Ocular Inflammation and Corneal Permeability Alteration by Benzalkonium Chloride in Rats: A Protective Effect of a Myosin Light Chain Kinase Inhibitor

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PURPOSE. The aim of this study was to evaluate the interest of an ophthalmic eyedrop preparation containing a myosin light chain kinase (MLCK) inhibitor, ML-7, in the treatment of ocular surface. The local protective effect on the inflammation and the increase of corneal permeability induced by benzalkonium (BAK) was evaluated.

METHODS. An ocular instillation of 10 μ L BAK at a concentration of 0.1% in PBS was performed on rats. The eyes were rinsed with sterilized water, 10 minutes after BAK preceded by instillation at T -24, -12, and -0.5 hours of 10 μ L of ML-7: 100 μ g (10 μ L) into a gel form vehicle. All animals were sacrificed 6 hours after BAK instillation. The eyes were isolated for study in a masked manner. The ocular surface inflammation was assessed by measuring the inflammatory cell infiltration by a histologic quantitative analysis and for total ocular myeloperoxidase (MPO) activity. The tight junction permeability was tested.

RESULTS. Instillation of 0.1% BAK increased the inflammation of the eye. The quantitative analysis showed an increase in the number of eosinophil and neutrophil polynuclears, and MPO activity. Pretreatment with ML-7 reduced inflammation ($P < 0.05$). The vehicle alone produced no notable effects. BAK instillation also thickened the fluorescent corneal front on frozen sections, indicating an increase of tight junction permeability. Pretreatment with ML-7 suppressed BAK-induced alterations of paracellular permeability while the vehicle had no visible effects.

CONCLUSIONS. Our study indicates that the inhibition of corneal cytoskeleton contraction by an MLCK inhibitor prevents BAK-induced ocular inflammatory response, and that ML-7 may be a new and original preparation in the treatment of ocular surface pathologies.

Keywords: ocular surface, dry eye, benzalkonium chloride, inflammation, paracellular permeability, tight junction, actin, myosin, MLCK, ML-7

Dry eye is an ocular surface disorder with a complex interplay of aggressive agents.¹ The anterior segment of the eye, the corneal and conjunctival epithelia, protects the eye against external aggressors, the ocular surface being a transitional mucosa between the deep ocular medium and the external environment. In fact, this epithelium is a competitive barrier between fluid loss and penetration of pathogens. It also protects the eye from abrasions.²

To be effective, the cells constituting this epithelium must adhere tightly to each other and also must adhere to subjacent cellular components. Considering the vulnerable position of the epithelium at the external surface of the eye, the response of the epithelium to any aggressor is immediate and effective.²

This ocular epithelium is the only site of exchange between the external medium of the eye and the internal medium. Water and electrolyte transport of small molecules use a transcellular route. The absorption of large molecules, and the passage of antigens and toxins occur through the paracellular route at the level of tight junctions (TJs) located between epithelial cells.³⁻⁷ These TJs form a paracellular seal between the lateral membranes of adjacent cells. They are composed of at least three families of transmembrane proteins (occludins, claudins,

and adhesion proteins) and a cytoplasmic plaque consisting of many different proteins that form large complexes. The transmembrane protein mediates cell adhesion, and constitutes the intermembrane and paracellular diffusion barrier.⁸ The cytoplasmic plaque of TJs is formed by different types of proteins that include adaptors, such as the zonula occludens (ZO) proteins and the proteins that contain PDZ domains, as well as regulatory and signaling components.⁸ There is a high density of cytoskeletal actin and myosin filaments, which surround the corneal epithelial cells near the apical region of the cellular borders at the level of the TJs.⁷ The disruption of the perijunctional actin-myosin filaments allows for an increase in the epithelium penetrability. Myosin light chain (MLC) contraction is regulated by the opposite actions of MLC phosphatase and MLC kinase (MLCK). MLC phosphorylation by MLCK triggers a contraction of the cytoskeleton (actin-myosin filaments) and subsequently an opening of intercellular TJs, giving rise to an increase of paracellular permeability favoring the entry of allergens and toxins.^{8,9}

Corneal and conjunctival epitheliums always are exposed to many aggressors, known to alter this competitive barrier. Different factors, such as temperature, humidity, ultraviolet

irradiation, bacteria, virus, fungi, allergens, contact lens wear, photorefractive surgery, or preservatives, can be responsible for corneal epithelial cell disruption linked to some alterations of corneal paracellular permeability. Some factors also can be determined genetically, such as the Gougerot-Sjögren syndrome.^{10,11}

Furthermore, the permeability of the ocular surface epithelium can be altered by preservatives that are present in eyedrops or antiseptic substances, such as quaternary ammonium salts. Benzalkonium chloride (BAK), a component of all multidose eyedrop formulas, such as those used in the treatment of glaucoma, is known to induce the lysis of cell membranes at the ocular surface, even at very low doses.^{12–17}

In addition, alterations of conjunctival and corneal permeability can occur after a trauma to the ocular surface, during the healing phases. Consequently, alterations of the anterior eye segment paracellular permeability result in acute or chronic dehydration of the ocular surface.¹⁸

This alteration of the epithelial TJs also can lead to sensitization due to the entry of microorganisms, allergens, or a chemical molecule, responsible for allergic and inflammatory phenomena often accompanied by pain leading to a chronic pathology.¹⁹

Therefore, the purpose of our study was to determine the protective effect of a selective MLCK inhibitor, ML-7,^{20–22} on the inflammation and the increase of corneal permeability induced by BAK and its implication on the regulation of the paracellular permeability linked to MLCK activation, which provokes epithelial TJ opening. We specifically looked for the direct effect of this MLCK inhibitor on the corneal barrier function.

METHODS

Chemicals

ML-7, an MLCK inhibitor, was obtained from Sigma Aldrich Chimie (L'Isle D'Abeau Chesnes, France). ML-7 is a 1-(5-iodonaphthalene-1-sulphonyl) - 1H-hexahydro-1, 4-diazepine.

Animals and Procedures of Benzalkonium Chloride and ML-7 Administration

Four groups of male Wistar rats (Janvier, Le Genest St Isle, France), weighing between 300 and 350 g, were used, as well as BAK + sodium carmellose, BAK + ML-7, PBS + sodium carmellose, PBS + ML-7; PBS and sodium carmellose being the solvents for BAK and ML-7, respectively.

The rats were housed in polycarbonate cages with lights (12/12 hour cycle) set at a temperature of 20°C to 22°C. The rats were fed with standard pellets (Safe 003, Epinay sur Orge, France). All procedures were performed in accordance with the relevant recommendations for animal care according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals received a local application of ML-7 (Sigma Aldrich Chimie), 24 hours, 12 hours, and 30 minutes before chemical induction of ocular inflammation. Thus, each eye was treated with 100 µg ML-7 in 10 µL eyedrop solution (sodium carmellose 4 mg/0.4 mL) or with 10 µL unmodified eyedrop solution.

Inflammation Induction

At 30 minutes after the third application of ML-7 or of the unmodified eyedrop solution, each eye was treated with 10 µL 0.1% benzalkonium chloride (Sigma-Aldrich, Steinheim, Ger-

many) in PBS or 10 µL unmodified PBS. After 10 minutes, the eyes of all the rats were rinsed with 250 µL sterile water.

Eye Excision

At 6 hours after the application of BAK or PBS, the animals were anesthetized with pentobarbital (80 mg/kg intraperitoneally [IP]; CEVA, Libourne, France) and sacrificed by decapitation. The eyes then were enucleated and frozen immediately or after surface biotinylation (TJ permeability test). All observations were performed in a masked manner.

Measurement of Polynuclear Eosinophil Infiltration

The polynuclear eosinophil leukocytes were stained with Direct Red and counted in the venous plexus region of the sclera. Immediately after excision, the eyes were embedded in a protective tissue freezing medium (Tissue Tek OCT compound; Sakura Finetek, Inc., Torrance, CA), frozen in liquid nitrogen, and stored at –80°C. Then, 6 µm thick slices were prepared with a cryostat and fixed in cold acetone for 10 minutes. After being dried, the slices were rehydrated by successive baths in toluene (5, 3, and 2 minutes), then in a 100% ethanol solution (3 and 2 minutes), a 95% ethanol solution (3 and 2 minutes), and a 50% ethanol solution (2 minutes). The sections then were bathed for 20 minutes in a staining solution of 0.03% Sirius red in 50% ethanol (Direct Red 75 dye content 30%; Sigma-Aldrich), rinsed with running water for 5 minutes, and mounted in an aqueous medium (glycerol/PBS, 50/50 vol/vol).

The eosinophils, bright pink stained on an illuminated background, were counted in the venous plexus region of the sclera under a Nikon Eclipse 90 I microscope equipped with a Nikon DXM1200F digital camera (both from Nikon Instruments Inc., Melville, NY). The area of the zone to be counted was determined with Nikon Lucia image analysis software (release 4.8; Nikon Instruments Inc.) and counts were expressed as the number of eosinophils per mm².

The results obtained for the four experimental groups were compared using a one-way ANOVA, followed by a Bonferroni multiple comparison test with statistical significance set at $P < 0.05$.

Measurement of Polynuclear Neutrophil Infiltration

Neutrophil polynuclear cells were specifically labeled by immunocytochemistry using an antimyeloperoxidase (MPO) monoclonal antibody as primary antibody, a horseradish (HRP)-conjugated secondary antibody, and an HRP-diamino benzidine (DAB) reaction as a staining step.

The cold acetone-fixed sagittal frozen sections (6 µm thick) first were incubated with hydrogen peroxide (0.6% in methanol) during 30 minutes to inhibit endogenous peroxidases. Nonspecific linking sites were saturated by a solution of normal goat serum (2% in PBS-Tween-1% BSA) during 10 minutes. Sections then were incubated with primary anti-MPO antibody (IgG1 Mouse Monoclonal [8F4] to MPO; Abcam, Cambridge, MA), 2000-fold diluted in Tween-PBS-1% BSA, overnight, 4°C.

After having rinsed with Tween-PBS, incubation with secondary antibody (stabilized goat anti-mouse HRP-conjugated; Pierce, Rockford, IL) (2000-fold diluted in Tween-PBS-1% BSA) was performed for one hour at room temperature.

Sections then were incubated with an HRP-chromogen substrate solution (3,3'-DAB kit; MP Biomedicals, Aurora, OH) for 5 minutes at room temperature.

FIGURE 1. Application of 10 μ L BAK (0.1%) in the eye led, after 6 hours, to a significant increase in the number of direct red-stained polynuclear eosinophils in the venous plexus of the sclera, as shown with the arrows. This polynuclear eosinophil infiltration was significantly inhibited by local application of ML-7. Effect of ML-7 on polynuclear eosinophilic infiltration induced by benzalkonium chloride in the sclera venous plexus following benzalkonium chloride treatment in rats (mean $\pm$ SEM, $n = 8$). * $P < 0.05$, significantly different from BAK. + $P < 0.05$, significantly different from Vehicle.

Sections were counterstained with Mayer's hematoxylin (20 seconds), dehydrated, and mounted in Depex medium. Counting was done using a Nikon DXM1200F digital camera (Nikon Instruments Inc.) as with eosinophils.

Measurement of TJ Permeability – Surface Biotinylation

The permeability of TJs in the cornea was evaluated by biotinylation of surface proteins. The chosen biotinylation reagent was water-soluble and contained an aminocaproyl spacer group, which lowered steric hindrance during avidin coupling. Immediately after excision, the eyes were incubated for 30 minutes at room temperature with gentle stirring in a solution containing sodium biotinamido-hexanecarboxylate and 3-sulfo-N-hydroxysuccinimide at 1 mg/mL in PBS (Sigma-Aldrich). The eyes then were rinsed three times with PBS, embedded in a protective tissue freezing medium (Tissue Tek OCT compound; Sakura Finetek, Inc.), frozen in liquid nitrogen, and finally stored at -80°C .

Six μ m thick slices were prepared with a cryostat and fixed in cold acetone for 10 minutes. After being dried out, the slices were labeled for 30 minutes in the dark with avidin D-FITC (Vector Laboratories, Inc., Burlingame, CA) 250-fold diluted in PBS-Tween containing 1% BSA, then rinsed three times for 5 minutes with PBS-Tween in the dark. The slices then were mounted in a fluorescent medium (Cappel fluorostab embedding medium; MP Biomedicals, Inc., Aurora, OH) and examined under a Nikon Eclipse 90 I fluorescence microscope equipped with a Nikon DXM1200F digital camera (both from Nikon Instruments Inc.). The images were analyzed with the Nikon Lucia image analysis software (release 4.8; Nikon Instruments Inc.). As no significant differences in corneal thickness were observed between the different groups (102 ± 10 , 110 ± 9 , 115 ± 13 , and 124 ± 8 μ m for BAK + sodium carmellose, BAK + ML-7, PBS + sodium carmellose, and PBS + ML-7 groups, respectively), the depth of fluorescence labeling reflected the permeability of external corneal epithelial TJs to the biotinylation reagent.

Measurement of MPO Activity

The activity of MPO, which is found in polymorphonuclear neutrophil granules, was assessed according to the method of Bradley et al.²³ Samples of the eyes were suspended in a potassium phosphate buffer (50 mM, pH 6.0) and homogenized in ice. Three cycles of freeze-thaw were undertaken. Suspensions then were centrifuged at 10,000g for 15 minutes at 4°C . Supernatants were discarded and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (HTAB, 0.5% wt/vol, in 50 mM potassium phosphate buffer, pH 6.0). These suspensions were sonicated on ice, and centrifuged again at 10,000g for 15 minutes at 4°C . The supernatants obtained were diluted in potassium phosphate buffer (pH 6.0) containing 0.167 mg mL^{-1} of O-dianisidine dihydrochloride and 0.0005% of hydrogen peroxide. Myeloperoxidase from human neutrophils (0.1 units per 100 μ L) was used as standard. The

FIGURE 2. Effect of ML-7 on the number of MPO-immunoreactive cells (neutrophils) accumulated in the sclera veinous plexus following benzalkonium chloride treatment in rats (mean $\pm$ SEM, $n = 8$). * $P < 0.05$, significantly different from BAK. + $P < 0.05$, significantly different from Vehicle.

kinetic changes in absorbance at 450 nm, every 10 seconds over 2 minutes, were recorded with a spectrophotometer (spectromètre de masse GC-MSn, Thermo Polaris Q; Thermo Fisher Scientific Inc., Waltham, MA). One unit of MPO activity was defined as the quantity of MPO degrading 1 μ mol hydrogen

peroxide $\text{min}^{-1} \text{ml}^{-1}$ at 25°C. Protein concentration was determined with a commercial kit using a modified method of Lowry (Detergent Compatible Assay; Bio-Rad, Marnes la Coquette, France). MPO activity was expressed as units per gram of protein.

Statistical Analysis

Data were presented as means $\pm$ SEM. To compare the groups, we used the Student's impaired t -test and the Bonferroni multiple comparison test. Statistical significance was accepted at $P < 0.05$.

RESULTS

Effect of Local Application of ML-7 on Polynuclear Infiltration Induced by Corneal Instillation of BAK

The instillation of 10 μ L 0.1% BAK in the eye led to a highly significant increase in the number of inflammatory cells as determined by the significant increase of Direct Red stained polynuclear eosinophils in the venous plexus region of the sclera, showing evidence of a severe ocular inflammation (Fig. 1).

FIGURE 3. Effect of ML-7 on the increase of permeability to the fluorescent dye induced by BAK. The application of 10 μ L BAK (0.1%) in the eye led, after 6 hours, to an opening of corneal epithelial TJs as manifested by deeper penetration of the fluorescence and by its diffusion. The influence of BAK was inhibited by ML-7, which suppressed this increase in the diffusion and thickening of the fluorescent zone.

FIGURE 4. Quantitative analysis showing that ML-7 significantly protects the corneal barrier in rats against the strong alteration of the paracellular permeability after 0.1% BAK instillation. The corneal barrier is evaluated by deeper penetration of the fluorescence ($n = 6$ for PBS and PBS ML-7, $n = 11$ for BAK + ML-7, $n = 20$ for BAK). * $P < 0.05$, significantly different from BAK. + $P < 0.05$, significantly different from PBS.

This polynuclear eosinophil infiltration was inhibited significantly after local application of ML-7 100 µg in a carbomer gel. The vehicle (unmodified carbomer gel) produced no notable effects (Fig. 1).

Similarly, after 6 hours, instillation of 10 µL 0.1% BAK led to a very significant increase of MPO activity in the sclerous venous plexus. The vehicle (unmodified carbomer gel) had no notable effects (Fig. 2).

These BAK increased MPO immunoreactivity cells were inhibited significantly by a pretreatment with 100 µg ML-7 in a carbomer gel. Histologic observations confirmed these results. (Fig. 1).

Effect of Local Application of ML-7 on TJ Permeability Alteration Induced by Corneal Instillation of BAK

On the frozen sections, the permeability study showed that instillation of 10 µL 0.1% BAK induces an alteration of TJ opening as demonstrated by a thickening of the fluorescent marker at the external of the cornea (Figs. 3, 4).

Pretreatment with 100 µg ML-7 in a carbomer gel protected against this alteration of paracellular permeability induced by BAK instillation (Figs. 3, 4).

These observations are confirmed by a quantitative analysis showing that ML-7 in a carbomer gel significantly protected against this alteration of paracellular permeability after 0.1% BAK instillation. The vehicle (carbomer gel) had no notable effects on paracellular permeability (Figs. 3, 4).

DISCUSSION

Dry eye syndrome is a chronic lack of sufficient lubrication and moisture of the ocular surface linked to inflammations affecting the cornea and conjunctiva.

Among the aggressors responsible for dry eyes, such as environmental factors, exposure of the ocular surface to preservatives (antiseptic substances) provokes significant disorders. The most well known preservative salts on the market are the quaternary ammonium salts, such as BAK, which is an ingredient of multidose eyedrops approved for the treatment of glaucoma. The said preservatives, by inducing free radical release²⁴ and apoptosis of ocular cells,^{24,25} reach the corneal epithelium and stimulate the infiltration of

inflammatory cells into the conjunctiva.^{16,26,27} Severe damages to ocular surface, such as ulcerations, large epithelial defects, and neovascularizations, can occur.²⁵ The administration of BAK induces changes similar to the dry eye syndrome in humans accompanied by a decrease in the amount of tears, an increase of the corneal fluorescein, and the rose Bengal score.²⁵ BAK also can affect cell membrane permeability, causing lysis of cell contents and allowing vital substances to escape.²⁸

It is now well demonstrated that BAK accelerates the desquamation of corneal epithelium cells with a concomitant depletion of intracellular ATP. Among the varied effects of ATP depletion, phosphorylation of regulatory light chain of myosin II (MLC) has been reported and it has been demonstrated clearly that the exposure of corneal epithelial cells to BAK leads to MLC phosphorylation,²⁹ which contracts the cytoskeleton of epithelial cells, thus, breaking down the corneal barrier integrity. Similar effects are noted in the presence of histamine.³⁰ This barrier loss contributes to the propagation and exacerbation of the inflammation.³¹

Furthermore, aggressors, such as BAK, can cause a decrease in the expression of the zonula occludens protein (ZO-1), a key compound of TJs,³² or alter the organization of the actin cytoskeleton in the apical region of the cell.³²

Our results confirmed that 0.1% BAK administration causes side effects on the corneal membrane through MLC phosphorylation. BAK 0.1% significantly provokes eye inflammation. We observed an increase of MPO immunoreactivity in the sclera venous plexus. We also noted a significant increase in the number of infiltrated eosinophil polynuclears. Pretreatment of rat eyes with ML-7, an inhibitor, significantly reduces the number of infiltrated neutrophil and eosinophil polynuclears ($P < 0.05$).

Concomitantly to the corneal inflammation, following 0.1% BAK administration, we observed a significant increase of the paracellular permeability in the corneal epithelium, as demonstrated on frozen sections of rat cornea after biotinylation and amplification by avidine fluorescein. This increase of paracellular permeability is prevented by ML-7, which attenuates MLCK activity, suggesting that it limits the entry of allergens and pathogens.⁹ Consequently to this, ML-7 limits the ocular inflammation response, as seen previously, at the level of the gut colonic epithelium.⁹

Furthermore, similar protective effects of ML-7 are described using ethanol as a barrier aggressor. ML-7 treatment attenuates the ethanol-mediated increase of paracellular permeability and MLCK activity.³³

In conclusion, ML-7, an MLCK inhibitor, by preventing the deleterious effects of BAK preservatives on corneal cytoskeleton and the consecutive inflammation, may be a new and original preparation in the treatment of ocular surface pathologies, such as dry eye.

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