Preservative Alteration of Corneal Permeability in Humans and Rabbits

Isotonic, neutral buffered solutions of benzalkonium chloride or chlorhexidine digluconate were applied topically to one eye of rabbits or human subjects. Contralateral control eyes received phosphate buffered saline as placebo. One-half hour later, the tear film of both eyes was loaded with nonpreserved sodium fluorescein. Anterior chamber fluorescence levels were measured at 1 hr intervals to determine corneal permeability changes attributable to preservative action. In rabbits, corneal permeability increased with rising preservative concentration. Benzalkonium chloride 0.01% increased anterior chamber fluorescence level 1.8 (±0.2 SEM) times over control eyes, while chlorhexidine digluconate 0.01% caused 1.5 (±0.2 SEM) to one ratio of fluorescence in treated/untreated eyes. In human subjects, neither preservative produced significant permeability change at 0.01% concentration. However, benzalkonium chloride 0.02% caused 1.23 (±0.08 SEM) permeability increase. The results support the hypothesis that rabbits are more
In rabbits, corneal epithelial disruption occurs with clinically used concentrations of benzalkonium chloride. Epithelial alteration has been demonstrated by increased fluorescein penetration, permeability to inulin, electrophysiologic measurement, and scanning electron microscopic evaluation. Of these methods, measurement of fluorescein penetration is the only one that is suitable for human subjects, since the invasive dye is considered noninjurious.

Of the total transcorneal resistance, over one-half is provided by the outer squamous epithelial layer of the cornea and its tight junctional complexes, or zonula occludens, so that diffusion of ionic solutes must normally occur by a pathway across the cells. Disruption of this layer, detectable by scanning electron microscopy, has been shown to cause decreased electrical resistance accompanied by increased ionic flow. It is reasonable to assume that fluorescein penetration characteristics detectable in vivo would serve to indicate the same morphologic changes as decreased electrical resistance and topographic surface changes seen by scanning electron microscopy in vitro. A comparison of effects in rabbits and human subjects is thus possible.

Marsh and Maurice measured fluorescein permeability alteration in human eyes in response to nonionic detergents, and noted that decreased corneal resistance occurred at concentrations slightly below those producing marked irritation. In this paper, their technique has been modified to prevent the direct interaction of the anionic fluorescein with cationic surfactants. This permits the corneal permeability change due to benzalkonium chloride and chlorhexidine digluconate to be compared in rabbit and human corneas.

**Materials and Methods.** Preparation and delivery of formulations: Solutions containing benzalkonium chloride or chlorhexidine digluconate in concentrations of 0.05%, 0.02%, 0.01%, 0.005%, and 0.0025% were prepared in phosphate buffered saline, pH 7.4 ± 0.1, at osmolality of 300 ± 5 mOsm. Each test eye received two drops of 0.05 ml each applied to the superior corneal limbus in rapid succession and allowed to flow across the corneal surface. Contralateral control eyes received two 0.5-ml drops of phosphate buffered saline only. Overflow was allowed to spill across lids or drain through the nasal punctum.

Either commercially available fluorescein 5% injection, or 3.34% isotonic fluorescein in sterile water, was used for topical application. Unused fluorescein was discarded daily, to guard against bacterial contamination of unpreserved solution. Doses of 2–5 µl were applied to both eyes of human subjects and rabbits, using a Gilmore 0.2-ml micrometer syringe with a flexible polyethylene tip.

**Experimental procedure:** Rabbits were examined for corneal surface erosions before testing and during fluorescein application. Rabbits received 0.1 ml of preservative in one eye and buffered saline in the contralateral eye. Beginning 30 min later, rabbits received five doses of 5 µl fluorescein each, in both eyes, at 2-min intervals. This investigation adhered to the ARVO Resolution on the Use of Animals in Research.

Human subjects were between 18 and 35 years of age, with no recent history of contact lens wear or use of topical ocular preparations. Informed consent was obtained from each subject after the nature of the procedure had been explained fully. The protocol was approved by the Stanford Medical Center human subjects experimentation committee. All subjects were pretested 1 week before the start of the study with fluorescein applied to both eyes. Anterior chamber fluorescence was measured after 2 hr, to ensure that contralateral fluorescein levels were within 20%. Preservative and test solutions were applied without subject knowledge of composition. Fluorescein was applied in five doses of 2 µl each, at 2-min intervals, beginning 30 min after administration of test and placebo solutions. All subjects were asked to register any unusual sensation or discomfort accompanying instillation of solutions in either eye. Participants were invited to return after a washout interval of 1 week, to be tested with new solutions. The highest concentration of each preservative, 0.05%, was not used in human subjects, since irritation of eyes could result.

**Measurement technique:** Fluorescein permeability levels were measured in aqueous humor at 2 and 3 hr after preservative application, by a simple fluorophotometer attached to a slit lamp. Some rabbits also were measured at 5 and 6 hr after dosing. The device utilized a fiber optic bundle to conduct light from the rear ocular image plane to a photomultiplier detector and amplifier/meter. Excitation and barrier filters were of the interference type. The device was calibrated using freshly prepared phosphate buffered solutions of diluted fluorescein.

A chart recorder coupled to the amplifier/meter measured both background fluorescence and fluorescein levels for each eye of each subject, providing a document of fluorescence levels, while the operator placed the end of the fiber optic sensing device at the proper position for measurement. The operator could not determine the fluorescence level being recorded during measurement.
Since the background fluorescence level measured included elements of photomultiplier dark current, ambient room light, and ocular autofluorescence, the total background was subtracted from each signal level measured, before the test results were normalized. The fluorescein permeability ratio (FPR) was defined as:

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FPR = \frac{(\text{EXP} - \text{BKGD})}{(\text{CONTROL} - \text{BKGD})}
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where \(\text{EXP}\) and \(\text{CONTROL}\) are the total measured anterior chamber fluorescence of the treated and untreated eyes, and \(\text{BKGD}\) is the measured ocular fluorescence plus photomultiplier dark current without fluorescein addition.

**Results.** **Fluorescein dynamics:** The anterior chamber fluorescein levels in three rabbits following tear film fluorescein application are shown in Figure 1. The experimental eyes pretreated with 0.05% benzalkonium chloride showed levels of fluorescein elevated over control eyes at all times measured. While the time and amplitude of peak concentrations in the aqueous humour varied considerably between rabbits, the ratio between treated and control eyes in any one animal was relatively uniform over the measured time interval. The peak concentration of fluorescein in aqueous humour occurred between 2 and 4 hr, in both rabbits and humans. The results were in agreement with an earlier study.10

The anterior chamber was considered well-stirred by thermal convection currents after an interval of 2 hr, and so measurements were routinely taken between 2 and 3 hr after fluorescein addition for computation of fluorescein permeability ratios. Several fold variation in anterior chamber concentration occurred in both rabbits and humans, resulting in the requirement for normalization of data by using contralateral eyes as controls in all cases.

**Preservative response—Benzalkonium chloride:** The permeability changes in response to benzalkonium chloride are seen in Figure 2A. Some increase in rabbit corneal permeability over normal was detected at concentrations as low as 0.0025%, and significant increases \((P = 0.05)\) occurred at all higher concentrations. At 0.02%, permeability was more than double that of control eyes, and 0.05% caused several fold permeability increase one-half hour after application of the preservative. Benzalkonium chloride is commercially used as a bacteriostatic agent at concentrations of 0.004%–0.02% in ophthalmic prescription drugs11 and in many over-the-counter (OTC) products.

Some human permeability increase was seen in response to benzalkonium chloride at 0.02%, yet fluorescein concentrations in aqueous humour were elevated less than 25% over normal \((P \leq 0.10)\). One subject out of six showed no detectable increase in permeability at this concentration.

**Chlorhexidine digluconate:** In Figure 2B are seen the permeability changes caused by chlorhexidine. Rabbit corneal permeability exhibits much less change than in response to benzalkonium chloride, and significant increase occurs only at levels of 0.005% or higher. At 0.05%, however, fourfold increase in permeability occurs. Currently, chlorhexidine is used at 0.0025–0.004% concentration for bacterial prophylaxis in some contact lens and tear replacement solutions.12 Human corneal permeability showed no significant modification on single application of 0.01–0.02% chlorhexidine digluconate.

**Ocular sensitivity:** Subjects generally did not notice discomfort in either eye. However, several subjects felt a difference in sensitivity when fluorescein was applied 30 min after benzalkonium chloride at 0.01% or 0.02%, and in each case the eye receiving preservative was identified as stinging or feeling "cool." This may indicate that preservatives increase short-term sensitivity to irritation, perhaps by alteration of permeability characteristics, or by stimulation of cellular repair processes.

**Discussion.** **Interpretation of permeability increase:** Several mechanisms might explain increased transcorneal penetration in response to surfactant preser-
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A. BENZALKONIUM CHLORIDE

B. CHLORHEXIDINE DIGLUCONATE

Fig. 2. Fluorescein permeability ratio (FPR) for two surfactant preservatives. Each plotted point is the mean of six or twelve measurements. Vertical bars signify standard error of the mean (SEM). A (+) indicates a result significant by the paired Student's t-test ($P \leq 0.05$), whereas (+) indicates ($P \leq 0.10$). Unmarked data points were not significantly different from controls at the 0.10 level of confidence. A, Benzalkonium chloride increases permeability far more greater in rabbits than in human subjects. A significant permeability increase was demonstrated at 0.02%. Humans were not tested at higher concentrations than 0.02% since strong irritation is already known to result at such levels. B, Fluorescein Permeability Ratio (FPR) for Chlorhexidine digluconate. Chlorhexidine showed less permeability increase in rabbits than did benzalkonium chloride. Human permeability increase was not significant at 0.02% or 0.01%.

Fig. 3. Davson-Danielli model of plasma membrane showing mode of intercalation of benzalkonium chloride molecules (zig-zag) of C-14 length. Shorter or longer chain lengths do not bond as strongly, probably due to insufficient hydrophobic chain length or difficulty of insertion. Chlorhexidine, a di-guanide, has two charged ends that cannot intercalate into the membrane in the same fashion as benzalkonium chloride, and hence has much lower binding. The type of insertion illustrated here may be presumed to be stable over time, so that multiple doses might allow accumulation of preservative in the membrane, causing additive toxicity.

benzalkonium chloride. A doubling of corneal fluorescein permeability occurs at the same concentration of benzalkonium chloride, which has been shown by scanning electron microscopic examination to cause cellular uplifting. The severalfold increase in permeability at the 0.05% concentrations of both surfactant preservatives also corresponds well with the previously observed complete removal of the squamous epithelial layer. This mechanism does not explain the slight increases in corneal permeability accompanying lower concentrations of preservatives, however.

Another explanation of increased transcorneal permeability involves membrane modification of the apical cell layer, such as might occur by molecular intercalation of detergents directly into the bilaminar lipid layer of the plasma membrane. Artificial bilaminar lipid membranes have been constructed, and they are modified by the action of polar molecules that intercalate into the membranes, forming channels for increased passive transport of ionic species. At low concentrations, benzalkonium chloride might intercalate in this manner, allowing decreased ionic resistance. The quantitative work of Cadwallader using red blood cell hemolysis supports this theory, demonstrating that long hydrophobic carbon chain length in benzalkonium chloride increases membrane disruption. A molecule with good membrane “fit,” such as benzalkonium chloride illustrated schematically in Figure 3, increases membrane permeability even at very low concentrations, and remains intercalated for long periods of time once positioned. This mechanism would help explain cumulative effects of multiple doses of quaternary amines.

Chlorhexidine digluconate does not have this ability.
to “fit” into the membrane as shown schematically in Figure 3 for benzalkonium chloride. Its molecular configuration may explain its low-membrane binding constant. Notably, chlorhexidine binds one seventh as strongly to contact lenses as benzalkonium chloride.

Comparison of rabbits and humans: Rabbits are more sensitive than humans to both surfactant preservatives tested. Therefore, measurements of ocular permeability and surface damage are not directly transferable from rabbits to humans. This observation agrees with the experiments of Buehler and Newmann,13 who found the rabbit eye much more susceptible to damage from topical drops of benzalkonium chloride than those of human or monkey. They found that if exposure to preservatives was limited to the cornea only, the toxicity was similar between species. This result suggested to them that the nictitating membrane could act as a reservoir for preservatives, increasing both time of exposure and volume of test fluid retained in the tear film. In a recent scanning electron microscopic comparison of benzalkonium chloride and chlorhexidine digluconate, cats and rabbits showed similar toxic response.6 Although the cat lacrimates like the human, the presence of a nictitating membrane in both cat and rabbit may explain the similarity of their response to preservatives.

Clinical significance of findings: It has been accepted since Swan’s investigation published in 1944 that benzalkonium chloride at high concentrations is damaging to corneal epithelium.15 The present study indicates that human corneal response to single topical preservative doses is less than for rabbits. However, greater epithelial damage might accompany the use of appliances such as contact lenses, or the instillation of drops at frequent intervals such as for dry eye treatment. Some subjects also are susceptible to recurrent erosion for a variety of reasons and may best avoid all preservatives topically instilled.

Methods that allow direct testing of human subjects for slight alteration of corneal epithelial permeability, possibly indicating damage and superficial erosion, should prove clinically useful in the future for screening of patient preservative tolerance, as well as allowing more interpretations to be made from test data derived from animal subjects.

Key words: corneal permeability, benzalkonium chloride, chlorhexidine digluconate, rabbit, human

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References