IN VITRO BIOLOGY OF CORNEAL EPITHELIUM AND ENDOTHELIUM*

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THIS THESIS OUTLINES THE *IN VITRO* PHENOMENA OF CELLULAR CONTACT INHIBITION as it pertains to the corneal epithelium and endothelium. Additionally, certain aspects of *in vitro* corneal epithelial and endothelial biology, especially cellular migration and relationships to substrata, are explored. Such a study may be expected to provide a more solid foundation for the formulation of principles in some aspects of corneal wound healing.

HISTORICAL REVIEW

CONTACT INHIBITION AND CELL LOCOMOTION

Abercrombie and Heaysman,[1,2] building on earlier work by Willmer,[3] Holtfreter,[4,5] Abercrombie and co-workers,[6] Weiss,[7,8] and Twitty,[9] elucidated the features of contact inhibition using chick heart fibroblasts cultured in liquid medium. They noted a significant inverse relationship between the rate of movement of a cell and the number of other cells with which it came in contact during the observed movement. Fibroblasts avoided moving over each other's surface due to contact inhibition, causing the cells to form a monolayer and to migrate predominantly radially from an explant, resulting in a circular growth pattern. Outgrowth into an area ceased after junction had been established between opposite sheets of cells (that is, cells from another explant coming from an opposite direction). Later, Abercrombie and Ambrose[10] showed by time-lapse microcinemaphotography that fibroblasts moved on a plane surface with their leading pole modified to form a thin ruffled membrane. When a ruffled membrane of one fibroblast touched any part of the peripheral surface of another fibroblast, the ruffling stopped and movement of the cell in the direction of the membrane ceased.

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Martz and Steinberg[11] described six (and perhaps seven) cellular contact inhibitions: inhibition of overlapping (which results in monolayering); of colony expansion; of cell speed (nuclear translocation); of ruffling; of orthogonal movement (proposed to explain spontaneous parallel alignment of cells); of neighbor exchanges; and perhaps of cell division (more operationally termed postconfluence inhibition of cell division). They felt that only the first three phenomena should be termed contact inhibition, because only those three had evidence eliminating action-at-a-distance. They further pointed out that contact inhibition of overlapping is not displayed by invasive neoplastic cells with respect to normal cells, so that invasive cells overlap freely upon normal cells, although not necessarily upon one another.

Contact inhibition of movement has been demonstrated between cells of different types, that is, ectodermal cell (corneal epithelium) and mesodermal cell (corneal endothelium).[12] This contact inhibition found in rabbits also has been found in dogs.[13] Similar findings have been shown between dermal fibroblasts meeting an epidermal epithelial sheet[14,15,16] and between mouse sarcoma cells and coherent endothelial sheets[17] so that monolayering was maintained with negligible nuclear overlap.

The expression of the phenomenon of contact inhibition is not absolute and may be modified readily by environmental factors. For example, using chick fibroblasts, Carter[18] showed that contact inhibition of movement *in vitro* could be modified by changing the physical properties of the substratum to which the cells were attached. As pointed out above, neoplastic (sarcoma) cells were not inhibited by normal cells.[17,19,20,21] Oldfield[22] observed that normal leukocytes were able to move over normal heart fibroblast-like cells and had a high nuclear overlap on the latter. Leukocytes, while not malignant, normally are invasive *in vivo*. Ceccarin and Eagle[23] showed that alterations of pH *in vitro* modify greatly the expression of contact inhibition. Also, Arya and associates[24] demonstrated that the addition of lysosomal preparations to cultured rabbit corneal endothelial cells caused multilayering of the cells, seemingly releasing the endothelial cells from contact inhibition.

Contact inhibition necessarily must be an important factor in determining the extent and direction of cellular locomotion. For example, as pointed out above, contact inhibition resulted in monolayering and in a circular growth pattern. Weiss[25] has reviewed the guiding principles of cell interactions and cell locomotion. He pointed out that locomotion has two components: motion or motility, that is, the power to move; and dislocation, that is, a shift from one place to another. Motion can take

place within a cell without the cell actually moving, and, conversely, a cell can be displaced without motility, as in the case of passive convection by an external force. A living cell suspended in fluid is essentially spherical. When a cell meets an interphase, it tends to flatten out to a disc shape due to a protoplasmic contractile wave which courses mainly in the plane of flattening, giving rise to ruffling or undulation of its "membrane." Metabolic energy is required to produce contractile waves, which tend to be rhythmic and coordinated. Active extension of the cell, that is, formation of pseudopodia, requires some adhesion between the peripheral fringe of the cell and an appropriate substratum. The shape of the cell depends on the differential adhesion between cell and substratum around the free margin of the cell, that is, equal adhesion all around produces a round, flat cell, whereas a cell with only two adhesion points between itself and the substratum becomes axially elongated into a bipolar, spindle cell. A cell then can be motile and change its shape, but locomotion of the cell only results if its center is in progressive translocation; if the translocation follows a steady course in a given direction, it is oriented (otherwise the locomotion is random). In a freely mobile cell, that part of the mobile fringe which comes in contact with another cell ceases to extend activity, that is, contact inhibition. Thus, if epithelial or endothelial cells make contact, they tend to form attachment sites and to grow out with a uniform front; whereas fibroblastic cells do not form attachment sites and tend to grow out away from each other not forming a uniform front.

When diploid fibroblasts are grown in culture, growth at first is logarithmic. As the density of the cell sheet increases so that confluency (complete cell to cell contact) is approached, the rate of cell division decreases markedly and thereafter continues at a rate sufficient only to maintain the total cell population.[26,27,28,29,30] Raff and Houck[31] initiated rapid cell proliferation (after about a two-hour lag period) by scraping a hole in human fibroblast confluent monolayers. When the cells approached confluency (about 4 days) so as to cover the "miniwound," the growth rate decreased. Small amounts of undialyzed or dialyzed serum were essential for cell division but not for cell migration. Pardee[32] proposed that the cell surface membrane was the controlling factor in deciding whether a cell will continue to proliferate or will go into the resting state. Interference with the normal regular periodic changes of the surface membrane (which occur during the cell division cycle) by cell-cell surface contacts prevented the normal progression of events and thereby changed the metabolic pattern so as to put the cells into a nonproliferative state. Middleton[33] demonstrated contact inhibition of loco-

motion in chick embryo pigment epithelium.

Abercrombie[34] has proposed several theories to explain why cells fail to move over one another's upper surfaces. One cell may act as a simple mechanical obstacle to the continued movement of the other. Firm, lateral, intercellular adhesions[35-37] or the passage of a signal between cells upon lateral contact, or both, might arrest locally locomotory surface activity and, consequently, cell movement. The adhesion of a cell to the inanimate substratum may be relatively stronger than its adhesion to the upper surface of another cell, with the result that cells preferentially adhere to the former.[11,18,38,39] Also, it is possible that the upper cell surface is totally nonadhesive, in contrast to the lateral edges, preventing other cells from adhering to it and moving on it. DiPasquale and Bell[40] suggested that the inability of the upper cell surface of cultured epithelial cells and fibroblasts to support spreading may be a general phenomenon. They note that inert particles and cell processes do not adhere directly to the upper cell surface but can initiate adhesions to the surface of a cell's free margin, demonstrating a variation of adhesive properties over a cell's surface. Human epidermis,[41] however, as well as many other epithelial cells cultured *in vitro*,[12,13] grow in a stratified, multilayered manner.

As pointed out above, cellular locomotion depends upon adhesion of the cells to the substratum. Weiss[42] has demonstrated that certain cells adhere to certain substrata and not to others. In addition, by altering the medium, that is, adding serum, he could make trypsinized cells adhere to substrata which they would not adhere to without serum in the medium. Khodadoust and associates[43,44] have shown that regenerating corneal epithelium requires an underlying basement membrane to establish a tight adhesion to the underlying tissue. Flaxman and co-workers,[41] Abercrombie and Heaysman,[2] and Carter[18] have noted that the behavior of cells *in vitro* is regulated by the strength of the attachments of cells to each other and to the substratum upon which they grow. Although many external factors have been known to influence attachments of cells to various substrata,[45] the actual morphologic observations of the attachment zone were not described until the report in 1968 by Flaxman and co-workers.[41] They showed, using human epidermal basal cells and human fibroblasts, that an extracellular material is present at the cell-substratum interface. The material seemed to implement the attachment of cells to the underlying substratum. In their study, the epithelial cells grew *in vitro* as a multilayered epithelial outgrowth, whereas the fibroblasts grew as a monolayer.

As cells move *in vitro*, cell-to-cell contact is made. With contact,

electronic coupling occurs.[46-49] Although dividing cells (*in vitro*) appear to lose intimate contact with surrounding interphase cells when examined by light microscopy, O'Lague and co-workers,[50] have shown in fibroblasts *in vitro*, and Cavoto and Flaxman[51] have shown in epithelial cells *in vitro* that electronic coupling persists. Electron microscopic observations[50] have shown that thin protoplasmic connections exist between dividing and interphase fibroblasts. These points of contact between the cells could be the sites of persistent gap junctions which appear to mediate electrical coupling between interphase cells. Cavoto and Flaxman[51] concluded that dividing epidermal cells become less "tightly" attached to their substratum and to their neighbors, but that total dissolution of contacts did not occur so that low-resistance pathways for the passage of electric current persisted during metaphase and telophase.

Locomotion of cells in tissue culture not only is dependent upon adhesion of the cells to the substratum and upon contact with other cells but also upon the nature of the substratum and the medium. For example, in 1918 Matsumoto[52] demonstrated that the corneal epithelium of the frog showed different types of locomotion in plasma, over solid supports and over fiberlike supports. Nohira[53] observed that frog corneal epithelium, cultivated in serum, grew differently along the connective-tissue plate of the cornea than when it left the cornea and grew into the medium. Takeuchi[54,55] showed that injured chick corneal stromal wound bed would not serve as a substratum for chick corneal epithelium of young embryos but would serve as a suitable substratum for chick corneal epithelium of old embryos. Adding colcemid (colchicine)[56,57] to the culture medium inhibited cell migration but not cell mobilization, whereas adding chloroquine to the medium[57] inhibited cell mobilization but not cell migration. Conversely, alpha-chymotrypsin[58] and epidermal growth factor[59,60] stimulated proliferation of cells.

CORNEAL EPITHELIAL OUTGROWTHS

In 1939, Thygeson, wishing to study the effects of viruses on epithelium, wrote that a review of the literature failed to disclose adequate data on the cultivation of human conjunctival and corneal epithelium.[61] He tried plasma-clot, semifluid, and fluid techniques. He found that in the plasma-clot technique a slowly developing epithelial growth, preceded by a migration of wandering cells (not further described), was usually visible after 24 hours incubation; fibroblasts began to appear in about 48 hours and eventually overran the epithelial growths. The semifluid technique yielded a high percentage of epithelial growths (29 of 38 attempts) with a rate of growth considerably more rapid than in the plasma-clot technique.

He had poor success with the fluid technique. He stated that he was able to obtain a pure strain of corneal epithelium from an excised bulla of an eye with bullous keratopathy.

Fowle and Ormsby[62] cultured human and monkey corneal epithelium on a shallow plasma clot covered with a fluid medium. They were able to obtain pure cultures of human corneal epithelium by removing the original explant of tissue before fibroblasts appeared. In addition, by using trypsin, they were able to subculture monkey corneal epithelium. In 1958, Stocker and co-workers[63] developed a technique of separating the cornea into epithelial, stromal, and endothelial layers and culturing them *in vitro*. Smith and co-workers[64] used a modification of Stocker's technique to cultivate rabbit corneal epithelial cells. In 1964, Favata[65] cultured guinea pig corneal epithelium. Adachi and Pollack[66,67] cultured rabbit corneal epithelial and fibroblastic cells. They found that the epithelial cells appeared earlier and disappeared earlier than fibroblasts, which gradually replaced the epithelial cells. By studying many enzymatic reactions (acid and alkaline phosphatases; adenosis triphosphatase; lipase; monamine oxidase; lactic, malic, and succinic dehydrogenases; and cytochrome oxidase), they found that the corneal epithelial cells were, by and large, stronger in enzymatic reactions than corneal fibroblasts.

Sugiura and Kuwanabe[68] showed that cultured rabbit corneal superficial explants could grow out not only epithelial cells and fibroblasts but also dihydroxy-phenylalanine (DOPA) positive polygonal cells identified as melanocytes. Sarker and associates[69] succeeded in establishing a line of epithelial cells from the cornea of a bull. They had failed previously, with repeated attempts, to continuously subculture rabbit and human corneal epithelium, although they had considerable success with corneal stroma and endothelium. They found that the corneal epithelium retained its epithelial morphology through 13 passages (265 days) but then started to look somewhat fibroblastic.

In 1968, Bülow and Ehlers[70] cultured rabbit and guinea pig corneal epithelium. They found that proliferative activity was intense in very young cultures two to four days old. Five to ten days after explantation proliferation was evident as a spotwise, scattered, rapid phenomenon, which gradually ceased. After two weeks in culture, no proliferation was noted. They also reported a non-enzymatic blackening of cultured cells by incubation with DOPA. In 1970, Ehlers[71] performed a comparative study on mammalian corneal epithelium (non-tissue culture study). In his discussion, he noted that although cells of different morphology were seen in the epithelium, no proof of the existence of several functionally

different cell systems had been given in the literature or in his studies. Ellingson and Yao,[72] using a double Rose chamber technique, cultured epithelium from rats, kangaroos, opossums, Chinese hamsters, albino rabbits, albino rats, and rhesus monkeys. Newsome and co-workers[73] established pure populations of human epithelial cells *in vitro*. Even after multiple serial passages, the cells retained their typical structural specializations. Further, they gave evidence for the presence of HL-A antigens on cultured epithelial cells.

Although most studies of cultured corneal epithelium have failed to demonstrate different biologic functions, recent work suggests that corneal epithelium may synthesize collagen. Dodson and Hay[74] demonstrated that embryonic chick corneal epithelium secreted ^{3}H-proline into the substratum. By electron microscopy they showed that the radioactivity was associated with striated fibrils and sheets that had the repeat period and interband pattern typical of native collagen. Gnädinger[75] showed that the regenerating epithelium of adult rabbit corneas was capable of synthesizing precursors (hydroxyproline) of collagen. They stated that the question remained whether the synthesis of hydroxyproline-containing peptides served only to rebuid the epithelial basement membrane or also contributed to the reformation of stromal collagen. Hay and Revel[76] have demonstrated fibrillogenesis by epithelial cells in developing avian cornea. Luenberger and co-workers[77] suggested that regenerating epithelium of the rabbit cornea synthesized both collagenoid proteins for the rebuilding of the basement membrane and native collagen fibrils. Gnädinger and Luenberger[78] demonstrated that cultured corneal epithelial cells of adult mammalians, which were not influenced by living mesenchymal cells, had a genetic code for producing different kinds of collagen: collagenoid proteins to rebuild the basement membrane and native collagen fibrils. This might play an important role in the healing of corneal wounds.

Dohlman[79] has reviewed the function of corneal epithelium in health and disease. He pointed out the role of the epithelium in stromal ulceration, particularly the elaboration of collagenase by damaged epithelial cells. Slansky and co-workers[80,81] showed that cultured epithelium from human corneal ulcers released collagenase. Fetal calf and chick serum was shown to inhibit mammalian collagenase, but rabbit and human serum did not.[82] Increased lysis of collagen gels by tissue from alkali-burned corneas was noted.[83] Brown and co-workers[84] investigated collagenolysis activity of alkali-burned corneas using a tissue-culture assay technique. The study showed substantial collagenase production from tissues removed from the immediate vicinity of the corneal ulcers or perforations.

Brown and co-workers[85] have demonstrated that intralamellar corneal junctions of small quantities of harvested collagenase from the ulcerated tissues of alkali-burned corneas produced full-thickness ulcers in intact alkali-burned corneas, but had no noticeable tissue destruction after intralamellar injection into normal corneas. Brown and Weller[86] showed that the known *in vitro* inhibitors of collagenase, cysteine and sodium edetic acid, but not calcium edetic acid, were effective in preventing ulcers in alkali-burned corneas. In order for corneal epithelium to produce collagenase, the stroma had to be injured as well.[87-90] If one damaged the periphery of the cornea, increased collagenase synthesis took place by normal cells at the central cornea.[91] Corneal collagenolysis was augmented by hydrocortisone or dexamethasone,[87] but was inhibited by chelating agents, including cysteine and histidine,[92] and by D-penicillamine.[93] Harris and Krane[94] have recently reviewed the entire subject of the collagenases. It is interesting that collagenase obtained from skin wound margins is released by both epithelium and mesenchymal (granulation) tissue.[95] The collagenases obtained from epithelium and mesenchymal cells had somewhat different properties.[96] Mesenchymal cell collagenase had a broader pH optimum (6.7 to 7.2) and was inhibited by cysteine, whereas activity of the epithelial collagenase actually was enhanced by 0.1 M cysteine, a most unusual finding for mammalian collagenases.

The effect on corneal epithelium by adding drugs to the tissue culture medium has been studied. Weimar and Fellman[57] showed that chloroquine inhibited cell mobilization but not cell migration; conversely, colchicine inhibited cell migration but not cell mobilization. Epidermal growth factor[59,60] and alpha-chymotrypsin[58] were shown to stimulate epithelial growth. Krejci and Harrison[97,98] showed that epinephrine caused degenerative changes in cultured rabbit, dog, and human corneal epithelium within 48 hours. They further showed in rabbit and human cultured corneas that the strongest cytotoxic effects were produced by neostigmine bromide and carbachol; epinephrine and echothiophate iodide were slightly less damaging; intermediate degrees of toxicity were shown by guanethidine sulfate and a preparation of pilocarpine hydrochloride with epinephrine; demacarium bromide and pilocarpine were least toxic. Krejci and Krejcova[99] showed that the combined effects of topical corticosteroids and various antiglaucoma drugs indicated significantly reduced resistance to damage of cultured corneal epithelial cells, so that the cells showed more damage than when corticosteroids were not used. Weimar[100] showed that uncouplers of oxidative phosphorylation, oxidase inhibitors, sulfhydryl group inhibitors, fluorides, and several types of nitrophenols had no effect on the uptake of neutral

red by corneal epithelial cells. The uptake was inhibited by low temperatures, caffeine, and urethane. Evidence was presented which suggested that the neutral red was taken up by lysosomes of both cell types. Weimar and Haraguchi[101] showed that riboflavin derivaties, which have many structural similarities to neutral red, inhibited neutral red uptake by normal corneal epithelial cells.

Additionally, Gnädinger and associates[102] have shown that cultivated and abraded epithelial cells from rabbit cornea yielded homogenates which hydrolysed acetylcholine. The material responsible for splitting acetylcholine most likely was acetylcholine esterase as described by Peterson and associates.[103] Finally, Manski and Whiteside[104] demonstrated that corneal cell culture (epithelium and endothelium) apparently involves an unmasking of metabolic dependent antigens.

CORNEAL ENDOTHELIAL OUTGROWTHS

Binder and Binder,[105] studying regeneration of the corneal endothelium by analyzing flat mounts of corneal endothelium, suggested that amitotic but not mitotic division was present in the normal rabbit endothelium and that amitotic and mitotic division were present in regenerating rabbit endothelium. The concept that the cells of the normal endothelium do not divide by mitosis had been noted by many authors including Ballowitz[106-108] Nagano,[109] Cogan,[110] Stocker,[111] and Thomas.[112] In 1961, however, von Sallman and associates[113] refuted the conclusion that no mitosis occurred in the endothelium of the growing eye. They showed that in the rabbit corneal growth is accompanied by mitosis in the endothelium, predominantly in the peripheral portion. The mitotic index decreased with age and approached a value of zero in the full-grown animal. Oh and colleagues[114] confirmed von Sallman's work.

Matsui[115] in 1929 first cultured corneal endothelium (from rabbit). In 1958, Stocker and co-workers[63] devised a technique of separating mechanically the cornea into endothelial, stromal, and epithelial layers. In 1962, Sarker and co-workers[116] for the first time continuously subcultured corneal endothelium from the rabbit. In 1963, Smith and associates[64] also were able to subculture rabbit corneal endothelium. Slick and associates[117] used trypsin and a modified Rose chamber to culture rabbit corneal endothelial cells. In 1965, Mannagh and Irvine[118] devised a technique for culture of human corneal endothelium. Lowry[119] found that a rapid outgrowth, high mitotic rate, and monolayer covering of the growth surface with polygonal cells were characteristic of rabbit corneal endothelial explant cultures. Electron microscopy of the cultured cells showed well-developed desmosomes and intracellular fibrillar sys-

tems not present in freshly excised endothelium; the structures were considered to be adaptations to growth in a three-dimensional substrate. Cultured cells contained increased amounts of glycogen and decreased rough-surface endoplasmic reticulum and mitochrondria in comparison with freshly fixed tissue. Histochemical reactions specific for lactic dehydrogenase demonstrated high activity in cultured cells but not in freshly excised endothelium. No mention of a newly formed basement membrane (Descemet's) was made.[119] Newsome and associates[73] established pure populations of human endothelial cells *in vitro*. Even after multiple serial passages, the cells retained their typical structural specializations. Further, they gave evidence for the continued presence of HL-A antigens on cultured endothelial cells. In 1974, Perlman and Baum[120] demonstrated a tissue culture method for the mass production of rabbit corneal endothelium. With this technique, sufficient corneal endothelial cells could be obtained for biochemical investigation.

Very little is known about the biosynthesis of Descemet's membrane.[121] Perlman and Baum[122] observed synthesis of a basement membrane in monolayer cultures of rabbit corneal endothelium. The membrane resembled Descemet's in appearance, staining properties, and collagen content. Cameron and associates[123] showed incorporation of ^{14}C proline and ^{14}C lysine *in vitro* into basement membrane collagen by Descemet's membranes isolated from rabbit corneas. The size of the ^{14}C collagen was estimated by gel filtration in agarose-SDS after reduction with mercaptoethanol and found to be consistent with the size of basement membrane collagen precursors synthesized by other cell types.

Whether or not endothelium can convert to a fibroblastic-like tissue has been investigated. Morton and co-workers[124] and Brown and Kitano[125] suggested that a denuded posterior corneal area is first overgrown by endothelium which forms a fusiform scar. In 1970, Inomota and associates[126] showed that with good apposition in normal corneal grafts, the scar area was covered by endothelial cells which formed a new Descemet's membrane. If apposition was poor, multiple layers of endothelium sometimes formed. Michaels and co-workers[127] produced retrocorneal fibrous membranes experimentally in rabbits without disrupting Descemet's membrane. Their light and electron microscopy suggested stongly that the cellular and extracellular components of the retrocorneal fibrous membrane were derived from metaplastic endothelium. Fine and Yanoff[128] pointed out that endothelium is really mesothelium. Mesothelia frequently are considered capable of readily undergoing alteration into fibroblasts. In 1973, Matsuda and Smelser[129] showed that in healing central rabbit corneal wounds, at the posterior part of the incision,

endothelium proliferated and formed a membranous tissue which produced both normal-appearing collagen fibers and Descemet's membrane-like material.

WHOLE CORNEAL (ORGAN) CULTURES

Cintron[130] demonstrated that whole chick embryonic cornea could best be maintained as an organ culture if serum was used as part of the nutrient media. Summerlin and collagues[131] showed that human, chicken, rabbit, and guinea pig corneas could be maintained, and remain clear, as an organ culture up to one month. They suggested that these corneas could be used as allografts (rabbit to rabbit) and xenografts (chicken to rabbit), demonstrating viability of such stored corneas as well as antigenic modification. Benezra and Sachs,[132] however, were unable to confirm the findings of Summerlin and colleagues. Doughman and associates[133] in an electron microscopic study of the endothelial cells of the human organ cultured cornea showed that the cells form a complete covering over Descemet's membrane and maintain their ultrastructural integrity.

Cameron and associates[12] grew full-thickness explants of rabbit corneas in organ culture. The migratory behavior of both epithelium and endothelium was observed under different experimental conditions. Following explantation, epithelial migration along the cut surface of the stroma began within six hours. By 24 hours, the epithelium reached the cut edge of Descemet's membrane and made contact with the endothelium at this point. Further epithelial advancement was then completely inhibited for up to 30 days following contact with the endothelium. Likewise, there was no migratory endothelial movement. If the endothelium was mechanically removed from the posterior corneal surface just prior to culture, the epithelium subsequently moved onto Descemet's membrane and completely encircled the explant after only 48 hours *in vitro*. Prior removal of epithelium permitted the endothelium to behave in a similar manner. However, endothelial migration was not completed until 96 hours *in vitro* because of a 48-hour delay before movement began. The results showed that epithelium and endothelium mutually inhibited each other's forward movement and suggested an important role for this inhibitory interaction during corneal wound healing. Glickstein and associates[13] confirmed the findings of Cameron and associates,[12] using dog corneas.

EXPERIMENTAL PLAN

The historical review raises many questions. There are too many questions to answer in one study. I therefore have confined my experimental plan

to the exploration of four main areas: (1) the proper culture medium for corneal tissue; (2) the effect of serum on *in vitro* tissue growth; (3) the *in vitro* interrelationships between corneal epithelium and endothelium; and (4) the biology of cultures of whole corneas (organ cultures). Ideally, I hoped to find a culture medium with a minimum number of added factors in order to have as few variables as possible in analyzing corneal growth patterns. The makeup of the medium becomes especially important in determining the effects on the cultured tissue of added substances, that is, drugs. Towards the same end, I hoped to find a culture medium which would support corneal growth without having to add serum with all of its unknown factors. After determining which culture medium to use, I hoped to study interrelationships between corneal epithelium and endothelium cultured in the medium. Additionally, I planned to observe whole (full thickness) corneas cultured in the medium.

METHODS AND MATERIALS

PRIMARY CORNEAL EPITHELIAL OUTGROWTHS

Adult Dutch female rabbits or adult albino rabbits were killed by asphixiation in a closed CO_2 chamber or by fracture of the cervical spine. The eyes were enulceated immediately, using a sterile technique. The eyes were rinsed in sterile modified Eagle's minimal essential medium (MEM) containing 10% fetal calf serum and 100 units each of penicillin, streptomycin, and mycostatin (MEM-plus). The corneas were removed by sharp dissection and rinsed a second time in MEM-plus. The excised corneas were placed epithelial side down on a paraffin surface, under 1 to 2 ml of MEM-plus, and secured with a hypodermic needle. By tangential illumination, the cut edges of Descemet's membrane could be identified easily and stripped from the underlying stroma with gentle traction, using a jeweler's forceps. The stripped Descemet's membrane with its attached endothelium was used for primary corneal endothelial explants (see below). Epithelial explants were then prepared by punching out 1 mm buttons from the epithelial-stroma tissue. The buttons were allowed to swell for 1 to 2 hours in MEM-plus; they assumed a cylindrical appearance by the end of the time period. The epithelial surface was shaved off with a razor blade, excluding as much stroma as possible. The resultant epithelial explant (with minimal stroma attached) was placed epithelial side up, held in place by a clot comprised of one drop of chick plasma and one drop of chick embryo extract, on a sterile glass coverslip in a 35 mm petri dish. One specimen was placed in each quadrant of the coverslip. The preparations were air-dried at room temperature for 45 minutes and then covered with 2 ml of MEM-plus, with either 4% or 10%

fetal calf serum added. The specimens were incubated at 37°C in 5% CO_2 in high-humidity-air atmosphere. Daily observations by phase contrast microscopy were performed. The cultures were sacrificed and fixed in a glutaraldehyde-formaldehyde mixture[134] at appropriate intervals (zero time up to 15 days). The coverslips which contained the explants and outgrowths were stained with hematoxylin. After removing the explants, the coverslips were mounted on a glass slide and the outgrowths were studied by light microscopy.

Primary corneal epithelial explants were prepared as described above. Two methods were used to place the explants on coverslips as alternatives to the clot method described above. In one method, the primary corneal epithelial explants were placed immediately upon preparation onto glass coverslips, epithelial side up, and put in 35 mm petri dishes. Because no clot was used, the explants in both methods were held in place by a second coverslip placed on top of the first. MEM-plus without serum was then added. During the culture period, the uppermost coverslip was removed at different time intervals from 2 hours to 2 days after placement in the culture in order to determine the optimal time to remove the top coverslip without causing the explant to float off of the bottom coverslip into the medium, and to promote an even diffusion of the medium about the tissue (if the top coverslips were left in place the entire 7 days, medium could not flow properly between the coverslips to bathe evenly the explants).

Corneal epithelial cell growth characteristics in each of four tissue culture media were compared so as to choose the optimal medium. Primary corneal epithelial explants were placed in a clot in 35 mm plastic petri dishes by the procedures outlined above. Two ml of modified Eagle's minimal essential medium (MEM), Dulbeccos' medium, medium 199, or medium F-10 were added. The specimens were incubated at 37° C in 5% CO_2 in high humidity air atmosphere. Approximately 100 explants were cultured in each medium. The culture dishes were observed directly daily, by phase contrast microscopy. At appropriate intervals (20, 24, 30, 48, 52, and 72 hours) the outgrowths were sacrificed by fixing with a glutaraldehyde-formaldehyde mixture.

Additional studies were performed by altering the percentage of fetal calf serum. In one series, no serum, 1, 3, 5, 7, 10, 15, or 20 per cent fetal calf serum was added to MEM, and the epithelial explants were held in place with a clot as described above. In a second series, no serum, 4, 7, or 10 per cent fetal calf serum was added to MEM, and the epithelial explants were held in place between coverslips, without a clot, as described above.

PRIMARY CORNEAL ENDOTHELIAL OUTGROWTHS

Stripped Descemet's membrane with its attached endothelium was prepared from adult rabbits as described above. The Descemet's membrane-endothelial tissue was then quartered. From time to time, the Descemet's membrane-endothelial tissue was allowed to coil into a cylindrical roll, placed between two slices of rabbit liver, frozen in a cryostat, and then sectioned. This procedure was performed to check that Descemet's membrane was free of attached keratocytes and that the endothelium was not lost. In order to minimize its natural curling tendency, the quartered Descemet's membrane-endothelial tissue was held at three equidistant points on its circumference in three drops of chick plasma and three drops of chick embryo extract on a glass coverslip until a clot formed. The procedure produced some curling between the fixed points. The resultant curls, however, opposed each other where they joined at the fixed points so that a central flat triangular surface was left, oriented in the clot with the curled edges up and the endothelial surface down. The clot was allowed to air-dry at room temperature for 45 minutes and 2 ml of MEM-plus with 4% or 10% fetal calf serum was added. The specimens were incubated at 37°C in 5% CO_2 in high-humidity-air atmosphere. Daily observation by phase contrast microscopy was performed. The cultures were sacrificed and fixed in a glutaraldehyde-formaldehyde mixture at appropriate intervals (zero time to 15 days). The coverslips which contained the explants and outgrowths were stained with hematoxylin. The coverslips then were mounted on a glass slide and the outgrowths were studied by light microscopy.

SERIAL CULTURES OF PIG ENDOTHELIUM

Primary corneal endothelial explants were prepared from 6-month-old pigs, as described in the previous section. After 16 days of growth, the endothelium was subcultured by the following method. The culture fluid was removed. The cells were covered with 0.25% trypsin at 37°C for 10 minutes in a shaker. The fluid was transferred to a centrifuge tube and centrifuged at 600 *g* for 10 minutes. The supernatant fluid was discarded. The cells in pellet form were resuspended in 6 ml MEM. The resultant suspension was divided into three 35 mm petri dishes with coverslips and incubated at 37°C in 5% CO_2 in high-humidity-air atmosphere. A total of 6 subcultures were performed.

EPITHELIAL-ENDOTHELIAL COLLISION STUDIES

Primary corneal epithelium and endothelial explants from adult albino rabbits were prepared as described above. An endothelial explant was

placed in a clot in the center of a coverslip in a 35 mm culture dish. Epithelial explants (2 to 4) were placed at an appropriate distance (2 to 6 mm) from Descemet's membrane, held in place by a clot. The preparations were cultured in MEM-plus, with 4% or 10% added fetal calf serum as described above.

An alternative method of preparing the explants was used. Instead of using coverslips, the explants were placed directly on sterilized ethylene oxide millipore filters in a 35 mm petri dish. Because the filter was opaque, the progress of the transparent outgrowths could not be evaluated. Parallel cultures on coverslips were observed by phase contrast microscopy to gain an indication of the approximate rate of growth of the culture, and of the appropriate time to fix the outgrowths. Following fixation with the glutaraldehyde-formaldehyde mixture, the portion of the filter paper to be examined was cut into a rectangle with the long axis perpendicular to the cellular interface. The filter paper with its attached explants and outgrowths was oriented vertically in a paraffin block and sectioned.[135]

FULL THICKNESS CORNEAL ORGAN CULTURES

Excised corneas from adult Dutch female rabbits, adult albino rabbits, adult dogs, adult pigs, and adult humans were prepared as described above under *PRIMARY CORNEAL EPITHELIAL EXPLANTS*. Corneal explants, 2 mm in diameter, were then "punched out" with a trephine blade. The specimens were placed free-floating in plastic petri dishes containing 2 ml of MEM-plus, with added 4% or 10% fetal calf serum, and incubated at 37°C in an atmosphere of 5% CO_2 in air. Additionally, adult albino rabbit, dog, and human corneas were cultured in MEM-plus free of serum. The culture fluid was changed every three days. The dishes were agitated daily to prevent explants from attaching to the bottom surface of the dish. Explants which did become attached were dislodged with a sterile hypodermic needle. At appropriate intervals, specimens were fixed in a glutaraldehyde-formaldehyde mixture, and paraffin was embedded by hand to minimize trauma to the tissue. Specimens were sectioned at 8 μ and stained with hematoxylin and eosin or periodic acid-Schiff.

In some experiments, the endothelium was removed by vigorously rubbing the posterior surface of the cornea with sterile cotton-tipped applicators prior to explantation *in vitro*. The efficacy of this method in completely removing the endothelium was confirmed by light microscopy. The endothelium-deficient explants were then cultured in the same

manner as explants in which the endothelium was undisturbed. In other preparations, the epithelium and a small portion of anterior stroma were removed by sharp dissection prior to incubation, leaving the endothelium intact.

MITOTIC INDEX METHOD

Colcemid (2 μg/ml) was added to the media four hours prior to sacrificing epithelial outgrowth cultures. The mitotic index (MI) was calculated by the formula MI = number of mitosis ×100/total number of cells according to Chopra and co-workers.[136] In order to obtain the figures, an area count was made initially. One ocular of the microscope contained a 1 cm × 1 cm grid which was divided into 1 mm squares. Using 40× magnification, a count was made of the number of small squares covering the cells in the outgrowth; the results of the count represented the total area. Sample nuclear counts were then performed at 400× magnification. The counts were done in eight different locations in the outgrowth; four in the periphery and four centrally, at approximately the 12, 3, 6, and 9 o'clock positions. The average nuclear count was then calculated. The product of the average nuclear count and the area count, determined above, yielded the total number of cells per outgrowth. Under the same 400× magnification, the number of mitotic figures in the entire outgrowth was counted. A standard deviation was calculated for the four outgrowths on each coverslip. Additionally, colcemid (2 μg/ml) was added to the media four hours prior to sacrificing endothelial outgrowth cultures and full thickness corneal organ cultures. The resultant preparations were studied for the presence of mitotic figures.

^{3}H-THYMIDINE UPTAKE STUDIES

At appropriate intervals, ^{3}H-thymidine (specific activity 20 Ci/m mole) was added to the corneal epithelial outgrowth cell cultures for one hour at a final concentration of 8 μ Ci/ml of culture fluid. After one hour the cultures were rinsed through three baths of Grey's balanced salt solution and then fixed in 10% formaldehyde. For autoradiography, the cultures were dehydrated, air-dried, and the coverslips containing the outgrowths dipped in Kodak NTB 2 emulsion. Exposures were carred out at 4°C for 2 weeks. The ^{3}H-thymidine uptake studies were performed on corneal epithelial outgrowths cultured with and without serum and held in place with a clot or with a coverslip.

RESULTS

PRIMARY CORNEAL EPITHELIAL OUTGROWTHS

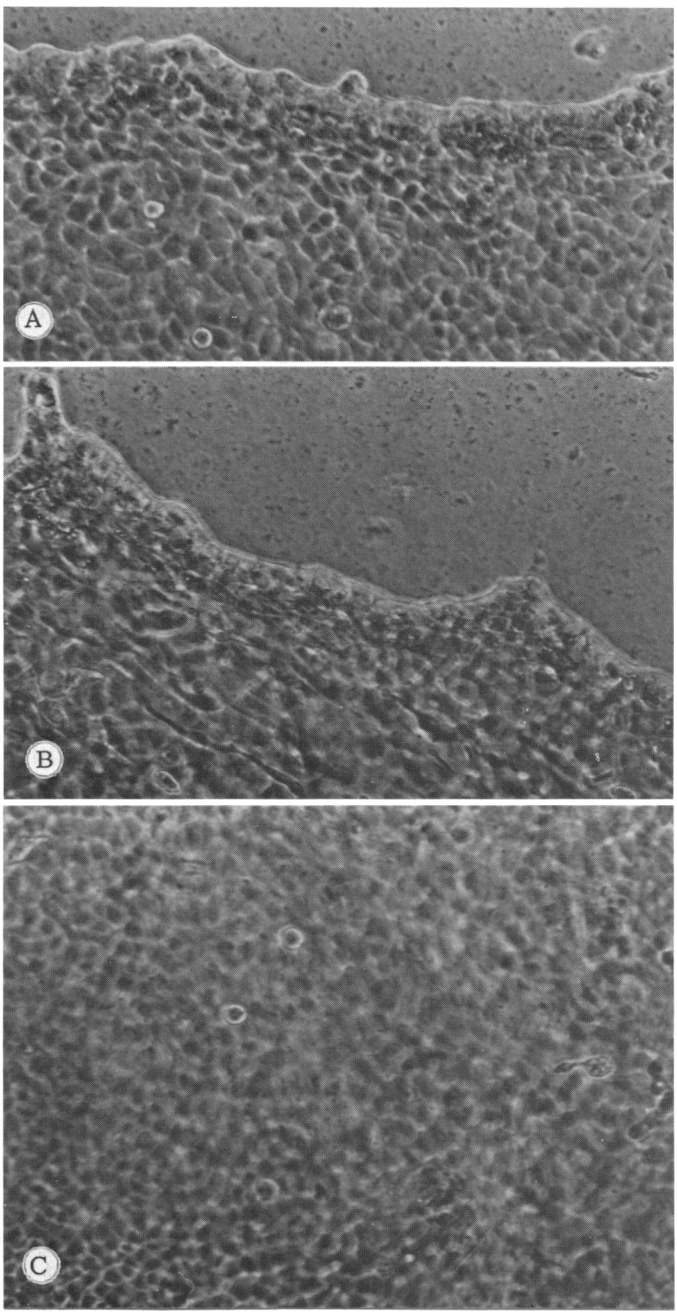
Standard Method With Clot:

Although the epithelium grew readily in both 4% and 10% fetal calf serum over the 15 day observation period, the cells seemed better preserved, less vacuolated, and more compact with 4% serum. The cells grew out as a multilayered sheet.

Up to 12 to 18 hours there was very little outgrowth from the explant. Sometime from 12 to 24 hours, an outgrowth of cells, about 4 to 5 cell diameters wide, appeared at one or more points at the margin of the explant. Initially, the outgrowth took the form of finger-like projections, with smooth advancing edges. Between 24 and 48 hours, the outgrowth completely surrounded the explant and the finger-like projections became blunted and less prominent (Figure 1). From 48 to 96 hours, proliferation of the outgrowth was most intense. The advancing edge of the confluent sheet of epithelial cells became much smoother (Figure 2). Mitotic activity was plentiful but appeared in groups (Figure 3); that is, if one mitotic figure was found, generally others could be seen in the same area, whereas in other areas, no mitotic figures could be found. Growth after 5 days — up to 7 or 8 days — appeared to slow down considerably from the rapid growth observed from 2 to 4 days. During the 5 to 7 or 8 day period, cells behind the advancing edge seemed to enlarge, become vacuolated, and even "drop out" leaving empty spaces. Other cells rounded up and floated off of the coverslip into the media. These rounded up, presumably desquamated, cells contained tiny, pyknotic nuclei (or no nuclei) and usually were semi-attached to the underlying coverslips by find strands, presumably cytoplasm. The free-floating, or semi-attached, small round cells increased with the age of the culture. From 7 to 8 days up to about 10 days, growth appeared sporadic and spotty. More free-floating round cells were found. Vacuolated and enlarged outgrowth cells, presumably degenerating, were plentiful. From 10 to 12 or 14 days, the outgrowth seemed to reach a steady state with little or no further growth. Vacuolization and enlargement of out-

FIGURE 1 *(overleaf)*

A and B: Epithelial outgrowth, cultured without serum, shows smooth advancing edge. C: Cells behind advancing edge form cohesive sheet. (All rabbit, all phase contrast, ×100)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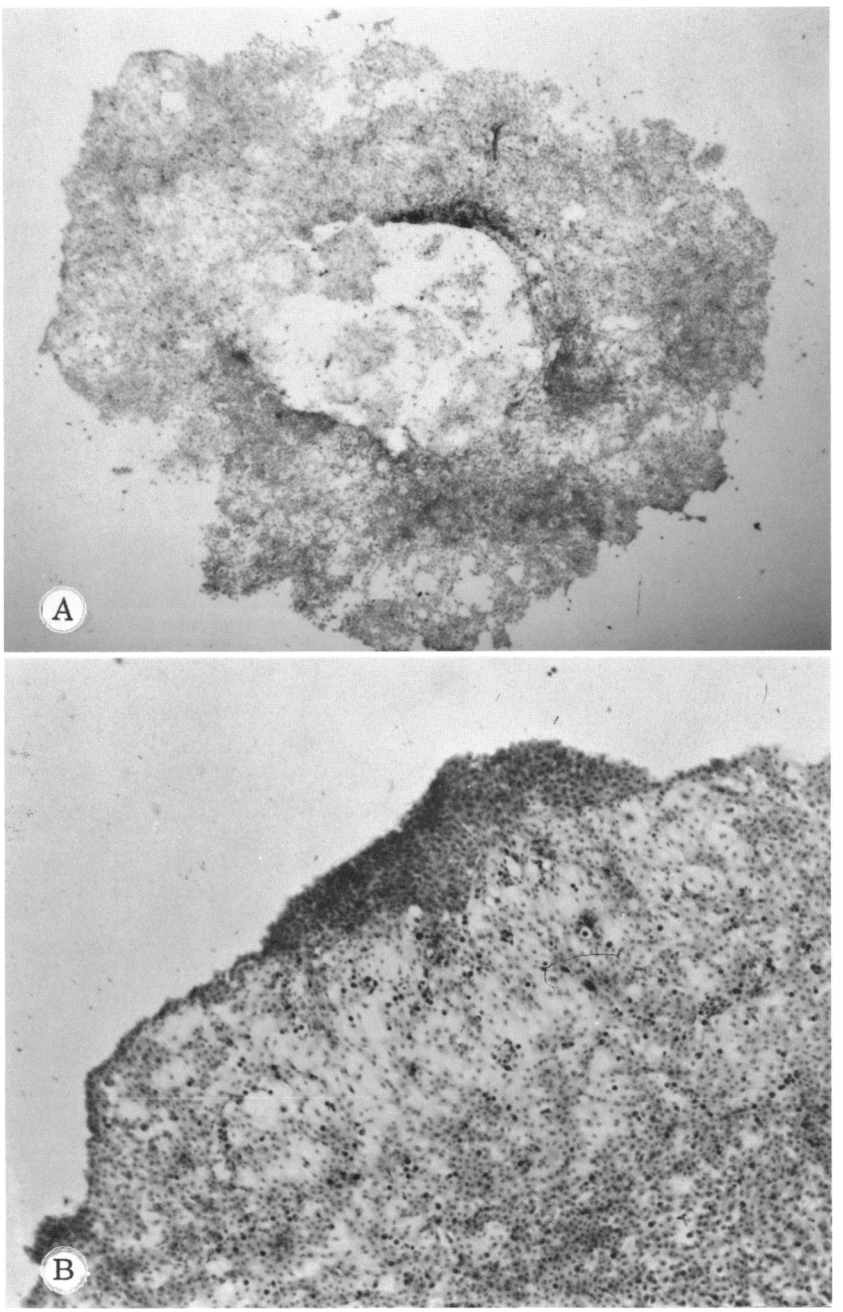
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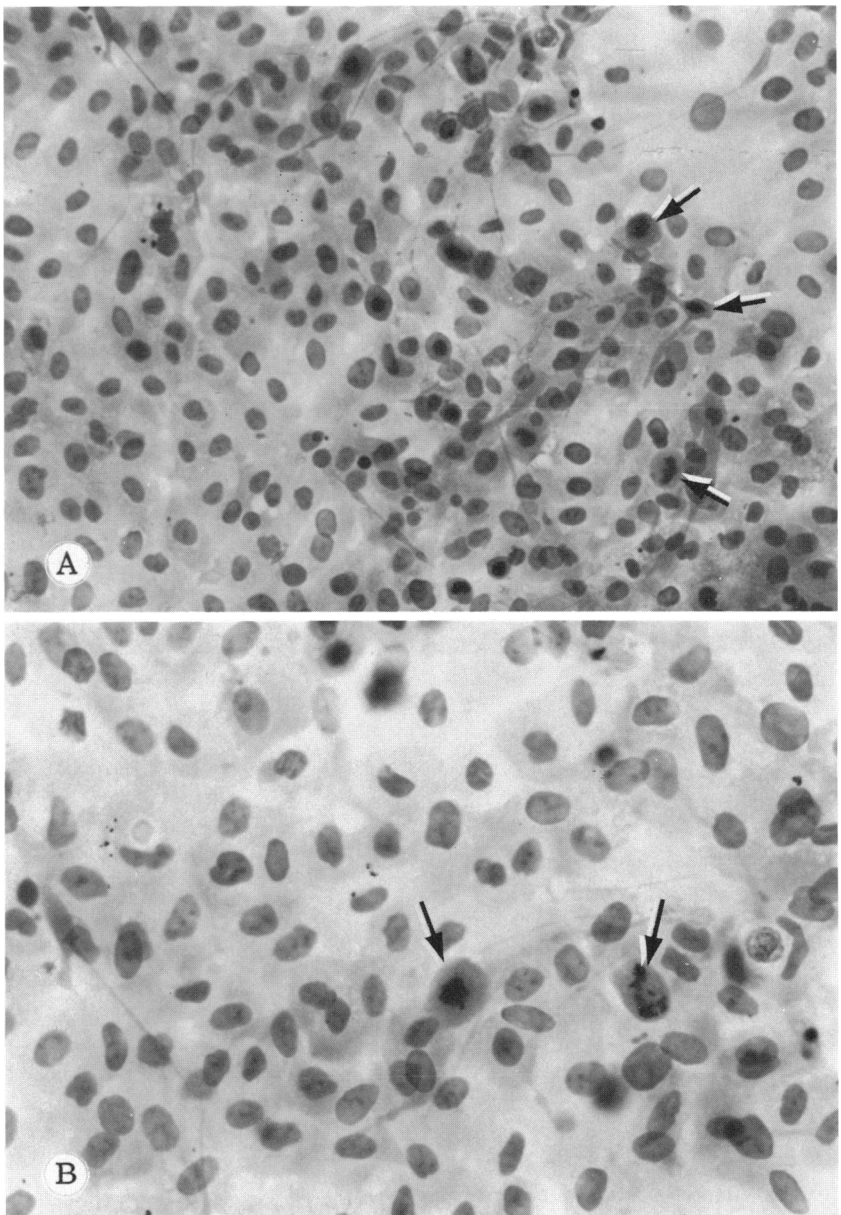
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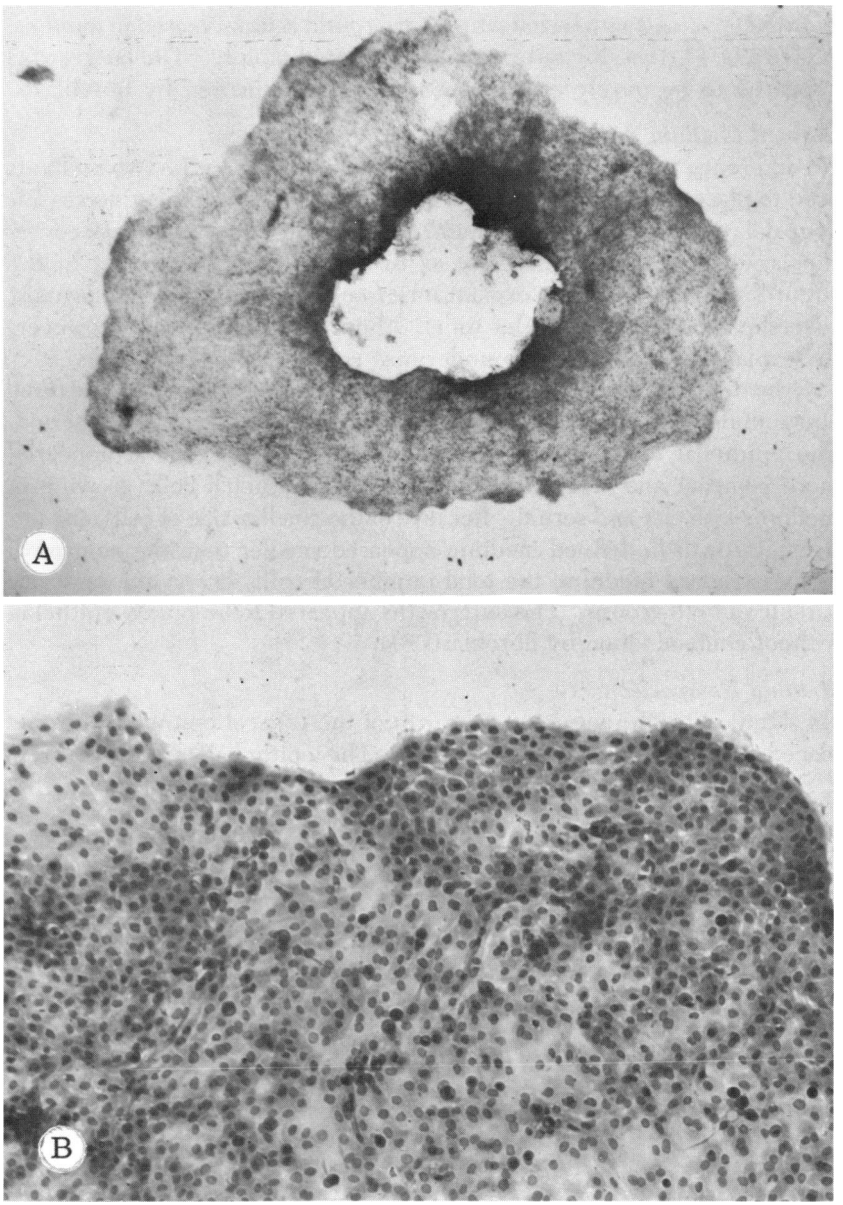
A
B

growth cells along with free-floating small round cells increased in number. After 12 or 14 days, the outgrowth deteriorated rapidly. The outgrowths appeared to be purely epithelial without contamination by fibroblasts.

Defined Medium Technique:
No difference in ultimate outgrowth was noted between the two methods used to place the explants on coverslips (immediate placement versus 24-hour-delay placement). It was found that the uppermost of the two coverslips could be removed as early as two hours after placement in the culture media, leaving the explant intact and adherent to the undermost coverslip, at least 50% of the time. About half of the time, however, the explants floated off of the undermost coverslip into the media.

Growth of the epithelium (Figure 4) was comparable to that described above under the standard method, during the 7 day observation period. The epithelial cells growing in defined medium in general appeared more compact and less vacuolated than the epithelial cells growing in medium with clot and serum. Because of the smaller size of cells, the entire outgrowth in defined medium appeared smaller than the outgrowth in the standard medium; the total number of cells, however, was comparable in both groups. The outgrowths appeared to be purely epithelial without contamination by fibroblasts.

Medium Comparison:
No significant differences in outgrowth of the corneal epithelial explants placed in clots could be determined. The epithelial outgrowth grew

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FIGURE 2 (*page 589*)
A: Epithelial outgrowth, cultured with 10% serum, at 70 hours. Space in center due to removal of explant prior to mounting on slide. B: Edge of outgrowth is smooth and shows variations in structure. (A, rabbit, hematoxylin, ×8; B, rabbit, hematoxylin, ×28)

FIGURE 3 (*page 590*)
A and B show that miotic figures (*arrows*) tend to occur in groups. Epithelial outgrowth at 70 hours cultured with 10% serum. (A, rabbit, hematoxylin, ×176; B, rabbit, hematoxylin, ×252)

FIGURE 4 (*page 591*)
A: Epithelial outgrowth, cultured without serum, at 70 hours. Space in center due to removal of explant. B: Edge of outgrowth is smooth and shows somewhat less variation in structure than with 10% serum (see Fig. 2B). (A, rabbit, hematoxylin, ×8; B, rabbit, hematoxylin, ×69)

TABLE I: MITOTIC INDEX OF PRIMARY RABBIT CORNEAL EPITHELIAL OUTGROWTHS ALL GROWN FOR 72 HOURS IN DIFFERENT CONCENTRATIONS OF SERUM

% Fetal Calf Serum	Number of Outgrowths	Mitotic Index ± S.D.
0	2	13.20 ± 2.63
1	3	24.93 ± 1.09
3	2	7.92 ± 0.47
5	3	15.00 ± 5.66
7	4	13.08 ± 2.36
10	3	9.36 ± 2.28
15	8	1.33 ± 0.74
20	6	1.77 ± 0.66

TABLE II: MITOTIC INDEX OF PRIMARY RABBIT CORNEAL EPITHELIAL OUTGROWTHS GROWN FOR DIFFERENT TIME PERIODS WITHOUT SERUM OR IN 10% SERUM

Hours of Growth	Number of Outgrowths	Mitotic Index ± S.D. Without Serum	Number of Outgrowths	Mitotic Index ± S.D. in 10% Fetal Calf Serum
50	8	5.38 ± 2.71	7	10.58 ± 4.22
60	4	17.02 ± 4.20	8	4.90 ± 1.28
70	4	16.60 ± 0.00	4	8.40 ± 1.10

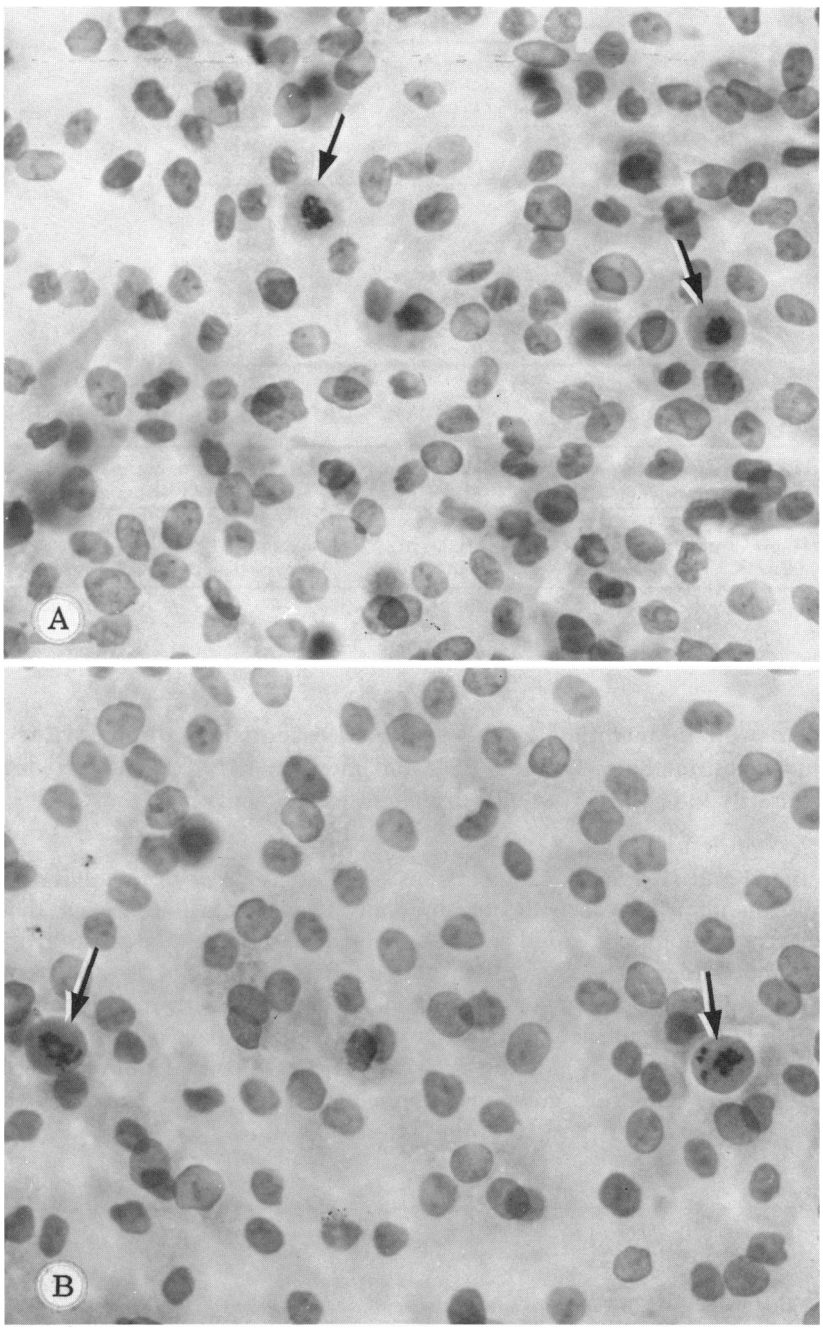
quite well in modified Eagle's minimal essential medium (MEM), Dulbeccos' medium, medium 199, and medium F-10. It was decided arbitrarily to use MEM in all subsequent experiments.

Effect of Serum:

Tables I and II show the comparisons of mitotic indices in two different experiments. The data do not allow any firm conclusions except that cultures without any added serum certainly have, by and large, as great a mitotic index as cultures with added serum. The outgrowths from explants with no serum, or serum concentrations less than 7% showed excellent cytological detail. Good growth for up to 7 days occurred without serum, but rarely did the growths survive beyond 12 days. With 4% serum, however, good growth occurred up to 2 weeks. With concentrations of 7% and 10%, the outgrowths showed fair to good cyto-

FIGURE 5 *(overleaf)*

A and B show that mitotic figure (*arrows*) tend to occur in groups. Epithelial outgrowth at 70 hours cultured without serum. (A and B, both rabbit, both hematoxylin, ×252)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logic detail and good growth up to 2 weeks. With concentrations of 15% and 20%, the outgrowths showed fair to poor cytologic detail and good growth for up to 7 days but poor growth beyond 12 days.

The corneal epithelial outgrowths between coverslips showed good growth and mitotic activity without serum (Figure 5) as well as with serum (Figure 3). The outgrowths in no serum and 4% serum seemed by light microscopy to be less vacuolated and better preserved than the outgrowths in 7% and 10% serum.

Tritiated thymidine studies showed labeling in outgrowths cultured without serum as well as with added serum.

It was concluded that the optimal method for culturing corneal epithelium was in defined medium of MEM without serum. If a somewhat more prolonged growth of epithelium was desired, MEM with serum (up to about 10%) seemed best.

PRIMARY CORNEAL ENDOTHELIAL OUTGROWTHS

Light microscopic observation of numerous stripped Descemet's membrane-endothelial tissues failed to show any attached stromal cells, that is, fibrocytes (keratocytes).

Up to 48 hours there was little or no outgrowth from the explant. The earliest outgrowths tended to start at the three points where the folded edges of Descemet's membrane met. Large angulated cells, similar to fibroblasts, but with thicker, more sharply turned processes, grew out from the explant (Figure 6A). Not until about 72 hours was the explant surrounded by outgrowth which assumed a sheet-like growth pattern. Unlike epithelial sheet growth, the advancing edge of the endothelial sheet never assumed a smooth edge, but remained sharply angulated and serrated with individual advancing endothelial cells well demarcated (Figure 6B). The advancing edge could be confused with fibroblasts. The individual endothelial cells at the advancing edge, however, appeared different than fibroblasts (as described above). Moreover, the cells in the sheet behind the advancing edge did not at all resemble fibroblasts, but rather were smaller and more closely packed than the peripheral cells, had poorly defined cell borders, appeared cohesive, and migrated

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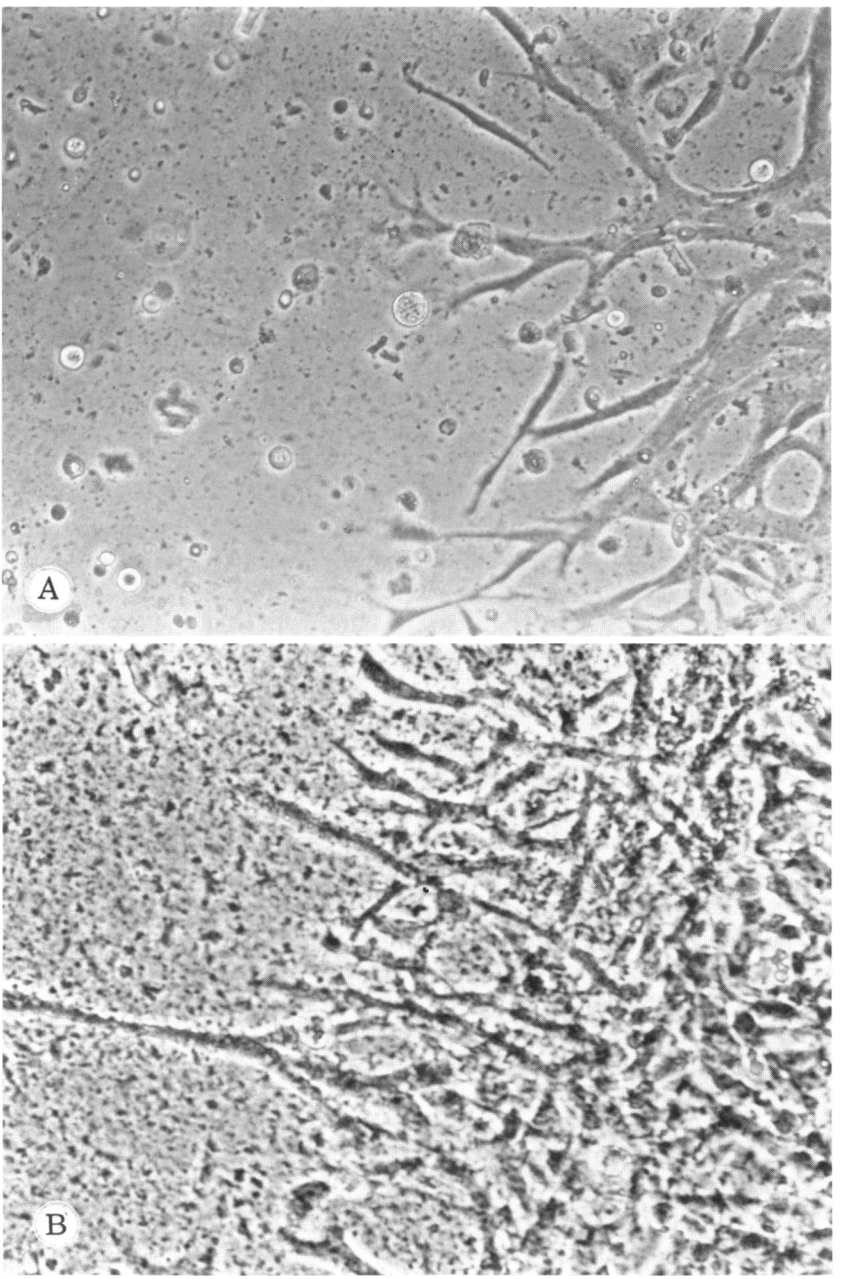
FIGURE 6 *(overleaf)*

A: Endothelial cells at advancing edge of outgrowth are similar to fibroblasts but have thicker, more sharply turned processes. (Rabbit, phase contrast, ×100) B: Endothelial cells at advancing edge of outgrowth remain sharply angulated and serrated after 4-days growth. (Rabbit, phase contrast, ×100)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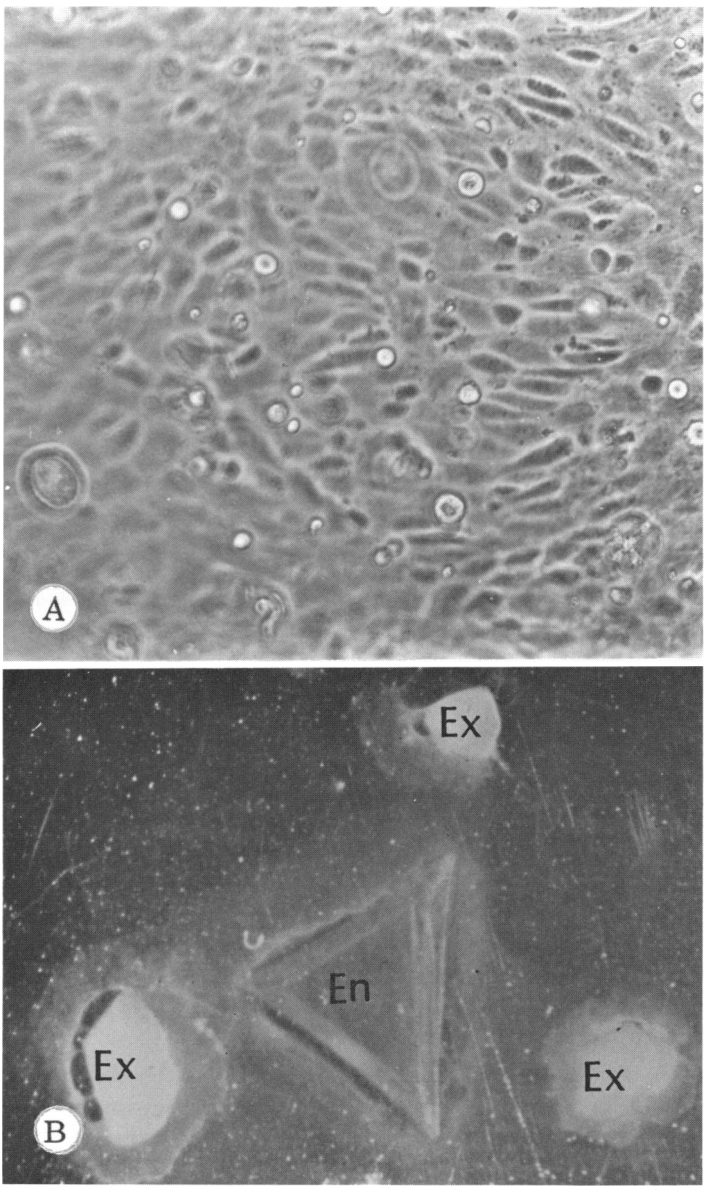


FIGURE 7

A: Endothelial cells behind advancing edge form cohesive sheet of cells. (Rabbit, phase contrast, ×100) B: Endothelial explant (*En*) is surrounded by three epithelial explants (*Ex*). Outgrowth, faint white area, surrounds each explant after 72 hours culture period. (Rabbit, phase contrast, ×5)

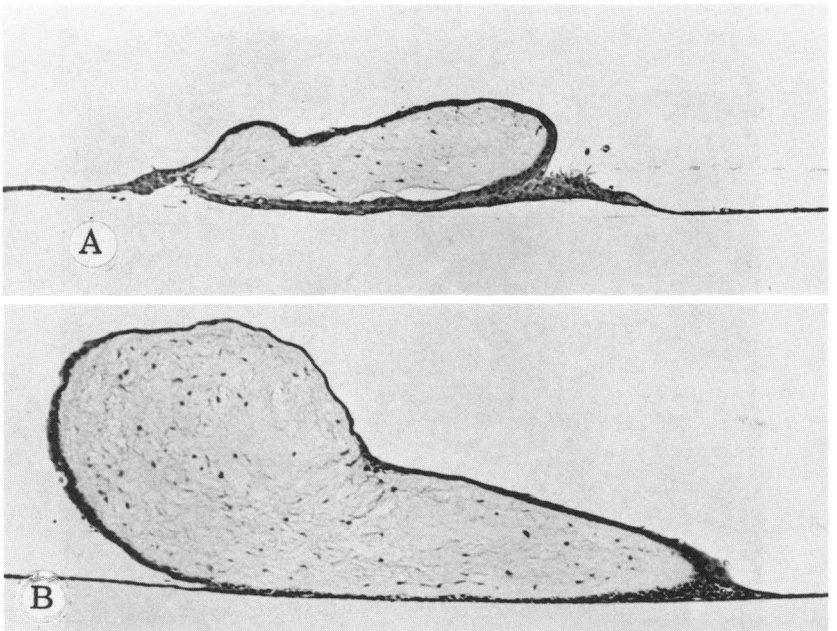


FIGURE 8
A, after 3 days in culture, and B, after 7 days in culture, both show epithelial-stromal explant completely surrounded by multilayered epithelium. Only multilayered epithelium presented in outgrowth. (A and B, both rabbit, both hematoxylin and eosin, ×40)

radially in sheets (Figure 7A). They showed none of the "fascicular pattern" characteristic of fibroblast growth. The growth pattern of the endothelial cells appeared to show a steady state of progression, unlike the acceleration-deceleration pattern of epithelial cells. Additionally, after about 2 weeks the endothelial cells covered completely the bottom of the culture dish and did not show a tendency to degenerate. Mitotic figures were seen frequently. As in epithelium, the mitotic figures tended to be found in clusters or groups. The outgrowths appeared to be purely endothelial without contamination by fibroblasts.

There appeared to be little difference between the cells cultured in 4% or 10% added fetal calf serum, although an impression was obtained that the cells seemed more compact and less vacuolated with 4% serum. The cells grew out as a monolayer.

SERIAL CULTURES OF PIG ENDOTHELIUM

All 6 subcultures of pig endothelium grew well. The total time of growth from the original explantation to the sacrificing of the sixth subculture was

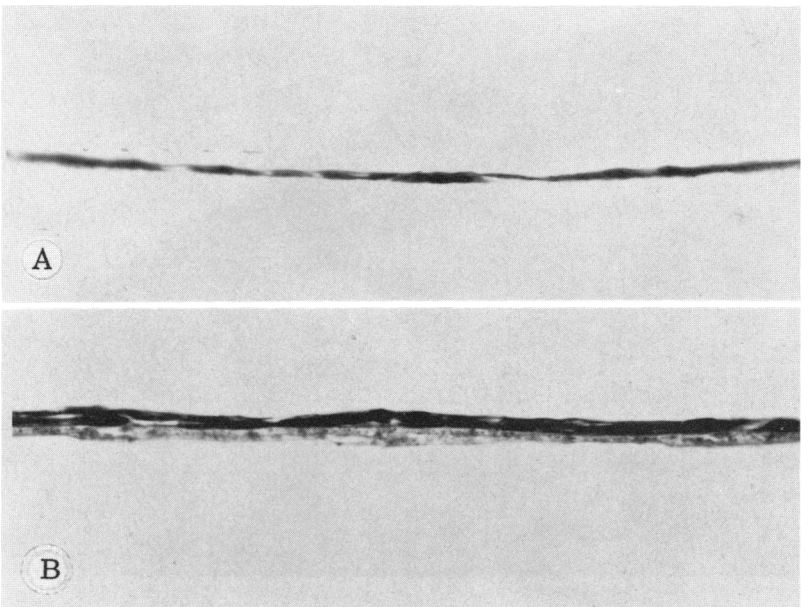


FIGURE 9
A: Endothelium present in monolayered form after 3 days in culture. (Rabbit, hematoxylin and eosin, ×252) B: Periodic acid-Schiff (PAS)-positive membrane is present under monolayered endothelium after 7 days in culture. (Rabbit, periodic acid-Schiff, ×252)

132 days. The initial growth as well as all subcultures was fairly uniform and quite similar to that seen with rabbit endothelium. There was very little, if any change in the structure of the endothelial cells from start to finish.

EPITHELIAL-ENDOTHELIAL COLLISION STUDIES

Epithelium, in a multilayer, and endothelium, in a monolayer, grew out (Figure 7B) as described above. The smooth leading edge of the epithelium and the angulated leading edge of the endothelium could be observed by phase contrast microscopy as they approached each other. The actual point of contact was difficult to delineate. Once contact was made, it was impossible to determine by phase contrast microscopy where the epithelial-endothelial junction was.

Studies of the outgrowth attached to filter paper showed that epithelium completely encircled the epithelial explant so that fibrocytes were trapped inside (Figure 8). No periodic acid-Schiff (PAS) positive membrane could be identified under the multilayered epithelial outgrowth up to the 7

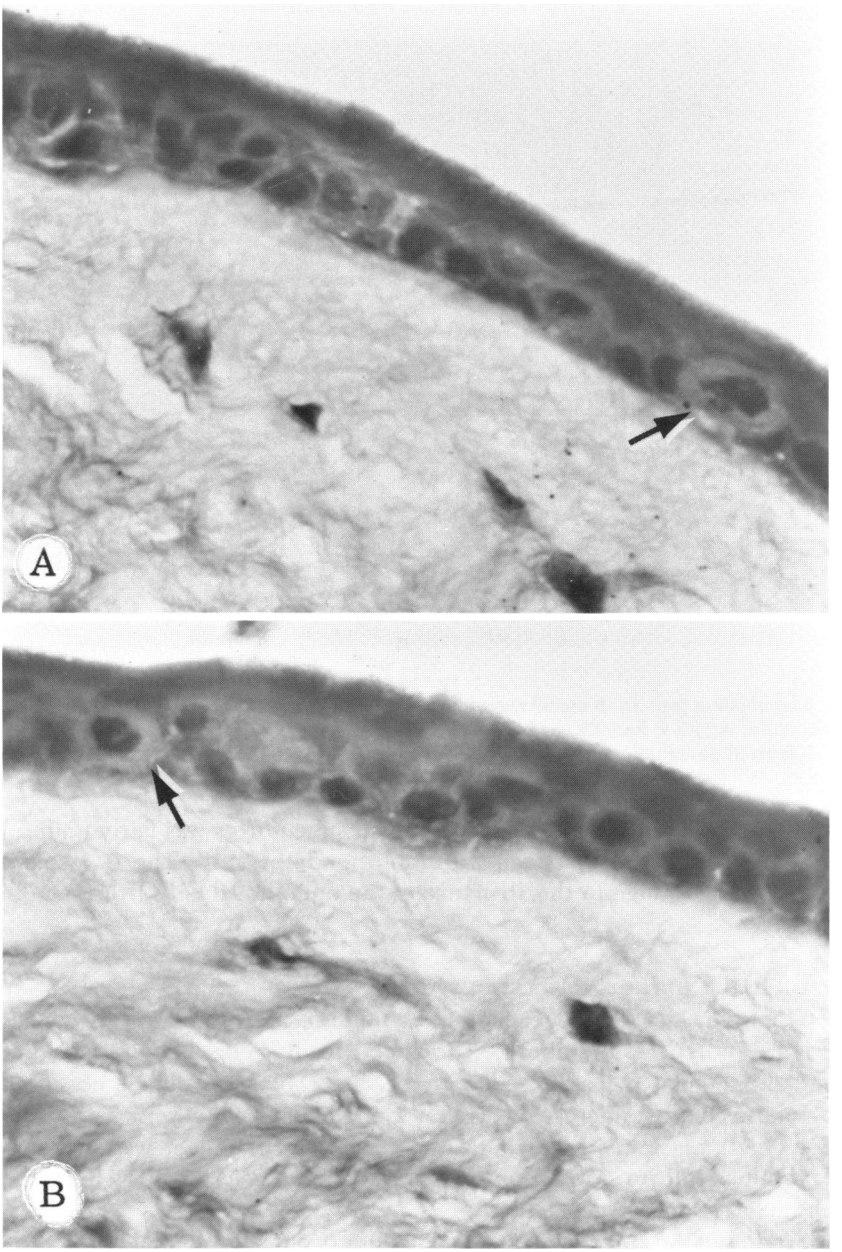


FIGURE 10

A and B show mitotic figures (*arrows*) in epithelium surrounding explant. Both in culture 5 days. (A and B, both rabbit, both periodic acid-Schiff, ×252)

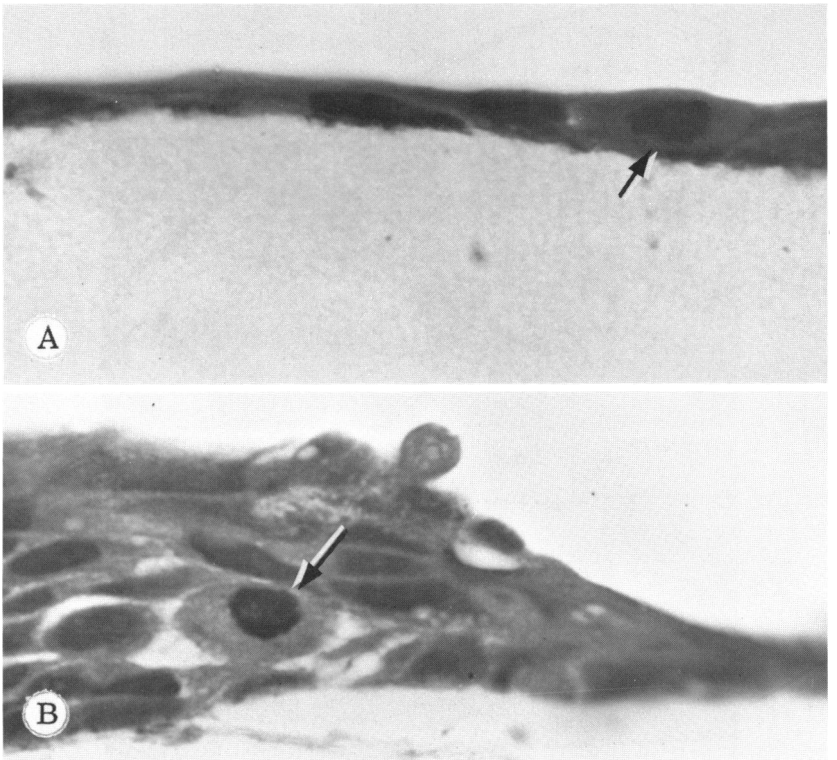
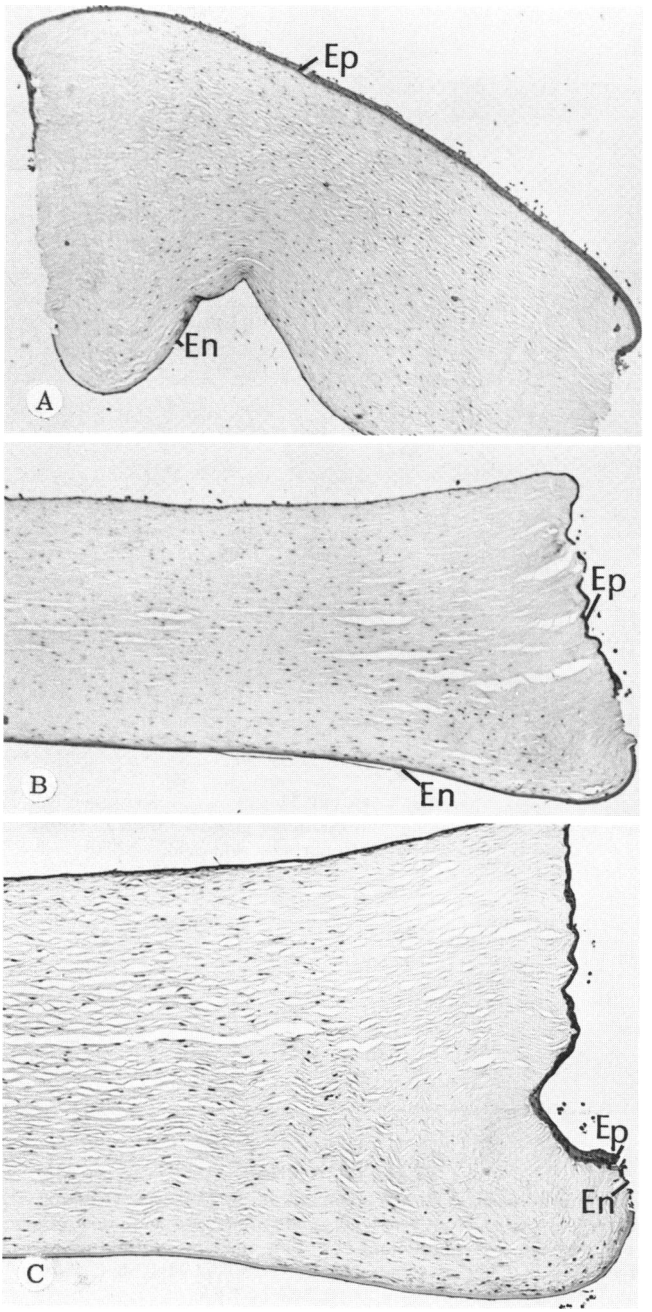


FIGURE 11
A and B show mitotic figures (*arrows*) in epithelial growth. Both in culture 5 days. (A and B, both rabbit, both periodic acid-Schiff, ×850)

FIGURE 12 *(overleaf)*
A, B, and C show full-thickness cornea organ culture at 24 hours, 41 hours, and 48 hours, respectively. Note relatively acellular stromal zone near cut edge. *En*, endothelium; *Ep*, epithelium. (A, dog, hematoxylin and eosin, ×21, SEI 74-1065; B, dog, hematoxylin and eosin, ×28, SEI 74-1066; C, dog, hematoxylin and eosin, ×40, SEI 74-1068)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En
A
Ep
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En
Ep
En
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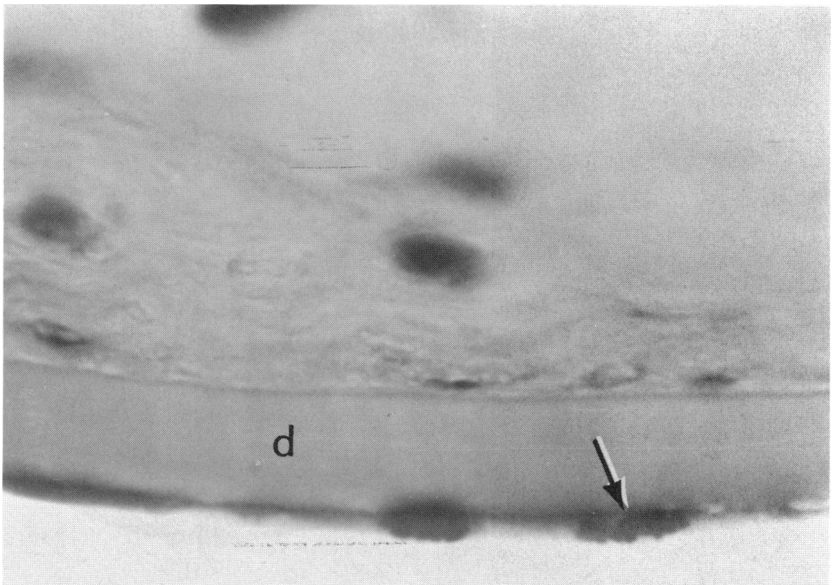

FIGURE 13
Endothelium shows mitotic figures (*arrow*) after 48 hours in culture (*d*, Descemet's membrane). (Rabbit, hematoxylin and eosin, ×1103)

days observation period, suggesting that the epithelium had not made any basement membrane. No fibrocytes could be identified in the Descemet's membrane-endothelial tissues. The endothelium grew out as a monolayer (Figure 9A). By 7 days a definite PAS-positive membrane was present under the endothelium, suggesting new basement membrane production (Figure 9B).

In the explants that received colcemid prior to sacrificing, mitotic figures were frequent and found easily in the epithelium. Unfortunately, the endothelium in this series was not preserved well during processing. The epithelial specimens sacrificed after 3 days of culturing showed mitotic figures all around the explant and at all levels from base to surface of the epithelium (Figure 10). The small amount of outgrowth could not be preserved in the sections. After 5 and 7 days of culturing, mitotic figures were still present all around the explants and at all levels of the epithelium. Additionally, the outgrowth showed plentiful mitotic figures in all areas and at all levels (Figure 11).

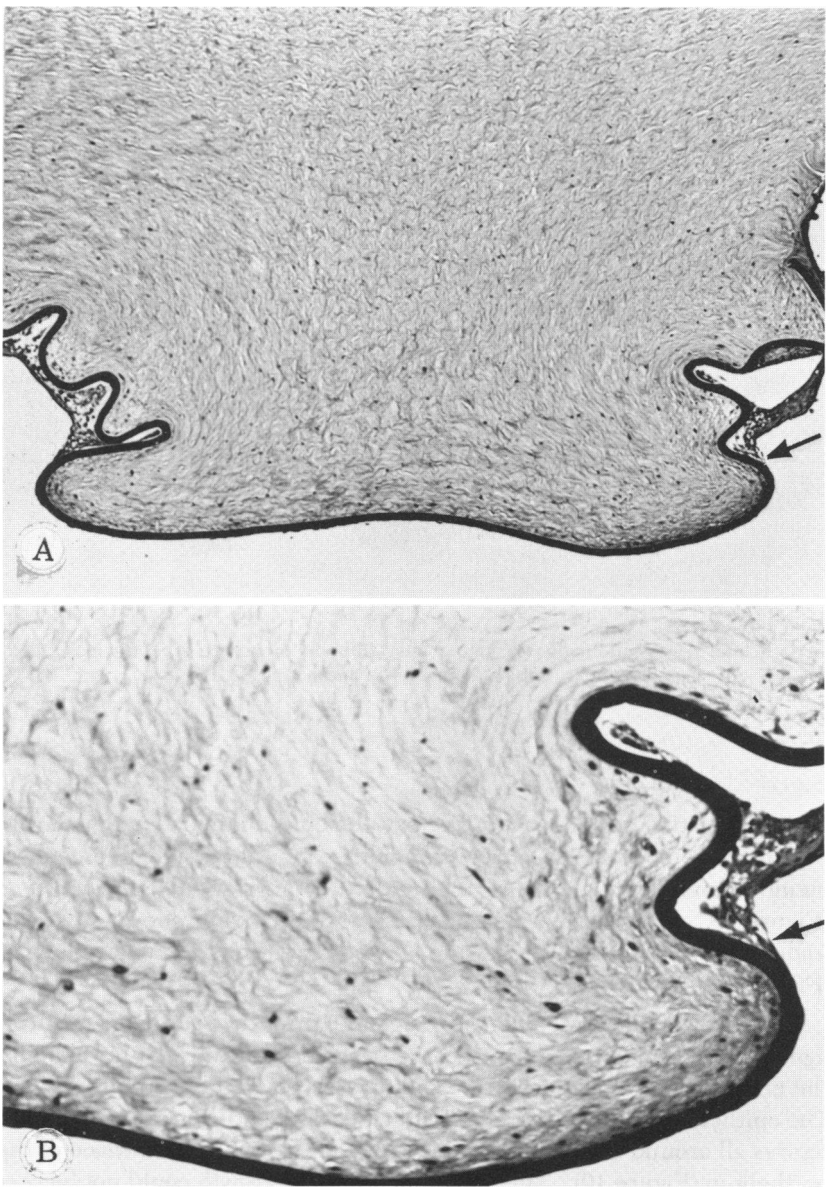

FIGURE 14

A: By 48 hours, epithelium has migrated down side of whole corneal tissue culture to make contact with endothelium (*arrow*). No further migration has occurred after 6 days. B: Higher magnification of epithelial-endothelial junction (*arrow*). (A, dog, periodic acid-Schiff, ×40; B, dog, periodic acid-Schiff, ×101)

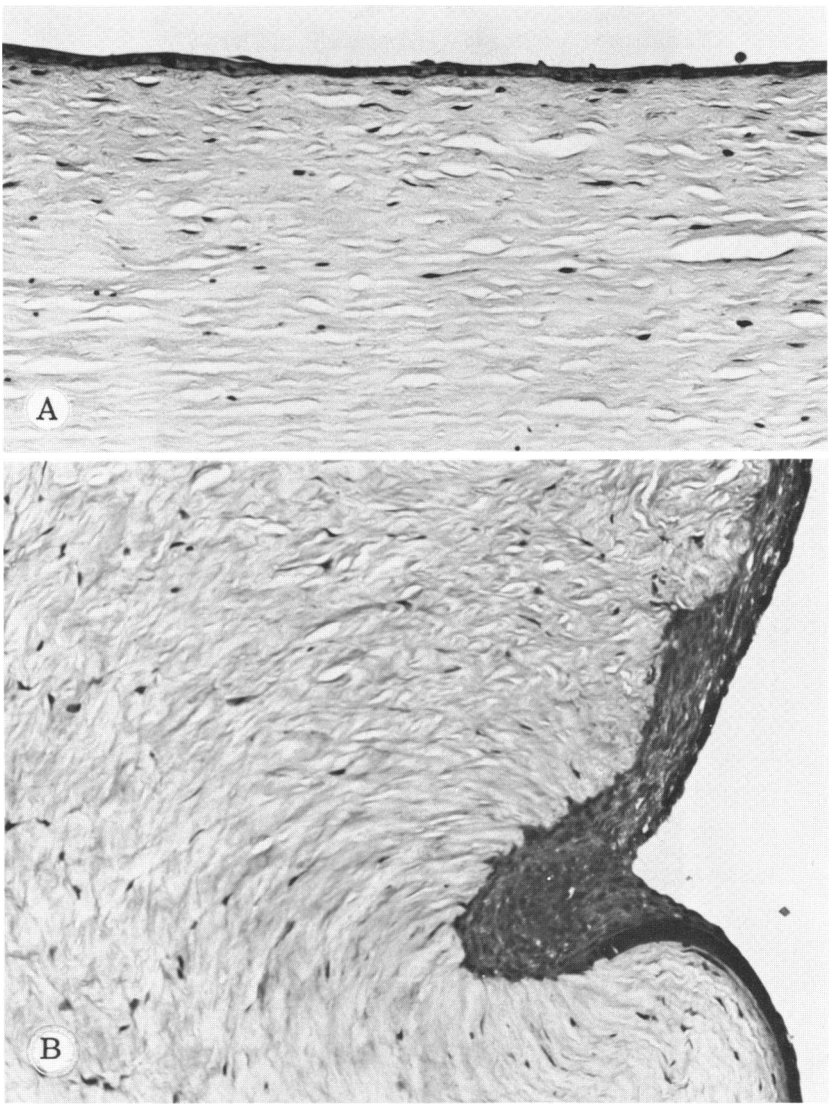

FIGURE 15

A: Epithelium one to two layers thick over smooth anterior surface of full thickness corneal tissue cultured for 6 days. (Dog, hematoxylin and eosin, ×101) B: Endothelium has become multilayered over ragged cut edge of whole corneal tissue cultured for 15 days. (Dog, periodic acid-Schiff, ×101)

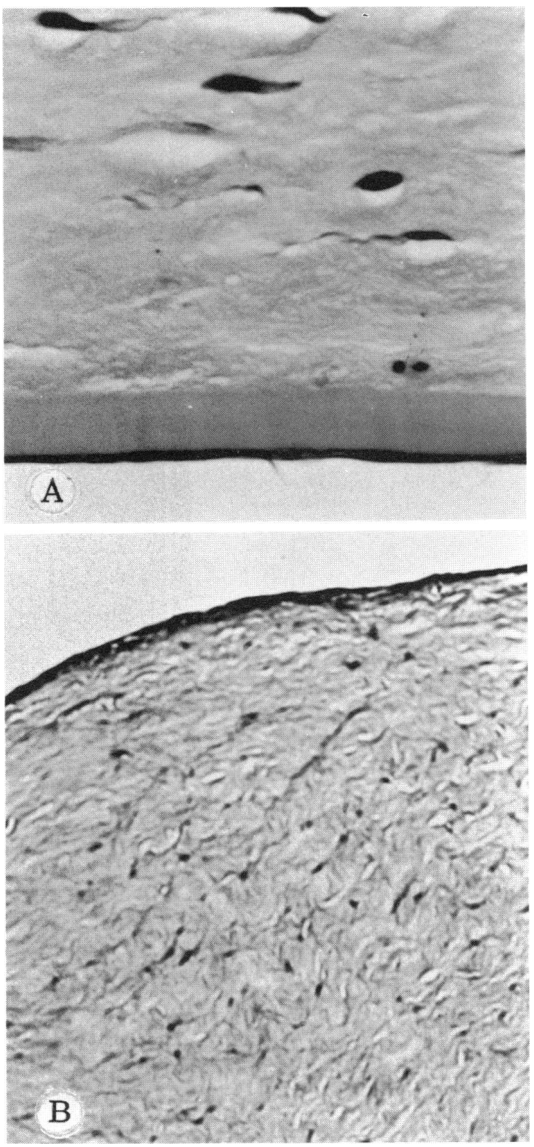

FIGURE 16

A: Endothelium monolayered over Descemet's membrane (*d*) after 9 days in culture. (Dog, hematoxylin and eosin, ×252) B: Endothelium monolayered over relatively smooth anterior surface of whole corneal tissue cultured for 15 days (epithelium removed prior to placement in culture). (Dog, periodic acid-Schiff, ×40)

FULL THICKNESS CORNEAL ORGAN CULTURES

The rabbit, dog, and human corneal organ cultures all showed the same type of growth pattern. Following immersion in the culture fluid with the added serum, the corneal stroma began to swell and within 24 hours attained a maximal thickness of three to four times normal. Concurrent with the swelling, the cut edge of Descemet's membrane curled inward toward the stroma, generally assuming a triangular "cocked hat" appearance. At about 24 hours, the leading edge of corneal epithelium had migrated over approximately 30% to 50% of the lateral cut surface of the swollen stroma (Figure 12). By 48 hours, the epithelium had arrived in the area of the cut edge of Descemet's membrane. At this point, subsequent epithelial behavior was determined by the presence or absence of the endothelium. In the presence of endothelium (Figure 13), which itself showed minimal migratory activity by 48 hours, the epithelium generally failed to show any further forward movement over Descemet's membrane (Figure 14). After six days in culture, the epithelium had not advanced past its point of contact with endothelium relative to its position to Descemet's membrane. At the point of contact, epithelial cells appeared to pile up to a depth of four to five layers in contrast with the one or two layered state seen during the early stages of migration. The epithelium remained four or five layers thick on the roughened sides of the full thickness explant, but thinned to one or two layers over the smooth anterior surface (Figure 15A). Up to 30 days no further movement of the epithelium was observed relative to its position with respect to Descemet's membrane. By contrast, in the absence of endothelium, the epithelium advanced unimpeded over Descemet's membrane to completely encircle the explant within 48 hours. After approximately six days in culture, the endothelium-free explants showed the typical multilayered epithelial cell epithelium over the entire extent of Descemet's membrane.

Additionally, in those preparations in which the epithelium and a portion of anterior stroma were removed prior to culture, leaving the endothelium intact, the endothelium was observed to migrate off Descemet's membrane onto the lateral cut surface of the stroma following a lag period of 48 to 72 hours. By 96 hours, the endothelial monolayer completely

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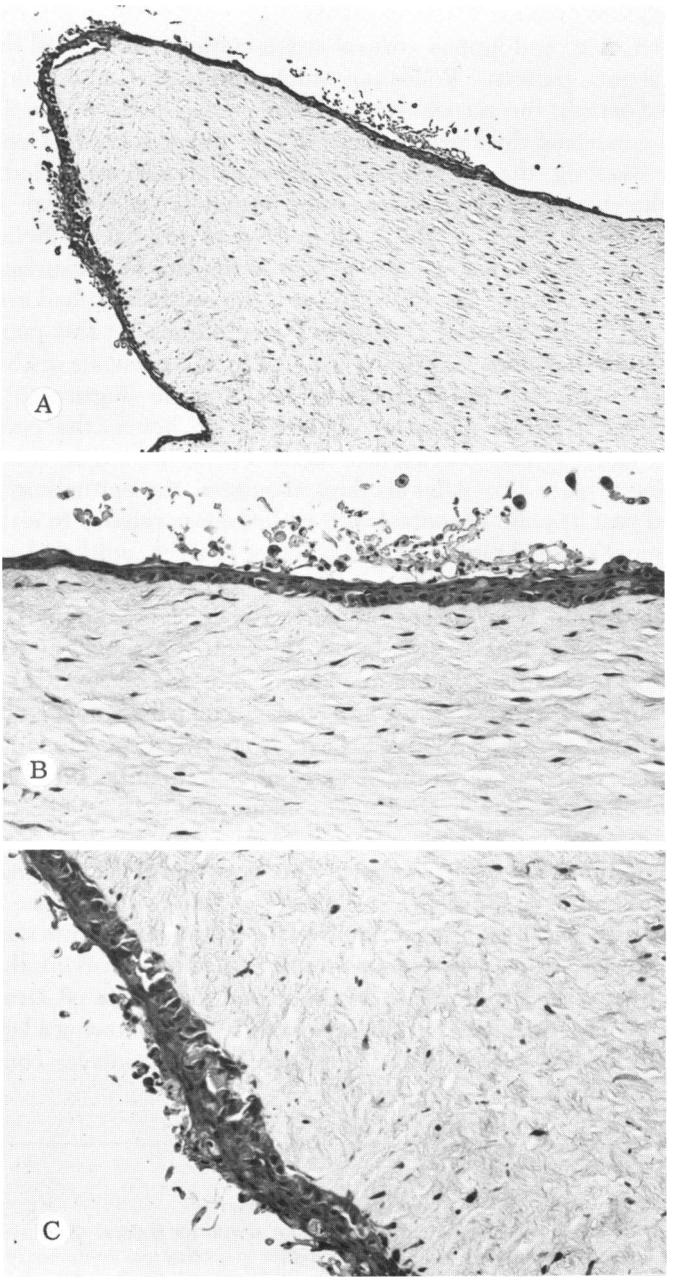
FIGURE 17 *(overleaf)*

A: Dog whole corneal tissue cultured without serum for 6 days. Epithelium has started to keratinize. B and C show higher magnifications to demonstrate epithelial keratinization. (A, hematoxylin and eosin, ×40; B and C, hematoxylin and eosin, ×101)


A
B
C

surrounded the corneal stroma. Only the adult albino rabbit and human corneas were grown for seven days. The endothelium in those instances became multilayered along the ragged edges (Figure 15B) but always remained monolayered over Descemet's membrane (Figure 16A), and tended to remain monolayered over the relatively smooth anterior surface (Figure 16B).

In the stroma itself, by 24 hours, the keratocyte nuclei had disappeared in a zone approximately 40 μ adjacent to the cut surface (Figure 12). Three to five days later, keratocyte nuclei were again found in this region but were not as plentiful as in other areas. No focal accumulation of keratocyte nuclei was noted in any area of the explant. By 9 to 14 days, keratocyte nuclei had disappeared from the posterior half of the stroma. By 30 days, keratocyte nuclei were present only in the upper quarter of the stroma.

The adult albino rabbit, dog, and human corneas cultured in media free of serum grew essentially as described above up to about 3 or 4 days. Starting around the fourth or fifth day, however, the epithelium started to show keratinization (Figure 17), which increased to the seventh day. No full thickness corneal explants were cultured in serum-free media for longer than 7 days.

DISCUSSION

A considerable amount of the preliminary tissue culture work was done with modified Eagle's minimal essential medium (MEM). In order to be sure that MEM was appropriate for corneal tissue culture work, three other media were compared. No significant differences were observed. It was decided, therefore, to continue the work with MEM.

In the past, most investigators have used serum in the culture fluid because of the widely held assumption that *in vitro* cells require serum for proliferation, if not for survival.[137-144] Raff and Houck[31] showed that serum was essential for human fibroblast division but not for migration. Conversely, Flaxman and Harper[145] demonstrated human epidermal cell division but no migration in organ cultures without serum. Levine[144] also was unable to show keratinocyte movement without serum. Weimar and Fellman[57] developed a method for an organ culture system in a wholly synthetic, known medium, that is, a defined medium, using MEM with supplemental glutamine (2mM/l) and Earle's non-essential amino acids (0.1 mM/l) but no serum. They showed rabbit fibroblast mobilization and migration but could not demonstrate mitotic figures. With a modification of this defined medium, Weimar and Haraguchi[58] showed that rabbit

corneal epithelium migrated partway down the cut edge of a corneal wound in a corneal organ culture study. Other tissues may act differently in medium without serum. For example, explants of mouse vagina show new DNA synthesis and mitosis when grown in defined medium without serum, and even show keratinization.[146] Also, human mammary gland grown in defined medium without serum shows DNA synthesis and mitosis.[147]

The value of being able to study a tissue under defined conditions is readily apparent. Serum contains many unknown factors, both growth-promoting and growth-inhibiting, thus presenting the researcher with a number of unknown variables that are not desirable in highly critical work. Gnädinger and associates[102] used 10% serum in their studies of acytylcholine-splitting activity of cultivated corneal epithelial cells. Adachi and Pollak[67] used 15% serum in studying the effect of many different enzymes on cultured rabbit corneal cells. In studying the effects of antiglaucoma drugs[97,98] and combined effects of corticosteroids and antiglaucoma drugs[99] on corneal epithelium, 15% serum was added to the culture media. Savage and Cohen[59] showed that epidermal growth factor stimulated proliferation of corneal epithelium in organ cultured material. Although they did not add serum, they did add 5% chick embryo extract. In the study of Bülow and Ehlers,[70] where they described the morphology and DOPA reaction of cultivated corneal epithelial cells, 20% serum was added. Even in the studies of Weimar and Fellman,[57] Weimar,[100] and Weimar and Haraguchi,[58] the defined medium had supplemental materials in addition to MEM.

From a biological point of view the work described in the present study, using MEM without supplemental materials, shows that rabbit epithelial corneal explants and rabbit and human corneal whole explants (organ cultures) appeared to continue most of their normal functions. For at least the first 7 days the epithelial cells migrate and proliferate. Because stimulation of migration and DNA synthesis and mitosis occur under defined *in vitro* conditions, the present corneal epithelial explant and organ culture systems may offer important advantages not previously available in studies of human corneal epithelium, especially relating to effects of ophthalmologic drugs on ocular tissues.

If serum is to be used, the generally suggested concentration of 10% probably is not the optimal amount. The present study indicates that a lower percentage, probably 4% fetal calf serum, yields better results than 7%, 10% or even higher percentages. In 4% serum, the epithelial cells as well as endothelial cells most closely approximate their *in vivo*

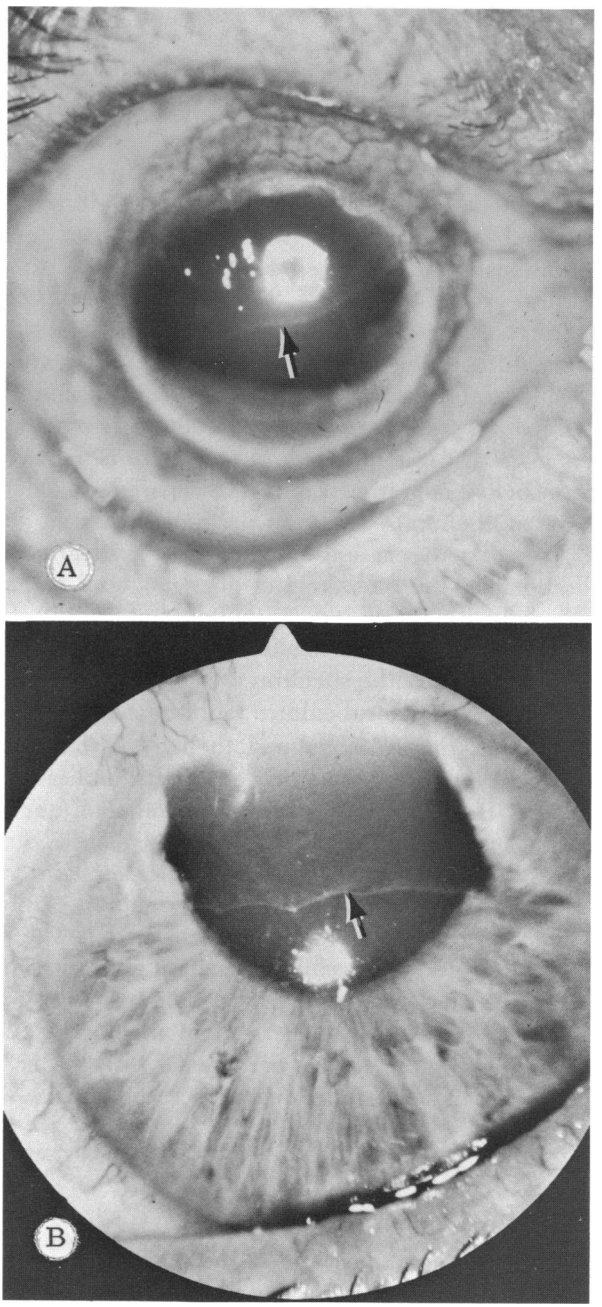
corneal counterparts. Active epithelial and endothelial migration and mitosis occur with 4% serum.

Epithelium, unlike the monolayered growth of corneal endothelium and keratocytes, proliferates in tissue culture (epithelial explant or organ culture) as a multilayered cohesive sheet of cells. As occurs *in vivo*, the superficial cells tend to desquamate and then float free in the medium. Although the epithelial cells grow readily in tissue culture, they do not grow indefinitely but have a finite period of growth of approximately 2 weeks. Additionally, in the present study, attempts to subculture the rabbit corneal epithelium failed. Sarker and co-workers,[69] although able to subculture serially epithelial cells from the corneal of a bull, also could not subculture rabbit corneal epithelium. Newsome and associates[73] were able to subculture serially human corneal epithelium.

Endothelium does proliferate in tissue culture as a monolayer. Its growth differs from that of epithelium in that there is a prolonged delay of up to 48 hours before migration occurs (as compared to a delay of from 12 to 24 hours for epithelium). Whereas epithelium has an acceleration-deceleration growth pattern, endothelium has a steady-state growth pattern. Additionally, the endothelium does not tend to have a finite growth period of about 2 weeks like epithelium but rather tends to grow continuously, limited only by the size of the culture dish. Furthermore, pig corneal endothelium can be sucultured continuously (no attempt was made in the present study to subculture rabbit or human endothelium).

It was hoped that the epithelial-endothelial collison studies would demonstrate contact inhibition when the epithelial and endothelial outgrowths collided. However, the epithelial-endothelial junction formed when contact was made could not be identified by phase contrast microscopy. Parallel studies carried out on filter paper for light microscopic examination did not give the necessary information. Unfortunately, the cultures were sacrificed before contact was made. The filter paper technique, however, did give useful information about basement membrane production. No basement membrane could be identified under the corneal epithelial outgrowth, but a basement membrane (Descemet's) could be identified under the corneal endothelial outgrowth. This observation of endothelial basement membrane secretion agrees with similar

FIGURE 18 *(overleaf)*
A and B show epithelial downgrowth in 2 different patients. Note how epithelium has grown over only posterior aspect of superior cornea, ending in a horizontally linear line (*arrows*). (A and B from Yanoff and Fine[148])


A
B

observations made by Pearlman and Baum.[120,122] The phenomenon of contact inhibition was not pursued in the present epithelial-endothelial collision study because of its ease of observation with the use of corneal organ cultures.

Clinically, epithelial downgrowth (ingrowth) on the posterior corneal surface following cataract extraction involves, as a rule, the superior two-thirds of the cornea, sparing the inferior third. Moreover, the line of demarcation between epithelial downgrowth and uninvolved cornea tends to be horizontally linear in character, frequently corresponding to the major corneal fold of the surgical corneal flap (Figure 18). It is puzzling why the epithelium doesn't grow right over the whole posterior cornea instead of sparing the inferior third. Presumably, during the manipulation of the corneal flap during delivery of the cataract, mainly the superior portion of the cornea is traumatized. The trauma can occur from instruments and cataract rubbing against the posterior surface of the superior cornea and from folding and bending of the superior corneal flap. The corneal endothelium would be the most likely part of the cornea to be injured (that is, as noted by postoperative striate keratopathy). There would be a fairly-well demarcated boundary, then, between uninjured inferior corneal endothelium and injured superior corneal endothelium.

As shown in the present study, healthy corneal endothelium of human, rabbit, dog, and pig inhibits migration of corneal epithelium. In only one instance did contact inhibition not occur in a human corneal organ culture, resulting in the epithelium surrounding completely the corneal explant. This failure occurred in a cornea with central corneal guttata and, therefore, a diseased endothelium. The experimental situation suggests that if the corneal endothelium is healthy *in vivo* it can inhibit epithelial growth. The inhibition would explain the sparing of the inferior cornea by epithelial downgrowth. The epithelium is able to grow over (or replace) the surgically injured superior corneal endothelium but is inhibited by the noninjured inferior endothelium. Perhaps if there is proper closure of the corneal wound, epithelium still may grow down the cut edge of the wound. If the epithelium contacts healthy endothelium, however, it probably would be inhibited from further growth. If the endothelium is not healthy, however, or if covered (that is, by iris or vitreous incarcination), the epithelium probably will not be inhibited and will grow into the eye, resulting in epithelial downgrowth.

In those rabbit and human corneal organ cultures grown in MEM without serum an unexpected finding occurred. After about four days, the normally nonkeratinized corneal epithelium became keratinized. Thus, an experimental model to study corneal keratinization was produced.

Preliminary studies adding vitamin A to the MEM medium without serum suggest that vitamin A protects against the development of keratinization. Much more work will have to be done with this experimental model.

CONCLUSIONS

1. Modified Eagle's minimal essential medium (MEM) proves to be as successful as three other commonly used tissue culture media in allowing corneal epithelial explants to proliferate.
2. Corneal epithelial outgrowths and the epithelium and endothelium of corneal organ cultures migrate and proliferate in MEM without added serum. Defined media, therefore, can be used for experimentation on corneal tissue culture growth.
3. Corneal epithelium grows in tissue culture as a multilayered, cohesive outgrowth with a smooth advancing edge.
4. Corneal endothelium grows in tissue culture as a monolayered, cohesive outgrowth with a sharply angulated and serrated advancing edge.
5. Pig corneal endothelium can be serially subcultured with ease.
6. Epithelium can be differentiated easily from endothelium in epithelial-endothelial collision tissue culture studies. When the two tissues made contact, however, it was impossible by phase contrast microscopy to demonstrate the epithelial-endothelial junction because of limitations of the experimental design.
7. In corneal organ cultures epithelium and endothelium inhibit each other mutually on contact so that no further migration of either tissue takes place. If endothelium or epithelium is removed prior to culturing, the remaining tissue surrounds completely the cultured corneal explant.
8. In whole corneal organ culture, with time, the endothelium shows some release from contact inhibition (when epithelium is removed prior to culture) so that the normally monolayered tissue becomes multilayered (in areas).
9. If serum is not added to the MEM, the corneal epithelium becomes keratinized after about four days of culturing, thus providing an experimental model to study corneal epithelial keratinization.

SUMMARY

Four main areas are explored: (1) the proper culture medium for corneal tissue; (2) the effect of serum on *in vitro* tissue growth; (3) the *in vitro* interrelationships between corneal epithelium and endothelium; and (4)

the biology of cultures of whole corneas (organ cultures). Modified Eagle's minimal essential medium (MEM) proved to be an excellent culture fluid. Corneal tissue could be grown in MEM without serum or clot, thus providing a defined culture medium. The *in vitro* biology of outgrowths of multilayered corneal epithelium and monolayered corneal endothelium are discussed. Contact inhibition between epithelium and endothelium is demonstrated in whole corneal (organ) cultures.

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