Guinea pig corneal and conjunctival surfaces were examined by transmission electron microscopy and cytochemistry. Some specimens of cornea and conjunctiva were examined morphologically; others were stained with ruthenium red or tannic acid before examination to enhance the detection of glycoproteins at cell surfaces. The epithelia were covered by microvilli and on the cornea also by microplicae. These surface projections were the shortest (150 nm) over the central cornea, and became progressively longer (~300 nm) over the tarsal and fornical conjunctiva. There was a filamentous cell coat (glycocalyx) on the microvilli and microplicae that was best demonstrated in specimens stained with tannic acid. The glycocalyx extended approximately 300 nm from the tips and lateral surfaces of the microvilli and microplicae. Although there were slight local variations in its thickness, the maximum thickness of the cell coat was uniform over the cornea and conjunctiva. Heavy deposits at the cell surface after ruthenium red staining indicated that the cell coat contained many highly charged polyanions. The density of the ruthenium red stain obscured the fine structure of the filaments in the cell coat. The glycocalyx forms a scaffolding that is believed to bind mucus, with its content of immunoglobulins, by weak chemical interactions to the epithelial surface. Therefore, the microvilli, microplicae, and glycocalyx that were demonstrated in this study provide the structural framework that supports and binds a complex of related factors, including tears, mucus, and immunoglobulins, that have the common function of protecting the eye. Invest Ophthalmol Vis Sci 24:570-576, 1983

The epithelia of the cornea and conjunctiva provide vital barriers between the delicate structures beneath them and the external environment. It is at this surface that invading microorganisms and foreign substances, both immunogenic and inert, first impinge upon the eye. Like epithelial cells at other sites, they are covered by microvilli (and on the cornea also by microplicae) upon which there is a filamentous glycocalyx. Over this, mucus glycoproteins are believed to form the inner layer of the tear film. Because of the importance of the epithelia in the protection of the eye, studies using electron microscopy and cytochemistry were undertaken to analyze the structures at the outer surfaces of the corneal and conjunctival epithelia.

Materials and Methods

Pathogen-free Hartley strain guinea pigs were obtained from Charles River Breeding Laboratories, Wilmington, MA. Immediately before experiments, the animals were killed by intraperitoneal injection of 400 mg of sodium pentobarbital (Somlethol, MedTech, Inc., Elwood, KS). The corneal and conjunctival epithelia were fixed briefly by dropping fixative onto the eye. Specimens were then dissected and treated as described below.

Tissue for Morphologic Studies

Specimens for morphologic study were fixed for periods ranging from 2 to 12 hrs at 4 C in 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4). They were then postfixed for 2 hrs in cold 2% osmium tetroxide in Veronal-acetate buffer (pH 7.4) with 1% sucrose, and stained in block with Kel- lenberger’s uranyl acetate for 1 hr.

Ruthenium Red Staining

After primary fixation for 2 hrs in glutaraldehyde as above, some specimens were washed in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) with 7% sucrose, then immersed in 2% osmium tetroxide in Veronal-acetate buffer (pH 7.4) with 1% sucrose, and stained in block with Kel- lenberger’s uranyl acetate for 1 hr.

Tannic Acid Staining

Additional specimens of cornea and conjunctiva were stained with tannic acid by a modification of the Simionescus’ method. Specimens were fixed for 12 hrs at 4 C in 1.5% glutaraldehyde in buffer (0.1 M sodium cacodylate-HCl, pH 7.3) with 1% sucrose. They were then washed in buffer without sucrose, fixed for 1 hr in 1% glutaraldehyde with 2% tannic acid at 4 C, washed, postfixed in cold 1% OsO and.
stained in block with Kellenberger's uranyl acetate stain.9,10

Subsequent Processing
All specimens were dehydrated in ethanol and propylene oxide and embedded in epon. Thin sections were cut with a diamond knife on a Sorvall MT-2 microtome, stained with aqueous uranyl acetate and Reynolds' lead citrate (or with the latter alone) and examined in a Siemens Elmiskop IA electron microscope operating at 80kv with a 50-μm objective aperture.

Tissues Selected for Examination
Epithelial cells of the cornea have a half-life of approximately 7 days,13,14 and other epithelial cells may have an even shorter life span.15 Because of this rapid turnover, many of the superficial cells of the cornea and conjunctiva are about to be sloughed. In preliminary examinations of our specimens, we found many epithelial cells that were detached partially from the underlying tissues. They were dense, details of their internal structure were obscure, and they appeared to be dying. Therefore, we selected only the lighter, apparently more viable cells of the epithelial surface for analysis.

Results

Microvilli and Microplicae
Although the epithelium of the external eye is a continuous layer, it varies from stratified squamous on the cornea (Fig. 1) to stratified columnar in some areas of the conjunctiva (Fig. 2). The squamous cells at the surface of the cornea are flat and contain a paucity of organelles other than microfilaments, ribosomes, and elongated nuclei that lie parallel to the cell surface. By contrast, the tarsal and fornical conjunctival cells are richly endowed with endoplasmic reticulum, Golgi complexes, and mitochondria, suggesting greater synthetic and metabolic activities.

The projections on the epithelial surfaces reflect some of the structural differences of the underlying tissues. Surface projections are relatively short (100-200 nm) over the central cornea. Microvilli become progressively longer until they reach maximal length on the fornix and tarsus of the conjunctiva. On the cornea, the surface projections are spaced irregularly. In this region, the width of each microvillus is variable (200-300 nm); many are broader at the base and taper upward to rounded tips. Interspersed randomly among the corneal microvilli are broader ridges (~400 nm) called microplicae, of approximately the same height.

On the conjunctival tarsus and fornix, the microvilli are longer (~300 nm), and are more regularly and closely spaced than elsewhere. In sections cut perpendicular to the cell surface, they are erect and parallel. Bundles of filaments within the microvilli (Fig. 2, inset A) extend down into the superficial cytoplasm (Fig. 2, inset b). The corneal limbus is a transition zone between cornea and conjunctiva. As one might expect, the size and structure of limbal microvilli are intermediate between corneal and conjunctival microvilli. The goblet cells of the tarsus and fornix have microvilli that are similar in size to those of neighboring epithelial cells.

Glycocalyx
When examined at high magnification, the microvilli and microplicae display a fuzzy covering of filamentous material, the glycocalyx. In specimens prepared for morphologic examination, the glycocalyx appears thin on the cornea where surface projections are shortest, and thicker on the conjunctiva where microvilli are longest (Figs. 3–6). However, when specimens are stained with tannic acid (Figs. 7–10), the structure of the glycocalyx is revealed more
Fig. 2. Low power view of an epithelial cell from the conjunctival fornix, showing the size of the microvilli in relation to the whole cell. Note that the cell has abundant mitochondria (m), indicating high metabolic activity, and plentiful rough endoplasmic reticulum (er), indicating synthetic capability (X6,200). Inset A: Cross-section through a microvillus, demonstrating the internal bundle of microfilaments (X130,000). Inset B: The microfilaments of a microvillus are shown extending into the superficial part of the epithelial cell (X35,000).

clearly, and its filaments appear longer than in specimens prepared for morphologic examination. Although slightly variable from one region to another, the filaments reach a uniform maximum length over all of the cornea and conjunctiva. Connections of the microfilaments to the underlying unit membrane are clearly discernible when the tissue is stained with tannic acid. Angular bends and branches in the filaments are seen, as well as a beaded substructure that is not evident without tannic acid staining. Some filaments reach out far enough laterally to connect adjacent microvilli, and they fan outward from the tips of microvilli for a distance of approximately 300 nm.

Staining with ruthenium red obliterates the structure of the fine filaments of the glycocalyx (Figs. 11–14). Coarse aggregates of stained material are prominent along the outer tips of the microvilli and microvilli. Beneath these aggregates, the membranes
Figs. 3-6. Structure of the microvilli and glycocalyx on corneal and conjunctival epithelium routinely fixed in 3% glutaraldehyde for morphological examination. Fig. 3. Central cornea; Fig. 4. Corneal limbus; Fig. 5. Conjunctival tarsus; Fig. 6. Conjunctival fornix. The glycocalyx appears thinnest over the central cornea. The microvilli in this region are approximately half the height of those on the conjunctiva (all figs. x62,300).

of the cellular surfaces themselves are covered by a layer of dense stain approximately twice the thickness of the underlying unit membrane. Irregular clumps of material sometimes filled the spaces between microvilli of the tarsus and fornix (Figs. 12, 13). These densely stained substances are localized within 300 nm of the microvillar membrane, and thus within the region of the glycocalyx. There is no stained material beyond this area that might be interpreted as mucus.

Discussion

We found that the structures at the surfaces of the cornea and conjunctiva vary with their location. Microvilli and microfolds are short and heterogeneous in shape over the central cornea, but the microvilli are uniform and tall in the tarsal and fornical conjunctiva. In spite of the structural differences between the underlying cells, the glycocalyx approaches a uniform thickness in all parts of the epithelium of the outer eye.

The intestinal brush border is an instructive model for the microvilli of the eye. The intestinal cell coat, on which many studies have been made, is produced by the cells on which it is found.\textsuperscript{16,17} The glycocalyx rapidly turns over; when pulse-labeled, the label decreases after 6 hrs.\textsuperscript{1} It is continually renewed by the cells of the epithelium that are rich in the organelles needed for glycoprotein synthesis. The structure of the conjunctival cells indicates that they are metabolically and synthetically active and apparently capable of producing an extracellular coat. In contrast, the cells of the cornea do not appear to be so well equipped to maintain a glycocalyx. Except for ribosome-studded perinuclear cisternae and a few scattered cisternae of endoplasmic reticulum and free ribosomes, they appear to have relatively little machinery for protein synthesis, and Golgi elements are sparse. In fact, as the corneal cells move toward the epithelial surface, their internal structure becomes obscure, and presumably their viability decreases before they are shed. Nonetheless, the glycocalyx of the corneal cells is as prominent as that of the conjunctival cells.

Although a glycocalyx is believed to be a component of all cell membranes,\textsuperscript{18} there are only a few cell types (intestinal\textsuperscript{18} and toad bladder\textsuperscript{1} epithelia, for example) in which the filamentous substructure of the glycocalyx is seen as clearly as in the eye. The functions of the glycocalyx on the cornea and conjunctiva are not yet known, but they may well prove to be complex. When the intestinal glycocalyx was analyzed, it was found that the filaments of the cell coat
are carbohydrate side-chains of glycoprotein enzymes that are embedded in the cell membrane. The protein moieties of the enzymes are believed to be integral membrane proteins. The enzymes include sucrase, maltase, lactase, aminopeptidases, and alkaline phosphatases, which hydrolyze nutrients before their transport into the intestinal epithelial cells.

Some information concerning the glycocalyx of the external eye was obtained from the experiments with ruthenium red. This stain consists of small cationic molecules that bind to tissue by means of electrostatic linkages with polyanions. The intensity of the stain is roughly proportional to the charge density on the polyanions. Therefore, the thick abundant stain seen on the external eye indicates that the glycoproteins comprising the cell coat are highly acidic, perhaps as a consequence of a high density of sialic acid residues. Ruthenium red staining of the conjunctiva was previously demonstrated in the guinea pig and in man.

In the cornea and conjunctiva, the glycocalyx is doubtless of considerable importance in the initial interactions between invading bacteria and the epithelium. The glycocalyx may determine the specific sites on the epithelia where bacteria first adhere, the requisite first step in the initiation of an infection. It is well known that certain microorganisms invade either the cornea or conjunctiva selectively. For example, Chlamydia trachomatis, Hemophilus influenzae, Entero virus 70, and Newcastle disease virus consistently produce conjunctivitis, but only rarely infect the corneal epithelium. Indeed, H. influenzae subtype aegyptius is commonly found adherent to conjunctival epithelial cells in smears. Conversely, Pseudomonas species rarely cause purulent conjunctivitis, but are a serious cause of corneal ulceration. The specificity of these relationships indicates differences in the vulnerability of the two epithelia to infection, perhaps as a consequence of differences in their surfaces where the initial stages of microbial attachment and invasion occur.

Randomly intermingled among the microvilli on the cornea are surface projections called microplicae. Previous studies on the cornea have produced conflicting results concerning them. Pfister concluded that the microplicae seen by scanning microscopy are artifacts resulting from adherence of microvilli to the corneal surface. However, microplicae are reportedly present on the corneas of man and elasmobranches, and they form elaborate and beautiful patterns on the corneas of many teleosts. The function of these microprojections of the epithelium remains unclear. It has been proposed that they play a mechanical role in preventing gravitational flow of...
the tear film over the cornea, but others believe that the tear film is sufficiently stable that it does not require such support. It is evident that the microvilli and microplicae increase the surface of the epithelium and thereby its effectiveness in transport of small molecules across the epithelium. It has been calculated that the intestinal microvilli, which are similar to those of the conjunctiva, but longer, increase the surface area of that epithelium twentyfold.

Conjunctival microvilli, like those of the intestinal brush border, have cores of microfilaments that extend into the superficial cytoplasm and join a mass of horizontally disposed filaments called the “terminal web.” Analyses of isolated intestinal brush borders have revealed the presence of myosin, meromyosin, and calmodulin, and have shown that the brush borders contract in the presence of calcium and ATP. Using heavy meromyosin as a label, Gipson and Anderson demonstrated actin filaments in the microvilli of normal corneal epithelium and in the basal cell layer of wounded epithelium as well. They postulated that actin filaments play a role in the migration of corneal epithelial cells during wound healing.

Conjunctival microvilli may also have the ability to move, because they have cores of similar microfilaments. Contractility of microvilli on the conjunctival surface might serve the important function of spreading the tear film and maintaining its uniform thickness, essential for normal vision.

The inner layer of the tear film, overlying the glycocalyx of the epithelial cells, is believed to be mucus, which forms a protective barrier between the tissues and the extracellular environment. Mucus is a highly hydrated gel that theoretically acts as a molecular filter, permitting only small molecules such as amino acids and glucose to penetrate it whereas larger molecules are blocked at its surface. Mucus is believed to be weakly bonded to the glycocalyx because of the chemical similarities of the glycoprotein constituents. Because it is hydrophilic, mucus increases the wettability of the eye and enhances the spread and continuity of the tear film. In this way, IgA, a glycoprotein segment that resembles mucus glycoproteins; it is postulated that this hydrophilic moiety of the molecule is inserted into the mucus phase and that the remainder of the molecule is in the overlying fluid of the tear film. In this way, IgA is believed to form a monolayer at the fluid-mucus interface where it would be most efficient in opsonizing invading pathogens.

IgA has recently been localized on corneal cells with immunoperoxidase staining.
Before approaching the glycocalyx, invading organisms must penetrate the mucus barrier. Although mucus was not demonstrated in this investigation, it forms an important part of the complex outer covering of the eye. Mucus depends upon the glycocalyx and microvilli for support and renewal. Thus, these various components (tears, immunoglobulins, mucus, glycocalyx and microvilli) at the surface of the eye constitute a complex protective barrier that must be breached by foreign substances or invading organisms before inflammation or infection ensue.

**Key words:** cornea, conjunctiva, microvilli, glycocalyx, epithelia, eye, tears, mucus

**References**