A Technique for Obtaining Basal Corneal Epithelial Cells

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A technique has been developed for obtaining a cell suspension enriched (89%) in basal corneal epithelial cells. Eleven millimeter corneal buttons were removed and placed in culture medium containing low (10 μM) calcium. The posterior half of the stroma was removed with forceps. Three superficial cuts were made with a Bard-Parker blade on the anterior half of the cornea, which was then incubated for 18 hr at 35°C. Nonadherent cells were brushed off after the incubation and basal cells were harvested after a 1-hr incubation in Dispase II. Cell viability estimated by Erythrocycin β exclusion was 90%. Further evidence of viability was that the cells adhered to their native substrate, the denuded basal lamina. The authors protocol provides a method for analyzing the biochemistry of a known population of epithelial cells and makes available a defined source of cells for culture. Invest Ophthalmol Vis Sci 26:233-237, 1985

The availability of a suspension of basal corneal epithelial cells can facilitate studies on cell-substrate adhesion and biochemical characterization of a specific population of cells of the stratified epithelium. Such a preparation also will provide a defined source of cells for culture. Previously, investigators used preparations from full thickness epithelium for cell-substrate adhesion studies1 or to determine the composition of the extracellular matrix that is laid down by corneal epithelial cells.2 To obtain basal cells from epidermal epithelium, investigators have separated the epidermis from the dermis using either Dispase II or trypsin followed by further incubation of the epidermis in trypsin. The basal cells then are harvested by stirring the epidermis and decanting off cells3,4 or by culturing the epidermal cells in medium containing less than 1 mM Ca2+. Only basal cells proliferate under low calcium conditions.5 The percentage of basal cells present in several epidermal preparations was 85%.3,4,6

We report here a simple, rapid procedure for obtaining an enriched population of basal corneal epithelial cells that are viable and adhere to their native substrate, the basal lamina. The technique is based on the observations: (1) that only basal cells remain adherent to the basal lamina after 18 hr incubation in low-calcium medium7 and (2) that basal cells can be freed from the basal laminae after an incubation with Dispase II.8

Materials and Methods. All investigations involving animals reported in this study conform to the ARVO Resolution on the Use of Animals in Research. New Zealand white rabbits were killed by administering 5 ml pentobarbital (325 mg) intravenously. An outline was made on the cornea with an 11-mm trephine and corneal buttons were cut free with scissors. The posterior half of the stroma then was pulled away from the cornea with jewelers' forceps. Three evenly spaced, superficial cuts (1 mm in length) were made on the cornea with a small scalpel to facilitate the penetration of the medium. The anterior half of the cornea then was placed in culture medium containing a low concentration of calcium (10 μM) and incubated for 18 hr at 35°C in 5% CO2 and 95% air. As a control, corneas were incubated for 18 hr in defined culture medium (normal calcium levels) (Figs. 1a, b).9 After 18 hr, suprabasal cells were removed by brushing gently across the cornea with two strokes of a paint brush (size 5) (Fig. 1c). The corneas, with their adherent basal epithelial cells, were washed twice for 10 min in defined culture medium9 and then incubated for 1 hr in culture medium containing 1.2 U/ml Dispase II (Boehringer Mannheim Laboratory; Indianapolis, IN). To free the remaining cells, the cells were gently pipetted off the underlying surface. The cells then were spun at 1000 × g for 10 min, resuspended to a concentration of 3 × 105 cells/ml and filtered through a nylon cloth (120 μm) (Tetko Inc.; Elmsford, NY) to remove cell aggregates. The viability of the cells was estimated by Erythrocycin β (Sigma; St. Louis, MO) exclusion.10

To determine whether only basal cells remained after the incubation in low-calcium medium, control and experimental corneas were fixed and prepared for light and electron microscopy9 prior to the treatment with Dispase II. The total number of remaining epithelial cells on cross-sections of seven corneas were counted. From this total number of cells, the percentage of basal cells (cells adhering to the basal lamina) was calculated. The number of basal cells...
harvested from single corneas was determined by comparing the number of cells present in a 1-ml suspension of cells with the number of cells present on an 11-mm corneal button (assuming that the cell diameter is 20 \(\mu m\)).

To determine whether the harvested basal cells were functionally viable and would adhere and spread on their native substrate, the basal lamina, a cell substrate adhesion assay was developed. Stromas with freshly denuded epithelial basal lamina\(^9\) were trephined into pieces 3-mm in diameter. Petri dishes (30 X 60 mm) were coated with a layer of paraffin and a 3-mm trephine was used to make four wells in each dish. A 3-mm piece of stroma, denuded basal lamina side up, was placed into each well. A piece of a pipet tip 4 mm in length with a base diameter of

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**Fig. 1.** a, Light micrograph of a section of cornea after 18-hr incubation in defined culture medium. Note normal apical-basal stratification (X215). b, Light micrograph of a section of cornea after 18-hr incubation in 10 \(\mu M\) calcium-containing medium. Note the separation between the basal and suprabasal layers (X215). c, Light micrograph of basal cells remaining after suprabasal cells are brushed away (X215). d, Electron micrograph of basal corneal cells, showing apical blebbing (X1,722). e, Electron micrograph of hemidesmosomes along the basal lamina of cornea after incubation in low-calcium medium (X73,915). f, Phase contrast micrograph of harvested basal cells (X385). Basal cell possesses intact nuclear and cell membranes as seen in the inset (X2,430).
Fig. 2. a, Scanning electron micrograph of a 3-mm stromal piece used as a substrate in the adhesion assay. Cells are evenly distributed over the denuded basal lamina after 45 min (×46). b, Scanning electron micrograph of a cell after 15-min incubation. Basal cell is round and has not begun to spread along substrate (×35,389). c, Scanning electron micrograph of a basal cell that has spread along the substrate after 60 min (×3,409). d, Electron micrograph of a basal cell adhering to the denuded basal lamina after 45 min. Note electron densities (arrows) (×42,456). Entire cell in cross-section seen in inset (×4,098).

2.5 mm and top diameter of 3.5 mm was placed over the stromal segment in each well to form an incubation chamber; 8 × 10³ basal cells were pipetted into each chamber, which then was incubated for 0, 15, 30, 45, 60, or 90 min at 35°C. After incubation, the stromas with adherent cells were dipped 10 times in Ringer's solution, fixed in modified Karnovsky's, and processed for transmission⁹ and scanning electron microscopy (SEM). For SEM, the samples then were washed in 0.1 M cacodylate buffer and postfixed in 2% OsO₄ in cacodylate buffer (pH 7.4) for 1 hr. They then were dehydrated through an ethanol series and CO₂-critical point dried. Dried and mounted samples were coated uniformly with gold-palladium for 4 min at 22 mA. Photographs were taken on an AMR 1000A scanning electron microscope.

Results. The appearance of a cornea after incubation in defined culture medium for 18 hr can be seen in Figure 1a. The cornea displayed normal continuous apical-basal stratification with columnar basal cells. The appearance of a cornea after incubation in medium containing 10 μM calcium can be seen in Figure 2a. A delineation existed between the basal and suprabasal cells, spaces were present between cells, and only one layer of epithelial cells remained adherent to the basal lamina (Fig. 1b). After brushing, 89.3 ± 1.3% of the remaining cells were basal (Fig. 1c). These basal cells appeared columnar, demon-
strated apical blebbing, and maintained their cell substrate adhesion junctions (hemidesmosomes) (Figs. 1d, e).

Seventy percent of the basal cells attached to the basal lamina were harvested after the incubation with Dispase II. Surface blebbing was present around the entire surface of the basal cells (Fig. 1f). Nuclear and cellular membranes were intact. This was confirmed by the observation that over 90% of the harvested basal cells excluded the vital stain, Erythrocin β.

Further evidence that the basal cells were viable was obtained with an adhesion assay. After a 15-min incubation, basal cells adhered to the basal lamina and after 45 min, were distributed evenly over the stromal pieces (Fig. 2a). The basal cells were round after attachment (15 min) (Fig. 2b), but by 45 min lamellipodial extensions were present. The extent of cell spreading increased until the cells appear flattened at 60 min (Fig. 2c). Electron densities, which may be adhesion plaques (Fig. 2d), were present at 45 min.

In obtaining a cell preparation enriched in basal corneal epithelial cells, two major technical difficulties may be encountered. The motion of the brush to remove the suprabasal cells must be in one direction and must be done lightly so that the underlying basal cells are not damaged. The removal of the basal cells after the 1-hr incubation with Dispase II also must be conducted in a gentle manner so that the cell membranes are not disrupted.

Discussion. Our technique for obtaining a cell suspension enriched (89.3%) in basal corneal epithelial cells allows for the collection of cells that are viable and that retain the ability to adhere and spread along the basal lamina. The preparation makes available a defined source of cells for investigators who previously used full thickness corneal epithelium to examine the production of extracellular matrix components and the mechanism of cell substrate adhesion.12

We attempted to identify a basal cell marker, which could further determine the purity of the preparations. Rhodamine β (Sigma; St. Louis, MO), a dye that stains basal epidermal cells blue and keratinized cells red,11 did not differentiate between any of the corneal epithelial cell layers. Several lectins (Wheat germ agglutinin [WGA], Ricin communis agglutinin I [RCA120], Peanut agglutinin [PNA], Concanavalin A [Con A], Pismus sativum agglutinin [PSA], and Bandeiraea [Griffonia], simplicifolia lectin [BSL-1]) (Vector Lab; Burlingame, CA) were tried, and none bound preferentially to basal cells in intact rabbit corneal epithelium. An antibody to bullous pemphigoid also was used as it is known to bind preferentially to epidermal basal cells,12 but, again, we found no distinct binding pattern to intact rabbit corneal epithelium. Therefore, we have continued to use the number of cells remaining on the basal lamina after the incubation in low-calcium medium to assess the identification of our cells.

The technique for isolating basal cells resulted from the observation that only basal cells remain adherent to the basal lamina when intact epithelial sheets are placed on denuded epithelial basal lamina and incubated for 18 hr in low-calcium-containing medium.7 From these experiments we reported that the formation of new hemidesmosomes and desmosome maintenance were calcium concentration dependent.7 Investigators have shown that desmosome formation is also calcium dependent and formation occurs within 2 hr of the addition of 1 mM calcium to the medium.5 Unlike desmosomes, hemidesmosomes are maintained in medium containing only 10 μM calcium. Our observations indicate that although hemidesmosomes and desmosomes are morphologically similar, they possess different Ca2+ requirements for junction maintenance. The different requirements for junctional maintenance were key to the isolation of basal cells.

In summary, we have described a technique for obtaining basal epithelial cells by incubating corneas in low-calcium medium, which is followed by treatment with Dispase II. Our protocol provides a methodology for analyzing the biochemistry of a specific population of epithelial cells and should facilitate studies of corneal epithelial biology and disease.

Key words: basal cells, corneal epithelium, cell adhesion, basal lamina, basement membrane

Acknowledgment. The authors thank Ann Tisdale for technical assistance.

From the Eye Research Institute of Retina Foundation, and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts. Supported by National Institutes of Health (NIH) grant R01-EY03306 (IKG), and postdoctoral fellowships from NIH (F32-EY05614) and Society for Prevention of Blindness (VTR). Submitted for publication: July 2, 1984. Reprint requests: V. Trinkaus-Randall, Boston University Medical School, Department of Biochemistry, 80 East Concord Street, Boston, MA 02118.

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Recombinant Human Interferon αD in HSV-1 Recurrence in the Rabbit

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Recombinant human interferon alpha subtype D (RIFNaD) was effective in reducing the shedding of herpes simplex virus type-1 (HSV-1) induced by 6-hydroxydopamine iontophoresis followed by topical epinephrine application in previously infected rabbit corneas. A treatment schedule of RIFNaD, two drops QID was superior to one drop BID. RIFNaD also appeared to be effective in reducing viral shedding. Rabbits treated with RIFNaD during two episodes of adrenergically induced HSV-1 shedding, but not during anticipated episodes of spontaneous shedding, did not show a significant reduction in shedding of virus. Interferon was present in significantly higher concentration in tear samples following treatment with RIFNaD as compared with RIFNaA.

Interferon (IFN) prophylaxis has been shown to inhibit both viral penetration and replication. Of the four subtypes of recombinant human alpha interferon (RIFNa) tested (A, B, D, and hybrid A/D), subtype D was the most protective against herpes simplex virus type-1 (HSV-1) in an in vitro infection of rabbit kidney cells. Using an in vivo rabbit model of primary ocular HSV-1 infection of the cornea, RIFNaD also has been shown to be more effective than RIFNaA in ameliorating the course of the disease. We have also shown RIFNaD to be effective in reducing the ocular shedding of HSV-1 McKrae strain following iontophoresis with 6-hydroxydopamine (6-HD) and treatment with topical epinephrine in previously infected rabbit corneas.

The antiviral efficacy of RIFNaD may be due to its persistence within the tear film and to influence adrenergically induced HSV-1 shedding.

Materials and Methods. All experiments conformed with the ARVO Resolution on the Use of Animals in Research. At the start of each experiment, all eyes were examined with the biomicroscope to be sure they were free of external disease.

Experiment 1: The corneas of 19 New Zealand White (NZW) rabbits were bilaterally anesthetized with topical proparacaine HCl 0.5%, scarified with a Lindner spatula, inoculated with 1 drop (0.006 ml) of HSV-1 McKrae strain (1.1 × 10⁷ plaque forming units (PFU/ml), and subsequently examined daily with the biomicroscope to document primary clinical disease. Forty days after viral inoculation all rabbits were anesthetized and iontophoresed bilaterally with 1% 6-HD at 0.5 mA for 3 min and begun on a course of epinephrine, as described by Shimomura et al. Six rabbits received topical RIFNaD, one drop BID (8 AM, 5 PM); six rabbits received topical PBS one drop QID. Therapy began on day 1 (one day prior to iontophoresis) and continued through day +3. The epinephrine and RIFNaD or PBS were administered at least 15 min apart. On days +3 and +4, the eyes were swabbed for virus isolation.

*RIFNaA, RIFNaD, and Li 8 Ab were supplied by Hoffmann-LaRoche Inc., (Nutley, NJ).