Layer-by-Layer Desquamation of Corneal Epithelium and Maturation of Tear-Facing Membranes

Joseph L. Sokol,* Sandra K. Masur,† Penny A. Asbell,* and J. Mario Wolosin*†

A method to devitalize single layers of apically exposed rabbit corneal epithelial cells through the use of digitonin is described. Devitalized cells exfoliate spontaneously as loosely cohesive, trypan-blue-stained layers, exposing underlying viable cells. Repeated application of this devitalization-exfoliation methodology results in the gradual elimination of each of the epithelial cells. The generation of corneal surfaces composed of the tear-facing membranes of all intraepithelial cell types—subsurface, wing, and basal—is thus attainable. Exposed surfaces were studied with respect to microanatomy, the binding of lectins, and the adherence of *Pseudomonas aeruginosa*. Microprojections (microvilli or microplicae) were absent in the basal cells but were present in all suprabasal layers, and increased gradually in density as cells approached the surface position. Wheat germ agglutinin and concanavalin A were found to bind to the tear-facing membranes of all suprabasal cell layers. The tear-facing membrane of the basal cells, in contrast, was not labeled. Within each labeled layer, the magnitude of lectin binding differed markedly from cell to cell; lectin binding decreased as the cellular area exposed to the tear surface increased. *Pseudomonas* were found exclusively at microprojection-free cellular areas, suggesting that inhibition of attachment is linked to the ontogeny of these microprojections.


The apical membrane of the surface cells of the corneal epithelium is the first boundary between the external environment and the eye. The membrane is believed to play a vital role in the stabilization of the tear film.1 Its permselectivity is a main determinant of the overall ion transport properties of the epithelium,2,3 and it constitutes the primary barrier against pathogenic invasion of the eye.4 Since the corneal epithelium undergoes continuous, rapid cell renewal,5 these properties are dependent on the adequate development of polarity as the cells mature during migration from their germinal limbo-basal6 to the final surface position. Studies concerning the progression of this cellular maturation have been hindered by difficulties encountered in trying to separate cells within the epithelial strata. Recently, we devised a method that induces the devitalization and rapid exfoliation of rabbit surface epithelial cells without affecting the physiologic status of the remaining tissue.7

In this report, we describe a modification of this devitalization-exfoliation methodology which allows the exposure of cells deep within the epithelia. The method was applied to the study of the changes in microanatomy and lectin-binding patterns of the apical cell surface throughout stratification. The relationship of these changes to the ability of the corneal surface to prevent invasions by *Pseudomonas aeruginosa* also was examined.

Materials and Methods

Digitonin Treatments

All procedures were performed according to the ARVO Resolution on The Use of Animals in Research. New Zealand rabbits (2.5 kg) were sacrificed with a phenobarbital overdose, and eyes were then enucleated under aseptic conditions. Conjunctival and surrounding muscle tissue was trimmed, and the globe was rinsed extensively with saline (0.9% NaCl). Eyes were placed with the corneal side down over a scintillation vial containing a 20-μM solution of digitonin (80% pure; Sigma, St. Louis, MO) in saline, and stirred at 30–45 rpm by means of a 1-in magnetic bar. Solution contact with the eyes was limited to the corneal and the limbal surfaces.

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After a desired period of digitonin exposure, to stain devitalized cells, the eye was washed with saline and deposited with the corneal side down, on a vial containing 0.2% trypan blue (Sigma) in saline, for 2 min. The eye was then washed and, when indicated, placed with the corneal side up in a beaker filled with wax, molded to accommodate the posterior half of the globe. The beaker, containing a small amount of saline, was tightly capped with parafilm, to generate a humid chamber environment, and incubated at 36°C, for 15 min. After this incubation was completed, exfoliating cells were flushed away by a stream of saline delivered from a syringe. When needed, the digitonin exposure and trypan blue staining were repeated a second time. Subsequently, trypan-blue-stained corneas were excised around the limbus immediately after the trypan blue step and placed on a 60-mm petri dish containing 7 ml 1:1 mix of Dulbecco’s modified Eagle’s medium and Ham F12 (D/F 12; Sigma), buffered with 25 mM N-2-hydroxy-ethyl piperazine-N’-2 ethane sulfonic acid (HEPES; Sigma), pH 7.4. The petri dish was placed on a swirling incubator (60 rpm) at 36°C, and the incubation was continued until a skinlike layer of trypan-blue-stained cells was seen to detach spontaneously from the corneal surface. Detachment times increased with increasing cellular distance from the original surface. Based on these characteristic times, the following protocols for progressive depth of desquamation were established.

Removal of the surface cell layer was accomplished by a single 8-min digitonin treatment followed by a 30-min incubation of the excised cornea. Removal of two cell “layers” (a discussion of the concept of “layers” can be found in Results) was accomplished by either two 8-min digitonin treatments or by a single 14-min treatment. In both cases, the final incubation of the excised cornea was continued for 1 hr. A removal of three “layers” was accomplished by one 2-hr incubation following corneal excision. Lastly, removal of four cell “layers” and exposure of the basal cell monolayer was achieved by treating the cornea two times for 14 min followed by a final 3-hr incubation of the excised cornea.

Lectin Staining

Wedge-shaped specimens (each one sixth) were cut from the whole cornea and immersed for 30 sec in a phosphate (10 mM) buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and laid on a flat surface. A 2-µl drop of a fluorescein isothiocyanate (FITC)-conjugated lectin (Vector Laboratories, Burlingame, CA) solution (0.5 mg/ml in 0.02% BSA–PBS) was added to cover the epithelium. To prevent evaporation, drops of distilled water also were deposited on the flat surface, and the whole area was covered by a 60-mm petri dish. Fifteen minutes later the specimens were transferred to 60-mm petri dishes containing 5 ml PBS, and were swirled at 60 rpm for 15 min. Finally, they were placed with the epithelial side down on a glass cover slip and observed under epifluorescent illumination with an Olympus IMT-2 research inverted microscope. Labeling of the control corneal surface with ferritin-coated wheat germ agglutinin (WGA; Sigma) was performed in whole eyes as described for digitonin, with 1 ml of a 0.1 mg/ml lectin in 0.02% BSA–PBS solution contained in the cap of a small scintillation vial. The exposure was continued for 15 min.

Transmission Electron Microscopy (TEM)

Specimens were fixed in 2.5% paraformaldehyde/2.5% glutaraldehyde/2.5% acrolein in 100 mM cacodylate, pH 7.4, dehydrated through a 50–100% graded alcohol series, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

Scanning Electron Microscopy (SEM)

Corneal specimens were fixed in 2.0% paraformaldehyde + 2.5% glutaraldehyde in 100 mM Na cacodylate, pH 7.4. The fixed specimens were postfixed in buffered 1% osmium tetroxide for 1 hr, dehydrated through a 50–100% graded alcohol series and critical-point dried at 42°C at 1300 psi in an Omar SPC-900/EX critical point dryer, using liquid CO2 as the transition fluid. The tissues were then attached to silver stubs with silver paint, coated with a light coating of gold/palladium alloy for 1 min at 9.5 kV in a Hummer V sputter coater and examined using a Hitachi S-530 SEM operating at 10 kV, with a working distance of 7 mm and a fixed tilt angle of 30°.

Bacterial Cell Cultures and Corneal Inoculations

Stock cultures of Pseudomonas aeruginosa, maintained on Trypticase soy agar were obtained from patient samples typed in the microbiology laboratory at the Mount Sinai Hospital (New York, NY). The bacteria were transferred to, and grown overnight in 6 ml Trypticase soy broth at 36°C. The broth subsequently was centrifuged at 10,000 rpm for 10 min at 4°C, and the bacterial pellet was resuspended in a mixture of 3 ml saline + 3 ml Trypticase soy broth. The concentration of this inoculum was adjusted to 2.0 X 10¹¹ colony-forming units per ml by using a standard curve that relates variable counts to optical
density. For each inoculation, 10 μl were administered to the epithelial surface of a one-sixth wedge section of whole cornea. The inoculum was allowed to remain in contact with the epithelium for 15 min at room temperature (RT) while covered as described for the lectin stainings. The tissue was then immersed in 10 ml saline solution and agitated for 30 sec. The washed specimens were laid flat on a plastic surface and covered with 20 μl SEM fixative. After 15 min the wedges were transferred to a vial containing 2 ml fixative and incubated for 16 hr at RT. Superficial scars on the intact corneal epithelium were generated by advancing the beveled edge of a 28-gauge needle through the surface of the epithelium under gentle pressure.

Results
Effects of Exposure of the Corneal Surface to Digitonin

Exposure of the corneal surface to the 20-μM digitonin solution has been shown to induce cell devitalization, which results in a loss of transepithelial resistance and allows nuclear staining of superficial cells by trypan blue (Fig. 1). After a period of exposure of 8 min with 20-μM detergent, the whole monolayer of cells is stainable. After a 15-min incubation at 36°C, the stained cells exfoliate spontaneously (or upon minimal mechanical prodding) from the corneal surface. Despite the dramatic appearance of this induced exfoliation, persistent effects on physiologic properties are barely detectable. Within 1 hr the tissue regains a transepithelial resistance identical to that observed prior to the detergent treatment. Studies of resistance recovery have suggested that the action of digitonin occurs on a layer-by-layer basis. Exposures ranging between 2 and 8 min seem to affect only the outermost cell layer. An exposure of 14 min, however, leads to the same pattern of resistance recovery seen after two short (2-8-min) sequential treatments, a result that suggests that the longer treatment affects two cell layers.

These conclusions were confirmed in the current investigation by studying the appearance of digitonin-treated corneas in sagittal sections under light and electron microscopes. In these experiments, corneas were brought into contact with ferritin-tagged WGA prior to detergent treatment. The outer surface became ferritin-labeled, due to WGA binding sites (see below) present in this surface.

Under the light microscope (Fig. 2), a cornea treated for 8 min with digitonin and incubated subsequently for 10 min at 36°C showed a single monolayer of devitalized cells. The nuclei of these cells were extremely swollen, and nuclear contents appeared to have been lost (Fig. 2B). Another cornea, treated for 8 min but incubated subsequently for 45 min, showed a surface free of devitalized cells (Fig. 2C). A sheet of cells detached from this cornea after approximately 20 min of incubation. Detaching sheets were extremely fragile and dispersed rapidly into individual cells with the swirling motion used during the incubation. A control cornea is shown for comparative purposes (Fig. 2A).

Ultrastructural details of the same specimens are shown in Fig. 3. All cells at the surface of the control
cornea were decorated with ferritin. Cells exhibited a variability in electron density of cytoplasm, which probably corresponds to differences between newer and older decaying cells. Electron dense cells were rich in apical microvilli, whereas the low density cells had few microvilli. These differences in microvillar density correspond to the mosaic multitone pattern seen under SEM (see below). After the digitonin treatment, the cytoplasm of the devitalized cells was extremely swollen and had an empty appearance, with only remains of tonofilaments and organelles. Ferritin particles were visible on the distorted microvilli of these cells (Fig. 3B, top). The cells were clearly separated in the areas corresponding to the tight junctions. In some areas, desmosomes between live and permeabilized cells were still intact, whereas in others it was possible to visualize desmosomes in the process of disassembly (Fig. 3B, bottom). This desmosomal dissociation is fully consistent with the release of the devitalized cells a few minutes later. Underneath these dead cells, cytoplasmic profiles appeared normal. As in the methylene-blue-stained sections, the nuclei of the devitalized cells appeared devoid of contents. This appearance seems contrary to the intensive trypan blue nuclear staining shown in Figure 1. It is possible that the diluted protein content in the devitalized cells prevented efficient fixation and led to a washing-out of contents during postfixation processing, eg during sample dehydration. The ultrastructural studies indicated also that the devitalized, swollen cell bodies separated from each other. This is consistent with the observation that the detaching sheets could be preserved, but rather separated into individual cells with minimal attempts of handling. In the sample subjected to 45-min incubation, the surface was composed of cells of even cytoplasmic density. However, they could be distinguished from the original surface because no ferritin particles could be seen (Fig. 3C).

Fig. 3. Ultrastructural details of the effects of exposure of the ocular surface to 20-μm digitonin. Corneas were labeled with WGA-coated ferritin prior to the digitonin treatment described in Materials and Methods and were incubated for 10 min at 36°C in D/F12. (A) A representative area of the control rabbit cornea surface. Two superficial cells are seen: one cell displays a dense cytoplasm and elaborated microvilli, whereas in the other, the cytoplasmic density is much lower and microvilli appear to be absent. A glycocalyx decorated with ferritin can be seen over the surface of both cells. A tight junction (tj) is present at the cell-to-cell contact most proximal to the surface. Desmosomal (d) contacts with underlying cells are also visible. (B) Details of a cornea treated for 8 min with detergent and incubated for 10 min. (Center) Low magnification view. Highly swollen superficial cells in a clear state of disintegration are seen. The extreme swelling seems to have pulled the two cells apart, particularly at the previous tight junction location. (Top) A higher magnification of the microvilli-containing area, showing drastic distortion in their morphology and the preservation of ferritin decoration. (Bottom) A magnification of the interface between devitalization and vital cells. Dissociation of desmosomes (arrowheads) can be observed clearly. (C) The surface of a digitonin-treated cornea incubated for 45 min. A sheet of trypan-blue-stained cells has floated into the incubation medium 20 min after the start of the incubation. Microvilli containing a typical glycocalyx can be seen. Ferritin decoration is absent. Bar = 1 μm for all micrographs with the exception of (B, center), where it = 4 μm.
Light microscopic sagittal views of corneas exposed to digitonin for 14 min and fixed immediately thereafter are shown in Figure 4. The surface cells were extremely swollen; in particular, the nuclei achieved enormous size and were devoid of content. Cells below this layer also depicted profiles consistent with devitalization. The demarcation of vital and of devitalized cells was less clear than in the case of the sample treated for 8 min. Similar conclusions can be reached from the TEM observation (Fig. 5A).

The epithelial surface generated by the exfoliation of devitalized cells (after 40-50 min of 36°C incubation) had a normal appearance: abundant microvilli, a glycocalyx, and cell-to-cell apical interdigitation indicative of zona occludens could be seen. The glycocalyx in these freshly generated surfaces was seen to concentrate on the tips of the microvilli (Fig. 5C).

Unlike the case of the 8-min detergent treatment, the exfoliate obtained from the continuous 14-min treatment behaved as a relatively cohesive sheet. Small intact sections of these sheets were floated onto glass coverslips and were examined under Nomarski illumination (Fig. 6). At the exfoliate border it was possible to distinguish two or three superimposed cells.

Our objective in the current study was to determine whether the devitalization-exfoliation process could be repeated sequentially to accomplish the removal of any desired number of cell types. It was found that through the use of two sequential 14-min exposures, it was possible to generate corneas covered by a monolayer of basal cells at the central region. This is shown in Figure 7, which depicts eosin–hematoxylin-stained paraffin-embedded 4-µm thick sections of control cornea, cornea treated one time for 14 min, and cornea subjected to two sequential 14-min digitonin treatments (Fig. 7A, B, C, respectively). Hematoxylin staining of the epithelial cytoplasm was of similar intensity in the control and postdesquamation corneas.
Fig. 6. Normarski view of a sheet of devitalized epithelial cells that exfoliated spontaneously after exposure of the ocular surface to digitonin for 14 min and incubation of the cornea at 36°C for 1 hr. Bar = 20 µm.

It should be mentioned at this point that although it is customary to view this tissue as a five-layered structure, the cells of the corneal epithelium are not organized in perfectly superimposed layers; a true monolayer arrangement, defined by the presence of tight junctions, exists only at the exposed surface. Therefore, since two 14-min treatments led to the exposure of the basal cell layer, each 14-min treatment could be considered for practical purposes to induce the removal, on the average, of cells corresponding to two “layers”. This consideration, the morphologic observations presented above, and trial-and-error studies led to the development of the desquamation protocols described in Materials and Methods for the removal of between one and four “layers” of epithelium. The differences in incubation times were required because whereas each successive 6–8-min digitonin exposure led to the devitalization of a monolayer of outermost exposed cells (as attested to by the trypan blue staining), the time span between devitalization and spontaneous exfoliation increased as deeper aspects of the epithelium were exposed. Attempts were made to accelerate cell release by the use of a forceful fluid stream at times shorter than that required for spontaneous detachment. However, SEM visualization demonstrated that such measures resulted in the rupture of the devitalized cells, with adherent fragments of basolateral membranes left behind.

Structural and Chemical Features of Tear-Facing Membranes

The digitonin treatments resulted in the sequential generation of four new artificially created corneal surfaces. Their structural and chemical features were studied in order to map cell membrane maturation along the stratification axis. Viewed by SEM (Fig. 8) the untreated surface exhibited the well described “three tone mosaic” pattern of dark, light, and intermediate cells. This heterogenous refractive pattern

Fig. 7. Eosin–hematoxylin stained 4-µm thick sagittal sections of cornea. (A) Untreated; (B) after one 14-min digitonin treatment and exfoliation; and (C) after two 14-min digitonin treatments and exfoliations. Bar = 20 µm.

Fig. 8. SEM of untreated and digitonin-treated ocular surfaces. (A) Untreated surface. The dissected cornea was incubated in D/F12 at 36°C for 1 hr before fixation. (B) Surface after the removal of a single layer of superficial cells; (C) surface after removal of the two most superficial cell layers; and (D) surface after removal of the three most superficial cell layers. Bar = 20 µm.
has been ascribed to differences in the density and size of surface microprojections (microvilli and microplicae) between old and newer emerging surface cells. After one 8-min digitonin treatment, the post-exfoliation surface was composed entirely of cells with a homogenous density of microprojections leading to a monotonic surface reflection pattern. After one 14-min treatment, the density of surface microprojections was decreased markedly when compared with that seen in the light surface cells (Fig. 9). Upwardly protruding, smoothly surfaced filipodic extensions with the appearance of excess membrane were seen frequently at the cell borders. The density of microprojections was decreased further in the surface exposed by an additional 8-min treatment; cell surfaces exhibited either moderate microprojection density or nearly smooth cell contours (Figs. 8, 10).

Nuclear profiles bulging under the tear-facing membranes also were common, suggesting that in the newly exposed cells the apical membranes tend to adopt a flat configuration rapidly, stretching over a nucleus that is still rounded. The two sequential 14-min treatments left a distinct surface composed of cells of relatively small frontal diameter (Fig. 10). The density of surface elaboration here was minimal. At the corneal periphery, however, the surface was found frequently still to be covered with flattened, mosaic-type cells exhibiting microvilli.

Next, the distribution of lectin binding sites was examined. Six lectins were used: WGA, concanavalin A (ConA), peanut, soybean, Ulex europeus, and Dolichus biflorus. Observations of the patterns of binding and binding inhibition were confirmed by repeating each experiment a minimum of two times. Only WGA and ConA were found to bind to the intact corneal surface. The patterns of staining observed were similar for these two lectins. For simplicity, only the WGA results are shown. At the intact surface, cells stained with a variety of intensities, leading to the generation of a mosaiclike pattern (Fig. 11). The extent of staining appeared to correlate with the size of the exposed surface areas of the individual cells. Large surfaces stained faintly; small angularly shaped surfaces intercalated between several of the larger cells stained more intensely. In some of the larger cells, while the surface remained completely free of lectin binding, nuclear staining was evident. This staining pattern indicates that those cells were probably old surface cells that already had become nonviable. Indeed, similar nuclear staining by WGA was seen after permeabilization by digitonin (data not shown).

Fig. 9. High-magnification SEM of the ocular surface. (A) The tear surface of a light cell from an intact cornea. (B) The tear surface from a cell exposed by removal of the two most superficial cell layers. Bar = 20 µm.
A mosaic appearance for both WGA and ConA was obtained also on the surfaces generated by single 8- or single 14-min treatments. The inverse relationship between cell size and staining intensity observed for the intact surface remained valid. Frontal cell diameters in these artificially generated surfaces were smaller than in the normal surface, and an increase in the average intensity of staining was noticeable.

The sugar specificity of the lectin binding was tested by inclusion of monomer target sugars. WGA binding was prevented by sialic acid or N-acetyl glucosamine, each at 400 mM. Siaic acid at 100 mM or N-acetyl glucosamine at 200 mM were unable fully to prevent WGA binding. ConA binding was prevented by inclusion of 200 mM mannose. Peanut, Ulex europeus, and Dolichos boforus lectins were unable to bind to these surfaces. Soybean lectin stained the lateral outer perimeter of the membranes after removal of two cell layers (data not shown). This binding was prevented by 200 mM N-acetyl-D-galactosamine.

At the monolayer of basal cells, little staining of the exposed tear-facing membranes was observed, but the cell periphery was stained by four lectins—WGA, ConA, soybean, and peanut. The level of resolution of light microscopy precluded determination of the exact binding location. No paracellular barrier between the basal cells exists at the point at which lectin staining was performed (J. M. Wolosin, unpublished observations). Therefore, the peripheral staining may reflect binding to lateral cell membranes. Two observations demonstrated that this binding was specific and not a result of physical entrapment. First, fluorescence was abolished when the respective monomer target sugars were included in the lectin solution (400 mM galactose was used for peanut lectin). Secondly, no fluorescence was noted with the two remaining lectins, Ulex europeus and Dolichos boforus.

The FITC–WGA binding studies assessed the extent of reproducibility of the desquamation protocols, especially in regard to the preparation of a basal cell monolayer. In this particular procedure, significant variability could result from the large numbers of sequential steps involved in the preparation, or from differences in the number of cell layers in individual specimens. Nevertheless, we consistently found (n = 8) that the two sequential 14-min digitonin treatments led to the generation of the characteristic lateral staining patterns of basal cell monolayers, shown in Figure 11D, over most of the corneal surfaces. Variations in this pattern were observed. First, with the fluorescent method, it was possible occasionally to visualize individual or small groups of flattened cells covering the basal layer. The lectin stained the tear-facing surface of these flattened cells and infiltrated below them to stain the lateral borders of the basal cells. Thus, with a 100× objective with a 1.25 numerical aperture, the two corresponding fluorescent images could be visualized independently in the same field of view by changing the focal plane by about 5 μm. Second, in some areas cells exhibited nuclear staining indicating devitalization. Third, consistent with the SEM observations, when the central cornea was covered by viable basal cells, ie, when there appeared lateral border staining of small front diameter cells with no nuclear staining, the limbal area was covered by mosaiclike surface-stained cells. This difference originates probably in a difference in the number of cell layers present in central and peripheral cornea.

Pathogens such as Pseudomonas aeruginosa are not normally able to attach to the mature corneal surfaces. However, diffuse binding under the same experimental conditions has been shown to occur when a superficial wound exposes inner epithelial surfaces. Therefore, the deposition of Pseudomonas aeruginosa on the tear-facing membranes of the inner cells also was investigated. Preliminary studies tested whether the strains available, to us except to the above, were able to adhere to the exposed tear-facing membranes of the intact corneal tissues. Two observations demonstrated that this binding was specific and not a result of physical entrapment. First, fluorescence was abolished when the respective monomer target sugars were included in the lectin solution (400 mM galactose was used for peanut lectin). Secondly, no fluorescence was noted with the two remaining lectins, Ulex europeus and Dolichos boforus.
areas. An overall tendency of the bacteria to bind near or at the cellular borders was noted, consistent with the observation that microprojections develop there last. The bacteria deposited more or less homogeneously throughout the surface on corneal tissues containing only the basal cells. However, even at this depth, a few microprojection-endowed cells refractory to deposition were seen.

**Discussion**

Digitonin, a cholesterol-dependent detergent, induces the generation of defects in cellular membranes and thereby allows the exchange of large solutes with the extracellular medium. This effect can be limited successfully to the most exposed membrane, leaving other aspects within a given cell unscathed. The physiologic outcome of the treatment depends upon the composition of the medium. In cell-like medium, (high [K⁺], sub-μM [Ca²⁺]) cell viability can be maintained. In the absence of these conditions, cell viability is lost. In the current study, these properties of digitonin were exploited to remove ocular surfaces. The histologic and ultrastructural studies clearly indicate that it is possible to devitalize and induce the exfoliation of outermost exposed cells, on a layer-by-layer basis. Even though the histologic studies suggested cell swelling during the digitonin exposure period, the overall devitalization-exfoliation process had no overt effect on the morphology of the remaining viable tissue.

The gradual removal of all suprabasal cells opens new avenues for the study of a number of features of the cell stratification and maturation process. In the current report, we address a number of issues pertaining to the anatomic and biochemical development of the tear-facing membrane and its resilience to pathogen attachment. Relevant information on exfoliative activity also was obtained.

The rapidity of exfoliation after devitalization exhibited a characteristic pattern consistent with the interactions occurring normally between the surface and subsurface cells; the outermost cells exfoliated very shortly after their devitalization. As the cell-to-cell interface at which exfoliation occurred was moved further away from the normal outer surface, the capacity for rapid release of dead cells decreased. Thus, the exfoliative capacity of the tissue seems to develop gradually with stratification.

SEM of the control surface and of the four corneal surfaces generated by the detergent treatment demonstrated that the development of microprojections at the tear-facing membranes begins very early in the stratification process, and that a gradual build-up in the density of these membrane projections continues.
as the cells migrate towards the surface. The observation that formation of the microprojections is initiated at the center of the cell is consistent with the site of reappearance of microvilli in surface cells recovering after exposure to ultraviolet radiation. It is conceivable that some of the microanatomy observed was generated during the time elapsed between devitalization and exfoliation. However, a high density of membrane microprojections in the first and second subsurface cell layers also was observed in the experiments in which cells were detached forcibly by a fluid jet after incubation times of only 15 min.

Our investigation of lectin staining yielded a number of surprising observations. On the intact surface of the tissue, the mosaic pattern could be correlated conveniently with the three-tone pattern seen under the SEM: cells lacking surface staining are probably old cells approaching their normal desquamation point and becoming nonviable. The mosaic pattern present on surfaces generated by exposure to digitonin could not be explained as readily. Since SEM of the surface generated by the removal of one and two cell layers exhibited a fairly homogenous refraction pattern and a similarity in surface microprojection density, we expected to see uniform intensities of lectin binding. Instead, a multitone mosaic pattern was evident as we uncovered deeper cells within the epithelium.

The inverse relationship between frontal cell size and staining levels can be tentatively explained. Gipson et al have observed the generation of a high density of WGA and ConA binding sites in apical surfaces in migrating rat cells. The high WGA binding density seen in the cells exposing a small cross-sectional outer surface area may be related to this phenomenon. The small profiles likely represent newly emerging surface cells which were undergoing motile processes to reach their surface position. As the cells achieve a stable expanded configuration, decreasing staining might result from internalization of binding sites, changes preventing WGA from reaching these same sites, or merely an apparent reduction in binding sites due to their spreading over a larger area. Lectin binding to sagittal sections of the corneal epithelia of various species has been reported often. In these preparations, both surfaces of the cell membrane are exposed, and the basal and tear-facing membranes of adjacent cells are closely opposed. This arrangement tends to hinder identification of the exact binding location, such that straightforward correlation of this data with our study is difficult.

Binding of Pseudomonas aeruginosa to the adult mouse and rabbit cornea in vivo has been shown to occur exclusively at wound sites. A number of extraepithelial factors, including adherent mucus and immunoglobulin A (IgA), have been considered in these studies as contributing factors to the host’s ability to resist invasion. In the current study, corneas were exposed in vitro to Pseudomonas after extensive surface irrigation. The inability of the two strains to deposit on the surface demonstrates that resiliency to invasion is an intrinsic property of the apical surface of the epithelium. In fact, this resiliency was exhibited also by the tear-facing membranes of the cells located below the surface, indicating that the biochemically relevant membrane maturation events were completed even though anatomic changes were still occurring. These events take place apparently with the start of stratification. The deposition of Pseudomonas exhibited an unequivocal relationship with the surface microanatomy; only microprojection-free cells were susceptible to the bacteria. Even in an individual cell, when projections were present only centrally, deposition was restricted to the smooth areas at the periphery. However, anatomic features are unlikely to be directly responsible for cellular protection. Binding of Pseudomonas is thought to be mediated by specific surface sugar moieties. In the rat, apical WGA and ConA sites have been reported to decrease in density as cells reach the surface. Therefore, we hypothesized that as cell layers were removed, we would be able to establish a correlation between an increase in Pseudomonas adherence and increased binding of lectin. The experimental observations did not conform to that initial working hypothesis, at least for the six particular lectins studied. The first two subsurfaces exhibited binding of WGA and ConA. This binding seemed to decrease significantly in the basal cell layer. Pseudomonas, in contrast, was unable to attach to cells recognized by the lectins, but bound profusely to the tear-facing membranes of the basal cells. It is possible that a moiety recognized by a lectin other than those used in our study is involved. Another possibility is that a property other than sugar moiety expression is involved in mediating bacterial adherence. The glycocalyx coating of the corneal microvilli of surface cells avidly binds ruthenium red, indicating that the glycocalyx is highly negative in nature. This negativity can be attributed to terminal sialic acid residues, which are responsible for the binding of WGA. Thus, it is possible that surface protection is afforded mostly by electrostatic shielding. This type of mechanism could well explain the correlation of bacterial adherence with microprojection density, since the TEM studies (Fig. 5C) suggest that the glycocalyx is concentrated on the microvillar tips.

As reported earlier, the selective devitalization of superficial cell layers facilitates the study of tight junction formation in the corneal epithelium. The
current results indicate that the expansion of this technology to achieve the devitalization and removal of all suprabasal cell layers can be useful for the study of a number of maturation processes occurring as cells migrate along the stratification axis.

Key words: cornea, exfoliation, apical membrane, scanning electron microscope, Pseudomonas aeruginosa

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