direct bearing upon ophthalmic solutions currently in use. EDTA is widely used, albeit at lower concentrations than used in this study and generally is formulated in conjunction with various types of preservatives. Commonly, these preservatives are highly water soluble and have been considered as having low permeability through the lipid barrier of the corneal epithelium. The present results suggest that under conditions of sufficient calcium chelation, either by high enough concentrations of one or more chelators, or frequent application at short intervals, preservatives indeed may enter anterior segment tissues. The effect of calcium removal on tissue permeability appears to be reversible in the corneal epithelium. Similar results have been obtained in perfusion studies of the corneal endothelium. The decreased drug concentrations found in the iris/ciliary body, however, were not reversible under the conditions of this study. This was either due to insufficient time for calcium to replenish this tissue or to the possibility that calcium is not able to penetrate from the precorneal area to the iris/ciliary body.

Key words: corneal permeability, EDTA, ocular kinetics, chelation, paracellular routes

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References


Presence of Langerhans Cells in the Central Corneas of Normal Human Infants

John W. Chandler, Michelle Cummings, and Thomas E. Gillette

Nine normal adult and seven normal infant human corneas were studied for the presence of dendritic epithelial Langerhans cells in a masked fashion. Epithelial flatmounts were separated from the underlying corneal stroma using EDTA. The epithelial Langerhans cell densities were determined in the limbus as well as the peripheral, pericentral, and central corneal regions following staining with ATPase. Segments of the flatmounts were also studied by immunofluorescence to confirm that the dendritic cells contained class II histocompatibility antigens. The limbus, peripheral, and pericentral zones of adult and infant flatmounts contained similar densities of Langerhans cells. However, the central corneal Langerhans cell densities in infants were significantly elevated as compared with those in adults. These results suggest that Langerhans cells are a constant constituent of the human central corneal epithelium during late gestation and early infancy. They further suggest that perturbations of the corneal epithelium are not required for the presence of Langerhans cells in the corneal epithelium. Invest Ophthalmol Vis Sci 26:113–116, 1985

The corneal and conjunctival epithelia of many species contain dendritic Langerhans cells (LC) with receptors for ATPase and antibody against class II histocompatibility antigens. These LC appear analogous to those in skin. The normal adult corneal epithelium contains fewer LC than the conjunctiva, and the central portion of the normal corneal epithelium is typically devoid of LC. Inflammation of the cornea is associated with an increased density of LC. Likewise, reepithelialization of the cornea following an abrasion is associated with an increased density of...
LC, which presumably has moved passively from the junction of the conjunctival and corneal epithelia.4 This report documents the presence of LC throughout the central corneal epithelia of normal human infants. This is a striking difference from the situation in normal adult corneas of several species.1–3

Materials and Methods. Human corneas: Adult and infant corneas were obtained from the Lions Eye Bank (Seattle, WA). Nine adult corneas (donors ages 54–87 years) and seven infant corneas (6-hr-old, 23-week gestation, and term infants 3 hr, 36 hr, 6 days, 2 weeks, 3 weeks, and 4 weeks old) were studied (Table 1). All infants died of congenital malformations or respiratory failure. There were no histories of intrauterine or infant infections. Five infants received silver nitrate ocular prophylaxis, two received erythromycin ointment. The clinical appearance of each infant cornea was normal. The epithelium was clear and intact in each instance.

Tissue procurement: Whole eyes were obtained using aseptic techniques. The human eyes were obtained within 6 hr of death and stored in moist chambers. They were used within an additional 12 hr. The murine and guinea pig corneas were obtained and processed immediately after death by cervical dislocation or ether anesthesia.

Preparation of epithelial flatmounts: Whole eyes were placed in an isotonic saline solution containing 20 mM EDTA and incubated at 37°C for 2 hr. Whole sheets of corneal with adjacent conjunctival epithelia were lifted from the whole eyes.5

ATPase staining: At least one-half of each epithelial sheet was stained for ATPase activity using a modification of the method of McKenzie and Squier.5,6 Briefly, epithelial sheets were rinsed in 0.2 M Tris buffer, fixed in cold cadoxylate buffered formaldehyde at 4°C for at least 2 hr, then rewashed in tris buffer. The epithelial sheets were then incubated in an ATP substrate composed of ATP (10 mg adenosine 5’ triphosphate disodium salt), 21 ml TRIS buffer, 2.5 ml 0.1 M MgSO4 and 1.5 ml of 2% lead nitrate at 37°C for 20 min. The sheets then were rinsed in TRIS buffer, developed in ammonium sulfide (20°C) for 5 min, and rewashed in distilled water.

Examination of ATPase stained flatmounts: The ATPase-stained epithelial flatmounts were mounted on glass slides with glycerol and cover slips. The slides for each species were numbered and subsequently read in a masked fashion. Specimens were examined at X40 magnification to determine the densities of ATPase positive dendritic cells by using an ocular grid that represented 50 μm2. Ten to 15 separate areas in various regions were examined to determine the ATPase positive cells/mm2. Adult corneas were received, processed, and read at the same time as the infant corneas. These were read in a random fashion. However, the larger sizes of the adult corneas prevented complete masking of the reader.

Immunofluorescence: Representative EDTA-separated epithelial flatmounts were fixed in absolute alcohol/glacial acetic acid (19:1) at 4°C for 12–15 hr or in acetone at 4°C for 20 min. After washing in 0.1 M phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) twice for 5 min, the epithelial sheets were immersed in FITC-conjugated monoclonal antibody against Ia (Becton-Dickinson; Sunnyvale, CA; clone 11–5.2) or against anti-human HLA-DR (Becton-Dickinson; Sunnyvale, CA; clone L243) diluted 1:10 in PBS-BSA and examined for immunofluorescence of dendritic cells to document the presence of class II histocompatibility antigens.

Definitions: The anatomic regions of the flatmounts have been described previously.3 The concentrations of these cells have been determined in three concentric regions of the cornea: central, pericentral, and peripheral, as well as in the bulbar conjunctiva at the limbus (ie, adjacent to the peripheral corneal region).

Results. ATPase-positive dendritic cells were a constant finding in all regions of the corneas and conjunctivae of newborn infants. These dendritic cells also expressed class II histocompatibility antigens as

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>Length of life</th>
<th>Cause of death</th>
<th>Densities of Langerhans cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Limbus</td>
</tr>
<tr>
<td>23 weeks</td>
<td>6 hours</td>
<td>Respiratory failure</td>
<td>50</td>
</tr>
<tr>
<td>Term</td>
<td>3 hours</td>
<td>Respiratory failure</td>
<td>154</td>
</tr>
<tr>
<td>Term*</td>
<td>36 hours</td>
<td>Cardiac abnormality</td>
<td>398</td>
</tr>
<tr>
<td>Term</td>
<td>6 days</td>
<td>Respiratory failure</td>
<td>402</td>
</tr>
<tr>
<td>Term*</td>
<td>2 weeks</td>
<td>Cardiac abnormality</td>
<td>364</td>
</tr>
<tr>
<td>Term</td>
<td>3 weeks</td>
<td>Cardiac abnormality</td>
<td>346</td>
</tr>
<tr>
<td>Term</td>
<td>4 weeks</td>
<td>CNS abnormality</td>
<td>386</td>
</tr>
</tbody>
</table>

* Received erythromycin ocular prophylaxis at birth. All other infants had silver nitrate ocular prophylaxis at birth.
evidenced by appropriate immunofluorescence with FITC-conjugated monoclonal antibodies. Table 2 lists the densities of the ATPase-positive cells in the various regions in the adult human corneas. These densities are comparable to those in other studies.\(^2,3\)

Newborn infants have densities of ATPase-positive dendritic cells that are comparable to adults in the limbal, peripheral, and pericentral regions (Table 2). In contrast, the densities of ATPase-positive dendritic cells were significantly greater in the central corneas of newborn infants, including an infant with a 23-week gestational age (Tables 1, 2). In addition, this higher density remains for the first several weeks of life in the absence of any known prenatal or postnatal infection.

**Discussion.** There have been conflicting reports of the presence or absence of ATPase positive dendritic cells bearing class II (Ia or D/R) histocompatibility antigens in the cornea.\(^2,3,7\) Some of these differences may be associated with species differences, genetic variations within a species, possible coexisting external eye infections or inflammations, or perhaps experimental techniques. A number of investigators have documented increased densities of Langerhans cells when the ocular surface is perturbed by a variety of mechanical or nonmechanical stimuli.\(^4\)

The only previous study that determined changes in the densities of ocular surface Langerhans cells demonstrated a gradual postnatal increase in mice of increasing age.\(^7\) Very few conjunctival Langerhans cells were noted at birth, however, gestation is very short in mice as compared with humans.

This study was carried out to look at the possible accumulation of Langerhans cells in the ocular surface as it is exposed to antigens following birth. Surprisingly, the ocular surface of a 6-hr-old infant following a 23-week gestation had Langerhans cells across its entirety. In fact, all infants had significantly increased densities of Langerhans cells in their central corneas when compared with adult corneas. The reasons for this increase remain unknown. Likewise, the low densities of Langerhans cells in the central corneas of healthy adults are unexplained. It seems reasonable to assume that Langerhans cells that reside in the ocular surface have a bone marrow origin like their epidermal counterparts. The latter Langerhans cells are believed to have a life span of approximately 3 weeks.\(^8\) The corneal epithelial layer is normally devoid of blood vessels. The conjunctiva and limbal regions represent vascularized tissues, which are contiguous with the corneal epithelium. The decreasing densities of Langerhans cells from the limbus to the central corneal region make it tempting to assume that the Langerhans cells are passively carried toward the central cornea with the normal movement of epithelial cells.

The statistically significant higher density of Langerhans cells in the central corneas of infants as compared with adults may be explained in several ways. One, there may be more rapid centripetal movement of infant corneal epithelium and thus more passive carrying of Langerhans cells. Second, it is possible that Langerhans cells have limited mobility from blood vessels, and the smaller infant eye allows greater numbers to reach the central corneas. Third, the ocular instillation of silver nitrate or erythromycin as prophylaxis against ophthalmia neonatorum might stimulate the migration of Langerhans cells. An experimental guinea pig model has been developed in our laboratory and preliminary experiments do not support this possible explanation (unpublished results). Fourth, there could be factors produced by adult corneal epithelial or stromal cells that inhibit the migration or reduce the life span of Langerhans cells in the corneal epithelium. Fifth, other factors that decrease the densities of Langerhans cells such as ultraviolet radiation may be operative in adults.

**Key words:** cornea, Langerhans cells, epithelia, immunology, ocular immunology

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**Table 2. Densities of ATPase positive dendritic cells in ocular surface preparations from adults and infants**

<table>
<thead>
<tr>
<th>Sites of counts</th>
<th>Adults (N = 9)</th>
<th>Infants (N = 7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limbus</td>
<td>269.7 ± 24.6</td>
<td>300.0 ± 139.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Cornea Peripheral</td>
<td>142.2 ± 25.8</td>
<td>165.0 ± 60.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Cornea Pericentral</td>
<td>57.2 ± 28.7</td>
<td>70.7 ± 22.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Cornea Central</td>
<td>7.7 ± 6.2</td>
<td>39.9 ± 13.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**References**

Anterior chamber injection of donor rabbit lymphocytes sensitized in vitro to recipient alloantigens leads to the development of small focal areas of endothelial cell destruction (pocks) on the recipient cornea. Damage may be observed through a specular microscope as early as 2 days after injection of sensitized lymphocytes. Recipients of unsensitized allogeneic or sensitized autologous lymphocytes demonstrate little or no endothelial damage and no pock formation. Flat endothelial preparations reveal focal destruction of the endothelium with multiple foci, many infiltrated and surrounded by mononuclear cells. This model provides controlled sensitization to a variety of histocompatibility and corneal antigens that may be responsible for initiation of graft rejection. Invest Ophthalmol Vis Sci 26:116-121, 1985

Maintenance of a functionally intact donor endothelium is of critical importance in keratoplasty. Allograft rejection may produce severe damage to this layer of the cornea, which results in hydration and clouding of the stroma and loss of vision. For these reasons, an understanding of the interactions between lymphoid and endothelial cells is of utmost importance.

A model of endothelial allograft rejection was reported previously in which pocks of focal destruction of corneal endothelium were produced by lymphocytes from a donor sensitized to the recipient by prior skin grafting.1 This reaction was shown to be allospecific, since it was demonstrated that only appropriately histoincompatible corneas suffered destruction. The model is invaluable for assaying interactions between lymphoid cells and corneal endothelium because the reaction can be observed and quantitated directly using a slit lamp. Sensitization by skin grafting, however, does not allow for the study of specific antigens involved in the initiation phase of the immune response.

The in vitro mixed lymphocyte reaction (MLR) has been previously reported to represent the initial stages of a cell-mediated response to foreign histocompatibility antigens.2 Lymphocyte proliferation that occurs during the MLR precedes the development of effector cells including cytotoxic T-cells specific for the histocompatibility antigens that initiated proliferation. The present report describes a simple, effective, totally in vitro system for the generation of cell-mediated immune responses, the effect of which can be assayed by direct observation in vivo.

Materials and Methods. Animals: All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research. A total of 32 outbred New Zealand white rabbits weighing 3-4 kg were used (Bunnyville; Littlestown, PA).

In vitro sensitization: Pairs of rabbits were anesthetized with an intramuscular injection of 44 mg/kg ketamine HCl (Ketalar, Parke-Davis) followed by a subcutaneous injection of 0.04 mg/kg atropine sulfate and 35 to 50 ml of blood was collected into a heparinized syringe by cardiac puncture. Equal amounts of whole blood and 3% pigskin gelatin (Sigma, Type II) in phosphate-buffered saline (PBS) were mixed and incubated at 37°C for 15 min to hasten erythrocyte settling. Peripheral blood mononuclear leucocytes (PBML) were obtained by overlaying the cell suspension above the erythrocytes on a Ficoll-Hypaque density gradient as described previously.3 PBML were harvested from the interface of the gradient, washed twice in PBS, counted and adjusted to 2 X 10^6/ml in RPMI 1640 (Gibco) supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS).

Culture conditions: Reciprocal one-way-mixed lymphocyte cultures (MLCs) were set up in 25-cm²