Rabbit corneal epithelial cell cultures were established from Dispase-treated anterior corneas. In culture medium containing cholera toxin, insulin and epidermal growth factor, these cells proliferated in vitro in the absence of any contaminating cells. Following subculture, cells retained epithelial morphology and the ability to synthesize cAMP in response to β-adrenergic stimulation, but lacked the ability to respond to serotonergic stimulation. Retention of the β-adrenergic system in culture serves as a functional epithelial cell marker; whereas expression of serotonergic responsiveness may be regulated by developmental or extrapithelial systems that are absent in these cell cultures. Invest Ophthalmol Vis Sci 24:1139-1143, 1983

Long-term cultures containing corneal epithelium have been used to study the biosynthesis of basement membrane components and keratins. These investigations used either a mixed epithelial-stromal explant in the presence of a selective medium or an irradiated feeder layer to support epithelial cell growth.

Our goal was to maintain differentiated corneal epithelial cells in culture in the total absence of other cell types. We have found that full-thickness epithelial sheets, removed by enzymatic treatment and grown in the presence of appropriate medium components, establish confluent cell layers of typical epithelial morphology which can be subcultured.

Native corneal epithelium synthesizes cyclic AMP (cAMP) when activated by either β-adrenergic or serotonergic agonists via independent pathways that stimulate chloride transport. We have studied the β-adrenergic and serotonergic responsiveness of cultured corneal epithelial cells as a measure of their differentiated state.

Materials and Methods. Primary culture: Adult New Zealand albino rabbits weighing 2–3 kg were killed by an intravenous overdose of Nembutal. The eyes were propoded and the corneas were removed by circimferential excision within the limbus. Descemet's membrane, endothelium, and posterior stroma were removed with forceps. The anterior cornea with intact epithelium was covered with Dispase II (1 unit/ml) in calcium and magnesium-free Hanks buffer and incubated at 37°C in a humidified CO2 incubator. One hour later, corneas were placed in culture medium and full-thickness epithelial fragments were gently peeled off with forceps.

Basal epithelial medium (BEM) consisted of equal volumes of HEPES buffered Dulbecco Modified Eagle Medium (DMEM-HEPES) and Hams F12 (F12) enriched with 5% fetal calf serum, 0.5% dimethylsulfoxide, and 1 μg/ml gentamycin. Hormone epithelial medium (HEM) was constituted by adding to BEM: 10 ng epidermal growth factor (EGF)/ml and 5 μg insulin/ml. Further addition of 0.1 μg cholera toxin (CTX)/ml gave supplemented hormonal epithelial medium (SHEM) that was routinely used for primary culture of epithelial fragments. Culture medium was changed three times weekly.

Subculture: Monolayers cultured in SHEM for 7–10 days were treated with Dispase (1 unit/ml) for 20 min at 37°C. The enzyme-containing solution was then gently decanted and replaced with BEM, HEM, or SHEM. A Pasteur pipette, with a flame polished tip, was used to disperse cell clumps. Aliquots of suspended cells were counted in a hemacytometer. Cultures were either split 1:4 at routine subculture or cells from a confluent 60-mm plate were used to inoculate two 24 well plates.

Growth curves: Cells derived from primary culture were plated in 24 well multiplates at 2–5 × 10^3 cells/well. One milliliter of appropriate culture medium was added to each well. At the indicated intervals, 100 μl of a commercial trypsin-versene solution was used to remove cells, and an aliquot of this suspension was counted in a hemacytometer.

cAMP synthesis: Cell subcultures were initiated in 24 well plates as described previously. SHEM was replaced with HEM immediately following Dispase treatment, and cultures were maintained for 7–10 days prior to determination of cAMP synthesis. The medium switch was necessary to remove CTX, which stimulates cAMP synthesis.
For the assay, culture medium was replaced with DMEM-HEPES containing 0.5 mM isobutylmethyl-xanthine (IBMX), and the culture plate was supported in a 37 °C water bath. Nialamide (0.1 mM) was present prior to and during incubation with serotonin. After 10 min, drugs were added to a final concentration of 10 μM and incubation was continued for 15 min. Medium was then removed by aspiration, and the adherent cell layer was dissolved with 250 μl hot 0.1 N KOH. The culture plate was heated briefly at 100 °C to promote lysis of the cells. After cooling, the lysate was removed and neutralized with an equal volume of 0.1 N HCl. Precipitated protein was removed by centrifugation at 2,000 × g for 15 min at 4 °C. The supernatant was removed and analyzed for cAMP by the acetylation modification of a commercially available radioimmunoassay. The pellet was dissolved in 1 N NaOH and used for determination of protein by the method of Lowry et al. Synthesis of cAMP by intact pieces of rabbit corneas was determined as previously described.

Phase contrast microscopy: Cultures were examined and photographed using a Nikon inverted phase contrast microscope.

Transmission electron microscopy: Cultures were fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer pH 7.4, postfixed for 60 min in 1.0% osmium Tetroxide, dehydrated, and embedded in Epon 812. Tissue sections were stained with uranyl acetate and Tetroxide, dehydrated, and embedded in Epon 812. Culture dishes and multiplates were from Falcon. CTX was from Calbiochem. Dispase (neutral protease grade II) was from Boehringer Mannheim. Insulin, EGF, and the cAMP assay kit were obtained from Collaborative Research. IBMX, serotonin (5-hydroxytryptamine) and dimethylsulfoxide were from Sigma. Methysergide was from Sandoz. Timolol maleate was obtained from Merck.

Results. Discrete islands of attached epithelial cells spread from tissue fragments within 24 hrs. By 1 week, the attached cells derived from a single cornea nearly covered the bottom of a 60 mm dish. When epithelial cells were released from the tissue culture dish following treatment with Dispase and trituration, the resulting cell suspension consisted largely of small clumps (2–20 cells) and single cells. When added to culture vessels containing SHEM, the cells reattached within 1 hr and spread within the next 12 hrs. Confluent monolayers were re-established within one week following a 1:4 split (Fig. 1A). Transmission electron microscopy showed some layering of the cultured cells, typical epithelial polarity, and intercel-

lular desmosomal attachments (Fig. 1B). No stromal fibroblasts have been observed in these cultures.

Corneal epithelial cells in culture increased in number as shown in Figure 2. Cells from a primary culture were treated with Dispase and 3 × 10³ cells were plated into each of 24 wells containing 1 ml SHEM. Cell counts, in quadruplicate, were made on days 1, 3, 5, 9, and 14. Approximately 80% of the inoculated cells were viable on day 1. Cells increased in number for about 9 days and division ceased when there was 1.9 × 10⁴ cells/well (1.0 × 10² cells/mm²). We estimate a population doubling time of 72 hrs for these cells.

Table 1 indicates the ability of the various factors to stimulate corneal epithelial cell division. In these experiments, 4 × 10³ cells were introduced into each of 12 wells, with the indicated additions. Cell number was determined in triplicate on days 1 and 14. Basal medium alone did not support cell division and, in fact, cell number declined without supplements. In the presence of the hormones, insulin and EGF, cell number increased fivefold (2–3 cell doublings) over the experimental period. Cholera toxin alone did not enhance cell division, but seemed to prolong survival of cells, as no decrease in cell number was observed. The combination of CTX and hormones, however, strongly enhanced final cell number with four population doublings occurring over the 2-week experimental period. Cholera toxin, therefore, increased final cell density and possibly the response to epithelial mitogens.

Table 2 demonstrates the response of subcultured corneal epithelial cells to the β-adrenergic agonist, isoproterenol (10 μM) in the absence and presence of the β-adrenergic antagonist, timolol (10 μM). Cells were initially grown in SHEM, and at the indicated time, cells were plated into 24 well plates containing HEM. Medium was changed three times over the next week in order to remove residual CTX, which itself stimulates cAMP synthesis, thus obscuring the agonist effect. This subculture and medium exchange resulted in near normal basal levels of cAMP synthesis, rather than the grossly elevated levels seen in cultures exposed for even a short time to CTX. Cultured epithelial cells synthesized cAMP in response to isoproterenol, and this synthesis was blocked completely by timolol. Timolol alone had no effect on cAMP synthesis (data not shown). These cells, therefore, maintain their typical β-adrenergic response pathway in culture.

Table 3 demonstrates that subcultured corneal epithelial cells do not increase their synthesis of cAMP in response to serotonin (10 μM). In intact corneas incubated in vitro, this agonist increased cAMP synthesis; this action was specifically blocked by the se-
Fig. 1A. Epithelial monolayer 1 week after second subculture. Compact cells cover the culture dish. Phase contrast. Bar = 100 microns.

B. Transmission electron micrograph of cells fixed 1 week after second subculture. Several layers of cells cover the substrate, numerous desmosomes are present between cells, and microvilli occur at the apical surface of the cells. Bar = 1 micron.
rotonergic antagonist, methysergide. Thus, corneal epithelial cells in culture lack the ability to respond to a serotonergic stimulus, while retaining a specific adrenergic response.

Discussion. We have demonstrated that rabbit corneal epithelial cells remain viable following digestion of corneas with the neutral bacterial protease, Dispase. The full-thickness epithelial sheet obtained by this method includes intact basal cells. These cells presumably mediate attachment of epithelial fragments and provide a mitotically active population while the superficial cells are shed and removed by the initial medium changes. Phase contrast microscopy demonstrates the epithelial appearance of these cells in culture. Electron microscopy illustrates the maintenance of normal cell polarity and cell-cell contact, features that are necessary to the normal physiology of the corneal epithelium.

The growth medium we have developed for these cultures is based on the nutrient mixture of 1:1 DMEM:F12, which supports growth of a variety of differentiated cells. Undoubtedly, fetal calf serum supplies many additional factors to our preparation.

Table 1. Mitogenic response of cultured corneal epithelial cells

<table>
<thead>
<tr>
<th>Additions to basal medium</th>
<th>Day 1</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Insulin/EGF</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>CTX</td>
<td>4</td>
<td>4.3</td>
</tr>
<tr>
<td>Insulin/EGF, CTX</td>
<td>4</td>
<td>35</td>
</tr>
</tbody>
</table>

* Mean values of triplicate determinations.

Table 2. β-adrenergic response of corneal epithelial cells

<table>
<thead>
<tr>
<th>Drug addition</th>
<th>Cultured cells</th>
<th>Intact corneas</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25.5 ± 4.3*</td>
<td>7.1 ± 0.8†</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>121.0 ± 19.7*</td>
<td>130.0 ± 8.0†</td>
</tr>
<tr>
<td>Isoproterenol + timolol</td>
<td>22.9 ± 3.8*</td>
<td>8.2 ± 0.6†</td>
</tr>
</tbody>
</table>

* n = 12. † n = 6.

Table 3. Serotonergic response of cultured corneal epithelial cells

<table>
<thead>
<tr>
<th>Drug addition</th>
<th>Cultured cells</th>
<th>Intact corneas</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10.6 ± 1.0*</td>
<td>7.1 ± 0.8†</td>
</tr>
<tr>
<td>Serotonin</td>
<td>11.2 ± 2.3*</td>
<td>24.0 ± 2.0†</td>
</tr>
<tr>
<td>Serotonin + Methysergide</td>
<td>9.3 ± 0.8*</td>
<td>6.5 ± 0.8†</td>
</tr>
</tbody>
</table>

* n = 13. † n = 6.

Only EGF, insulin, and CTX are included as potential mitogens. Whereas FCS contains insulin, the amount included in this formulation is far in excess of the normal serum values and may be interacting with somatomedin receptors. As in other systems, CTX potentiates the mitogenic response of cultured corneal epithelial cells to EGF. CTX itself is not mitogenic in this system, but may, via cAMP, increase adhesion and thereby plating efficiency of subcultured cells. We are currently investigating the role of CTX in cell substrate adhesion, as this mechanism may also be involved in CTX modulation of corneal epithelial wound closure in vivo.

Normal corneal epithelium responds to catecholamine stimulation via β-adrenergic receptor mediated activation of adenylate cyclase. Cultured cells grown under the conditions described here, maintain this response pathway following subculture. The ability of the potent β-adrenergic antagonist, timolol, to block isoproterenol-stimulated cAMP synthesis demonstrates the specificity of the β-adrenergic response. The serotonergic response pathway, however, is apparently lacking in cultured corneal epithelial cells. Perhaps regulation of this pathway is under developmental control and our cultured epithelium represents an immature or de-differentiated population. Alternatively the serotonergic response pathway may be located in a corneal cell type, for example a nerve cell, that is absent in culture.

In summary, we have established a system for the growth and subculture of rabbit corneal epithelial
sheets. The cells retain their epithelial morphology and synthesize cAMP in response to β-adrenergic but not serotonergic stimulation.

**Key words:** epithelium, cornea, rabbit, cell culture, β-adrenergic response, growth factors, cholera toxin, cAMP, serotonergic response

**Acknowledgments:** The authors thank Sally Ledgard for technical assistance and Yasuo Ishii for electron microscopy.


**References**


**A Comparison of the Ocular Anti-inflammatory Activity of Steroidal and Nonsteroidal Compounds in the Rat**

P. Bhattacherjee, R. N. Williams, and K. E. Eokins

The anti-inflammatory activities of steroidal and nonsteroidal compounds have been evaluated in the rat model of ocular inflammation induced by subcutaneous injection of lipopolysaccharides. Dexamethasone sodium phosphate, BW755C, flurbiprofen, indomethacin, and benoxaprofen were administered orally or topically for 24 or 48 hrs. Oral administration of dexamethasone, BW755C, and flurbiprofen inhibited iris-vasodilatation and leukocyte accumulation in the anterior chamber in a dose-dependent manner. Indomethacin and benoxaprofen were active only at high doses. Topical administration of these compounds inhibited the inflammatory responses in a similar manner. The inhibitory effect on leukocyte accumulation by these compounds was greater than their effect on vasodilatation. BW755C, a phenyl pyrazoline derivative, which is an inhibitor of both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism was the most active nonsteroidal compound and had an anti-inflammatory profile similar to dexamethasone. The results of this study also indicate that the model of rat ocular inflammation induced by subcutaneous injection of endotoxin can be used satisfactorily for comparative evaluation of anti-inflammatory agents. Invest Ophthalmol Vis Sci 24:1143–1146, 1983

The anti-inflammatory drugs such as aspirin and indomethacin, inhibit the enzyme, cyclo-oxygenase, which converts arachidonic acid into prostaglandins. Unlike the aspirin-like drugs, the anti-inflammatory corticosteroids reduce the formation of both cyclooxygenase and lipoxygenase products of arachidonate metabolism by preventing the release of the precursor acid, arachidonic acid. This additional inhibition of leukotriene formation might be responsible for the wider profile of anti-inflammatory activity exhibited by the corticosteroids.

Although effective clinically as anti-inflammatory agents, corticosteroids have a large number of undesirable side-effects when used either systemically or topically. A compound having the ability of a corticosteroid to prevent the formation of the products of