Tear Lipocalin Captures Exogenous Lipid from Abnormal Corneal Surfaces

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PURPOSE. The cornea is protected by apical hydrophilic transmembrane mucins and tears. In pathologic states the mucin barrier is disrupted, creating potential for meibomian lipids to adhere more strongly. Undisplaced lipids create an unwettable surface. The hypothesis that pathologic ocular surfaces alter lipid binding and the ability of tear proteins to remove lipids was tested.

METHODS. Corneas with pathologic surfaces were studied for lipid adhesion and removal by tears. Capture of fluorescence-labeled phospholipids by human tears was assessed by steady state fluorometry. Tear proteins were separated by gel filtration chromatography and analyzed for bound lipids.

RESULTS. Contact angle measurements revealed strong lipid adherence to corneas submerged in buffer. Lower contact angles are observed for lipids on completely de-epithelialized corneas compared with intact corneas ($P = 0.04$). Lipid removal from these surfaces is greater with whole tears than with tears depleted of tear lipocalin ($P < 0.0005$). Significantly fewer lipids are captured by tears from Bowman's layer than from epithelial-bearing surfaces ($P < 0.025$). The only tear component to bind the fluorescence-tagged lipid is tear lipocalin. The histology of a rare case of dry eye disease demonstrates the dominant features of contemporaneous bullous keratopathy. Lipid sequestration from this cornea by tear lipocalin was robust.

CONCLUSIONS. Lipid is captured by tear lipocalin from corneas with bullous keratopathy and dry eye. Lipid removal is slightly abrogated by greater lipid adhesion to Bowman's layer. Reduced secretion of tear lipocalin documented in dry eye disease could hamper lipid removal and exacerbate ocular surface pathology. (*Invest Ophthalmol Vis Sci.* 2010;51:1981-1987) DOI:10.1167/iovs.09-4622

The corneal epithelium is a critical barrier from environmental stresses. The cornea lacks a blood supply. Nutrients diffuse through tears to sustain the epithelium. The superficial cells that comprise the apical epithelium of the cornea have a unique microvillous architecture that is coated with hydrophilic transmembrane mucins, MUC1 and MUC16.¹ Mucins serve to increase the wettability of the corneal surface by decreasing the surface tension of tears.² If the mucinous sur-

face of the cornea is covered by exogenous lipids, diffusion of nutrients may be impaired because aqueous tears, with their high surface tension (~ 70 dyn/cm), cannot displace lipid that has a lower surface tension.^{2,3} Furthermore, some lipids, including fatty acids, are known to induce apoptosis in a variety of epithelia.^{4,5} Lipids, including fatty acids, are abundant in the meibomian glands of the eyelids and are a major component of tears.⁶ Lipids form a surface layer on tears that slows surface evaporation.⁷ An aqueous layer is interposed between the outer lipids and the apical epithelium of the cornea. This layer contains soluble mucins and proteins. Approximately 21% of the tear protein mass is composed of tear lipocalin, the predominant lipid-binding protein in tears.^{8,9} Tear lipocalin avidly binds lipids (K_d in the micromolar range). The lipid-binding characteristics of tear lipocalin, including the types of native lipids bound and the identification of the internal and external binding sites, have been intensively studied.¹⁰⁻¹² The lipid-binding energy landscape of tear lipocalin has been delineated by site-directed tryptophan fluorescence.¹³ Tear lipocalin rapidly sequesters lipids from the corneal epithelium with intact mucins.¹⁴ However, in common pathologic conditions such as bullous keratopathy and dry eye diseases, the mucin/epithelial barrier is disrupted because the apical cell layer exfoliates.¹⁵⁻¹⁷ Breaches in the mucin coating of these corneas are largely related to loss of MUC16.¹⁵ The loss of the hydrophilic protective surface could theoretically result in altered surface properties of the cornea and could plausibly alter the ability of tear lipocalin to remove lipid from the surface. The ability of tear lipocalin to capture lipid from epithelial surfaces at risk for lipid adherence was tested. The propitious collection of a cornea from a patient with well-documented dry eye disease and with bullous keratopathy provided an unusual ocular surface specimen for study. The only previous histology of the cornea in dry eye disease was a single autopsy report by Sjögren.¹⁸

MATERIALS AND METHODS

Collection of Human Tears and Corneas

The research was performed in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from donors of human tears after explanation of the nature and possible consequences of the study; the procedures were approved by the institutional review board. Human tears were collected from healthy volunteers and pooled as previously described.¹⁹ Human corneas, used for controls, were obtained either fresh or after fixation in 10% formalin from exenteration specimens; patients had no ocular symptoms, and the corneas had no visible abnormalities. Corneas from patients with bullous keratopathy or dry eye were obtained fresh immediately after surgery or after fixation in 10% formalin. Formalin was removed from the fixed corneas by six 15-minute washes in 10 mM Tris buffer, pH 7.2, that contained other ions in concentrations similar to those of tears (133 mM NaCl, 24 mM KCl, 0.8 mM CaCl₂, 0.61 mM MgCl₂). Fluores-

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cence-labeled phospholipid 2-(6-(7 nitrobenz-2-oxa,1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD C6-HPC) was obtained from Molecular Probes (Eugene, OR).

Preparation of Corneas

Six corneal buttons, 3-mm diameter each, were trephined (Skalar Instruments, West Chester, PA). For denudation studies, approximately half the normal corneal surface of a freshly received, surgically excised healthy eye was denuded of epithelium by gentle removal with forceps as a sheet of epithelium before application of lipid to both denuded and intact areas. Trephines from each area were taken after lipid application and rinsing. Separate corneas were used for the partially denuded experiments. Partial removal of superficial epithelium was accomplished by a modification of an impression cytology technique. A 4-mm diameter mixed cellulose membrane (Millipore, Billerica, MA) or a glass slide was gently impressed against the corneal epithelium of the whole eye for approximately 20 seconds, removed, and immediately fixed in 95% ethanol and stained according to a rapid hematoxylin and eosin protocol.²⁰

For histopathology, corneas were fixed in formalin processed routinely for paraffin embedding. Five-micron sections were stained with hematoxylin and eosin periodic acid Schiff stains.

Purification of Tear Proteins

Tear proteins were separated from a portion of the pooled human tears by gel filtration (Sephadex G-100; Sigma-Aldrich, St. Louis, MO), as described.^{9,21} During this purification, fractions that passed through a dextran column (DEAE-Sephadex; Sigma-Aldrich, St. Louis, MO) (tear lipocalin binds with the resin) were collected and combined with the remaining G-100 fractions depleted of tear lipocalin and reconstituted in the buffer to the initial volume described. Tricine PAGE was performed to confirm that the resultant mixture was depleted of tear lipocalin, as previously indicated.^{9,19} Tear lipocalin, lysozyme, and lactoferrin were purified from pooled tears by gel filtration and ion exchange chromatographies.^{9,22} Recombinant tear lipocalin was expressed in *Escherichia coli* BL21-(DE3) cells transformed with the lipocalin-1 (lcn-1) cDNA and purified as published.²³ Purification was confirmed with SDS tricine gel electrophoresis.

Contact-Angle Measurements to Assess the Lipid Binding to Corneal Surfaces

Contact-angle measurements of droplets of oleic acid were compared for both denuded and epithelialized corneal surfaces. Oleic acid (500 μ L) was mixed with a minute (<0.01 mg) granule of oil red O dye, and a 0.5 μ L droplet of the mixture was deposited on the cornea surfaces that had been allowed to dry in ambient conditions for 3 minutes. The corneas were covered with buffer mimicking tears, as described, and the lipid remained in place after equilibration for 30 minutes. All experiments were performed at 20°C. The droplet profiles were imaged with a digital camera (Nikon, Tokyo, Japan) attached to a dissecting microscope head (Bausch & Lomb, Rochester, NY) on a rotatable boom stand. The contact angles were measured from the vertex at the three phase lines along the tangency of the drop (Fig. 1A) with the angle tool on ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The means of the left and right angles on all droplets were calculated ($n = 6$ corneas). The measurements were analyzed using the Mann-Whitney U test. Contact angle measurements of pure oleic acid, without oil red O dye, were also performed with similar results.

Application and Removal of Fluorescent Lipids from the Cornea

For these experiments, the substrate NBD C6-HPC was prepared with Tween 20 (Fisher Scientific, Pittsburgh, PA) to prevent the formation of stable bilayers in aqueous solution.²⁴ NBD C6-HPC and Tween 20

FIGURE 1. Contact angle, θ , measurements of oleic acid placed on corneas submerged in buffer. (A) Typical experiment with lipid droplets on cornea. *Left*: contact angle for intact epithelium measures 81°. *Right*: contact angle for denuded epithelium measures 59°. (B) Graph shows angles (mean $\pm$ SD) that were significantly greater for intact epithelium (81° $\pm$ 13°) than for oleic on completely denuded epithelium (65° $\pm$ 10°). $P = 0.04$.

were dissolved in ethanol, dried under argon flow to produce a thin film, and vortexed vigorously for 2 minutes in buffer. The final concentration of NBD C6-HPC was 1.12 mM with 20 mol% Tween 20. In general, 6 μ L of 1.1 mM NBD-C6-HPC was applied uniformly to the corneal epithelial surface before trephination. Excess lipid was removed by gently rinsing, dropwise, the corneal surface with 10 mM Tris buffer, pH 7.2, which contained other ions in concentrations present in tears (133 mM NaCl, 24 mM KCl, 0.8 mM, CaCl₂, 0.61 mM MgCl₂). Spectra were monitored until there was no detectable fluorescence in the rinse solutions. Corneas were placed in a 3-mm customized chamber and overlaid with 200- μ L solutions of buffer, tears, or tears depleted of tear lipocalin, as previously described.¹⁴ Gentle mixing was performed before each measurement of fluorescence. A plot was made comparing fluorescence with time. Measurements were made for approximately 90 minutes or until saturation was achieved.

Steady State Fluorescence Spectroscopy

Steady state fluorescence measurements were made on a spectrofluorometer (Fluorolog τ -3; Jobin Yvon-SPEX, Edison, NJ). Spectral parameters were chosen for NBD C6-HPC ($\lambda_{ex} = 420$ nm; $\lambda_{em} = 480$ –650 nm). Bandwidths for excitation and emission measured 2 nm and 3 nm, respectively. Emission measurements were performed in ratio mode to ensure that possible excitation light intensity deviation was not a factor in fluorescence.

The differences between fluorescence after incubation with tears and tears depleted of tear lipocalin were evaluated with analysis of variance. A t -test statistic was determined for the difference between two population means. For NBD removal from corneas, $n = 6$ for each data point. Hypotheses for testing were formulated as $u_1 - u_2 = 0$ for H0 and $u_1 - u_2 \neq 0$ for HA, as described.²⁵ Because samples with complete epithelial denudation and intact epithelium were derived from the same cornea with identical lipid application and rinsing, no

FIGURE 2. Photomicrograph shows histologic section of cornea after superficial cell removal by an impression membrane (hematoxylin and eosin; original magnification $\times 250$). Apical epithelial cells are focally removed (arrow) or partially lifted (asterisks). Inset: cytologic appearance of cells from a typical experiment (hematoxylin and eosin; original magnification, $\times 400$).

normalization of fluorescence was needed. However, fluorescence from solutions of partially denuded corneas was normalized to that of the paired intact epithelium for peak fluorescence.

Separation of Tear Components after Lipid Removal

Size-exclusion chromatography was used to detect the protein fraction(s) responsible for lipid removal. Major tear proteins were separated in tear protein solutions incubated over corneas laden with fluorescence-labeled lipid. Fractionation was accomplished by gel filtration (Sephadex G100; Sigma-Aldrich), as previously described, except that the column size was 0.7×50 cm and that elution was performed at 1 mL/h.⁹ Absorbance at 280 nm and fluorescence spectra (with parameters as described) were used to correlate protein peaks with labeled lipids (NBD C6-HPC). Protein fractions associated with fluorescence were analyzed by tricine PAGE, as indicated.

RESULTS

Lipid Binding to the Cornea

The contact angle experiments demonstrate the relationship between surface tension at the interface of lipid and the type of cornea surface contacted (Fig. 1). Lipid placed on intact corneal epithelium showed significantly greater mean contact angles (mean, 81°) than lipid placed directly on Bowman's layer (mean, 65° ; epithelial denudation; $P = 0.04$). A representative experiment is shown in Figure 1 (inset). Lipid remained adherent to the corneal surfaces after several hours, and the contact angles were unchanged. Oleic acid alone revealed contact angles similar to those spiked with dye for photographic purposes and remained without change for at least 6 hours.

Influence of Epithelial Denudation on Lipid Removal by Tear Lipocalin

Model systems presenting various degrees of de-epithelialized surfaces were first investigated. The effect of partial removal of superficial epithelium was verified by histopathology (Fig. 2). Corneas with intact, superficial, or complete epithelial denudation were compared for removal of lipid by tears (Fig. 3). Capture of NBD C6-HPC from corneal surfaces with intact epithelium was similar when surfaces were partially denuded (Fig. 3). However, the retrieval of lipid from corneal surfaces with denuded epithelium was significantly lower at each time point ($P < 0.025$). The gel filtration profile showed fluorescence peaks (Fig. 4) that coincided with tear lipocalin by gel electrophoresis (Fig. 4, inset, lane 3).

Tear Lipocalin Captures Exogenous Lipid from Corneas with Bullous Keratopathy

Histopathology of the corneal surfaces in cases of bullous keratopathy showed the varied features of exfoliation, subepithelial bullae, and epithelial thinning. Superficial cell layers appeared to be markedly thinned in areas (Fig. 5). The surfaces were tested for removal of fluorescence-labeled phospholipid (NBD C6-HPC) by tears and tears depleted of tear lipocalin (Fig. 6). The fluorescent characteristics of NBD have been published; fluorescence is enhanced by ligand binding.¹⁴ No difference was noted in surface retrieval by tear lipocalin for fixed versus fresh corneas.¹⁴ Fluorescence of tears overlying the corneas ($n = 6$) laden with lipid increased rapidly with whole tears and was significantly greater at each time point ($P < 0.0005$) than the lipid removal with reconstituted tears without tear lipocalin. Furthermore, the sequestration of lipid by tears was equally robust from the bullous keratopathy surface compared with normal cornea surfaces (Fig. 6). Figure 7 shows the

FIGURE 3. Fluorescence-labeled lipid NBD C₆-HPC removal from corneas ($n = 6$ for each group) by tears with varying degrees of artificial denudation compared with intact epithelium. (◆) Intact epithelium. (▲) Partial denudation of superficial epithelium. (■) Complete epithelial removal. Fluorescence intensity, in counts per second (cps), was measured at $\lambda_{em} = 524$ nm. Mean intensities were not significantly different between intact and partially denuded epithelium. Intensity was significantly lower for completely denuded epithelium than for intact epithelium ($P < 0.025$) at each time point. Error bars show $\pm$ SD.

FIGURE 4. Gel filtration of tears used to remove lipid from denuded epithelial surfaces of cornea. ($\blacktriangle$) Fluorescence peak ($\lambda_{em} = 524$ nm) is plotted. ($\blacksquare$) Absorbance at 280. (inset) Coomassie-stained SDS tricine 10% acrylamide gel. Lane 1, tears 4 μ L; lane 2, fractions 30–36; lane 3, fractions 23–27; lane 4, fractions 14–21. TL, tear lipocalin; Lf, lactoferrin; Ly, lysozyme. Left: masses shown for prestained protein ladder standards, 20 and 50 kDa.

chromatographic separation of the tears overlying the cornea. Fractions were monitored for both protein absorbance (280 nm) and fluorescence. The peak of fluorescence from NBD C6-HPC coincided precisely with a single protein absorbance peak (Fig. 7). Fractions analyzed by gel electrophoresis demonstrated only tear lipocalin in the fractions of this peak (Fig. 7, inset, lane 4)

Removal of Lipid from a Cornea Manifesting Dry Eye Disease

The hypothesis that tear lipocalin garners lipids from corneal surfaces manifesting dry eye disease was preliminarily tested in a patient with bullous keratopathy and dry eye disease. Dry eye disease had been documented by bilaterally decreased Schirmer tests (3 and 2 mm), reduced tear break up times (5 and 4.3 seconds) and enhanced fluorescein staining (grades 2 and 1) by the Oxford system for the right and left eyes, respectively.

The histopathologic view of a cornea with dry eye disease is shown in Figure 8. The features of bullous keratopathy predominate in this cornea. Lipid removal by purified tear proteins was tested (Fig. 9). In this experiment recombinant tear lipocalin was used to ensure no contamination was present from other tear proteins. Addition of lysozyme and lactoferrin caused little change in fluorescence of the solution, whereas recombinant tear lipocalin resulted in rapid removal indicated by a rapidly increased slope in fluorescence in the overlying solution. Gel filtration profiles again confirmed that the lipid was bound to tear lipocalin.

DISCUSSION

The major finding of this study is that tear lipocalin robustly removes exogenous lipid from a variety of abnormal ocular

surfaces. The need for removal of lipid from the cornea is demonstrated by the adhesive interactions reflected in the contact angle experiments. Applied in this case, Young's equation relates the contact angle θ of a lipid droplet on a solid surface (cornea) to three surface tensions: that between the lipid and overlying media or tear buffer (γ_{LM}), that between the lipid and the corneal surface (γ_{LS}), and that between the surface and media (γ_{SM}): $\cos \theta = (\gamma_{SM} - \gamma_{LS})/\gamma_{LM}$. Despite the limitations of these analyses for nonideal surfaces, the consistent contact angles between different corneas with similar surfaces suggest that the limitations are negligible for a general comparison. Several clinically relevant features emerge. The contact angle ($<90^\circ$) and the convex shape of the lipid droplet on the cornea submerged in tear buffer reflected substantial hydrophobic forces between the lipid and both types, epithelial and denuded, of corneal surfaces. The lipid is not displaced after a long period on either the denuded or the epithelialized corneas, hence rendering both surfaces unwettable. An unwettable surface is deprived of water-soluble nutrients diffusively delivered by tears. All the surfaces created in this study may be found in ophthalmic disorders. Corneal abrasions, of full or partial thickness, are commonly encountered in contact lens wearers and from minor trauma. Complete removal of lipids from these surfaces is desirable.

The statistically significant difference in contact angles between the denuded and the intact epithelial surfaces provides insight into surface characteristics encountered in disease. The lower contact angle of lipid on the denuded surface layer reflects a lower critical surface tension of lipid in contact with Bowman's layer. Implied is a greater adhesive interaction of lipid with Bowman's layer than with the corneal epithelium. The greater adhesion of lipid to Bowman's layer explains the slower retrieval of lipids by tear lipocalin on denuded surfaces than on intact epithelium (Fig. 3).

FIGURE 5. Photomicrograph of bullous keratopathy showing the features of exfoliative epitheliopathy, including epithelial thinning, subepithelial bullae (asterisk), and loss of superficial cells (arrows). Periodic acid-Schiff; original magnification, $\times 250$.

FIGURE 6. Removal of fluorescent lipid NBDC₆HPC from corneas ($n = 6$ in each group) of controls without disease (▲), those with bullous keratopathy treated with tears (◆), and bullous keratopathy treated with tears without lipocalin (■). Error bars show $\pm$ SD. At all times from 15 to 90 minutes, the amount of fluorescence removed was statistically greater ($P < 0.0002$) for tears versus tears without tear lipocalin. Fluorescence intensity, counts per second (cps), was measured at $\lambda_{em} = 524$ nm.

The unique microphysical architecture of the normal corneal epithelium in the presence of surface transmembrane mucins may present a relatively complex hydrophilic surface to the lipid compared with the smoother Bowman's layer, which is composed mainly of collagen and is devoid of mucins. However, no difference was detected in the retrieval of lipids by lipocalin between partially denuded apical epithelium and intact epithelium. Tiffany^{26,27} found that the wettability of rabbit corneal surfaces appeared unrelated to mucins except that the lack of mucins promoted rapid desiccation of the surface and reduced wettability. However, in these studies, Tiffany^{26,27} thoroughly removed mucins by abrasive or chemical means, whereas in our experiments we purposely sought to partially remove apical epithelium bearing mucin to create a model that more closely approached the histologic appearance of corneas in disease. We purposely avoided contact angles in partially denuded and bullous surfaces because departure from ideal surfaces makes interpretation difficult.

Lipid retrieval by tear lipocalin has clinical relevance for meibomian lipid surface contamination in common ocular disorders that feature partial- or full-thickness epithelial defects such as dry eye disease, corneal abrasions, and bullous keratopathy. Lipid adherence to the cornea can create an unwettable surface. Lipid contamination may also produce cytotoxicity in a variety of epithelia, including ocular surface cells.^{4,5}

Bullous keratopathy, as a major indication for penetrating keratoplasty,^{28–32} is an important disorder to test lipid removal by tear lipocalin on abnormal ocular surfaces. The clinical and histopathologic manifestations of bullous keratopathy are well studied. The clinically painful surface epithelial defects and punctate fluorescein staining are associated with an exfoliative epithelial keratopathy characterized by subepithelial bullae, epithelial thinning, loss of apical epithelium, and breaches in ocular mucin expression.³³ Altered expression of molecules involved in cellular adhesion, tenascin C, and B6 integrin have been noted in bullous keratopathy.^{34,35} Bullous keratopathy serves as a model relevant to dry eye disease because subepithelial bullae, epithelial thinning, and exfoliation of cells were dominant findings in the only histologic description to date of a cornea with dry eye, a patient who underwent autopsy by Sjögren.¹⁸ Experimental murine models of dry eye disease show corneal epithelial thinning, apical cell desquamation, and increased expression of involucrin.^{16,17,36} Clinically performed confocal microscopy shows opaque cells on the human corneal surface, but correlative histology has never been performed.³⁷ The expression of the specific ocular mucin, MUC16, is diminished in the conjunctiva of non-Sjögren dry eye syndrome, and the number of superficial cornea cells with MUC16 expression is reduced in bullous keratopathy.^{38,39} Overlap of histopathologic and immunophenotypic features of the corneal epithelium of dry eye and bullous keratopathy may point to a common biological pathway for both desiccating and hydropic stress. Our case with simultaneous bullous keratopathy and dry eye is unusual because of the rarity of histology reports of patients with dry eye. Specific pathologic changes in epithelium attributable to dry eye disease were not discerned. Both diseases may show similar or at least overlapping histologic features. Alternatively, changes of moderate dry eye disease might have been overshadowed by the features of severe bullous keratopathy.^{15,40} It is likely that the opaque cells recently described clinically by confocal microscopy³⁷ are apoptotic or partially exfoliated cells that were seen in our

FIGURE 7. Gel-filtration profile of tears (■) that were used in experiments to test the removal of fluorescence-labeled phospholipid from the cornea with bullous keratopathy. Absorbance peaks of lactoferrin, tear lipocalin, and lysozyme are shown from left to right. Peak of fluorescence (▲) coincides with the absorbance peak of tear lipocalin. Inset: Coomassie-stained SDS tricine 10% acrylamide gel of pooled fractions shown in chromatograms. Lane 1, standards; lane 2, tears 4 μ L; lane 3, 13–19; lane 4, 21–24; lane 5, 31–37; lane 6, tears without TL. TL, tear lipocalin; Lf, lactoferrin; Ly, lysozyme. Left: masses shown for prestained protein ladder standards, 20 and 50 kDa.

FIGURE 8. Histologic view of a cornea with dry eye superimposed on bullous keratopathy. Microbullae with partial exfoliation of apical epithelium (asterisks), loss of Periodic acid-Schiff–positive staining in superficial layers of epithelium (black arrow), and hydropic epithelial changes are associated with epithelial thinning and disruption of Bowman's layer (green arrow). Periodic acid-Schiff; original magnification, $\times 250$.

case. Isolated cases of dry eye disease would be ideal to elucidate the precise pathologic epithelial changes in dry eye disease, but such surgical specimens have yet to be collected.

Given that penetrating keratoplasty is not performed for dry eye disease alone, the coexistence of dry eye disease and bullous keratopathy provided a rare opportunity to preliminarily determine whether lipid retrieval by human tear components is negated by the dry eye surface. Although animal models could be tested, tear protein components vary greatly among species, and most animal lipocalins in tears have little homology to human tear lipocalin.⁴¹ In this experiment purified tear lipocalin removed the lipid, whereas the other major tear components, lysozyme and lactoferrin, had little effect.

Although tear lipocalin is the major lipid-binding protein in human tears, a phospholipid transfer protein has also been described with some potential for lipid binding.⁴² However, at $<0.2\%$ of the concentration of tear lipocalin ($74 \mu\text{M}$), phospholipid transfer protein is a relatively minor component in tears ($0.137 \mu\text{M}$).⁴³ Contamination of lipocalin-containing fractions in gel filtration is unlikely because phospholipid transfer protein elutes at 160 kDa.^{43,44} Tear lipocalin elutes in gel filtration between 35 and 45 kDa.^{21,45,46} The use of purified recombinant tear lipocalin in the dry eye experiments precludes contamination by other tear components. The evidence provided in this study from gel filtration of the recovered tears after lipid removal demonstrates that tear lipocalin is responsible for lipid removal from all surfaces studied. Consistent lipid removal from normal and abnormal corneal surfaces suggests tear lipocalin functions effectively in preventing lipid contamination in a wide range of ocular surface conditions, including dry eye diseases. Hyposecretion of tear lipocalin and of other tear proteins and fluid can be expected from diverse pathologic conditions affecting the lacrimal secretory pathway. These include rare congenital absence of the lacrimal gland or

alacrima,⁴⁷ iatrogenic conditions such as radiation-induced lacrimal gland atrophy and fibrosis,⁴⁸ and conditions that interrupt the lacrimal neural reflex arc such as orbital tumor removal⁴⁹ and LASIK.⁵⁰ Sjögren's syndrome is the most prevalent condition (0.1% – 14.9%) and presumably results from immunologic destruction of the acinar epithelium.^{51–53} Tear protein secretion has been investigated thoroughly only in Sjögren's syndrome. Most studies concur that the concentration of tear lipocalin is decreased in Sjögren's syndrome either selectively or in concert with other proteins.^{54–56} The relevant implication is that decreased tear lipocalin secretion could hamper the predominant mechanism to remove lipid contamination from a surface that is prone to desiccation because of decreased tear flow.

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FIGURE 9. Removal of NBDC₆HPC from a cornea of a patient with dry eye and bullous keratopathy. At time 0, lysozyme and lactoferrin were added (final concentrations, 4 mg/mL and 1.3 mg/mL, respectively). Recombinant tear lipocalin (final concentration, 1.3 mg/mL) was added after 30 minutes (arrow). Fluorescence intensity, counts per second (cps), was measured at $\lambda_{\text{em}} = 524 \text{ nm}$.

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