Electrophysiologic and morphologic effects of ophthalmic preparations on rabbit cornea epithelium

Neal L. Burstein* and Stephen D. Klyce

The effects of several components of ophthalmic preparations on isolated rabbit corneas were studied by continuous electrophysiologic monitoring followed by fixation for scanning electron microscopy (SEM). Benzalkonium chloride (0.001 percent), thimerosal (0.0004 percent), and amphotericin B (0.0025 percent) all briefly increased ion transport, then greatly decreased epithelial resistance. Severe disruption of surface cell layers occurred simultaneously with resistance decrease. Silver nitrate (0.00017 percent) stimulated transport with less accompanying morphologic damage. Tetracaine (0.05 percent) disrupted epithelial function and caused exfoliation of several cell layers. Chlorobutanol (0.1 percent) produced a nearly complete loss of the squamous cell layer. Chloramphenicol, epinephrine, and pilocarpine produced minor changes in structure and electrophysiology at full clinical concentration. It was concluded that low concentrations of preservatives in ophthalmic preparations disrupt the barrier and transport properties of the corneal epithelium.

Key words: corneal epithelium, ultrastructure, ion transport, preservatives, drugs.
Figs. 1A and 1B. Influence of 1 mg per 100 ml BAK on the epithelium. A, Low-power view of BAK treatment of 60 min. B, Stereo pair at higher power of A. Note the elongated microvilli and the absence of microplicae.

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933069/)

Fig. 1C. Effect of BAK added at † on epithelial PD and SCC. The cornea was fixed at † for the morphology shown in A and B.

(SCC), indicative of ion transport, and corneal potential (PD) were measured, allowing calculation of transcorneal resistance (R = PD/SCC). Scanning electron microscopy (SEM) was then used to evaluate the morphologic alterations accompanying electrical changes. Lower concentrations of agents were then used until threshold levels were reached, below which no damage could be detected by our techniques.

Materials and methods

Corneas removed from adult New Zealand White rabbits after administration of a lethal dose of sodium pentobarbital were mounted atraumatically in Lucite chambers. Corneal surfaces were bathed in a stirred, aerated solution at 34° C composed of 103.4 mM NaCl, 15.3 mM NaHCO3, 10 mM NaHCO3, 2.2 mM KH2PO4, 0.5 mM KH2PO4, 5.24 mM H2PO4, 0.61 mM MgSO4, 0.7 mM calcium gluconate, 26 mM glucose, and 20 mM tris(hydroxymethyl)aminomethane (Tris). In ion substitution experiments Tris was used to replace Na+ and SO4²⁻ was used to replace Cl⁻. The pH was 7.4 and solutions were 305 mOsm. PD and SCC were measured with automatic voltage clamps (H. Fein). Corneas were short-circuited for 57 seconds of each minute for measurement of ion transport and PD was determined during the remaining 3 seconds.

Corneas were equilibrated in chambers for 60 to 90 min, at which time stable potentials of 25 to 35 mV were observed. Test compounds were
Fig. 2. Influence of 0.4 mg. per 100 ml. thimerosal added at † on the epithelium. A and B, Influence on surface morphology. C, Influence on PD and SCC. Cornea fixed at † for A and B.

then added to the tear-side bathing solution. Agents studied were benzalkonium chloride (BAK, alkylbenzyldimethylammonium chloride, Winthrop), thimerosal (ethylmercurithiosalicylate, Sigma), amphoterin B (Pungzone, Squibb), AgNO₃ (Baker), pilocarpine (Alcon), epinephrine bitartrate (Sigma), chlorobutanol (Parke-Davis), chloramphenicol (Ophthochlor, Parke-Davis), and tetracaine (Alcon). Solutions of appropriate dilution were left in contact with the epithelial surface for 30 to 110 min.

Corneas were rapidly removed from the Lucite chambers after treatment and fixed with 4 percent glutaraldehyde (Ladd) in 0.1M K₂PO₄ buffer (pH 7.3) for 2 hr. or more, rinsed in buffer, and post-fixed in 1 percent OsO₄ also buffered. Acetylcysteine 20 percent (Mucomyst, Meade-Johnson) was used as a rinse when the upper corneal surface was otherwise obscured by mucopolysaccharides.8 Dehydration by increasing concentrations of acetone was followed by critical-point drying with CO₂ as a transition fluid. Tissues were then mounted on aluminum studs by means of colloidal carbon cement and sputter-coated with 20 to 40 nm. of Au. The epithelial surface was examined with a Coates and Welter field-emission
Fig. 3. Influence of tetracaine on epithelial surface structure. A, At 0.5 mg. per 100 ml. tetracaine for 1 hr. the surface is normal. B, At 5 mg. per 100 ml. for 1 hr., minor modifications appear. C, At 10 mg. per 100 ml. for 1 hr., most surface cells are absent and remaining are modified. D, At 50 mg. per 100 ml. for 1½ hr., the surface layer has been lost and intercellular attachments of deeper layers are compromised. E, At 50 mg. per 100 ml. for 1 hr., this stereo pair suggests total involvement of the epithelium in a progressive loss of intercellular attachments.
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Fig. 4. Influence of 0.17 mg. per 100 ml. AgNO₃ on the epithelium. A, Surface morphology of companion cornea fixed at the peak response of the SCC. B, Modification of the surface is found when the cornea was fixed at ↑ in C. C, AgNO₃ stimulation of epithelial PD and SCC. Same cornea as in B.

SEM at 15 keV. At least six corneas were evaluated for each drug concentration. Some of these were taken at the peak of stimulation rather than at the end of electrophysiologic change.

Results

Control corneas incubated 2 to 3 hr. before fixation exhibited stable values of PD and SCC. The morphologic appearance of the epithelial surface was largely normal, with flat cell surfaces covered by microvilli and microvilli. Circular structures were noted in 20 percent of the cell population, corresponding to "hole formation." Uplifting of about 1 percent of the cell population was observed after 2 hr. in vitro, and this figure increased after 3 hr. This reaction has not been observed in intact animals; it is believed to result from
Fig. 5. Effect of 100 mg per 100 ml of chlorobutanol on epithelial morphology after 1 hr. of exposure. A, Note the prominence of the intercellular ridges indicated by arrowheads which are characteristic of the exposed second cell layer. B, Stereo pair of two remaining surface cells.
cellular desquamation as a normal process in epithelial surface renewal. Since lids are not present to sweep away such cells in the physiologic chambers, the cells remain on the corneal surface. The observed rate of 0.5 percent cell uplifting per hour would produce complete removal of the surface-layer cells in 8 days, consistent with the reported turnover rate.9

Preservative agents. BAK, at a concentration of 0.001 percent, briefly increased corneal PD before total inhibition (Fig. 1C). The SCC increased biphasically, and corneal resistance decreased. At 1 hr. following BAK addition, the epithelial surface was severely disrupted (Fig. 1A). Cells of the surface layer were loosened or removed, exposing second- and third-layer cells. The plicate appearance of surface cells was lost, and some deeper cells had abnormally long microvilli (Fig. 1B).

Treatment with 0.0004 percent BAK caused a slow inhibitory phase after an hour's treatment, accompanied by an in-
increase in the number of cells with peripheral loss of microvilli and microplicae. At 0.0001 percent, BAK caused no discernible modification of electrical properties or surface morphology after 2 hr. incubation.

Thimerosal, at a concentration of 0.0004 percent, caused a slight initial inhibition of SCC followed by slow stimulation and finally complete inhibition (Fig. 2, C). The PD increased slowly, then decreased after 1 hr. of incubation. After 90 min. of treatment, nearly half the surface cells were lost or loosened (Fig. 2, A). The remaining surface cells were shrunken, with disrupted intercellular attachments (Fig. 2, B). Corneas exposed for shorter times showed less alterations of surface features; cell detachment was not observed before the drop in corneal resistance at about 45 min.

Partial denudement of surface microvilli was seen after 90 min. of treatment with 0.0001 percent thimerosal. There was a transient inhibition of SCC accompanied by slow inhibition of PD at this concentration. No significant effect was detected after 2 hr. of treatment with 0.00004 percent thimerosal.

**Medications.** Tetracaine, at a concentration of 0.0005 percent, produced a sustained 25 percent increase in SCC, with only a slight reduction of PD. Epithelial morphology was relatively normal after 1 hr. at this concentration (Fig. 3, A). At 0.005 percent concentration, tetracaine produced a slow inhibition of PD and also increased SCC. After 1 hr., some cells were seen in the process of detachment, with long, sparse microvilli (Fig. 3, B). At 0.01 percent concentration, PD was
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Fig. 8. Effect of amphotericin B on the epithelium. A, Surface morphology after 45 min. of treatment with 0.25 mg. per 100 ml. amphotericin B. Arrowheads point out rings which are possibly transmembrane holes. B, Surface morphology of cornea treated with 2.5 mg. per 100 ml. amphotericin B shown in C. C, Stimulation of epithelial PD and SCC by amphotericin B. Cornea fixed at t.

inhibited more than 50 percent, and SCC was increased. Many surface cells were rounded after 1 hr. of exposure (Fig. 3, C). Desmosomal attachments were lost, and the second layer of cells appeared beneath in many places.

At 0.05% concentration, tetracaine irreversibly inhibited epithelial PD and SCC to near-zero values in 30 to 45 min. After 30 min. of treatment, nearly all the first-layer cells were lost. The second layer had become detached in many places, exposing third-layer cells (Fig. 3, D). Nuclei in these cells stood out in relief, and the imprints of the detached surface cells could be seen on this layer. After 1 hr. of exposure to this concentration of tetracaine, intercellular attachments of several cell layers were disrupted, first- and second-layer cells had detached, and many of the
remaining cells were rounded (Fig. 3, E). Several further layers of cells were exposed.

AgNO₃, at 0.00017 percent, markedly increased both SCC and PD (Fig. 4, C). At the peak of stimulated current, 20 min. of exposure, no alteration of surface structure was detected (Fig. 4, A). After 110 min., an increase in desquamating cells was observed (Fig. 4, B), along with reduction of peripheral microvilli on the surfaces of epithelial cells.

Chlorobutanol, at 0.1 percent, stimulated SCC and decreased epithelial PD during a 60 min. incubation period. The morphologic appearance of this experiment suggests the nearly total loss of the surface cell layer (Fig. 5, A). Many "holes" are seen in the second cell layer exposed. Occasional surface cells were seen still attached, as in Fig. 5, B. Some peripheral attachments remaining on one of the cells in this SEM view are suggestive of desmosomal anchorage.

Nearly complete inhibition of PD was produced after 40 min. of treatment with 0.05 percent chlorobutanol, but influence on SCC was minor. The surface structure was similar to that of controls, with about 1 percent of surface cells detaching. At 0.005 percent, chlorobutanol had no detectable effects on corneal epithelium.

Epinephrine, at 0.1 percent, immediately decreased corneal PD and SCC (Fig. 6, B). Surface morphology was largely normal, except for the prominence of nuclear outlines (Fig. 6, A). These nuclear prominences are suggestive of a shrinkage of cell profile, causing the nuclei to stand out in relief. Surface morphology was otherwise normal. This concentration of epinephrine was several hundred times larger than that producing physiologic response and was used only to evaluate toxicity due to topical application.

Pilocarpine, at 0.1 percent, had negligible influence on electrical properties. After 1 hr. of treatment, a small percentage of the surface cells exhibited peripheral denudement of microvilli and microplicae, as previously described.⁴

Chloramphenicol, at 0.01 percent, produced no detectable perturbation of epithelial physiologic properties. The superficial epithelial cell surface was composed largely of microvilli, rather than presenting the preponderance of plicae previously presented in controls (Fig. 7).

Amphotericin B, at 0.00025 percent, caused a rapid stimulation of both SCC (from 2.5 to 12.5 μA/m²/hr.) and epithelial PD (from 19.5 to 40 mV.) for a sustained period of 45 min. The surface of this cornea showed decreased numbers of microvilli, and some cells were seen lifting from the surface. Other cells were wrinkled and appeared to have discharged their contents through ruptures in the unit membrane (Fig. 8, A). At 0.0025 percent, amphotericin B evoked a large biphasic increase in SCC and epithelial PD (Fig. 8, C). At the peak of SCC (40 min.) morphology was normal. After 110 min. of treatment, much of the surface layer was rounded and detaching, with loss of microplicae and abnormally long microvilli (Fig. 8, B).

Surfaces of second-layer cells were seen in some areas.

**Ion-substitution experiments.** BAK, thimerosal, amphotericin B, and AgNO₃ all stimulated SCC from four to 15 times the control level (Table I). It has been previously demonstrated that AgNO₃ stimula-

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**Table I. Agents that stimulate epithelial SCC and/or PD**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc. (mg./100 ml.)</th>
<th>PD₀ (MV)</th>
<th>PDₚ (MV)</th>
<th>SCC₀ (μA/cm²/hr.)</th>
<th>SCCₚ (μA/cm²/hr.)</th>
<th>No. of obs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thimerosal</td>
<td>0.4</td>
<td>28.9 ± 2.7</td>
<td>23.9 ± 2.8</td>
<td>4.76 ± 1.49</td>
<td>19.2 ± 2.5 (&lt;0.01)</td>
<td>6</td>
</tr>
<tr>
<td>BAK</td>
<td>1.0</td>
<td>21.5 ± 2.8</td>
<td>27.8 ± 2.0</td>
<td>3.47 ± 0.36</td>
<td>16.0 ± 1.0 (&lt;0.001)</td>
<td>6</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>2.5</td>
<td>21.4 ± 1.7</td>
<td>57.6 ± 1.9</td>
<td>4.2 ± 0.8</td>
<td>61.1 ± 9.3 (&lt;0.001)</td>
<td>7</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>0.17</td>
<td>20.3 ± 2.2</td>
<td>53.5 ± 3.4</td>
<td>3.5 ± 0.5</td>
<td>36.1 ± 1.2 (&lt;0.001)</td>
<td>6</td>
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</tbody>
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*Values are means ± S.E.M. (probability of chance influence) of the PD and SCC before treatment (₀) and at the peak of the response (ₚ).*
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Ion substitution experiments were therefore performed to evaluate the influence of BAK, thimerosal, and amphotericin B on epithelial ion transport. The results are shown in Fig. 9.

Chloride-free Ringer did not significantly influence the peak response of SCC following treatment with 0.001 percent BAK (Fig. 9, A) compared to complete Ringer (cf. Fig. 1C). However, peak response was reduced by two thirds with Na+‐free Ringer, indicating that the bulk of inward ion transport was due to inward Na+ transport. Epithelial PD depression was independent of ionic composition, suggesting nonspecific influence of BAK in lowering extracellular pathway resistance. This is consistent with the damage shown by SEM (cf. Figs. 1A and 1B).

Influence of 0.0025 percent amphotericin...
B on both PD and SCC was nearly abolished when Na⁺ was replaced by Tris in bathing solution; when Na⁺ was restored to the tear side, SCC was stimulated severalfold (Fig. 9, C). Response to amphotericin B in Cl-free Ringer was qualitatively similar to the response in complete medium (Fig. 9, D, cf. Table I and Fig. 8). Amphotericin B then appears to primarily stimulate Na⁺ transport.

Response to thimerosal at 0.0004 percent was not altered by Na⁺-free Ringer. However, Cl-free Ringer completely suppressed the thimerosal effect on SCC, and restoration of Cl⁻ caused immediate response (Fig. 9, E; cf. Fig. 2). Apparently, thimerosal stimulates Cl⁻ secretion across the epithelium, presumably by increasing Cl⁻ permeability of the squamous cell, in addition to its later nonspecific influence on the extracellular pathway (cf. Fig. 2).

Discussion

Most agents studied in this report produced modification of surface structure, changes in ion transport, and/or increased permeability of the cornea. Changes in surface structure, including decreased numbers of microplicae, abnormally long microvilli, and smooth or rough cell margins, did not directly correlate with physiologic parameters. Likewise, ion-transport modification occurred without producing surface modifications apparent by SEM observation. In contrast, permeability changes, evidenced by decreasing transcorneal resistance, were accompanied by disruption of the surface-layer cell margins, and often by exfoliation of the entire surface cell layer.

The damage evoked by chronic treatment with BAK, thimerosal, amphotericin B, tetracaine, and possibly chlorobutanol would be expected to produce stromal edema secondary to destruction of the squamous cell layer. All the preservative agents studied produced dose- and time-dependent damage, and their chronic instillation with medication might well be curtailed. Previous studies have shown far greater damage due to BAK than to other preservative agents, whereas we find effects from very small doses of thimerosal. This may be due to buffering of the mercurial compound by the tear film of the eye in vivo, resulting in less binding of the agent to the squamous cell layer.

Intracameraly administered amphotericin B can produce toxic effects, including retinal and endothelial detachment. The instability of membrane bilayers containing cholesterol to polyene antibiotics after stoichiometric binding has been documented. A specific action on a membrane sterol might be responsible for amphotericin B effects on permeability, since it has been demonstrated that inward Na⁺ transport in the frog corneal is also stimulated by amphotericin B.

Tetracaine and other local anesthetics have long been known to retard corneal wound healing. Leuenberger has demonstrated punctate corneal stippling, loss of microvilli and microplicae, and cell membrane disruption after repeated application of 0.5 percent tetracaine to rabbits. Single-dose topical application at this concentration to corneas in vivo does not result in apparent morphologic damage, perhaps due to dilution by the tear film. Cultured cells lose cytoskeletal microfilaments and microtubules, and detach from substrate after rounding, when exposed to 0.15 percent tetracaine. Phosphatases and oxidative enzyme levels are altered in corneal epithelium after tetracaine instillation. It is unclear whether these effects are all the result of a specific membrane action of the local anesthetics.

Pilocarpine, epinephrine, and chloramphenicol produced no detachment of epithelial cells during continuous exposure at concentrations one tenth that of topically applied medications. Medications used to treat chronic conditions or applied by slow-release devices warrant careful study as to their toxicity. The present study is thought to provide useful information in such evaluation by monitoring the time...
course of corneal electrophysiologic change and its correlation with morphologic damage.

We are grateful to Dr. Norman K. Wessells for the use of his SEM facilities and to Mr. Otto Bernegger for his excellent assistance in this study.

REFERENCES