sigma-Aldrich, St. Louis, MO), and rinsed with phosphate-buffered saline (PBS, Invitrogen-Gibco, Cergy-Pontoise, France) containing 1% bovine serum albumin (BSA; Sigma-Aldrich). Membranes were labeled with mouse anti-MUC-5AC antibodies, and a negative isotopic control (BD Biosciences, Franklin Lakes, NJ), counterstained with Alexa488 conjugated-goat anti-mouse IgG at a 1:1000 dilution (Dako, Glostrup, Denmark). After two washes in 1% BSA-PBS, the sections were counterstained with propidium iodide, mounted in antifade medium (Vectorshield; Vector Laboratories, Burlingame, CA) and analyzed under a laser scanning confocal microscope (model PCM 2000; Nikon).

Immunofluorescence staining, the sections were labeled with antibodies against von Willebrand factor, I-α, CD68, TCR-β/β, CD54, rat granulocytes and erythroid cells, and rat IgG as follows. The sections were fixed with 4% PFA for 5 minutes and then permeabilized with 0.01%-diluted Triton X-100 (Sigma-Aldrich) for 5 minutes. After they were rinsed with 1% BSA-PBS, seven sets of primary antibodies were added in PBS: rabbit anti-von Willebrand factor IgG1 (1:100; Dako), mouse anti-rat IgG1 (Serotec, Cergy, France) against I-α (clone MRC OX-6, 1:50 dilution), CD68 (clone ED1, 1:50 dilution), TCR-β/β (clone R73, 1:25 dilution), and CD54 (clone 1A29, 1:50 dilution); mouse anti-granulocytes and erythroid cell IgM (clone HIS48/87.8 C10, 1:20 dilution; Serotec); goat IgM against rat IgG (1:1000 dilution; Beckman Coulter) and their respective negative isotopic controls, mouse or rabbit IgG1 and IgM (BD Biosciences). After 1 hour of incubation, the sections were rinsed twice in 1% BSA-PBS and incubated again for 1 hour in the dark with Alexa488 conjugated-goat anti-mouse IgG at a 1:1000 dilution or Alexa647 conjugated-goat anti-rabbit IgG at a 1:250 dilution (Dako) for sections previously incubated with anti-Von Willebrand factor. After two washes in 1% BSA-PBS, the sections were counterstained with propidium iodide, mounted in antifade medium (Vectashield; Vector Laboratories), and analyzed under a laser confocal microscope (PCM 2000; Nikon).

Inflammatory cells were counted on hematoxylin-eosin sections from three rats per treatment using a 100 × 100-μm reticulum. Twelve areas were counted per section, and six sections were used for each treatment. Means and standard deviations were calculated for each treatment.

**Results**

**Histology and Immunofluorescence Staining**

After impression cytology and confocal microscopy, three rats per group were killed after an intraperitoneal injection of a lethal dose of pentobarbital sodium (Ceva Santé Animale, Libourne, France) on D11, 12 hours after the rats received the last eye drops. The eyes were enucleated with care to avoid damage from manipulation, and the tissue was embedded in OCT (Tissue-Tek, Miles Inc., Elkhart, IN) and frozen at −80°C. OCT-embedded frozen sections (10 μm thick) were cut with a cryotome (model CM 3050s; Leica Microsystems AG, Wetzlar, Germany) and stored at −20°C until staining. The sections were subjected to hematoxylin-eosin staining or immunofluorescence staining. For immunofluorescence staining, the sections were labeled with antibodies against von Willebrand factor, I-α, CD68, TCR-β/β, CD54, rat granulocytes and erythroid cells, and rat IgG as follows. The sections were fixed with 4% PFA for 5 minutes and then permeabilized with 0.01%-diluted Triton X-100 (Sigma-Aldrich) for 5 minutes. After they were rinsed with 1% BSA-PBS, seven sets of primary antibodies were added in PBS: rabbit anti-von Willebrand factor IgG1 (1:100; Dako), mouse anti-rat IgG1 (Serotec, Cergy, France) against I-α (clone MRC OX-6, 1:50 dilution), CD68 (clone ED1, 1:50 dilution), TCR-β/β (clone R73, 1:25 dilution), and CD54 (clone 1A29, 1:50 dilution); mouse anti-granulocytes and erythroid cell IgM (clone HIS48/87.8 C10, 1:20 dilution; Serotec); and goat IgM against rat IgG (1:1000 dilution; Beckman Coulter) and their respective negative isotopic controls, mouse or rabbit IgG1 and IgM (BD Biosciences). After 1 hour of incubation, the sections were rinsed twice in 1% BSA-PBS and incubated again for 1 hour in the dark with Alexa488 conjugated-goat anti-mouse IgG at a 1:1000 dilution or Alexa647 conjugated-goat anti-rabbit IgG at a 1:250 dilution (Dako) for sections previously incubated with anti-Von Willebrand factor. After two washes in 1% BSA-PBS, the sections were counterstained with propidium iodide, mounted in antifade medium (Vectashield; Vector Laboratories), and analyzed under a laser confocal microscope (PCM 2000; Nikon).

Inflammatory cells were counted on hematoxylin-eosin sections from three rats per treatment using a 100 × 100-μm reticulum. Twelve areas were counted per section, and six sections were used for each treatment. Means and standard deviations were calculated for each treatment.

**Statistical Analysis**

All statistical comparisons were performed with two-way analysis of variance (ANOVA), followed, if necessary, by multiple pair-wise comparisons using Bonferroni adjustment (Statview V for Windows; SAS Institute, Cary, NC).
conjunctivalization of the cornea. All rats on D0 and the NaCl-, 0.01% BAC-, and 0.1% BAC-treated eyes during the entire experiment were free of any corneal transparency defect but showed some punctate staining (score 0.5; Fig. 1C) according to the fluorescein test. The 0.5% and 0.25% BAC solutions induced maximum corneal surface damage at D3 after instillation, with scores of 4 and 3, respectively, as shown by the severe fluorescein uptake. These scores progressively decreased until D18 but the 0.5% BAC-treated group score did not return to normal. The neovascularization scores, as assessed by slit lamp evaluation increased from D3 to D15 and from D8 to D15 for rats receiving 0.5% and 0.25% BAC, respectively (Fig. 1D). Their respective scores peaked at 4 and 3 without decreasing until D52. The examination of 0.1% and 0.01% BAC-treated rats did not reveal any sign of neovascularization during the experiment period.

In Vivo Confocal Microscopy

**Corneal Thickness.** The mean corneal thickness on D0 was 139 ± 5 μm (Fig. 2). Corneal thickness increased significantly for 0.5% and 0.25% BAC at levels between 230 and 299 μm from D3 to D11. On D8, a significant difference was found between both groups. Corneal thickness remained significantly higher than the control until D52, although it decreased after treatment cessation. BAC at 0.1% tended to increase corneal thickness on D3 without reaching significance and returned to normal on D8. Neither BAC at 0.01% nor NaCl induced significant changes in mean corneal thickness.

**Detailed Analysis of Corneal Layers. Epithelium.** The normal superficial epithelium exhibited large, polygonal, squamous, heterogeneously reflective cells with mostly visible nuclei (Fig. 3A1). The 0.5% and 0.25% BAC treatments induced the loss of superficial epithelial cells between D3 and D11, leaving the main part of the corneal stroma denuded (Figs. 3D1). The damage was considered severe, as presented in Table 1. At both concentrations, recovery was incomplete after the treatment was stopped. Loss of cell borders, disappearance of nuclei, metaplasia, fusion of the cells, and abnormal reflectivity patterns were observed (Fig. 3C1) and scored as mild epithelial changes (Table 1). The 0.1% BAC treatment induced slight superficial epithelial alterations from D3 to D18, except on D8 when a mild alteration (Table 1) was observed with epithelial erosion evidenced by imaging the smaller, deeper wing cells remaining after superficial desquamation (Fig. 3C1). On D0, D2, D7, and D10, BAC at 0.01% induced immediate superficial epithelial changes whose cell structures were impossible to discriminate as early as 5 minutes after the instillation. This alteration was transient, however, as demonstrated by images of the epithelial structures on D3 and D8. On D11, cell borders and nuclei could not be imaged (slight epithelial changes), as an effect of the 11-day-long treatment (Fig. 3B1). After the treatment was stopped, the epithelium returned to the normal state.

**Stroma.** The 0.25% and 0.5% BAC solutions induced stromal disorganization, keratocyte loss, inflammatory cell infiltration (Fig. 3B2), and neovascularization (Figs. 3C2, 3D2). After stopping treatment, inflammation decreased (Fig. 4) but growth of blood vessels (Fig. 5) and disorganization of the stroma persisted.

**Mean inflammatory cell density in the stroma was calculated (Fig. 4).** 0.5% BAC-treated corneas showed a significantly increased cell density from 1522 ± 165 cells/mm² on D3 to levels as high as 3000 ± 400 cells/mm² on D8 and D11. BAC at 0.25% induced slightly less inflammation, with a significantly lower cell density on D8 and a peak of 2260 ± 1450 cells/mm² on D11. After treatment cessation, mean cell densities significantly decreased to 963 ± 390 and 503 ± 90 cells/mm² on D15, for 0.5% and 0.25% BAC-treated eyes, respectively. Then, from D18 to D52, inflamed areas were rare, and mean cell density remained low or almost normal. On D8 and D11, only one of the six 0.1% BAC-treated rats exhibited stromal inflammation that persisted until D24. From D8 to D24, density of hyperreflective dots was between 98 ± 45 cells/mm² and 293 ± 130 cells/mm². BAC at 0.01% disclosed normal stromal patterns at all times during and after the treatment.

During the 0.5% and 0.25% BAC treatments, in vivo confocal microscopy revealed growing stromal neovascularization from D0
to D11 in the central cornea (Fig. 5). On D3 and D8, the corneal stroma was highly inflamed, and the blood vessels did not exhibit distinct vascular walls. On D11, blood vessel nodes were counted, giving densities of $150 \pm 64$ and $165 \pm 84$ nodes/mm$^2$ for 0.5% and 0.25% BAC, respectively. The stromal analyses showed the persistence of neovascularization from D15 to D52 at nonsignificantly different levels between the two groups.

**Endothelium.** The two highest concentrations of BAC provoked cellular edema (Fig. 3B3) on D3 followed by partial (Fig. 3C3) to complete (Fig. 3D3) endothelial invisibility. The 0.5% BAC-induced endothelial corneal damage was considered slight/mild on D3, mild/severe from D8 to D24, mild on D31 and slight from D38 to D52 (Table 2). 0.25% BAC was slightly less aggressive, with damage considered as slight on D3, severe on D8, mild on D15 and D18, and absent from D24 to D52. Application of 0.1% BAC seemed not to inflict significant damage on the endothelial cells. Nevertheless, one of the six rats exhibited slight abnormal endothelial changes on D8 and D11.

**FIGURE 2.** Mean corneal thickness calculated using in vivo confocal microscopy during and after 11 days of BAC treatment. The data are presented as the mean ± SD. The analyses were performed on six rats per group from D0 to D11 and on three rats per group from D15 to D52. The mean corneal thickness was calculated based on the depth difference between the most superficial and the deepest corneal structure.

**FIGURE 3.** Examples of in vivo confocal microscopic images serving as reference for severity scoring with increasing BAC-induced corneal damage severity in the superficial epithelium, the stroma, and the endothelium. *Top row:* Corneal superficial epithelium showing (A1) a normal control-like profile (score −); (B1) cell border loss (score +); (C1a) abnormal shape and reflectivity pattern (score +++) and (C1b) cell border loss and microcytosis (score ++); and (D1) epithelial denudation with the limit of ulceration (arrow; D1c), the remaining epithelium (D1a) on the left, and the inflammatory denuded stroma (D1b) on the right (score +++). *Middle row:* Corneal stroma showing (A2) a normal control-like profile; (B2) numerous bright points corresponding to inflammatory cells; (C2) a neovascularization with bigger blood vessels and absence of inflammatory cells; and (D2) a flare stroma with neovascularization and inflammation. *Bottom row:* Corneal endothelium showing (A3) a normal control-like profile; (B3) cell border loss; (C3) partial visibility of cell structures; and (D3) invisible cell structures. Note that (C1a) and (C1b) are two different illustrations of the corneal superficial epithelium with a mild damage severity score (++). These images have the same scale as the other images but show only one half of the area, whereas other images show a 400-µm$^2$ area.
TABLE 1. Severity of Epithelial Corneal Damage after BAC Treatment for 11 Days

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<tr>
<th>BAC Dose</th>
<th>D0</th>
<th>D3</th>
<th>D8</th>
<th>D11</th>
<th>D15</th>
<th>D18</th>
<th>D24</th>
<th>D31</th>
<th>D38</th>
<th>D45</th>
<th>D52</th>
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<td>0.25%</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>0.1%</td>
<td>(3A1)*</td>
<td>(3B1)*</td>
<td>(5B1)*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.01%</td>
<td>(3A1)*</td>
<td>(3A1)*</td>
<td>(3A1)*</td>
<td>(3B1)*</td>
<td>+</td>
<td>+</td>
<td>+</td>
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- Normal; +, slight: cell border loss; ++, mild: at least three of these parameters: cell border loss, cell metaplasia, marked anisocytosis, microcysts, abnormal reflectivity pattern, epithelial erosion; +++, severe: denudation, ulceration. No corneal epithelial damage was observed with instillation of NaCl.

* Corresponding image in Figure 3.

that persisted until D18, with cellular swelling, presence of heterogeneously reflective cells, and occasional guttae. Such changes were scored as slight endothelial changes.

**Immunofluorescence Labeling of the Conjunctival Imprints**

Figure 6 shows conjunctival imprints labeled with MUC-5AC, analyzed with confocal microscopy. Control specimens exhibited numerous epithelial cells with small, red nuclei and green-stained goblet cells (Fig. 6A) at a density of 12.9 ± 7 cells/0.01 mm² (Table 3). The two highest doses of BAC induced goblet cell loss, as shown by the remaining traces of mucus manifesting as green filaments (Fig. 6D) or dots (Fig. 6E) on D11: 2.4 ± 2.5 goblet cells/0.01 mm² and 1.3 ± 1.5 goblet cells/0.01 mm² were counted for 0.25% and 0.5% BAC, respectively. BAC at 0.01% and 0.1% did not induce significant loss of goblet cells.

**Sub-G1 Peak Analysis in Conjunctival Brushings**

Results for sub-G1 peak are presented in Figure 7. Apoptosis rates of 12.4% ± 1.9% and 13.1% ± 2.6% were evidenced in the control group on D8 and D11, respectively. On D8 and D11, BAC induced a significant increase in epithelial cell apoptosis, whatever the dose used (P < 0.05 compared with control). The effects seemed to be dose dependent, with 24.3%, 25.6%, 33%, and 47.7% of apoptosis induced on D8 and 22.9%, 26.3%, 27.6%, and 35.6% of apoptosis induced on D11 for the four increasing BAC concentrations, respectively. Of interest, the apoptosis rate was slightly decreased on D11 compared to D8.

**Histologic Analyses**

The results of histologic analyses are presented in Figure 8. The normal cornea presented a visible and relatively thick epithelium with small cubic basal cells and large squamous cells undergoing desquamation. The stromal fibers were normally arranged, with few keratocytes. The 0.1% and 0.01% BAC-treated eyes did not show any sign of inflammation. Nevertheless, the superficial epithelium seemed to be thinner in both cases on D11, correlating with in vivo confocal imaging of superficial epithelial alteration/erosion. Both the 0.25% and the 0.5% BAC concentrations induced stromal neovascularization (Figs. 8D1, 8D2) and infiltration of inflammatory cells (Figs. 8E1, 8A2, 8B2, 8C2, 8E2). The basal epithelial layer exhibited alterations, with enlarged abnormal cells (Fig. 8D1), confirming the tomograph confocal microscopic observations. Blood vessels (Fig. 8A2, a), macrophages (Fig. 8A2, b), and polymorphonuclear cells (Fig. 8A2, c) were also identified by standard histologic analyses. Quantification of inflammatory cells in these sections correlated with that performed using the confocal microscope–associated software (Table 4). The 0.5% and 0.25% BAC concentrations induced a significant stromal inflammatory cell infiltration of 2711 ± 517 and 1537 ± 429 inflammatory cells/mm², respectively (P < 0.05, compared to control), while 0.1% and 0.01% BAC-treated sections were not significantly different from the control. Antibodies against rat IgG (Fig. 8A3) and CD54 (Figs. 8D5, 8E) showed positive immunofluorescent staining in the corneal stroma of 0.5% and 0.25% BAC-treated eyes. Anti-class II (OX-G, Fig. 8B2), anti-Cd68 (Fig. 8C2), H1S48...
TABLE 2. Severity of Endothelial Corneal Damage after BAC Treatment for 11 Days

<table>
<thead>
<tr>
<th>BAC Dose</th>
<th>During BAC Treatment</th>
<th>After Treatment</th>
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<tr>
<td></td>
<td>D0</td>
<td>D3</td>
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<tr>
<td>0.5%</td>
<td>–</td>
<td>++++</td>
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<tr>
<td></td>
<td>(3A1)*</td>
<td>(3B1/3C1)*</td>
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<tr>
<td>0.25%</td>
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<td>–</td>
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<td></td>
<td>(3A1)*</td>
<td>(3B1)*</td>
</tr>
<tr>
<td>0.1%</td>
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<td>–</td>
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<td>(3A1)*</td>
<td>(3A1/3B1)*</td>
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–, Normal; +, slight: cell border loss; ++, mild: partial visibility of cellular structures; ++++, severe: invisibility of cellular structures. No corneal epithelial damage was observed with instillation of NaCl.

* Corresponding image in Figure 3.

(Fig. 8B3), and anti-TCR α/β (Fig. 8C3) antibodies were found to be reliable in locating inflammatory cells (0.5% and 0.25% BAC treatment).

DISCUSSION

Quaternary ammonium with surfactant properties is widely used as preservative in ophthalmic preparations and is known to cause ocular irritation, especially over the long term.

This article proposes using a set of new technologies—that is, high-resolution in vivo laser scanning confocal microscopy, flow cytometry applied to conjunctival brush cytology samples, and immunolabeling of MUC-5AC-positive goblet cells on conjunctival imprints—as advantageous and complementary tools for exploring and grading toxic drug-induced ocular surface damage in rats in addition to standard clinical assessments. Maurer and Parker suggested that rats could constitute a good surrogate for rabbits in studies of surfactant-induced ocular irritation. Experimental studies involving in vivo tandem scanning confocal microscopy have already been conducted in rats and rabbits for the assessment of ocular surface toxicity. In our study, we used the recently developed confocal laser-scanning HRT II system combined with the Rosette Counter Module to examine superficial to deeper layers of the cornea in a rat model of BAC-induced toxicity. Using NaCl 0.01%, 0.1%, 0.25%, and 0.5% BAC applications over 11 days, we collected a great number of images of the epithelium, the stroma, and the endothelium of live animals. The most superficial damage was evaluated by examining and scoring BAC-treated epithelia, from slight (cell border loss/anisocytosis) to severe damage (denudation, ulceration). The density of inflammatory infiltrates within the stroma was also calculated as an important parameter. Stromal neovascularization was evaluated by counting blood vessel nodes, and we found that high concentrations of BAC may represent an easy and reliable model for new blood vessel formation. The deepest corneal layers, especially the endothelium, were also easily examined and classified by degree of severity. The results showed a dose-response relationship for BAC in rat-treated corneas, with low (0.01% and 0.1%) BAC concentrations inducing damage restricted to the epithelium, whereas the highest (0.25% and 0.5%) concentrations caused epithelial denudation as well as major stromal and endothelial damage. In vivo confocal microscopy revealed corneal injury from epithelial cell border loss to epithelial erosion with 0.01% and 0.1% BAC, respectively, whereas the stromal inflammation and neovascularization found in vivo at 0.25% and 0.5% concentrations were confirmed by immunofluorescent labeling on histologic sections. The imaging of highly reflective punctate material was correlated with the presence of numerous Ia- and CD68-positive cells as well as HIS48- and TCR α/β-positive cells. At these concentrations, we evidenced the presence of IgG and CD54 bound to inflammatory cells bearing Fc receptors and/or pre-activated cells as well as HIS48- and TCR α/β-positive cells. The results presented are the mean number of nodes/mm² ± SD. On D3 and D8, no blood vessels with distinct vascular walls could be counted. The 0.1% and 0.01% BAC concentrations as well as NaCl did not induce neovascularization. The results are thus not presented.

FIGURE 5. Neovascularization of the central corneal stroma quantified by in vivo confocal microscopy. The analyses were performed in six rats per group from D0 to D11 and in three rats per group from D15 to D52. Based on 12 images, the results are presented as the mean number of nodes/mm² ± SD. On D3 and D8, no blood vessels with distinct vascular walls could be counted. The 0.1% and 0.01% BAC concentrations as well as NaCl did not induce neovascularization. The results are thus not presented.
CD54 in either the soluble or membrane form. Hematoxylin/eosin coloration also demonstrated destructuring of the corneal stroma with loss of linear fiber organization and the presence of blood vessels, macrophages, polymorphonuclear cells, and dendriform cells. Quantification of inflammatory cells correlated with those findings, based on confocal microscopy. Only one 0.1% BAC-treated rat exhibited signs of inflammation and/or keratocyte lysis in the stroma, probably because of higher individual sensitivity. These results obtained in a chronic toxicity-induced model are consistent with previously published data showing differences in irritancy correlated with area and depth of initial injury.16 They related BAC concentrations with damage severity grades within each of the three corneal components (i.e., the epithelium, the stroma, and the endothelium) the most concentrated solutions inducing higher severity grades and an increased number of injured components from the most superficial to the deepest structures. They also evidenced incomplete corneal recovery, at least in the stroma, with persistent neovascularization and in the epithelium with anisocytosis, abnormal reflectivity, and shape patterns, for 0.25% and 0.5% BAC on D52, even long after toxic removal, thus showing the stability of this model over time.

The method of in vivo confocal microscopy presented the advantages of detailed examination of both deep and superficial corneal layers as well as of the limbus and conjunctiva.45 Although the use of fluorescein staining gave direct information on epithelial defects and the slit lamp examination allowed evaluation of corneal opacity and neovascularization, in vivo confocal microscopy completed these results by imaging cornea at a histology-like cellular level. Damage to the endothelium, stromal inflammation, and neovascularization reflected highly toxic effects, and the analysis of the most superficial cell layer, the epithelium, was a good sensor of slight toxicity-induced corneal damage. Our results showed that this method enabled discrimination of abnormal corneal patterns induced by various concentrations of BAC, with a dose dependency from the highest to the lowest concentration. It offered a large spectrum of complementary and reliable analyses and a much higher sensitivity than the Draize-derived test or even the slit lamp examination.

The conjunctival epithelium is one of the first tissues targeted in topical application of eye drops. One simple and minimally invasive46 method of collecting cells from the superficial layers of the conjunctival epithelium is the conjunctival imprint technique. It has been used in humans to detect goblet cell loss34 and inflammation (expression of HLA-DR, IL-6, IL-8, IL-10, and CD40).27–38 to discriminate between Th1 and Th2 pathways in ocular surface diseases (CCR4 CCR5),47 and it has shown that patients treated with preserved antiglaucoma drugs exhibit inflammatory markers detected by flow cytometry.34 We successfully applied the immunolabeling technique of conjunctival cells directly on conjunctival imprints in the rat model. We observed a significant reduction in the number of goblet cells after exposure to 0.25% and 0.5% BAC, 11 days after the beginning of the treatment. Of interest, clinical studies provided similar results in humans with various ocular diseases.48–50 This method is therefore a useful tool for the qualitative evaluation of the rat conjunctival epithelium; other markers such as inflammatory markers could be examined in the future.

In this study, we also applied flow cytometry to the analysis of rat animal conjunctival brushings for the follow-up of BAC-induced conjunctival changes. Applying flow cytometry to the analysis of human impression cytology specimens, despite the small number of cells collected, was developed in our laboratory in 1997.27–38 We have since demonstrated the efficiency of this tool in exploring ocular surface disorders and monitoring drug-related efficacy and/or toxicity in humans. However, when impression cytology was used in rats, the specimens were too poor to allow satisfactory analysis (data not shown). The conjunctival brush cytology technique was also described for humans in 1997. It allows collection of specimens with greater cell density, although remaining relatively more aggressive than impression cytology.40 We adapted this cell collection technique in rats because it offered a minimally invasive and rapid way to collect rat conjunctival cells, a good alternative to biopsy, providing up to 100,000 cells per specimen. We demonstrated that sub-G1 peak analysis provided interesting results concerning the apoptosis status of the conjunctival epithelium. Normal specimens showed approximately 12% apoptotic cells, which is consistent with the natural exfoliation of superficial epithelial cells. On D8 and D11, all tested BAC concentrations disclosed a significant and dose-dependent in-

| Table 3. Quantification of Goblet Cells on Impression Cytology Specimens on D11 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 0.1% BAC        | 0.25% BAC       | 0.5% BAC        |
| NaCl            | 12.9 ± 7        | 9.1 ± 5.9       | 2.4 ± 2.5       | 1.3 ± 1.3       |
|                  | 12 ± 6          | 13 ± 7          | 11 ± 5.3        | 12 ± 7          |

Results are presented as the mean number/0.01 mm² ± SD. Analyses were performed with specimens from three different rats. For each animal, two specimens were used, and four fields were counted.

* Statistically significant with P < 0.05 compared with PBS-treated eyes.
FIGURE 7. Sub-G1 peak analysis in conjunctival brushings using flow cytometry showing BAC-induced apoptosis with a dose-dependent effect on D8 and D11. Data are presented as the mean ± SD. The analyses were performed on three rats per group on D8 and three new rats per group on D11.

FIGURE 8. Histologic and immunohistological analyses of rat corneas treated with various concentrations of BAC on D11. Column 1: Corneal histologic section of (A) an NaCl-treated eye presenting a visible and relatively thick endothelium, normally arranged stromal fibers and few keratocytes; (B) a 0.01% BAC-treated eye with a thinner epithelium; (C) a 0.1% BAC-treated eye with a thinner epithelium; (D) a 0.25% BAC-treated eye showing abnormally enlarged basal epithelial cells, stromal disorganization, neovascularization, and inflammation; and (E) a 0.5% BAC-treated eye showing an abnormal epithelium, stromal disorganization, neovascularization, and inflammation. Column 2: (A) Corneal stroma of a 0.25% BAC-treated eye showing neovascularization (a), macrophages (b), and polymorphonuclear cells (c); (B) inflammatory infiltration as detected by 1a (OX-6) labeling (green) counterstained with propidium iodide (red nuclei) at the limit of the epithelial ulceration (0.5% BAC-treated eye); (C) infiltration of macrophages as detected by CD68 labeling in a 0.5% BAC-treated cornea; (D) stromal neovascularization of a 0.5% BAC-treated eye as evidenced by von Willebrand labeling; and (E) presence of 1a-positive cells in the stroma and probably in the lacrimal film of a 0.25% BAC-treated eye, as shown by green cells in the stroma and on the epithelium. Column 3: (A) inflammatory cell or IgG infiltration as detected by IgG immunolabeling (green; 0.5% BAC-treated eye). Antibodies directed against rat IgG recognized soluble IgG as well as B lymphocytes bearing IgG or inflammatory cells that can bind IgG through their Fc receptors. (B) Stromal granulocyte infiltration, as detected by HIS-48 labeling (0.5% BAC-treated eye). (C) Infiltration of T cells, as detected by TCR α/β labeling (0.5% BAC-treated cornea). (D) Stromal presence of ICAM-1-positive cells or soluble ICAM-1 as evidenced by CD54 labeling (0.5% BAC-treated eye). (E) Stromal presence of ICAM-1-positive cells or soluble ICAM-1 as evidenced by CD54 labeling (0.25% BAC-treated eye).
crease in epithelial cell apoptosis. The highly concentrated 0.25% and 0.5% BAC solutions seemed to reach a maximum apoptosis rate on D8, whereas the lowest ones, 0.01% and 0.1%, induced increasing apoptosis from D8 to D11. In 1998, Jester et al.\textsuperscript{17} proposed that differences in surfactant-induced ocular irritation were directly related to depth of injury in the rabbit cornea, with epithelial cell death induced by slight irritant and keratocyte cell death induced by the mild to severe irritants. In our study, the results might be explained by the depth and the strength of epithelial conjunctival cell injury. Increasing BAC concentrations may induce an enhanced apoptosis rate in the most superficial layers and/or apoptosis in deeper layers. Nevertheless, in contrast to impression cytology, that collects only superficial epithelial cell layers, brush cytology analyzed with flow cytometry cannot discriminate apoptosis in superficial layers from apoptosis in deeper layers. The decrease in apoptosis rates we found for high BAC concentration may be explained by partial epithelial regeneration and/or erosion of the superficial epithelial cells, leaving less damaged deep layers to be collected by brushing.

These results indicate that even at low toxic doses, sub-G1 peak analysis on brush cytology specimens constitutes a reliable and sensitive tool for the detection of apoptosis. Similar studies with other classes of chemicals or irritants should be conducted in the future so as to confirm that this tool is a good marker for the toxic/irritating potential of drugs. The use of brush cytology combined with flow cytometry could help better document the sequential changes occurring in the conjunctiva over time and yield reliable information on the apoptosis/cell death status of the epithelium.

To our knowledge, this is the first study in which sub-G1 peak analysis applied to conjunctival brush specimens in conjunction with in vivo confocal microscopy of the cornea and direct immunolabeling on conjunctival imprints have been used to assess the ocular irritation/toxic potential of a compound quantitatively. This set of techniques presents the advantage of evaluating not just a single component of the ocular surface but both the cornea and the conjunctiva, including both superficial and deeper layers. In the future, analysis of tear samples to evaluate cytokines may also complete this set of tools.\textsuperscript{51} With in vivo confocal microscopy, we examined not only the degree of toxicity, but also the reversibility or the persistence of the damage induced by our tested compounds. Our set of combined technologies offers a noninvasive and sensitive way to study and follow ocular surface changes in pathophysiological or therapeutic models, as well as in toxicologic studies, in which lower concentrations might be used to reduce pain in research animals. Research should be continued to develop further tools that can be used to detect early and minor signs of toxicity. As the anatomic and clinical correlation between ex vivo histologic sections and in vivo imaging has been well established, fewer animals are used because it is unnecessary to kill animals at different times during the experiments. This study is consistent with the international strategy aimed at reducing the number of animals used in experiments and refining animal toxicologic models, opening up a new field of research in the development of new analysis systems for the prediction of drug-induced ocular toxicity.

References


