An Improved Method for Isolation and Culture of Rat Retinal Pigment Epithelial Cells

Paula L. Mayerson, Michael O. Hall, Virginia Clark, and Toshka Abrams

Retinal pigment epithelial cells from normal, Long Evans (LE) and retinal dystrophic (RCS) rats can be grown in vitro (Edwards, 1977). An improved technique is described which permits a more rapid isolation of RPE cells, and routinely gives high cell yields (30,000–40,000/eye), excellent cell viability (95%), and high plating efficiencies (95–100%). Whole eyes are treated with hyaluronidase and collagenase followed by trypsin. These enzymes degrade components of the extracellular matrix, releasing sheets of RPE from adherent attachments to the retina and choroid. Trypsin was then used to dissociate the sheets into single cells. RPE cells are grown to confluence in primary culture. This technique permits RPE cell isolation from both normal and retinal dystrophic (RCS) rats, 8–15 days of age. Normal cells isolated by this technique consistently show excellent phagocytosis in vitro. Invest Ophthalmol Vis Sci 26:1599–1609, 1985

Vertebrate pigment cells have a dual embryonic origin. The neural ectoderm forms both neural crest derived pigment cells of the sclera, choroid and outer iris, and retinal pigment epithelial (RPE) cells. These two pigment cell types have different morphologies and growth characteristics, both in vivo and in vitro. Neural crest derived pigment cells (melanoblasts and melanocytes) are highly migratory, rapidly dividing, bipolar cells; while RPE cells are non-migratory, slowly dividing epitheloid cells.

RPE cells are unique in their rapid, cyclical phagocytosis of shed rod outer segment (ROS) discs. The shedding of ROS discs and their phagocytosis by the RPE begins at 12 to 15 postnatal days in the normal rat. Deficiencies in ROS phagocytosis, such as the mutation in rats of the Royal College of Surgeons (RCS) strain, result in retinal degeneration and eventual blindness. Deficiencies in phagocytosis can be detected with the in vitro assay of Chaitin and Hall. Using this assay, we have determined that phagocytosis by RPE cells is a highly specific process. In vitro RPE cell culture also provides a controlled environment to study the nature of the phagocytic process and of the defects inherent in retinal degeneration.

RPE cells have been isolated from human cadaver eyes by discarding the anterior segment, retina and vitreous, and incubating trypsin directly in the eyecup. This direct approach is ineffective for rat RPE which adheres firmly to both choroid and retina. Rat RPE cells were successfully isolated by Edwards who pre-incubated enucleated eyes for 6–24 hr in a balanced salt solution prior to treatment with 0.1% trypsin. This technique, while successful in our laboratory for the isolation of both normal and RCS rat RPE cells from 9–10-day-old rats, gives inconsistent cell yields, perhaps due to cell damage during the long pre-incubation. The same technique does not permit RPE isolation from rats older than 10 postnatal days. This paper presents an improved cell culture technique in which enucleated eyes are pre-incubated in enzymes specific for the degradation of extracellular matrix materials. Whole eyes are then incubated in trypsin and the RPE is dissected out, according to Edwards. This new approach has proved highly successful, giving consistently high cell yields, excellent cell viability, high plating efficiencies and good cell growth. These cultured RPE cells avidly phagocytize isolated ROS. This improved technique permits the isolation and culture of RPE cells from 8–15-day-old rats, 12–15 days being the time of onset of ROS shedding and phagocytosis in vivo.

Materials and Methods

Rats

Pigmented, retinal dystrophic (rdy/rdy, p+/p+) RCS rats were obtained from Dr. M. M. LaVail. The Long Evans (LE) strain of rat was used as a control animal. Breeding colonies of both rat strains were established in the Department of Ophthalmology, Jules Stein Eye Institute, U.C.L.A. School of Medicine, Los Angeles, California.

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maintained on a 12L:12D cycle and were killed during the light phase of the cycle. Rats were maintained and killed in accordance with the ARVO Resolution on the Use of Animals in Research.

### Isolation and Culture of Rat RPE Cells

Rats are killed 8–18 days after birth. Eyes are enucleated and rinsed three times in a balanced salt solution (BSS), pH 8.0, containing 50 μg/ml of garamycin (Schering, Corp.; Kenilworth, NJ) and 100 μg/ml of kanamycin (Flow Labs.; McLean, VA). Intact eyes are incubated consecutively at 37°C in two enzyme solutions. The first incubation is for 45–90 min in 1 ml/e of BSS, pH 7.0, containing 105 Units/ml of collagenase (CLS, Worthington; Freehold, NJ) and 50 Units/ml of testicular hyaluronidase (Sigma; St. Louis, MO). The second incubation is for 57–72 min in 1 ml/e of BSS, pH 8.0, containing 0.1% trypsin (DIFCO; 1:250). Both enzyme solutions are prepared just prior to use and sterilized through 0.22 μ filters (Millipore; Bedford, MA). Eyes are agitated every ten min during incubations in the enzyme solutions. At the end of the designated incubation time, eyes are placed in growth medium. Eyes do occasionally open in trypsin, prior to the designated incubation time. These eyes are immediately transferred to growth medium for dissection. Growth medium consists of minimal essential medium (GIBCO) containing 20% fetal bovine serum (GIBCO; Grand Island, NY), 1% L-glutamine and antibiotics as above.

Microdissection is, with slight modification, according to Edwards. Eyes are opened by a circumferential incision just below the ora serrata, and the anterior segment and vitreous are discarded. With the aid of a dissecting microscope, the retina is gently lifted off the eyecup and the RPE is peeled off from both the retina and choroid. Regions of RPE, which are not easily dissected off the retina, can be removed by further incubation in growth medium at 37°C for 1–2 hr. The isolated RPE are placed in fresh medium, cleaned of adherent choroidal and retinal fragments, and transferred to a conical 15 ml centrifuge tube.

RPE tissue is rinsed three times with calcium and magnesium-free (CMF), BSS, pH 8.0, and incubated at 37°C in 1.0 ml of 0.1% trypsin in CMF-BSS. Trypsinization times adjusted for rat weight, are 2.5 min for 20–30 g rats, 2.0 min for 10–20 g rats and 1.5 min for 6–9 g rats. After trypsin treatment, RPE tissue is mechanically dissociated into a single cell suspension by gentle trituration using a Pasteur pipet with a fire-polished tip. Three to four milliliters of growth medium are immediately added to stop trypsin activity. The cell suspension is centrifuged for two min at 1000 rpm and the supernatant is discarded. A half ml of growth medium is added for every eight to ten eyes, and the durnell-shaped cells are counted on a hemocytometer. Excellent cell viability and growth result when cells are plated at densities 90 to 160 cells per mm², as shown in Table 1. Lower plating densities often give lowered plating efficiencies. Cells were routinely plated at 30,000 to 35,000 per 18-mm glass coverslip for phagocytic assay and at 20,000 per 13-mm thermanox coverslip (LUX) for scanning electron microscopy and cell counting (Table 1).

Coverslips are placed in tissue culture dishes and RPE cells are diluted to their final concentration just prior to plating, to ensure a more even distribution of cells across each dish or coverslip. Cells should be allowed to settle for a few minutes prior to careful transfer to the incubator to minimize the clustering of cells in the center of the dish or coverslip. Growth medium is carefully added to all dishes containing coverslips after 24 hr of culture. Half of the medium in all cultures is replaced every two to three days. RPE cells plated at the densities suggested in Table 1 appear confluent (no obvious spaces are seen between cells viewed under inverted phase optics) in the most central area of the colony after three to five days in culture.

RPE cells can be subcultured using the same enzyme solutions described for RPE cell isolation from the eye. Cultures are rinsed three times with BSS, incubated for five min at 37°C with collagenase + hyaluronidase in BSS, rinsed three times with CMF-BSS and incubated for 4.5 min with 0.1% trypsin in CMF-BSS. Growth medium is added, and cells are dislodged by trituration, rinsed by centrifugation, and plated in growth medium. This method is unsatisfactory for the quantitation of cell growth, as cell recovery and viability are low after growth in tissue culture.

### Table 1. Suggested plating densities for RPE cells

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Number of cells</th>
<th>Volume of medium</th>
</tr>
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<tbody>
<tr>
<td>60 mm dish</td>
<td>220,000–250,000</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>35 mm dish</td>
<td>85,000–100,000</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>12 mm glass coverslip</td>
<td>37,000–50,000*</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>13 mm thermanox coverslip</td>
<td>20,000</td>
<td>60 μl</td>
</tr>
<tr>
<td>12 mm glass coverslip</td>
<td>17,500</td>
<td>50 μl</td>
</tr>
<tr>
<td>24 mm plate (16 mm)†</td>
<td>20,000*</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>96 well plate (6.5 mm)†</td>
<td>10,000*</td>
<td>0.15 ml</td>
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</table>

* Number of cells per well.
† Diameter of each well.
‡ Cells plated on coverslips were initially plated in a small volume of medium to allow cell attachment to the upper surface. Additional medium was added after 24 hr in vitro.‡
Light Microscopy and Cell Counts

RPE cells were plated at lower than normal density to allow sufficient time in vitro for determination of cell growth parameters. 15,000 cells, isolated from 9–10 day rats were plated in 0.6 ml of growth medium onto each 13-mm theranox coverslip; two to three coverslips were placed in each 35-mm tissue culture dish, and 2.5 ml of growth medium was added to each dish after 24 hr in vitro. Half of the growth medium was replaced every two to three days thereafter. Live cells were photographed with phase optics under a Nikon (Nikon; Tokyo, Japan) Diaphot-TMD inverted microscope. The growth characteristics of nine-day Long Evans (ETEC Corp.; Hayward, CA) and RCS rat RPE cells were determined by fixing RPE cell cultures after 1, 3, 4, 5, 7, 10, 14, 20 and 30 days in vitro. Two coverslips were fixed for each time point by immersion for one hr at room temperature in 2.5% glutaraldehyde in phosphate buffer, pH 7.3. Fixed cells were first stained with a Schiff’s reagent,26 which stains the perinuclear region of RPE cells pink, and then with Mayer hematoxylin,26,27 which stains nuclei blue. The double staining was necessary because normal rat RPE cells, which have been shown to become multinucleate during the first two postnatal weeks in vivo,28 also become multinucleate in vitro. The nuclei of rat RPE cells grown in vitro are not stained by Schiff’s, Giemsa or Crystal Violet, using procedures known to stain nuclei of other cell types.26

The staining procedure used is as follows: coverslips are successively fixed, rinsed and oxidized for ten min in 0.68% aqueous periodic acid; rinsed, stained for 5 min in cold Feulgen reagent; immersed for five min in 0.5% aqueous potassium metabisulfite; rinsed, stained for five min in Mayer hematoxylin, and finally dipped in a saturated aqueous solution of sodium bicarbonate. Stained colonies are dehydrated through xylene, placed on a clean glass slide, and coverslipped with mounting medium. Each coverslip is examined under a Zeiss (Carl Zeiss, Inc.; Oberkochen, West Germany) light microscope, and cells are counted at 156X with an ocular grid, the area being 0.50 mm² per field of view at this magnification. Cell counts are made every 1 mm across two perpendicular diameters of each coverslip, and the two sets of data points are used to determine cell distribution. Cell concentrations are highest in the center of each coverslip and lowest at the edges, especially at early time points.

Data were fit to a seven parameter polynomial of the form: \( y = ax + bx^2 + cx^3 + dx^4 + ex^5 + fx^6 + gx^7 \), using the BMDP statistical software program “PAR” for derivative-free non-linear regression.29 This statistical software package uses an iterative process to get the best least squares fit curve for the observed data. Values predicted from the best fit polynomial curve are then used to estimate the predicted number of cells, \( y_i \), within each 0.5 mm² unit area at various positions \( x_i \) across each coverslip, where \( r \) represents the distance in millimeters of the area counted from the edge of the cell colony. The center of mass and radius of each cell distribution is then determined by the equation: \( \text{radius} = \frac{\sum x_i y_i}{\sum y_i} \). The total number of cells per coverslip is calculated as the sum of each predicted \( y_i \) value multiplied by its radius (its distance from the center of mass of the cell distribution); or the Total Cell Number per coverslip = \( \sum (x_i - \text{radius}) y_i \). The total cell number counted per 0.5 mm² is corrected to counts per 1.0 mm². Average total cell counts, from two coverslips per time point, are fitted to an exponential growth curve of the form: \( n_t = n_{ao} - ae^{-kt} + be^{-ct} \), where \( n_t \) is the number of cells after \( t \) days in culture, \( n_{ao} \) is the number of cells at maximum growth density (the asymptotic cell count), \( a \) and \( b \) are parameters relating to initial cell number, and \( k \) and \( c \) are growth constants. The first part of the equation, \( n_{ao} - ae^{-kt} \), represents the difference between the actual cell count at time \( t \) and the final asymptotic cell count. The second part of the equation, \( be^{-ct} \), expresses any decrease in cell number during the first 24 hr due to less than 100% plating efficiency. The growth constants \( k \) and \( c \) are the most accurate representation of the growth rate. Each coverslip is assigned an arbitrary number prior to counting so that cell counts were unbiased. The goodness of fit of the observed values to the best fit exponential curve is determined by calculating a linear correlation coefficient of the log of the difference of each average observed value from its asymptotic value. Only cell counts from 3 to 30 days are included to eliminate the effect of cell loss during the isolation procedure as described by the term be^{-ct}.

Scanning Electron Microscopy

Confluent RPE cell cultures, on theranox coverslips, were fixed in 4% paraformaldehyde with 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.3 for one hr at room temperature. Coverslips were rinsed in phosphate buffer and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for one hr at 4°C. Fixed cultures were dehydrated through a graded series of ethanol and critical point dried with CO₂. Cells were sputter coated with gold-palladium and viewed using an ETEC scanning electron microscope.

Results

RPE Isolation

The RPE cell layer in the rat adheres firmly to both the choroid and the retina. Isolation of single RPE cells

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Table 2. Intact, enucleated rat eyes are successively incubated in a hyaluronidase and collagenase solution followed by trypsin*

<table>
<thead>
<tr>
<th>Rat age (days)</th>
<th>Av. Rat weight (g)</th>
<th>Hyaluronidase + Collagenase (min)</th>
<th>Trypsin (min)</th>
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<tr>
<td>8</td>
<td>17</td>
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<td>15</td>
<td>21</td>
<td>70</td>
<td>72</td>
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* This table gives suggested enzyme incubation times for average weight rats. These suggested times should be adjusted by 1–2 min for every gram difference between the average weight shown and the actual weight of the rat, larger rats requiring longer incubation times.

requires preincubation of intact eyes in two consecutive enzyme solutions, the first containing hyaluronidase and collagenase and the second containing 0.1% trypsin. Optimal incubation times were determined by incubating whole eyes, from 163 rats, for 30–90 min in each enzyme solution. Table 2 lists those times found optimal for maximum recovery of RPE cells from 8 to 15-day-old rats. Rat weight was also found to affect optimum incubation time, heavier rats requiring longer incubations in both enzyme solutions.

Table 2 demonstrates that optimal hyaluronidase + collagenase incubation times increase with every day of postnatal development, while trypsin incubation times vary only slightly for 8–12-day rats, but increase significantly at 14 days of age. The optimal trypsin incubation time varied appreciably throughout the year of experimental testing. This variability could not be accounted for by rat weight, size or diet. Optimal hyaluronidase + collagenase incubation times also varied due to loss of enzyme activity, hyaluronidase being labile during frozen, dessicated storage.

The average RPE cell yield from 50 dissections of 9–10 day old rats, was 29,200 ± 8,800 cells/eye. Higher yields of up to 60,000 cells/eye were obtained when extreme care was taken during dissection to obtain maximum cell yields and when adherent RPE was released by reincubating the retina for two hr in growth medium at 37°C. Cell viability, assayed by trypan blue exclusion, averaged 95%, and plating efficiency was in the range of 95–100%. These consistently high cell yields and excellent cell viability are an improvement over our previous yields of 18,400 ± 5,500 cells/eye using previously published techniques.23

Both normal and RCS rat RPE have been successfully isolated using the procedures just described. The technique gives consistently high cell yields and excellent cell viability for rats up to 15 days of age, the age at which light activated ROS disc shedding has begun in vivo.12 RPE cells have been isolated from 18 to 20-day postnatal rats, but cell yields are usually low and adherent retinal tissue is difficult to remove. Cells isolated by these procedures also show consistently excellent phagocytosis of freshly isolated rat ROS.19 Preliminary studies indicate that RPE cells maintain normal phagocytic abilities after 1 month in primary culture.

RPE Cell Culture and Growth Characteristics

RPE cells are dumbell shaped at isolation (Fig. 1).23 Figure 2 shows the growth of normal (Long Evans)

![Fig. 1. Freshly isolated normal and retinal dystrophic RPE cells have a characteristic dumbell shape. Normal RPE cells are shown on a hemocytometer at 95X (Fig. 1A) and at 38X (Fig. 1B).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933120/ on 04/09/2018)
Fig. 2. Normal (LE) and retinal dystrophic (RCS) RPE cells from 10-day-old rats were plated at 15,000 cells per coverslip and photographed with inverted phase optics. All magnifications are 85X. (A-D): LE cells after 1 day (A), 3 days (B), 5 days (C) and 7 days (D) in culture. (E-H): RCS cells after 1 day (E), 3 days (F), 5 days (G) and 7 days (H) in culture.
(Fig. 2a–d) and retinal dystrophic (RCS) (Fig. 2e–h) RPE cells isolated from 10-day-old rats, plated at low density (15,000 cells per 13-mm thermapan coverslip) and grown in culture for 1, 3, 5, and 7 days. The two genotypes show similar morphologies. At 24 hr both genotypes show adherent pigmented RPE cells scattered across the coverslip (Fig. 2a, c). Cell numbers have increased significantly by three days, the center of each coverslip showing confluent regions of contiguous cells interspersed among less dense regions (Fig. 2b, f). By 5 days of culture, most of the coverslip is covered with a continuous sheet of cells, only the most peripheral regions showing obvious intercellular spaces (Fig. 2c, g). At seven days, a sheet of tightly packed RPE cells covers the coverslip (Fig. 2d, h).

Figures 3 and 4 show the growth of RPE cells isolated from 10-day old normal (LE) rats, plated at 20,000 cells per 13-mm coverslip and grown in monolayer culture. Figure 3 compares the morphology and pigmentation of RPE cells from a single dissection which are either grown in primary culture (Fig. 3a–d) or subcultured after 3 days (Fig. 3e–h). Cells were photographed at 2 and 2 wk of culture with and without phase optics to demonstrate both cell morphology (Fig. 3a, 3c, 3e, 3g) and pigmentation (Fig. 3b, 3d, 3f, 3h), respectively. Primary RPE cells show a characteristic polygonal morphology and increased cell packing density from 2 to 4 wk in culture, with no apparent dilution in pigmentation (Fig. 3a–d). Subcultured RPE cells show a more flattened morphology with a lower cell packing density (Fig. 3e–h), despite the increased number of cells plated (three coverslips are subcultured onto two coverslips after three days in vitro). Thus, this subculturing technique is not optimal, for cell numbers are not expanded and cell morphology is altered.

The inability of trypsin alone to release cells from their substrate is probably due to the large amount of extracellular matrix elaborated by these cells in vitro. Figure 4 is a scanning electron micrograph showing extensive extracellular matrix deposition at the edge of a normal confluent RPE cell culture. Figure 5 shows the typical polygonal arrangement of RPE cells in the center of a primary culture that has just reached confluency. These cells are tightly packed and contain numerous short apical microvilli. RPE cells occasionally arrange themselves in other configurations, such as the rosette-like structure shown in Figure 6. Rosette formation is characteristic of dissociated embryonic cells which are attempting to reorganize and differentiate in vitro.30,31

Figure 7 shows the change in pigmentation in a single culture from 1 day to 3 months of growth in culture. Cell-packing density is highest in the center of the coverslip (Fig. 7a), due to surface tension at the edge and to the high density of the pigment granules which causes cells to swirl centrally during plating. RPE cells fail to redistribute, maintaining their original asymmetric distribution throughout the culture period. Figures 7b–e show cells in the central, most confluent region of a single cell culture at 1, 2, 3, and 4 wk in vitro. These cells show a gradual increase in pigmentation during their first month in vitro. Pigmentation continues to increase from 1 to 3 months of culture. Figure 7f shows an exceptionally well pigmented 3-month-old culture with foci of heavily pigmented RPE cells interspersed against a background of lighter pigmentation. Pigmented RPE cells have been maintained in primary culture for 6 months.

Growth curves were determined for both genotypes. Due to the inadequacy of the subculturing technique, accurate cell counting necessitated fixing, staining and counting cells in situ. Figure 8 shows the best fit growth curves for normal and retinal dystrophic rat RPE cells. The average observed values for total cell number per coverslip are shown for both genotypes after 0–30 days in vitro. Both exponential curves are excellent fits to the observed average cell counts, correlation coefficients being r = 0.954 for normal (LE) RPE cells and r = .959 for retinal dystrophic (RCS) RPE cells. The curves shown in Figure 8 each consist of three parts: (1) an initial decay or drop in cell number in the first 24 hr of culture, (2) an exponential growth phase, and (3) a final leveling off at the maximum cell count by 30 days in culture. The initial drop in cell number is not accurately estimated by the curve, the fit being least meaningful in this region. This inaccuracy is due both to the lack of data points between 0 and 1 days of culture to the fact that small errors in initial cell counts become noticeable when cells are diluted for plating. The apparent lower than normal cell viability during the first day of culture may in part be due to the low density at which cells are plated (15,000 rather than the suggested 20,000). A low plating density was used so that more time points on the growth curve could be measured for better estimation of growth dynamics. Both genotypes showed an eventual cessation of growth at approximately 67,000–70,000 cells per coverslip, due to contact inhibition.

The value k, of the growth curve equation, represents the best estimate of the rate of cell growth in the middle of the curve, or k is proportional to the normalized slope of the growth curve halfway between the minimum and maximum cell counts. The absolute value of k is also inversely proportional to the number of days required for the culture to increase its cell number by e (or 2.718) times its original number, not taking other factors into consideration such as the initial decay and the eventual leveling off of cell numbers. The growth constants for the curves shown in Figure 8 are k = −0.0813 ± 0.035 for retinal dystrophic (RCS) and...
Fig. 3. RPE cells, from 10-day-old LE rats, were plated at 20,000 cells per coverslip, grown for 2 and 4 wk in vitro, and photographed with and without the phase ring. All magnifications are 85X. (A-D) are primary cultures: (A) 2 wk, phase in; (B) 2 wk, phase out; (C) 4 wk, phase in; (D) 4 wk, phase out. (E-H) are subcultured cells: (E) 2 wk, phase in; (F) 2 wk, phase out; (G) 4 wk, phase in; (H) 4 wk, phase out.
Fig. 4. Scanning electron microscopy shows extensive extracellular matrix deposition at the edge of a normal RPE cell monolayer after 5 days in culture (2560X).

\[ k = -0.2216 \pm 0.091 \] for normal (LE) RPE cells. A previous experiment (data not shown) gave very similar growth constants of \( k = -0.0822 \pm 0.074 \) for retinal dystrophic and \( k = 0.1778 \pm 0.071 \) for normal RPE cells. Although this previous experiment had fewer time points than the data presented in Figure 8, the reproducibility of these growth constants suggests that the two genotypes do grow at different rates. The ratio between the mean values of \( k \) indicate that normal RPE cells grow 2.5X faster than retinal dystrophic RPE cells during the logarithmic phase of the growth curve. A statistical analysis of the difference between the growth curves of the two genotypes indicates that the standard error of the combined populations is 1.46, indicating that the hypothesis that the two genotypes show different growth rates has a confidence level of 0.86. Thus, RCS and LE RPE cells do show different growth rates, but the difference is not highly significant and must be interpreted with caution.

**Discussion**

Extracellular matrix materials, including collagen and hyaluronate, separate the major tissue layers of the eye. Retinal pigment epithelial cells adhere to the matrix materials which they secrete from their basal surface, both in vivo and in vitro. The apical surface of these cells contains numerous microvilli which, in vivo, are coated with an interphotoreceptor matrix. Rat RPE cells, in vitro, secrete a fibrous mat of matrix materials from the basal surface within the first two days of culture. RPE cell-matrix adhesions are quite strong, in vitro, for they are not disrupted by treatment with non-ionic detergent, or by mild trypsinization.

The RPE cannot be removed from freshly enucleated eyes which are untreated or treated with trypsin alone. Edwards first successfully removed RPE from intact rat eyes by soaking the eyes for 6–24 hr in BSS prior to trypsinization. Cell detachment was achieved, but with moderate cell yields and viability in our labora-

Fig. 5. The typical polygonal arrangement of RPE cells in the center of a primary normal RPE cell culture. 800X.

Fig. 6. RPE cells occasionally form rosette-like cell arrangements in primary culture. Normal RPE cells are shown at 400X.
Since testicular hyaluronidase has been shown to weaken substratum adhesions for RPE and other cells in vitro, \cite{32,33,39} we chose to pretreat freshly enucleated rat eyes with the two most readily available degradative enzymes for extracellular matrix, hyaluronidase and collagenase. This pretreatment, followed by a trypsin incubation, releases sheets of RPE which are easily dissociated into a single cell suspension by a subsequent short trypsinization. This new technique gives high cell yields, high cell viability, and excellent plating efficiency. RPE cells can be subcultured by incubation in hyaluronidase + collagenase, followed by trypsin. However, the total cell yield, as well as the viability of these cells is low. Since trypsin treatment alone does not disrupt cell-matrix adhesions in vitro,\cite{23} it is suggested that rat RPE cells contain membrane proteins, or produce matrix components, which are insensitive or inaccessible to trypsin and which function in cell-substrate adhesion.

Optimum incubation times in hyaluronidase + collagenase, and to a lesser extent in trypsin, were found to be age dependent in 8-15 day old rats, eyes from older rats requiring longer incubation times. Thus, the amount of extracellular matrix material beneath the RPE appears to increase with increased developmental age in vivo. Extracellular matrix deposition also occurs.
Fig. 8. Growth curves for normal and RCS RPE cells plated at 15,000 cells per 13-mm thermoplastic coverslip and grown in culture for 1 to 30 days. Cell monolayers were rinsed, fixed and stained with Schiff's reagent and Mayer's hematoxylin. Cells were counted within a 0.5-mm$^2$ grid every mm across two perpendicular axes of each coverslip. Cell counts from each coverslip were fit to a polynomial curve and the total cell count per coverslip was calculated. Each data point shown represents the total cell count from a single coverslip. The average counts from two coverslips were used to plot the best fit exponential growth curves for each genotype.

in vitro, at the basal surface of RPE cells. The large amount of matrix material secreted by these cells in vitro make it impossible to detach enough viable RPE cells to do accurate cell counting, even after pretreatment with hyaluronidase + collagenase and trypsin.

While LaVail and coworkers$^{40}$ reported that RCS rats show altered interphotoreceptor matrix at the apical surface of RPE cells, we observed no such differences in extracellular matrix at the basal RPE cell surface. RCS RPE cells in vitro showed the same degree of metachromatic staining and are isolated using the same times and techniques developed for normal RPE cells. The two genotypes also display similar morphologies in vitro.

RCS and normal RPE cells show different growth characteristics in vitro. Normal and RCS RPE cells plated at the same initial density reach the same maximum cell number but with different growth kinetics, normal (LE) cells growing faster than mutant cells. The growth rates ($k$) determined for LE and RCS cells, confirmed in two independent experiments, gave cell doubling times of 3.5 days for normal and 9.0 days for RCS RPE cells in primary culture. However, these results must be interpreted with caution due to the multiple statistical analyses necessary to determine growth rates from the raw data and the confidence level of 0.86 that the two populations show different growth rates. Also, even if the difference in growth rates is real, the interpretation of this difference is difficult since RCS and LE rats are not congenic strains.

Both LE and RCS have relatively long doubling times, being much slower than the one to two-day doubling times of most cell lines,$^{41}$ the 32 hr reported for human RPE cells,$^{20}$ or the 12-hr doubling time of neural crest derived pigment cells$^{42}$ in vitro. The slow doubling times of RPE cells could simply mean that only a small proportion of the cells are actually dividing. The latter is most likely since embryonic neuronal cells, prior to terminal differentiation, have short doubling times of eight to ten hr.$^{43-45}$

Normal RPE cells are characterized by their highly specific phagocytosis of rat rod outer segments, both in vivo and in vitro.$^{7,8,12,14,18,19}$ The culture technique presented in this paper yields normal RPE cells which show excellent binding and phagocytosis of rod outer segments in vitro.$^{19}$ This phagocytic process is defective in the RCS rat and this defect is maintained in vitro using this culture technique.

Key words: retinal pigment epithelium, retinal dystrophy, RCS rat, extracellular matrix, cell culture

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References