

EVALUATION OF THE TOXICITY OF BENZALKONIUM CHLORIDE ON THE OCULAR SURFACE

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Abstract

Benzalkonium chloride (BAC), the most widely used preservative in ophthalmic solutions, acts on the ocular surface through toxic or immunoallergic reactions. Due to its surfactant properties, this quaternary ammonium strongly decreases lachrymal fluid stability. It also causes toxic effects to epithelial cells and increases corneal permeability. In vivo, strong histopathological changes are observed after topical treatments with preservatives, including infiltration by inflammatory cells, similar to those induced in humans by long-term topical treatments. We designed a series of in vitro experiments confirming the toxicity of BAC,

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even at very low concentrations. In vitro, BAC induces cell necrosis at the concentrations found in most eye drops after a few minutes, and apoptosis when applied at lower concentrations. This apoptotic phenomenon is confirmed by 4',6-diamidino-2-phenylindole, diacetate (DAPI) coloration and with the use of flow cytometry. A decrease in cell size is observed with BAC at 0.001% and is confirmed by morphological assay. An overexpression of Apo 2.7 associated with an increase of sub G1 phase cells is also shown. BAC induces irreversible cytotoxic damages with some characteristics of apoptosis in a concentration-dependent manner.

Introduction

Long-term use of antiglaucoma drugs has been associated with toxic as well as inflammatory changes of the ocular surface. However, little is known concerning the accurate mechanisms of such toxic side effects in long-term and/or multitreated glaucoma patients. There is, however, growing evidence that long-term use of topical drugs may produce damage to conjunctival and corneal epithelial cells. Histopathological studies have confirmed that topically applied drugs may exert toxic effects to the corneconjunctival surface and induce chronic infraclinical inflammation, as shown by the presence of immunological changes and inflammatory infiltrates in multitreated eyes.

The origin of topical inflammation has not yet been clearly determined but benzalkonium chloride (BAC), which is used as a preservative in almost all antiglaucoma preparations, has shown strong evidence of toxicity to the ocular surface. It may act on ocular surface through toxic¹ or immunoallergic reactions.² The toxicity can be direct on epithelial and goblet cells or indirect on the lachrymal film, resulting from its tensioactive properties. Furthermore, repeated doses of preserved eyedrops can lead to a cumulative effect and preservatives transferred with ophthalmic devices will have prolonged contact with the epithelium. An iatrogenic disorder may result that could lead to chronic irritation and subconjunctival fibrosis, which could worsen keratoconjunctivitis sicca or increase the risk of failure of trabeculectomy in patients with glaucoma.^{3,4}

The effects of ophthalmic preservatives on corneconjunctival cells have been investigated by scanning electron microscopy,^{5,6} tandem scanning confocal microscopy,⁷ in vitro studies of cytotoxicity of preservatives to cultured corneal epithelial cells,⁸ quantitative evaluation of corneal epithelial permeability, and histological or cytological analyses after exposure to eyedrops of varying composition in humans⁹⁻¹² or animals. In vivo studies have been conducted after topical treatment with preservatives and in vitro experiments on human cultured conjunctival cells have also been done. Rare clinical studies have been conducted to compare preservative-free and preserved eyedrops. Conjunctival biopsies taken at the time of glaucoma surgery

have demonstrated a significant increase in immune cells and fibroblasts, possibly related to cumulative treatment with antiglaucoma drugs.⁹⁻¹¹ Impression cytological specimens from long-term-treated patients have shown abnormal induction of inflammatory markers by epithelial cells in about 50% of eyes.¹² These inflammatory and fibrotic anomalies may result in pseudophakic or significant reduction of success rate following glaucoma surgery.¹³

To understand the relative role of preservatives such as benzalkonium chloride in the toxicity of long-term use of antiglaucomatous drugs, we performed a series of *in vivo* and *in vitro* experiments.

Different *in vivo* studies have been conducted. The preservatives most often currently used in topical ophthalmic preparations were tested in rats (cetrimide, benzalkonium chloride, benzododecinium bromide, thiomersal, methylparahydroxybenzoate). Topical drops were administered in both eyes, three times daily for 1 month. Histopathological evaluations and indirect immunocytochemistry studies were performed. This study demonstrated strong toxic effects of preserved solutions. Preservatives induced corneal epithelial damages and limbal and conjunctival infiltration by immunocompetent cells.¹⁴ Comparison of the different preservatives showed no significant difference in the intensity of pathological effects between the five preservatives tested.

To address the eventual toxicity of beta blockers with or without preservative, another study was conducted in rats using immunocytochemistry. Benzalkonium chloride as well as unpreserved and preserved timolol were administered four times daily for

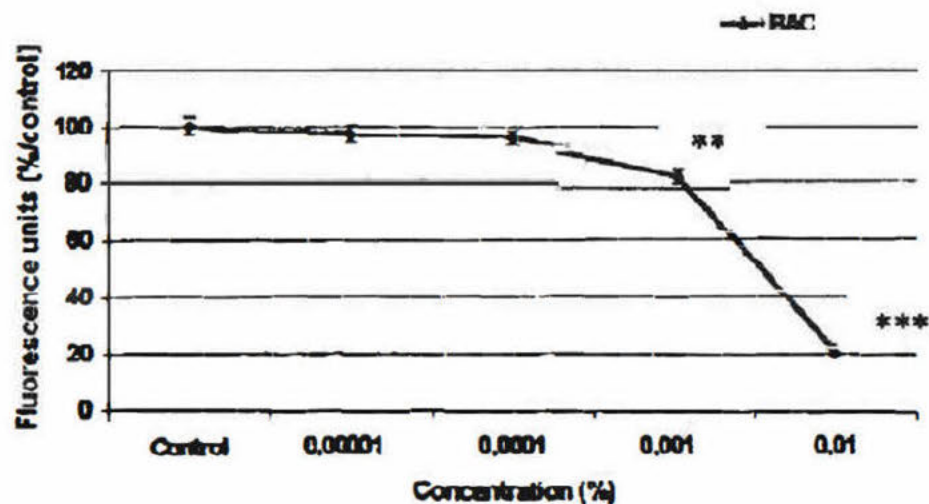


Figure 1. Evaluation of cellular viability after 15 min treatment with BAC. Results are expressed in variation from the control values: ** $p < 0.01$ as compared to control; *** $p < 0.0001$ as compared to control.

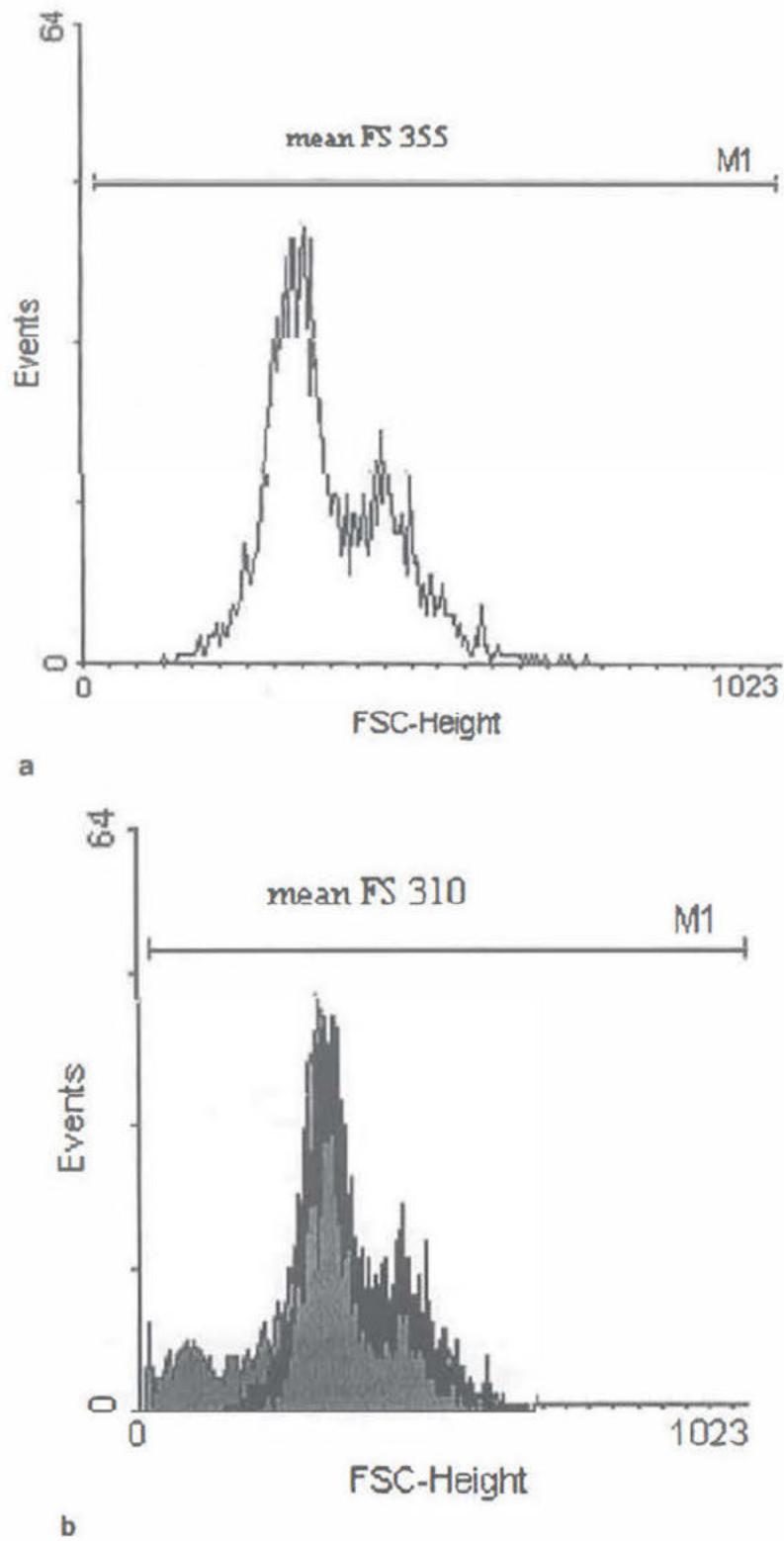


Figure 2. Cell size evaluation by flow cytometry after treatment with BAC. a. Control. b. BAC 0.001%. c. BAC 0.01%. d. BAC 0.1%.

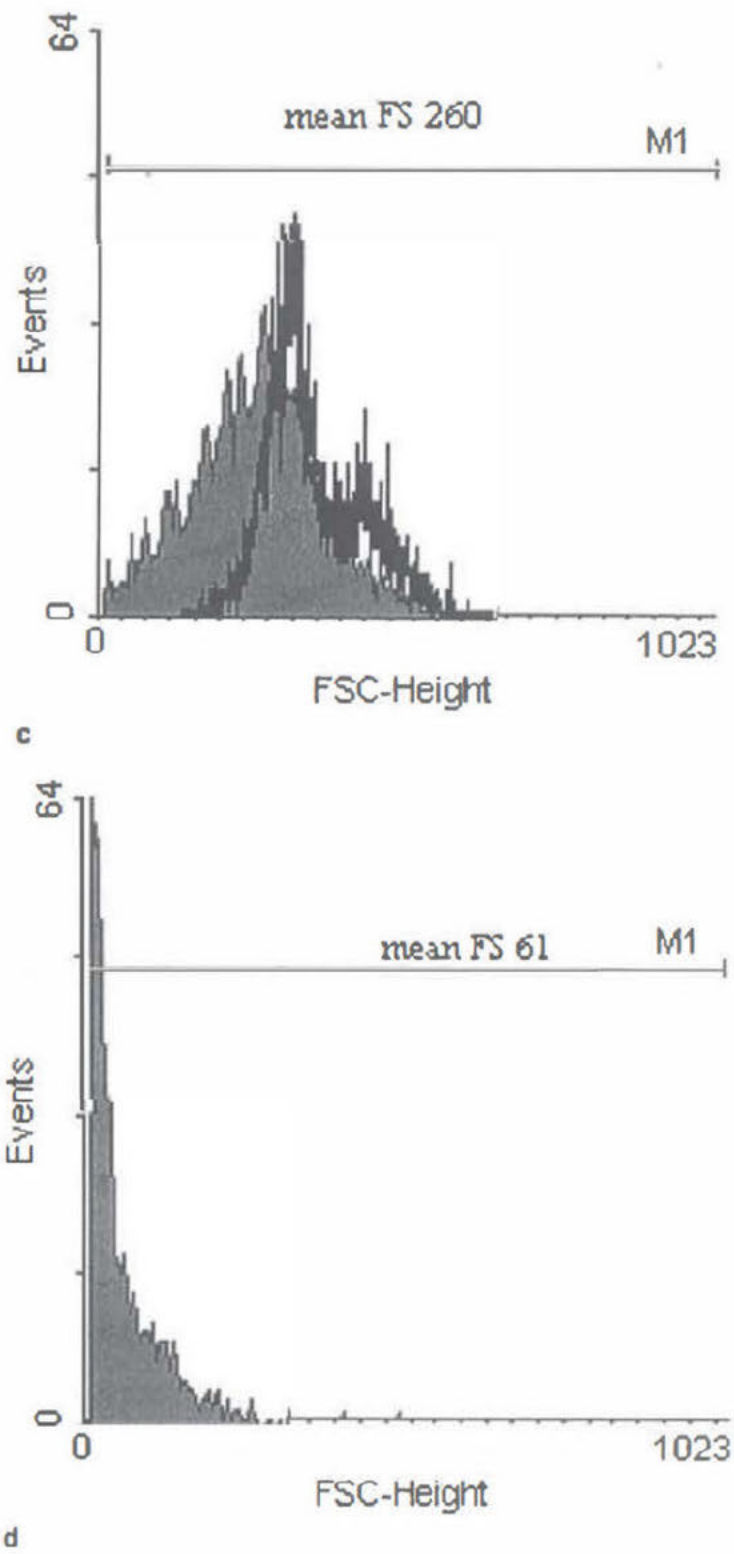


Figure 2. Continued

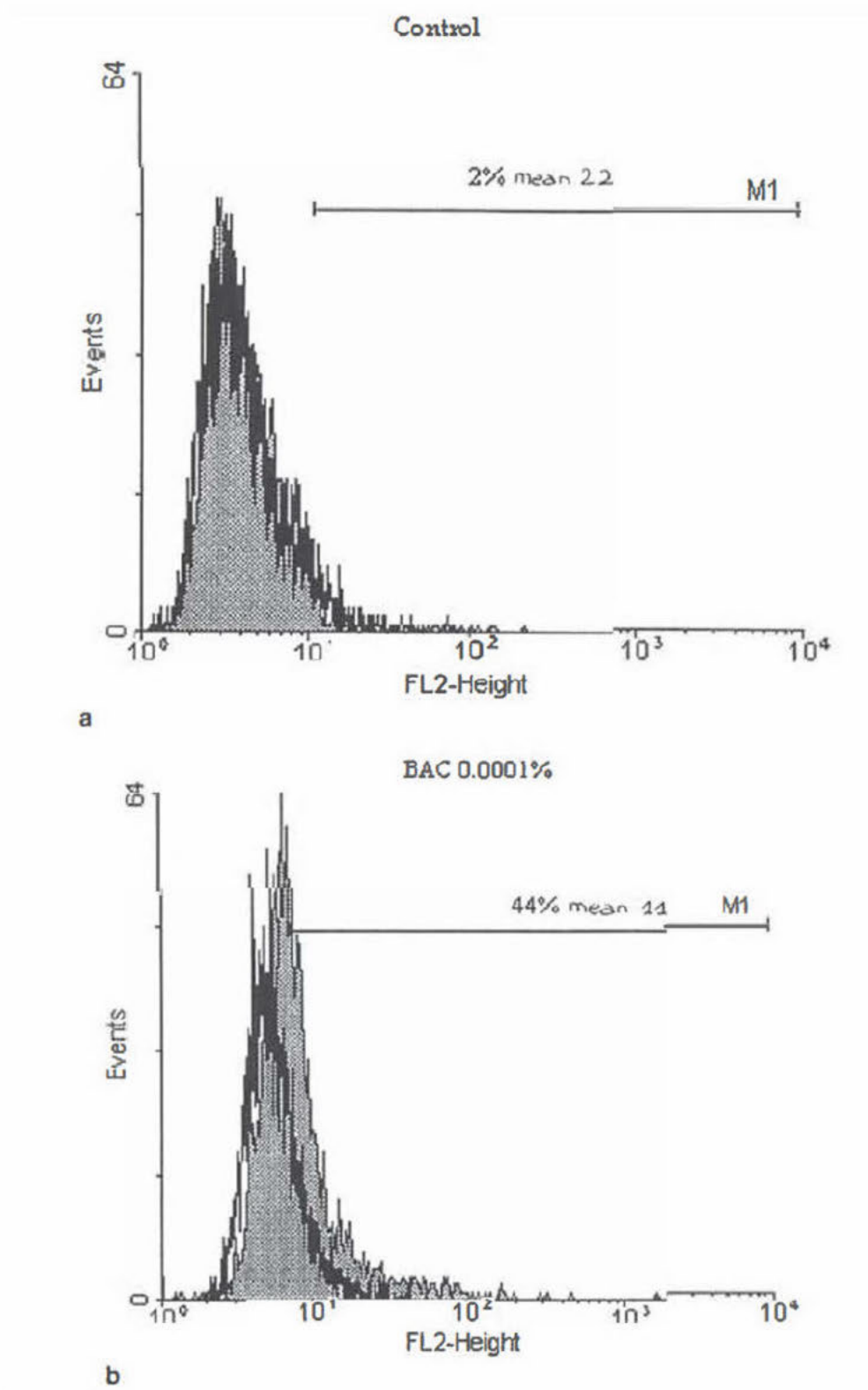


Figure 3. Apo 2.7 evaluation by flow cytometry after treatment with BAC. a. Control. b. BAC 0.001%. c. BAC 0.01%. d. BAC 0.1%.

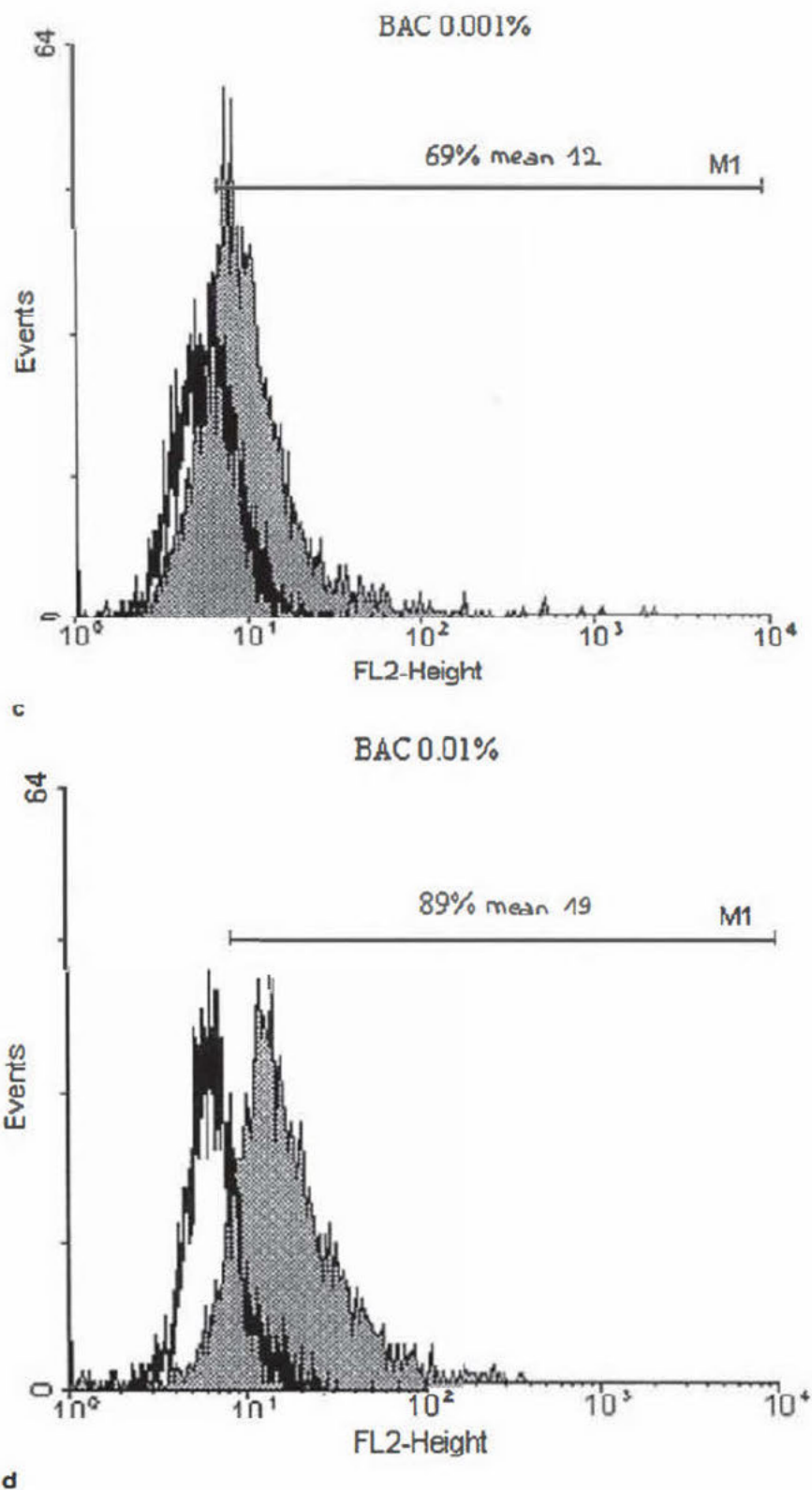
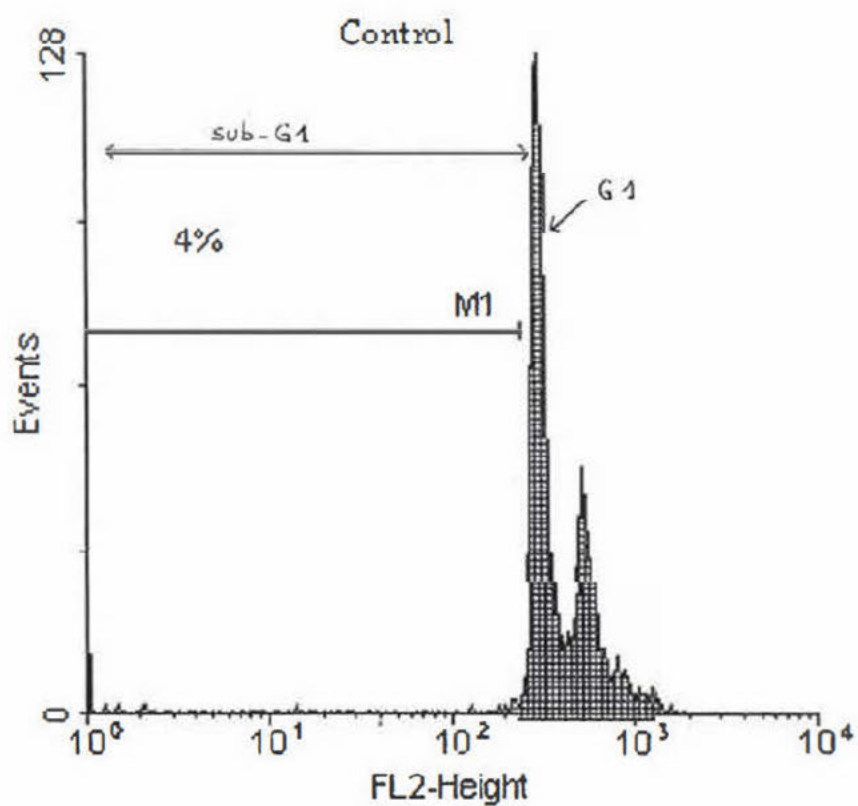
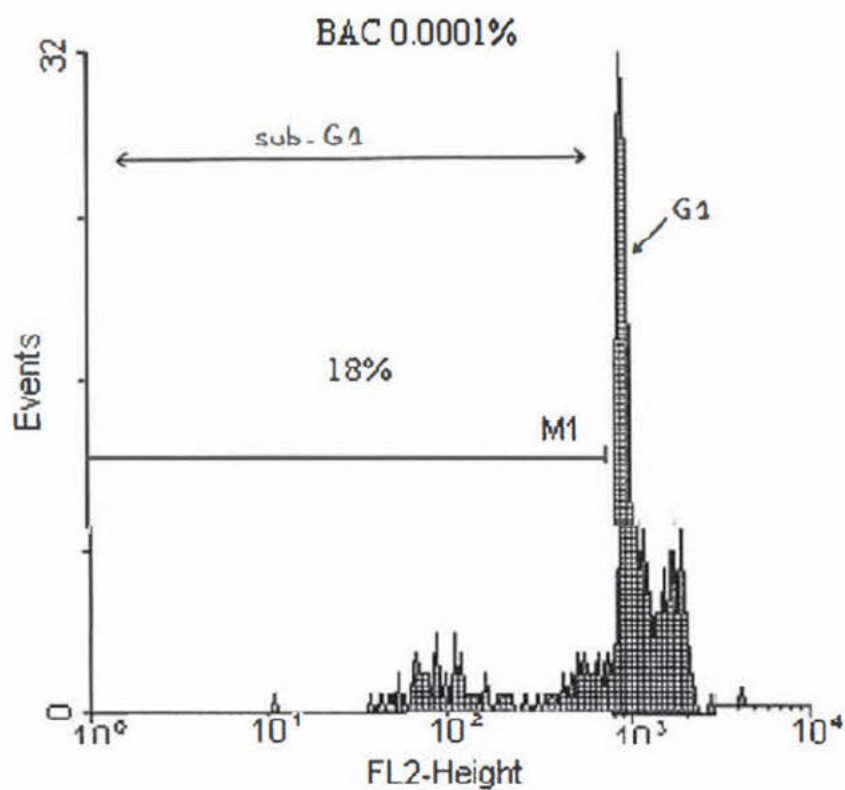


Figure 3. Continued



a



b

Figure 4. Cellular DNA quantity evaluation after treatment with BAC. a. Control. b. BAC 0.001%. c. BAC 0.01%. d. BAC 0.1%.

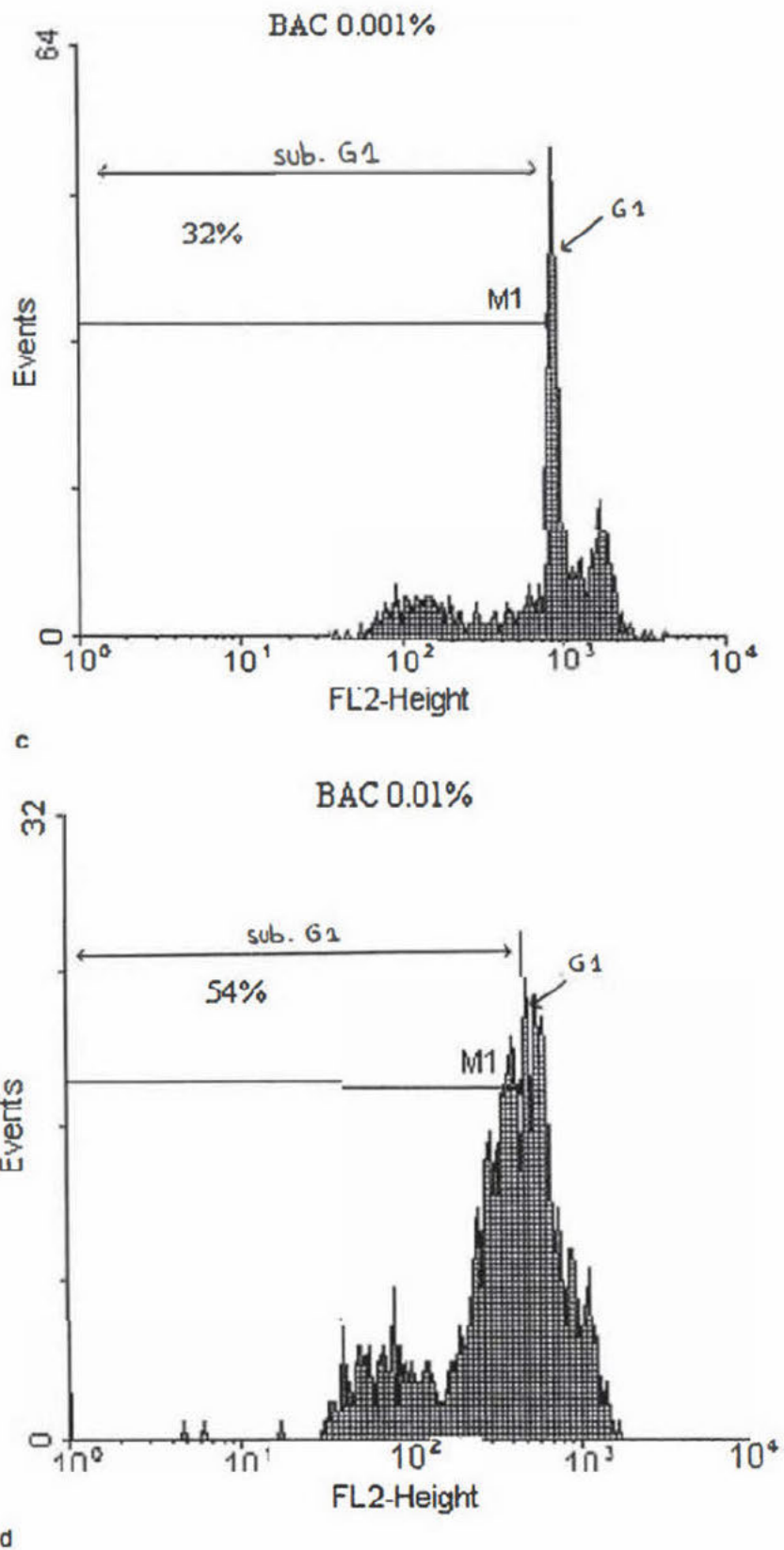


Figure 4. Continued

1 month. Ocular lesions were found with inflammatory infiltrates in the conjunctiva induced by both preserved timolol and benzalkonium chloride, unpreserved timolol did not result in significant infiltration compared to controls.¹⁵ The results of studies taken together confirmed that most of the preservatives used in ophthalmic eyedrops may induce significant histopathological and inflammatory changes in the ocular surface, and that they play a major role in the toxicity of chronically administered eyedrops.

The toxic effects induced by preservatives *in vivo* were confirmed by a series of *in vitro* experiments on a continuous human conjunctival cell line (Wong Kilbourne-derived human conjunctiva). The tests were performed using immunocytological and toxicological techniques and flow cytometry.¹⁶ Cell viability was evaluated using cold light cytofluorimetry. To confirm the apoptotic phenomenon induced by benzalkonium chloride, we used a DAPI coloration. Apo 2.7, a mitochondrial marker that appears early in apoptotic mechanisms, cellular DNA quantity, and cell size also were analyzed by flow cytometry. Cells were treated with different concentrations of benzalkonium chloride (from 0.0001% to 0.1%) over 15 mins.

Using a neutral red assay (Fig. 1), we observed a decrease in cell viability after a benzalkonium chloride treatment at a concentration of 0.001% ($p < 0.01$). Benzalkonium chloride 0.01% induced cell necrosis after a few minutes. At lower concentrations, no alteration in cell viability was observed but a DAPI coloration clearly showed apoptotic nuclei.

This apoptotic phenomenon was confirmed using flow cytometry. A 30% decrease in cell size was observed when we compared the control and the cells treated with BAC 0.01% for 15 min (Fig. 2). This result was also found using a morphological assay with cytoplasmic retraction, after treatment with benzalkonium chloride 0.01%, the concentration used in most eyedrops. An overexpression of Apo 2.7 was also shown. This expression was concentration-dependent (Fig. 3). Immunostaining confirmed these results. Furthermore, we analyzed cellular DNA quantity to detect apoptotic cells. These apoptotic cells had a lower DNA quantity than control cells. After treatment with BAC, there was a concentration-dependent increase of sub-G1-phase cells, which confirmed the apoptotic mechanism induced by BAC (Fig. 4).

These studies have proven that most of the preservatives used in eye drops may induce strong inflammatory, histopathological, and toxicological changes in the ocular surface.

In animal models, BAC causes epithelial toxicity and inflammatory infiltration of ocular surface structures. *In vitro*, BAC induces growth arrest and cell death in a dose-dependent manner at a concentration as low as 0.0001%. Cells die by necrosis after BAC treatment at high concentrations and by apoptosis if lower concentrations of BAC are applied. BAC could therefore be involved in ocular surface toxicological and/or inflammatory disorders observed in patients undergoing long-term topical treatments with preservative-containing drugs.

Very few clinical studies have been conducted to compare preservative-free

and preserved eyedrops. Studies have shown strong benefits of unpreserved solutions to the ocular surface. However, very few preservative-free ophthalmic solutions are now available. It is therefore of striking importance to become aware of preservative toxicity in order to develop in the near future many more unpreserved drugs, especially for long-term use and/or for patients with pre-existing ocular surface disorders.

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