Examination of Murine Tear Film

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PURPOSE. To define spatially any free aqueous layer in murine tear film.

METHOD. A pre-zeroed microelectrode was touched to the superficial corneal epithelium and then raised in steps of 1 μm through the murine tear film into the air and then retraced along the same path. Other murine tear films were partially probed with a spatial resolution of 0.1 μm. The reference microelectrode was placed in a fragment of 3% polyacrylamide gel equilibrated against 154 mM NaCl and located on the nasal quadrant of the scleral conjunctiva. Other murine corneas were quick frozen in melting isopentane and freeze substituted or pretreated with cetylpyridinium chloride and then examined by transmission electron microscopy.

RESULTS. The recorded electrical profiles of the tear film were reproducible in each preparation and showed a relatively uniform positive electrical potential throughout their whole thickness, except within 0.5 μm of the epithelial surface when the potential reversed to negative values. The thickness of mouse tear film averaged 7.4 ± 0.8 μm (mean ± SD, n = 40). The electron microscope images showed the murine tear film to have a relatively uniform positive electron density throughout the thickness.

CONCLUSIONS. Electrical profiles of the murine tear film presented no evidence of a separate free aqueous phase. The tear film is observed as an aqueous gel that includes anion-exchanging polyelectrolytes throughout most of its thickness, but within 0.5 μm of the epithelial surface, it changes to cation-exchanging polyelectrolytes. Electron microscope images provide some supporting evidence. (Invest Ophthalmol Vis Sci. 2003;44:3520–3525) DOI:10.1167/iovs.03-0178

The mammalian tear film arises from tear fluid that originates from the lacrimal glands and mucins that are attached in some way to the glycolalx of the corneal epithelium. The tear film is covered over its anterior surface by a lipid layer expressed by the meibomian glands in the lid margins. The tear film is covered over its anterior surface by a lipid layer expressed by the meibomian glands in the lid margins. The internal structure of the tear film first suggested by Wolff1 is the classic three-layer model of a free fluid phase sandwiched between an outer lipid phase and a mucin phase.

A fourth layer, the glycocalyx secreted by the corneal epithelial cells, was later added.2-4 Refinements to the classic model have been suggested in which additional layers are included. Along with the lipid, aqueous, and mucin layers, there are also suggested to be a polar lipid layer and an adsorbed mucin layer.5 Tear film mucins clearly play a role in the maintenance of tear film structure.6-12 but studies in mice bred without the MUC1 gene have so far failed to give unambiguous elucidation of their structural purposes in the tear film.13

In general, the various suggestions of subdivisions within the microscopic structures of the tear film include the assumption of a free-fluid layer. A biophysical study of the human precorneal tear film questioned the assumption of its possessing a substantial free-fluid phase.14 The data showed that tear samples collected in the conventional manner had a substantially lower viscosity than the precorneal tear film in vivo, and it was suggested that the human tear film includes a fluid flow-inhibiting matrix throughout its thickness with structural components that have a longer time constant of exchange than the soluble proteins of the collected tear fluid.

Chen et al.15 provided more direct evidence. Using electron microscopy to visualize the freeze substituted tear film, they reported that the rat tear film appeared throughout its thickness as a homogeneous layer consisting of fine network-like structures. The thickness of the tear film varied from 2 to 6 μm with a membrane-like layer covering its surface, which could be identified as the lipid layer. The tear film appeared to contain no free aqueous layer and the authors suggested that the three-layer theory should be modified.

The purpose of this study was to investigate the presence of any free aqueous layer in mouse tear film using microelectrode techniques with supportive evidence from transmission electron microscopy techniques. Microelectrodes were used to record the electrical profile through the tear film thickness. These recordings were expected to show a net electric voltage in the mucin layer, characteristic of all hydrophilic polyelectrolyte gels16 but to record, within the profile, regions of zero electrical potential corresponding to any free aqueous layer.

MATERIALS AND METHODS

Experiments were performed in 8-week-old laboratory CalpB strain white mice. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tear Film Electrical Profiles

Glass microelectrodes were pulled from filamented borosilicate glass capillaries of outer diameter 1.0 mm and inner diameter 0.58 mm (GC100F-15; Clark Electromedical Instruments, Reading, UK) using a horizontal microelectrode puller (PN-3; Narishige, Tokyo, Japan). Routinely, they were back filled with 154 mM KCl and had a tip resistance of 28 to 30 MΩ. Results obtained with these microelectrodes were checked with those of similar diameter back filled with 3 M KCl, and these high-salt microelectrodes always produced values similar to those filled with isotonic salt. Two freshly fabricated microelectrodes were used for each tear film profile measurement. They were connected across an electrometer (Dual Differential Electrometer FD223; World Precision Instruments, Sarasota, FL), with the output stored on a pen recorder. Recordings were made within an earthed Faraday cage.

The mouse was killed by intraperitoneal injection of 0.5 ml pentobarbital sodium and placed beneath the investigating microelec-
The experiments were all recorded on drying preparations and therefore procedures were adopted to monitor potential sources of error that might arise. Three murine tear films had their thickness monitored for 15 minutes with the reference electrode in a PAG fragment. No significant changes of tear film thickness were detected when using the 1-μm step probe method of analysis.

**Liquid Junction Potentials**

A potentially greater source of error arose from liquid junction potentials that could arise over a period from the drying PAG fragment or saline drop in which the reference electrode was placed. Liquid junction potentials (sometimes called diffusion potentials) would arise if there were significant changes in the concentration of salt in the reference solutions arising from evaporative losses. From the Planck equation, liquid junction potentials $V_i$ are related to changes in the concentration of NaCl in the reference solution by

$$V_i = 58 \left( \frac{\Delta \mu_{Na}}{\Delta \mu_{Cl}} \right) \log \frac{C^+}{C^-}$$

where 58 is a constant, at 22°C, which converts the value of $V_i$ to units of millivolts, $\mu_{Na}$ is the relative mobility of a sodium ion in solution of 0.682, $\mu_{Cl}$ is the relative mobility of a chloride ion in solution of 1.038 (both values are with reference to a standard value of 1 for potassium ions), $C^+$ is the initial concentration of salt in the reference solution and $C^-$ is the concentration of salt in the reference solution after time $t$.

The experimental rates of the changes of salt concentrations within the reference electrode phase of either the PAG fragments or the 1-μL drops of 154 mM NaCl were recorded as follows. The PAG fragment was weighed and then placed on the murine conjunctiva for 1 minute and then reweighed. The procedure was continued for 20 minutes, during which there was a linear loss of water corresponding, after 20 minutes, to 3% of the total. To check salt concentration changes within the 1-μL saline drop reference pools, the volume including 1.2 $\times$ 10$^3$ Bq $^{22}$NaCl was placed on the murine conjunctiva, and 100 mL was collected with a Hamilton syringe at intervals of 1 minute for 10 minutes. Only one sample was taken from each volume. The experiment was repeated three times for each determination. The samples were added to 10 mL of scintillation fluid (Ecosint; National Diagnostics, Atlanta, GA) and counted for 10,000 sample counts in a scintillation counter (model 1409; Wallac; Perkin Elmer, Boston, MA). Comparisons with 100-nL samples of fresh radioactive saline determined the increase in saline in the reference drop due to water losses.

From these measured changes in salt concentrations, it was possible to calculate from equation 1 the rate of development of the liquid junction potentials developed in either the PAG fragment or the saline drop. This prediction was checked experimentally as follows. A PAG fragment was placed on the conjunctiva and after 20 minutes. A second freshly prepared PAG fragment was placed on the conjunctiva, and any liquid junction potential that developed was measured in three preparations. A saline drop was placed on the conjunctiva, and after 5 minutes a freshly prepared PAG fragment was placed on the conjunctiva, and any liquid junction potential that developed was measured in three preparations.

**Electron Microscopy**

**In Vivo Cryofixation and Freeze-Substitution Method.** The mice were killed by an overdose of pentobarbital sodium. As before, the palpebral aperture was distended to the maximum, and the eyes were snap frozen by pouring over them a surplus of isopentane cooled to its melting point by prior immersion in liquid nitrogen. The carcass was quickly immersed in liquid nitrogen in an open trough where the frozen sciera was nipped with precooled bone forceps. In most attempts, a substantial fragment of the cornea broke off cleanly from the underlying frozen aqueous humor. The cornea plus tear film preparations were then transferred into a solution of 2% osmium tetroxide in acetone at −80°C for 20 hours and slowly warmed in steps.
of 10°C, with frequent solution changes over a period of 3 days until they were transferred to a 4°C refrigerator for 2 hours and finally to room temperature. The specimens were washed with absolute acetone three times and then embedded in epoxy resin. Care was taken throughout all the solution changes to ensure that the tear film remained uppermost on the corneal fragments and was minimally traumatized mechanically. In all, 12 preparations were taken through the procedure. For transmission electron microscopy, 80- to 90-nm sections were cut on a Cambridge Huxley ultramicrotome and stained with uranyl acetate and lead citrate. The specimens were observed in a transmission electron microscope (EM400; Philips, Eindhoven, The Netherlands) at 80 kV.

Cetylpyridinium Chloride Precipitates. The whole eye was dissected, with care taken not to touch the epithelial surface, and immersed in 1.5% glutaraldehyde containing 0.5% cetylpyridinium chloride and 1% sucrose buffered at pH 7.4 by 0.1 M phosphate buffer for 1 hour at room temperature. They were then poststained for a further hour in buffered glutaraldehyde and sucrose including 1% tannic acid and then dehydrated, embedded in epoxy resin, and prepared for electron microscopy as for freeze substitution.

RESULTS

The time from the death of the mouse to the start of electrical recordings was approximately 30 seconds. A full electrical profile recording in both directions, up and down, took a further few minutes. The electrical profiles were stable and reproducible for the first 15 minutes after death with the reference electrode in the PAG fragment placed on the conjunctival membrane. This period corresponded with the post-mortem period of stability of the first Purkinje image, which generally starts to deteriorate approximately 20 minutes after death. We recorded no significant tip potentials in our system or any significant baseline drift during the experimental period.

Typical reversal electrical profile traces are shown in Figure 1, where the electrical noise on the traces, using a PAG fragment for the reference microelectrode, was less than 0.2 mV. Electrical profile traces from each mouse investigated showed the same features as illustrated in Figure 1. The initial negative potential was the intracellular transmembrane potential of the squamous corneal epithelium. As the microelectrode was raised in 1-μm steps, a positive potential was recorded that remained at a similar value until the microelectrode went into open circuit. When the microelectrode returned on its downward traverse, the profile repeated well and showed a positive electrical potential of similar magnitude until the microelectrode reentered the epithelial cell. At this spatial resolution of 1 μm, the tear film generated a uniform positively charged profile with no detectable discontinuities. Profiles from 10 separate mouse tear films are shown in Figure 2A. The average tear film thickness was 7.2 ± 0.7 μm (mean ± SD, n = 10). In general, 9 of the 10 tear films were of similar thickness, and the remaining one, which was measurably thinner at 5 μm, also exhibited the lowest magnitude of the electrical potential. All the electrical profiles within the tear films showed a continuous and positive potential. Their mean potentials ± SEM were 4.7 ± 0.3, 9.6 ± 0.4, 5.6 ± 0.2, 13.5 ± 0.8, 4.6 ± 0.7, 3.6 ± 0.3, 2.7 ± 0.3, 2.8 ± 0.4, and 3.9 ± 0.3 mV. Individual electrical profiles appeared to increase their magnitude at the apical surface. Others showed the reverse tendency (Fig. 2A).

On average (Fig. 2B), there was no significant gradient in the

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933711/)

**Figure 1.** A microelectrode dynamic trace of murine tear film electrical profile from E, the epithelial intracellular potential; to T, the tear film potential; to O, the open circuit in the ambient air; and then the reversal back into the epithelium. The reference microelectrode was in a fragment of polyacrylamide gel equilibrated against 0.9% buffered saline on the nasal scleral conjunctiva. Dotted lines across the trace indicate where the sensory electrode was moved.

**Figure 2.** (A) Individual electrical profiles of 10 individual mouse tear films. (B) The mean profiles of mouse tear film. On average, there were no significant differences in the profiles with distance from the epithelial surface.
electrical profiles. There were, however, statistically significant differences in the average standing electrical potentials between different murine tear films. Similar profiles were obtained when the reference electrode was placed in a 1-μL drop of saline situated on the nasal quadrant of the mouse sclera. With these preparations, the recorded electrical profiles were not only much noisier, as would be expected on general principle, but they also showed a much briefer period of stability of only a few minutes (described later). It was, on occasion, possible to collect reproducible data on exit and entry through the tear film thickness, but usually before the whole profile could be collected, the recording was lost. In these preparations, the initial average tear film thickness was measured at 7.5 ± 1.0 μm (mean ± SD, n = 10) and typical standing electrical potentials varied between 7 and 14 mV. At the spatial resolution of 1 μm, we detected no region where the electrical potential was zero.

Substructures within the Murine Electrical Profiles

When parts of the murine tear film were sampled at a spatial interval of 0.1 μm, then throughout most of the tear film thickness from the air toward the surface of the epithelial cells, the finer resolution used to probe again showed no significant electrical gradients. Very close to the epithelial surface, the electrical potential reversed to give a negative potential. Typical data points, together with a summary of the thickness and magnitude of this periepithelial region is shown in Figure 3.

All the control experiments were accurate to 0.1 mV and gave no net electrical potentials. They were as follows: between a PAG fragment on the nasal sclera and another on the temporal sclera between; between a PAG fragment on the nasal sclera and another PAG fragment situated in the murine tear film; and between a PAG fragment and a drop of lacrimal fluid.

The rate of loss of fluid from both the PAG fragment and the saline drop are shown in Figure 4. The rate of fluid loss from the PAG fragment resulted in negligible liquid junction potentials after 15 minutes. In contrast, the saline drops showed significant rates of fluid loss and at 5 minutes, calculated from equation 1, liquid junction potentials of 3.5 mV should theoretically arise in the saline drops if the losses were due to evaporative water loss. Measurements of liquid junction potentials indicated that none arose in PAG fragments within 20 minutes of their placement on the scleral conjunctiva. Liquid junction potentials of 5.0, 5.2, and 5.5 mV arose after 5 minutes of placing a 1-μm drop of saline on the conjunctiva.

The transmission electron microscope images of the snap-frozen, freeze-substituted murine tear films all showed similar profiles (Figure 5A). At lower powers, it was possible to observe the relatively uniform coverage of the superficial epithelial cells by a layer of material approximately 1 μm thick. It was noted that irregularities in the innermost layer, caused by the presence of the epithelial microvilli were not reflected in the outermost layer, which appeared to be much smoother. The presence of an electron-dense layer at the outer surface of this material could be interpreted as the superficial lipid layer, easily observed in the living mouse tear film. It was notable that the layer included a number of apparently spherical electron dense bodies, typically of a diameter of 0.1 to 0.2 μm, with an uncertain identity.

Otherwise slight irregularities in the electron density of this layer might be associated with nano-sized ice crystal formations. In general, after scanning with a densitometer, the layer appeared to be of fairly uniform electron density except for the electron-dense spherical inclusions. The images gave no sup-
port to the concept of an original clear aqueous layer or of aqueous lakes contained within the layer.

Murine tear film pre-precipitated with cetylpyridinium chloride and stained with tannic acid also exhibited a supraepithelial layer approximately 1 μm wide (Fig 5B). In these preparations, the outer surface of the layer was notably irregular.

**DISCUSSION**

Electrical profiling of the murine tear film indicates that, at a spatial resolution of 1 μm, the profile showed a relatively homogeneous single phase 7 μm thick. This spatial resolution was too coarse to detect the inner glycocalyx layer or the outer lipid layer, but it would detect any substantial free aqueous layer. No such layer was detected.

Millivolt electrical potentials arise primarily from one of two sources in biological tissues: from Donnan potentials, which arise when the tips of the two microelectrodes and are immersed in different phases, each having a differing net fixed charge density, or from electrical potentials, which arise from current generators located between the two electrodes (e.g., the source of the intracellular membrane potentials or the pattern electroretinogram). The question as to the source of the tear film electrical potential is resolved by the control experiments described herein, in which no potential was noted between PAG fragments located on the nasal and temporal sclera, nor was there an electrical potential found between two PAG fragments when one was on the nasal sclera and the other on the tear film, which would have detected any current generator–derived potential. The tear film electrical potential is a Donnan potential generated by the fixed net electrical charges within the tear film. Similar biological Donnan potentials are commonly found in connective tissues and other extracellular spaces, such as the vitreous humor, and even in intracellular matrices, such as muscle myosin. They have recently been reviewed. The most noteworthy property of the murine tear film Donnan potential, reported herein, is that it seems to be unique, in that it shows a positive potential. All other reported extracellular biological gels generate negative potentials.

The relationship between the electrical potential, \( V \) (in units of mV) and the concentration of fixed charge \( Q \) and the local concentration of salt \( C \), which is around 154 mM in extracellular media, is calculated by

\[
Q = C(10^{1/87} - 10^{-1/87}) \tag{2}
\]

Applying equation 2 to the mean electrical potentials reported in the Results section indicates the fixed positive charge concentrations (in mM, or more exactly, milliequivalents per liter) in different murine tear films are 153 ± 16, 107 ± 5, 61 ± 8, 47 ± 10, 42 ± 3, 39 ± 3, 29 ± 4, 29 ± 5, and 15 ± 5. For comparison, the fixed (negative) charges in ox, rabbit, and human corneas under physiological conditions are much more tightly controlled at 40 ± 2 mM.

The presence of fixed positive charges throughout the murine tear film raises a question as to their identity. When we washed the corneal surface with 4 M guanidinium chloride and examined the washes’ contents, we found no sufficiency of positively charged protein to account for the observations of positive charge, but there were significant amounts of glucosamine and galactosamine in acid hydrolysates of the washes. Although these data are suggestive of a source of tear film positive charge, clearly more work is needed to eliminate possible intracellular contamination.

There was no clear relationship between fixed charge density and thickness in different mouse tear films, but it was noted that the tear film with the least fixed charge density was measurably thinner than the other tear films (Fig. 2). Clearly, in future biophysical experiments it would be interesting to explore the nature of the Donnan potential after exposure to different concentrations of salt, after changing the nature of the salt, and examining the pH titration curve. We have not yet found a way of replacing the fluid content of the tear film matrix that gives reproducible results.

The submicrometer investigations of the electrical profiles (Fig. 3) revealed the presence of a negatively charged region within 0.5 μm of the epithelial surface. We think that this layer may be related to the epithelial glycocalyx.

The electron microscope investigation indicated the presence of a fairly uniform-density layer lying over the squamous epithelium, which averaged 1 μm in thickness (Fig. 4A). At its outer surface was a consistently electron-dense layer that may
be identified with the superficial lipid layer. There were no regions of diminished electron density in the images, but, on the contrary, there were numerous inclusion particles that were spherical and electron dense, the origin of which is not known. From our extensive examinations of human tear film in the slit lamp, these inclusions are frequent, and we have supposed them to originate from environmental pollution. Other filamentous electron-dense regions in the tear film may have been associated with ice crystal damage. Ice crystal damage can readily be visualized in the deeper epithelial tissue. The thickness of the tear film in the electron microscope images (1 μm) is consistent with an original hydrophilic gel of thickness 7 μm, which has the water replaced by a hydrophobic solvent, when the dynamic interactions at the double layer that support the gel in solution are removed and the nonaqueous component of the gel contracts. The data are consistent with the fractional water volume of murine tear film occupying 85% of the volume. This is the thickness of the supraepithelial layer when the water is removed by precipitation with cetylpyridinium chloride (Fig. 5B). Figure 5A shows two main differences from the images reported by Chen et al. of rat precorneal tear film: the inclusion bodies, which are not reported to be present from the images reported by Chen et al. of rat precorneal tear film. Other filamentous electron-dense regions in the tear film may have been associated with ice crystal damage. Ice crystal damage can readily be visualized in the deeper epithelial tissue. The thickness of the tear film in the electron microscope images (1 μm) is consistent with an original hydrophilic gel of thickness 7 μm, which has the water replaced by a hydrophobic solvent, when the dynamic interactions at the double layer that support the gel in solution are removed and the nonaqueous component of the gel contracts. The data are consistent with the fractional water volume of murine tear film occupying 85% of the volume. This is the thickness of the supraepithelial layer when the water is removed by precipitation with cetylpyridinium chloride (Fig. 5B). Figure 5A shows two main differences from the images reported by Chen et al. of rat precorneal tear film: the inclusion bodies, which are not reported to be present from the images reported by Chen et al. of rat precorneal tear film.

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