ENDOTHELIUM OF THE HUMAN ORGAN CULTURED CORNEA: AN ELECTRON MICROSCOPIC STUDY

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INTRODUCTION

DURING THE PAST THREE DECADES, METHODS OF MAINTAINING GROWTH AND viability of mammalian tissues in cell and organ culture have been perfected. The behavior of skin in tissue and organ culture has been extensively studied.[1-14] Utilizing those techniques, Summerlin demonstrated that whole adult skin, both human and animal, maintains functional viability for extended periods of time in organ culture, and after such time, fails to be rejected when transplanted to an homologous host.[15-16] Summerlin also confirmed immunogenic modification of organ cultured skin through an analogous series of human allografts from mis-matched female donors through male recipients.[17] It has been pos-

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sible to adapt this phenomena to an animal model using isografts and allografts of cultured skin in mice.[16] Major mouse (H2) and human (HLA) histocompatability barriers have been crossed. The model even permits use of cultured skin across species barriers as well (xenografts).[18]

We have reported work from our laboratory demonstrating that human and animal corneas can be stored in organ culture for periods up to four weeks and appear to remain viable by criteria such as phase microscopic examination, light microscopic examination, and flat mount preparations of endothelium.[19] We have also reported the use of these corneas in allografts (rabbit to rabbit) and xenografts (chicken to rabbit) demonstrating viability of such stored corneas as well as antigenic modification.[20]

As an extension of our investigations of organ cultured corneal tissues, we have been studying the ultrastructure of human and cat corneas stored for varying lengths of time in organ culture. Our findings with cat corneas as well as human epithelium and stroma will be presented elsewhere. The purpose of this paper is to report our findings regarding the effect of organ culture on the ultrastructure of human corneal endothelium. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were utilized with emphasis on the effect of storage duration in organ culture and its comparison with moist chamber storage.

MATERIALS AND METHODS

Six pairs of human corneas were obtained within four hours after death of the donor and stored in moist chambers at 4 degrees C until received in the laboratory. Details of the clinical data regarding these corneas as well as the duration of organ culture can be seen in Table 1. The causes of death of the donors is listed in Table 2.

The human eyes used in this study were those which were donated to the Minnesota Lions Eye Bank. Moist chamber storage consisted of a saline moistened sponge in an air-tight jar in which the eye was placed at 4 degrees C. The eyes were prepared for organ culture by sectioning the cornea from the eye with a two to three mm rim of sclera as we have previously described.[19]

The organ culture technique that we utilized was a modification of the technique that we have previously reported.[19] The organ culture media was exactly as previously described. However, we did change the technique by placing the corneal button epithelial side down and elim-

TABLE 1. CLINICAL AND ORGAN CULTURE DATA REGARDING PAIRED ORGAN CULTURED CORNEAS

Donor #	Age (yr)	Postmortem Time* (hr)	Organ Culture Eye #1	Time (day) Eye #2
50	75	74	0	21
51	66	33	0	21
52	58	24	0	21
1	62	10	10	20
3	58	6	10	18
5	73	12	10	21

* Includes time between death and enucleation (within 4 hours in all cases) plus moist chamber storage at 4 degrees C.

TABLE 2. CAUSE OF DONOR DEATH

Patient #	Diagnosis
50	Cerebral Vascular Accident
51	Pulmonary Embolism
52	Metastatic Lung Carcinoma
1	Hodgkins Disease, Gram Negative Septicemia
3	Septic Shock, Renal Failure
5	Pulmonary Edema, Breast Carcinoma

inating the fibrinogen and thrombin that were originally used to hold the cornea in place. Otherwise, placement in the media, changing the culture media, and manipulation of the culture media were as previously described. Incubation of the corneal buttons was done in a water-jacketed tissue culture incubator at 37 degrees C with an atmosphere of 5% CO_2 and 95% air.

For transmission electron microscopy (TEM) whole corneas were fixed in 2.7% glutaradehyde and phosphate buffer (pH 7.2, 330 mOsM) for at least 24 hours. The corneas were then rinsed in buffer, cut into wedge-shaped pieces, and post-fixed in 2% osmium tetroxide for one and one half to two hours. The tissue was then rinsed in buffer and dehydrated in a graded series of alcohol and propylene oxide before being subjected to an overnight period of penetration in Spurr Low-viscosity embedding medium.[21] The tissue was embedded in flat molds to facilitate orientation of the tissue for sectioning. Thin sections for TEM were cut with a diamond knife on a LKB Ultratome, stained with uranyl acetate and lead citrate, and examined with a Forgflo EMU-4C electron microscope.

For scanning electron microscopy (SEM), the epon was washed off the endothelial surface of each cornea according to a modified method of

Cleveland and Schneider.[22] After polymerization of the tissue specimens overnight at 37 degrees C and for 48 hours at 60 degrees C, the tissue was glued to SEM specimen stubs, coated rotationally with gold palladium metal and, viewed with a Cambridge S4-10 scanning electron microscope.

RESULTS

Moist Chamber Storage

SEM examination of these three corneas (Figures 1a, 3a, 5a, 5b) demonstrates irregular hexagonal cells that vary in diameter between 10 and 25 microns. Except for patches of lysed cells, some with rounded nuclei (Figures 3a, 5b), the endothelial layer is intact with definable cell borders and bulges over the nuclear region. With increasing storage time in the moist chamber, cell lysis is increased in area and severity. (Figure 1a).

TEM confirms the SEM studies. In those corneas stored for 33 and 24 hours, (Figures 3b, 5c) the cells are flat (2.8 - 4 microns thick) with normal nuclei, intact flat posterior plasma membranes and normal cytoplasm with many mitochondria, endoplasmic reticulum, and ribosomes. TEM examination of the lysed cell (Figures 1b, 5d) demonstrate rounded nuclei, swollen mitochondria, and loss of cytoplasm (evidence of cell death). The intact cells stored 74 hours (Figure 1c) demonstrate early degenerative changes, that is, swollen mitochondria with loss of cristae, nuclear swelling, and irregular posterior plasma membranes.

Moist Chamber Storage Plus 21 Days Organ Culture

SEM (Figures 2a, 4a, 6a, 6b) demonstrates an intact endothelium over the entire posterior corneal surface in each instance. Due to incomplete rinsing of the specimens, a coating — presumably protein from the organ culture media — is present to some extent in some specimens. However, we are still able to identify cell borders. The cells are flat and large; some were 50-60 microns in diameter. Rounded cells, 15-20 microns in diameter (Figures 2a, 6a, 6b) were seen in some organ cultured corneas, especially those with prolonged postmortem storage time. Some of these cells showed possible attachment to the underlying surface (Figure 6a) and definite intercellular bridges (Figure 6b).

TEM (Figures 2b, 2c, 4b, 6c, 6d) demonstrates thin cells less than 1 micron. The nuclei are either flattened or appeared normal and the posterior plasma membranes are flat and intact. The cytoplasm is

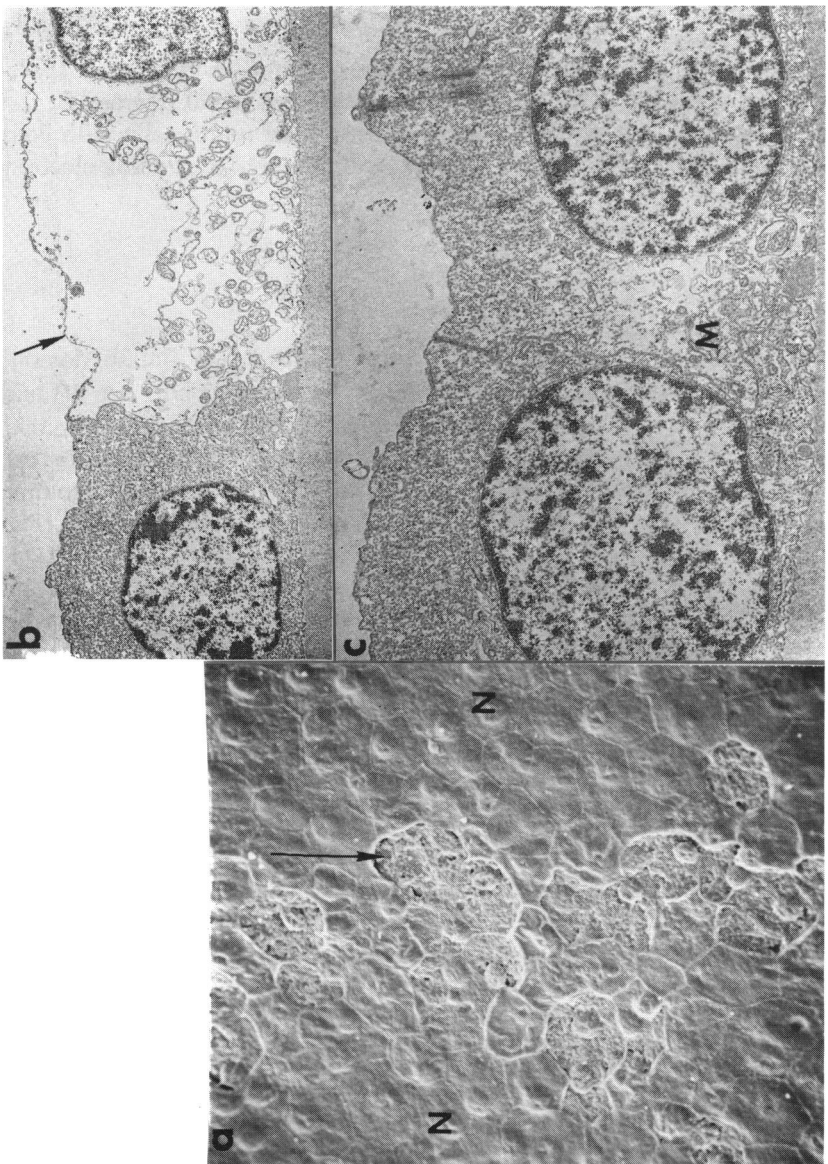


FIGURE 1

Donor #50. Postmortem time 74 hours. Moist chamber. a. SEM: Large patches of lysed and missing cells (arrow) scattered among intact cells. Intact cells show nuclear bulges (N). (× 535). b. TEM of lysed cell. (right) Remnants of the posterior plasma membrane (arrow) remain intact, but cytoplasm washed out. Remaining mitochondria swollen and disrupted. Cell on left still intact. Nuclei of both cells swollen, (× 4325). c. TEM of intact cell. Cells are approximately 7 μ thick with swollen nuclei, intact but irregular posterior plasma membranes and swollen mitochondria (M) (× 4500).

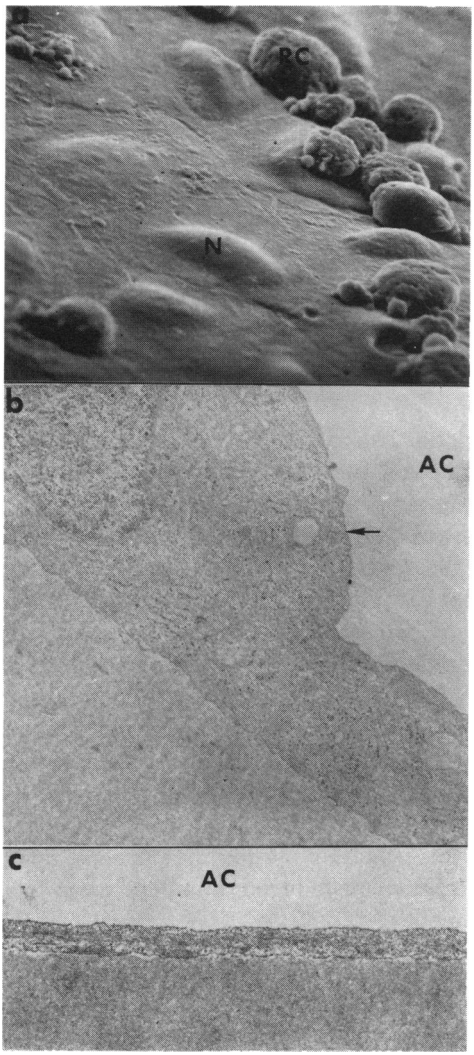


FIGURE 2

Donor #50. Postmortem time 74 hours, organ culture 21 days. a. SEM: Endothelial cells are large (50 μ) and flat with nuclear bulges (N) and completely cover Descemet's. Round cells (RC) 10-20 μ noted in some areas. (× 560) b. TEM: Section through cell with same diameter as round cell seen in 2a (arrow). Endoplasmic reticulum and ribosomes present. Mitochondria not identifiable. AC - Anterior chamber. (× 10,800) c. TEM: Endothelium very thin, less than 1 μ. Few organelles present. (× 10,800).

normal except for decreased numbers of mitochondria. The intercellular spaces are sometime dilated but the terminal bars are intact (Figure 6d).

In summary, SEM comparison of moist chamber vs organ cultured corneas shows that the endothelial cells seem to spread to cover the defects noted in moist chamber, become quite large, and as seen by TEM quite thin. There appeared to be a decrease in the number of mitochondria in the cytoplasm of the endothelial cells with organ culture, but otherwise, the endothelium was well preserved.

Ten Day Organ Culture vs 18-21 Days Organ Culture

10 Days

SEM (Figures 7a, 8a, 10a) demonstrates an intact endothelium over the entire cornea in each specimen. The cells are flat with continuous borders ranging in size from 10-40 microns. Folding and wrinkling of the posterior cornea is seen (Figures 7a, 11a) and multiple rounded cells are seen in the central cornea of donor #1 at 10 days (Figure 7a).

TEM of donor #1 (Figures 7b, 7c) demonstrates cells with dense bodies in the cytoplasm — the only human cornea in which we have observed this phenomena. This culture appeared infected at the time of fixation, (bacteriological studies were not done). The dimension of the cell in figure 7b coincides with the dimensions of the round cells seen by SEM. The cell seen in Figure 7a contains numerous small mitochondria, endoplasmic reticulum, and a flattened nucleus. TEM of the specimen from donor #3 (Figure 8b) and #5 (Figure 10b) demonstrates flat cells with normal nuclei, posterior plasma membranes, cytoplasm, and with the possible exception of reduced numbers of mitochondria, normal cytoplasmic organelles.

18-21 Days

The specimen from (donor #1 became infected — no specimen available.) SEM again demonstrates an intact endothelial cell layer over the entire posterior cornea. The cells range in size from 15-40 microns. (Figure 9a) Some wrinkling of the surface is noted (Figure 11a). No round cells are seen.

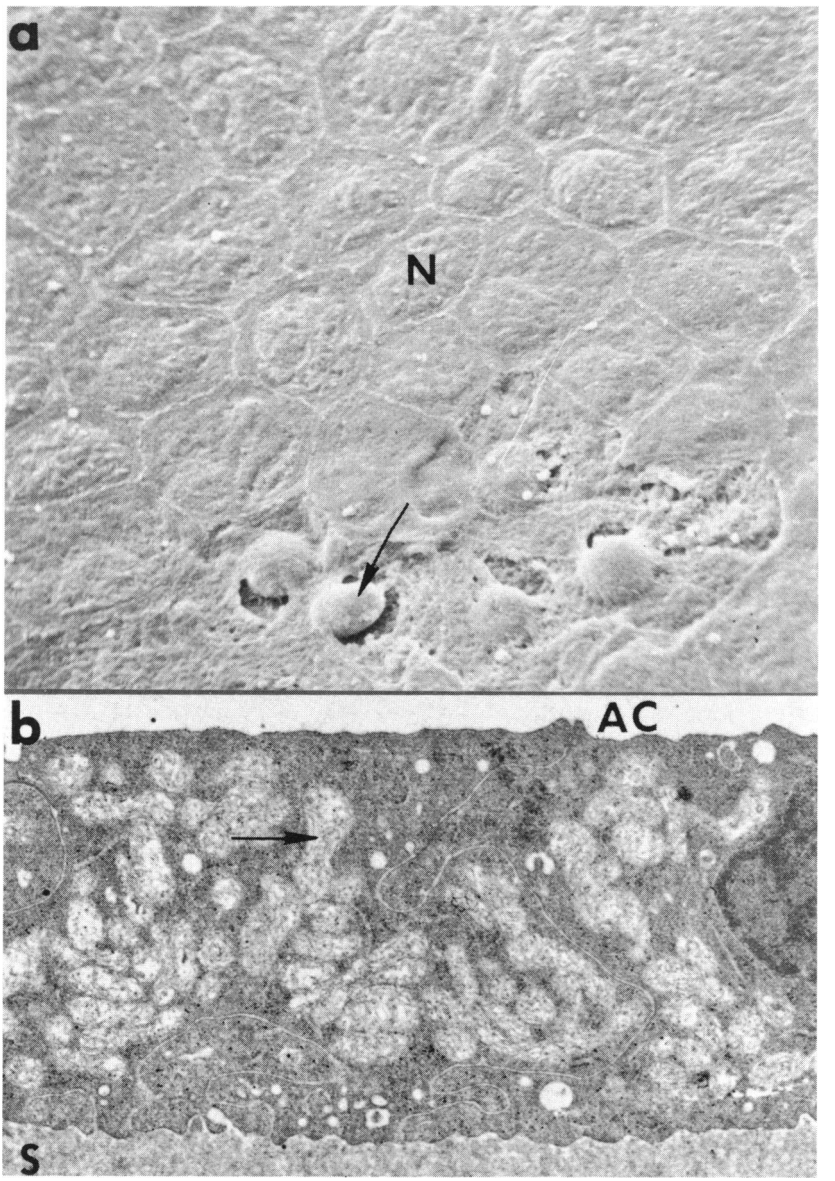
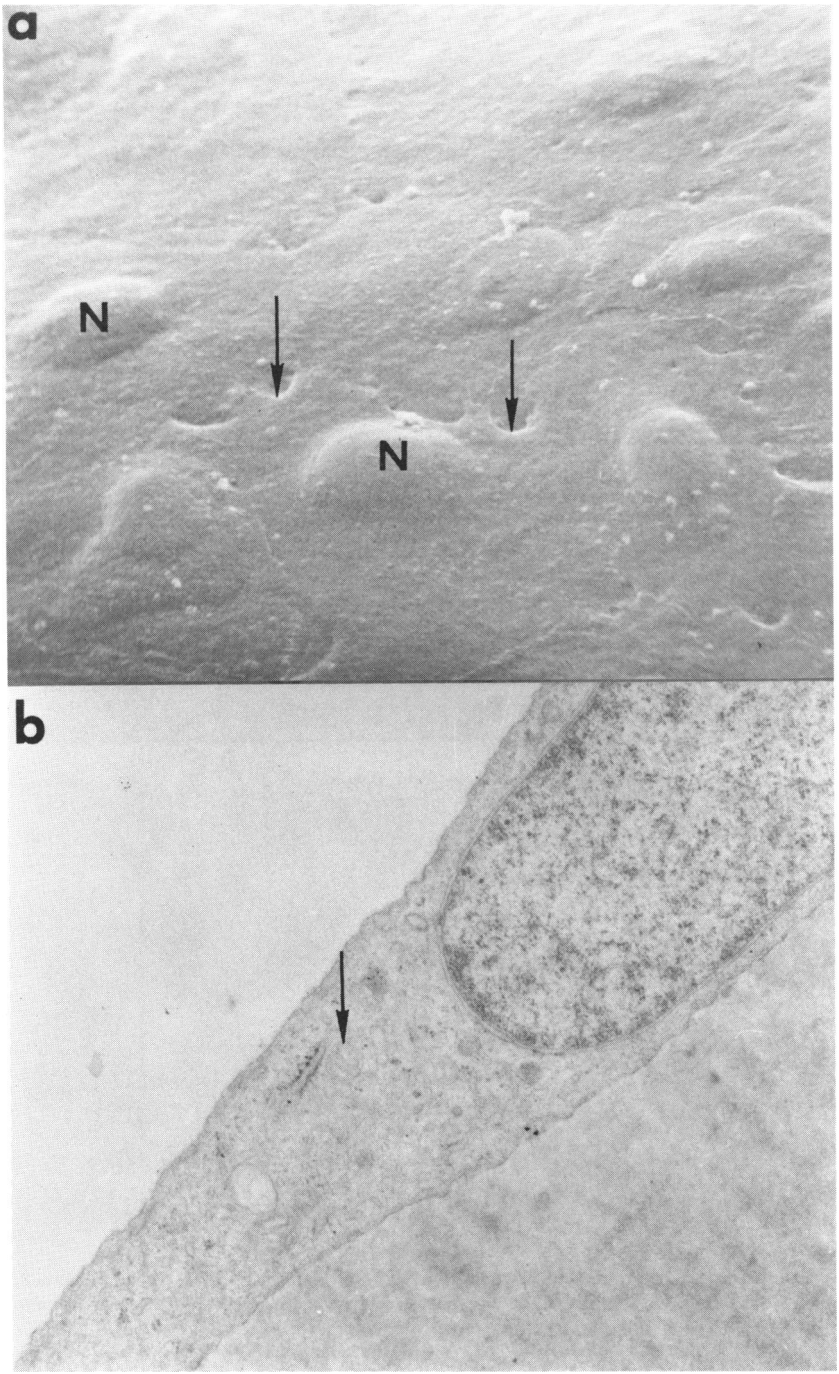


FIGURE 3

Donor #51. Postmortem time 33 hours. Moist chamber control. a. SEM: Most cells intact, 10-30 μ diameter, vary in size and shape. Areas of lysed cells (arrow) less in number and severity than donor #50 (fig. 1a). N — Nuclear bulge. (× 1000) b. TEM: Cells 3 μ thick with many mitochondria (arrow). AC — Anterior chamber, S — stroma. (× 3940).


a
N
N
b

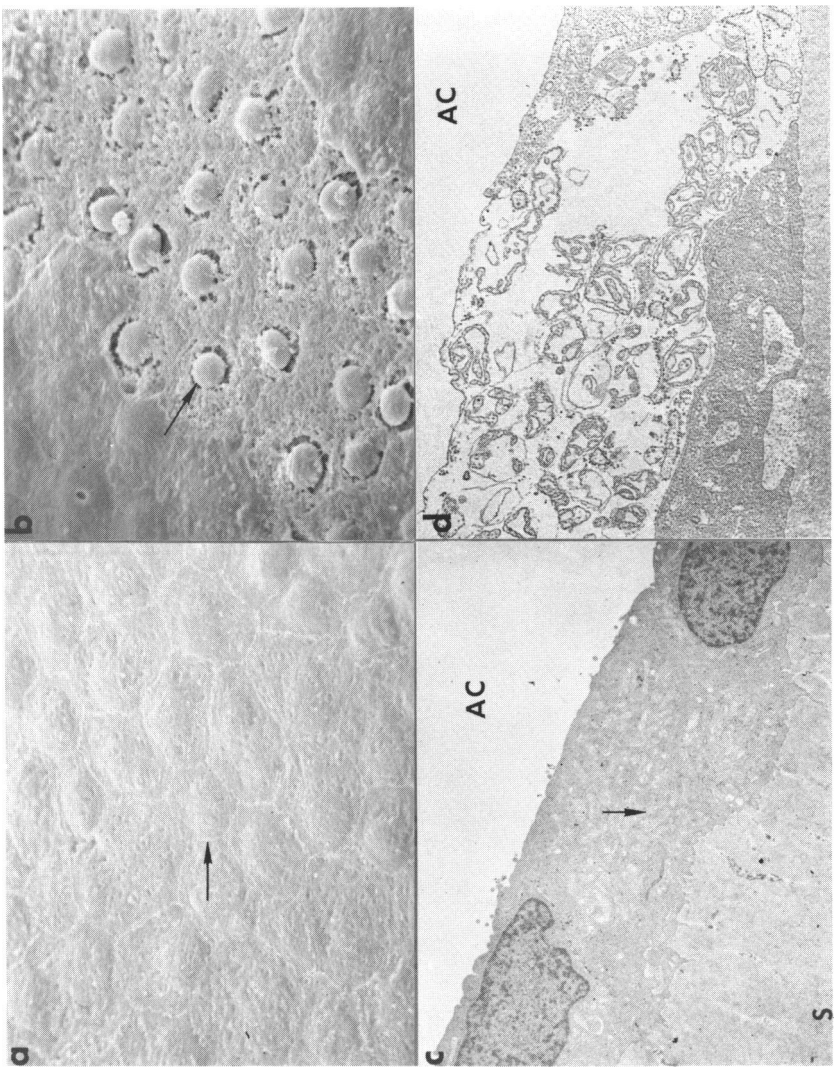


FIGURE 5

Donor #52. Postmortem time 24 hours. (Moist chamber control) a. SEM: Flat, irregularly hexagonal cells 10-25 μ diameter, well defined borders (arrow). (× 635) b. SEM: Patch of lysed cells with rounded nuclei. These areas of lysed cells were less in number and area than donors #50 and #51. (figures 1, 3). c. TEM: Intact cell 3-4 μ thick with mitochondria (arrow) endoplasmic reticulum and ribosomes present. AC — Anterior chamber. (× 3800) d. TEM: Degenerating cell on left. (× 3800).

FIGURE 4 *(opposite)*

Donor #51. Postmortem time 33 hours, organ culture 21 days. a. SEM: Endothelial cells are large (50-60 μ). Cell borders distinct (arrow). N — Nuclear bulge. (× 1000) b. TEM: Endothelial cells 1-3 μ thick, normal nuclei and posterior plasma membrane. Mitochondria (arrow) few in number. (× 19,600).

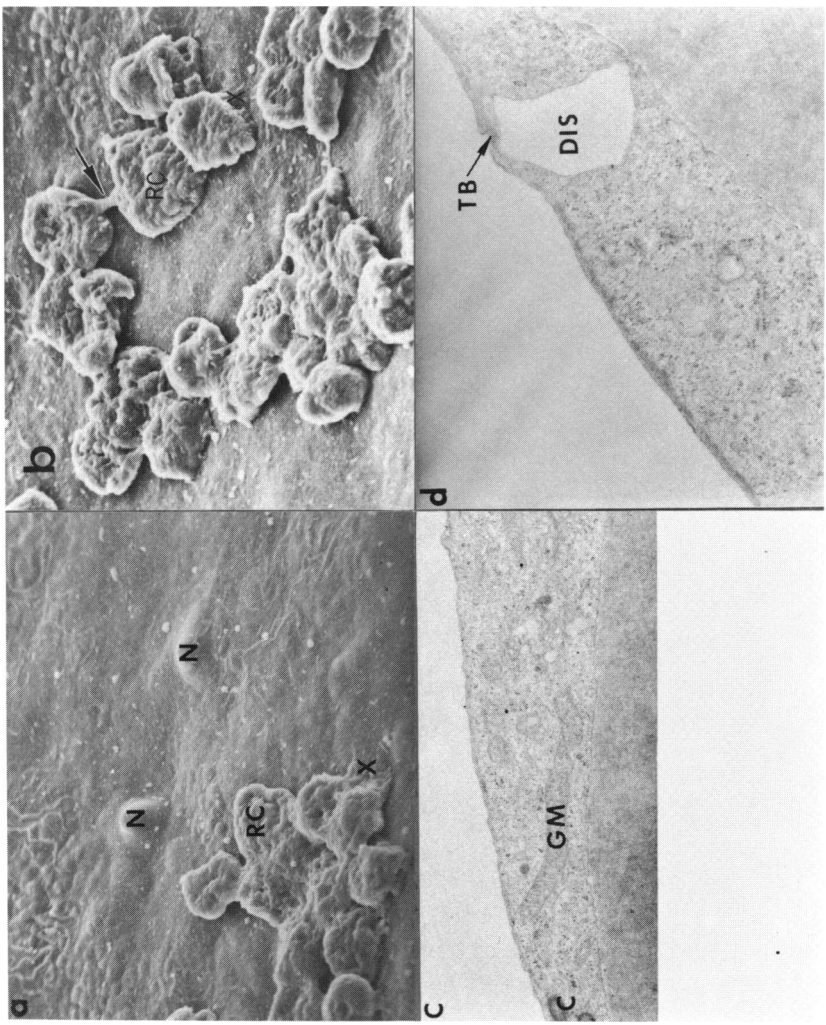
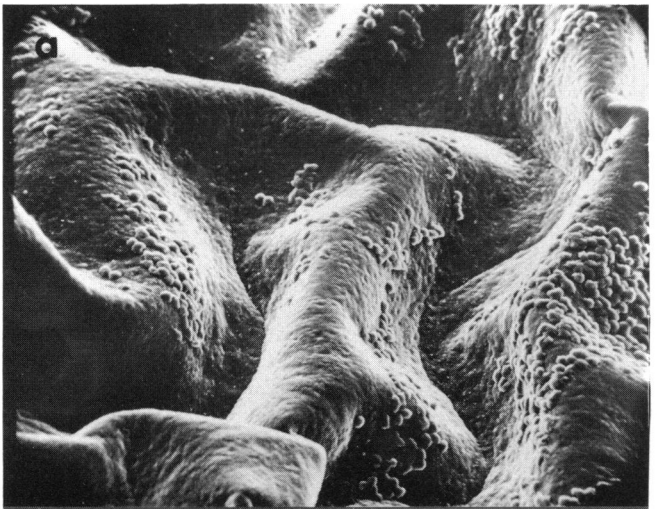


FIGURE 6

Donor #52. Postmortem time 24 hours, organ culture 21 days. a. SEM: Rounded cells (RC). Possible continuity (X) with flat endothelial cells. N — Nuclear bulge. (× 650) b. SEM: Note intercytoplasmic bridge (arrow) between two round cells (RC). × 650) c. TEM: Endothelial cell 1-2 μ thick. Centriole (C) and giant mitochondria (GM) are seen. (× 7,600) d. TEM: Dialated intercellular space (DIS) with intact terminal bar. (TB). (× 10,650).



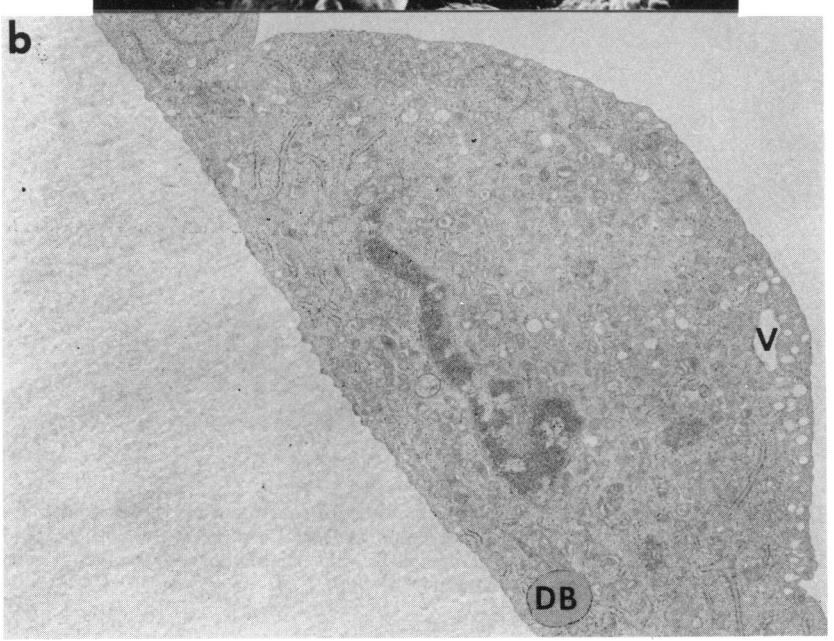


FIGURE 7

Donor #1. Postmortem time 10 hours, organ culture 10 days. a. SEM: Many folds in Descemet's membrane. Multiple scattered round cells (Dia. 10-25 μ overlying intact endothelial cell layer. ($\times$ 85) b. TEM: Cell is same diameter as round cells in a. (11 x 7 μ). Has abundant normal organelles, including mitochondria. Dense bodies (DB) and vacuoles (V) are seen in the cytoplasm. ($\times$ 7,800) c. TEM: Cells vary from 2-9 μ thick. Multiple dense bodies (DB) and vacuoles in cytoplasm. Endothelial cell on right has degenerative material in cytoplasm (X). Mitochondria and endoplasmic reticulum present. ($\times$ 7600).

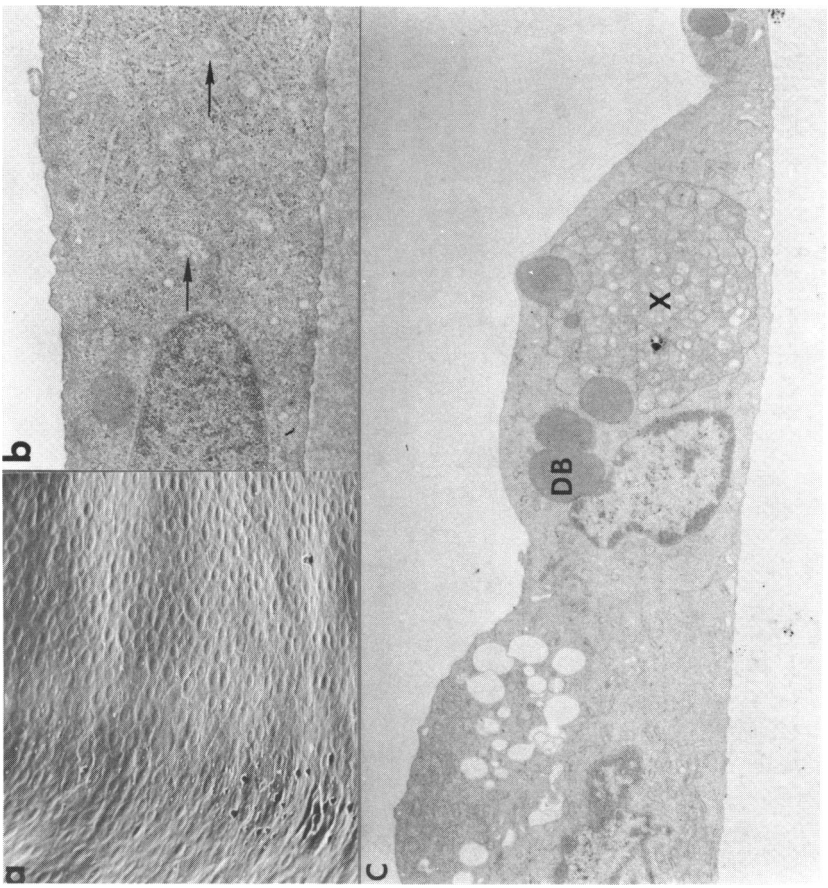


FIGURE 8

Donor #3. Postmortem time 6 hours, organ culture 10 days. a. SEM: Entire endothelial cell surface intact. No round cells seen. Some folds in Descemet's. (× 260) b. TEM: Endothelial cell 3.3 μ thick. Abundant organelles. Mitochondria (arrows) swollen. Nucleus normal (× 18,500).

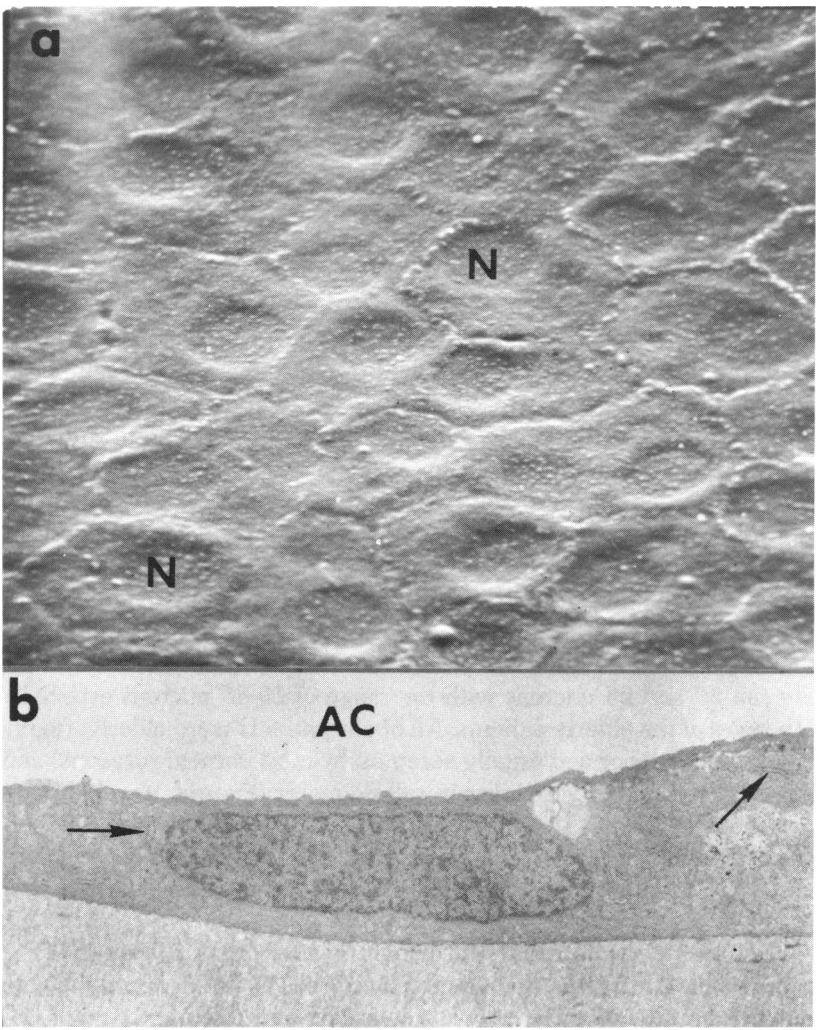


FIGURE 9

Donor #3. Postmortem time 6 hours, organ culture 18 days. a. SEM: Entire endothelial layer intact. No round cells seen. Cells 15-30 μ diameter. N — Nuclear indentations. ($\times$ 1200) b. TEM: Endothelial cells thin, 2.8 - 4.5 μ. Organelles present (endoplasmic reticulum — arrows). Mitochondria hard to identify. AC — Anterior chamber. ($\times$ 7500).

TEM (Figures 9b, 11c) demonstrates thin, intact cells with normal nuclei and cytoplasmic organelles. Mitochondria are difficult to find in figure 9b, but plentiful in figure 11c.

In summary, when one compares the endothelial ultrastructure of 10 day vs 18-21 days in organ culture, there is no discernible difference. In both 10 and 18-20 days the cells are of the same diameter by SEM and maintain ultrastructural integrity by TEM.

DISCUSSION

The value of TEM in studying endothelial morphology and correlating this with viability has been established in moist chamber[23, 24] and cryopreserved corneal tissue.[24, 25, 26, 27]

SEM, on the other hand, has only recently been employed to study the endothelium from the point of view of viability.[28] Its advantage is that it can survey the entire endothelium and viability, based on the structural integrity of the individual cells can be estimated.

Svedbergh and Bill recently described the normal SEM appearance of corneal endothelium in man and monkeys.[29] The moist chamber SEM studies in patients #50, 51, and 52 compare favorably with the findings of Svedbergh and Bill. They found the normal diameter cells to be between 20 and 25 microns with the range of 10-45 microns especially in the eyes of the elderly patients. All of our patients were elderly, (many older than the donor age usually accepted by most corneal surgeons) and demonstrated similar variability in cell diameter (Figures 10a, 11b, 3a). SEM (Figures 7a, 8a, 11a) demonstrated that in all organ cultured corneas there was a covering of endothelial cells over all of Descemet's membrane, whereas moist chamber specimens demonstrated patches of missing or lysed cells. (Figures 1a, 3a, 5b)

Measuring the endothelial cell diameters after organ culture by SEM as well as measuring the thickness of these cells by TEM would indicate that in some situations the cells are enlarging and thinning as if spreading to cover the area left by the lysed cells noted in moist chamber storage.

This would indicate a dynamic process where spreading endothelial cells "heal" defects in its own layer caused by cell lysis and death that occurs with increasing postmortem times. That lengthened postmortem time increases cell death has been previously described in moist chamber[26] and cryopreserved[24] corneas. This can be demonstrated in this study by comparing donors 50, 51, and 52 (postmortem times 24-74 hours) with donors 1, 3, and 5 (postmortem times less than 12 hours).

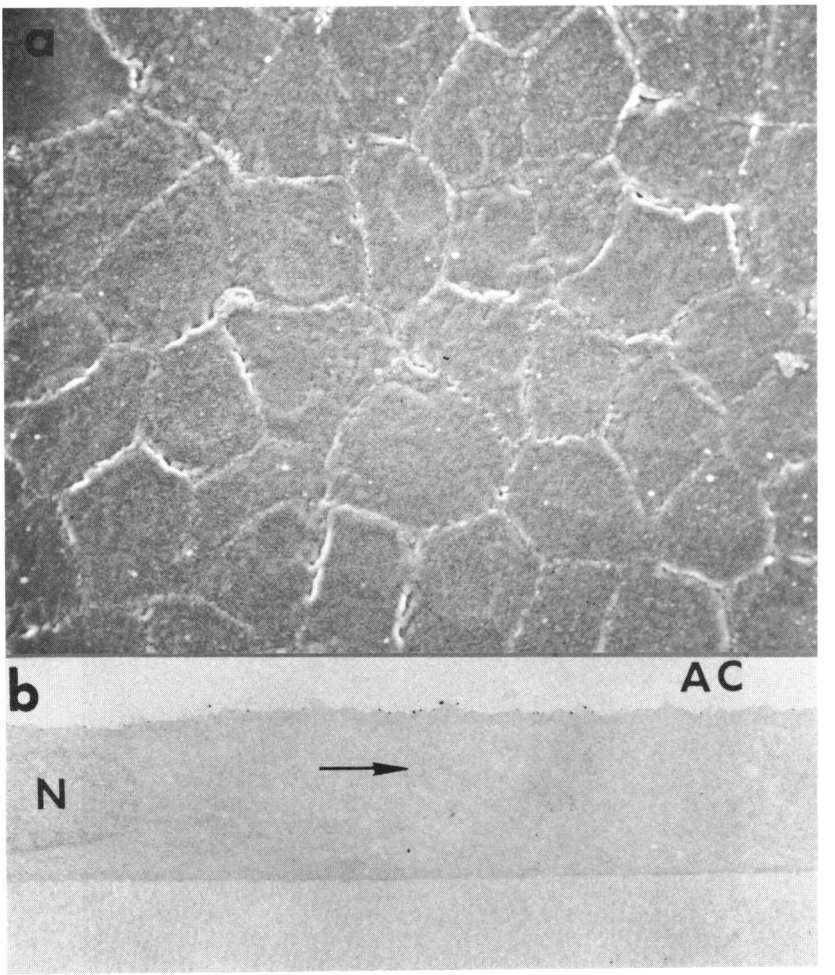


FIGURE 10

Donor #5. Postmortem time 12 hours, organ culture 10 days. a. SEM: Endothelial cell layer intact but coating obscures details. Cells 30-40 μ diameter. No round cells present. ($\times$ 650) b. TEM: Flat continuous cell layer 4-5 μ thick. Normal cytoplasm, nucleus (N) and endoplasmic reticulum. Few mitochondria (arrow). AC — Anterior chamber. ($\times$ 20,500).

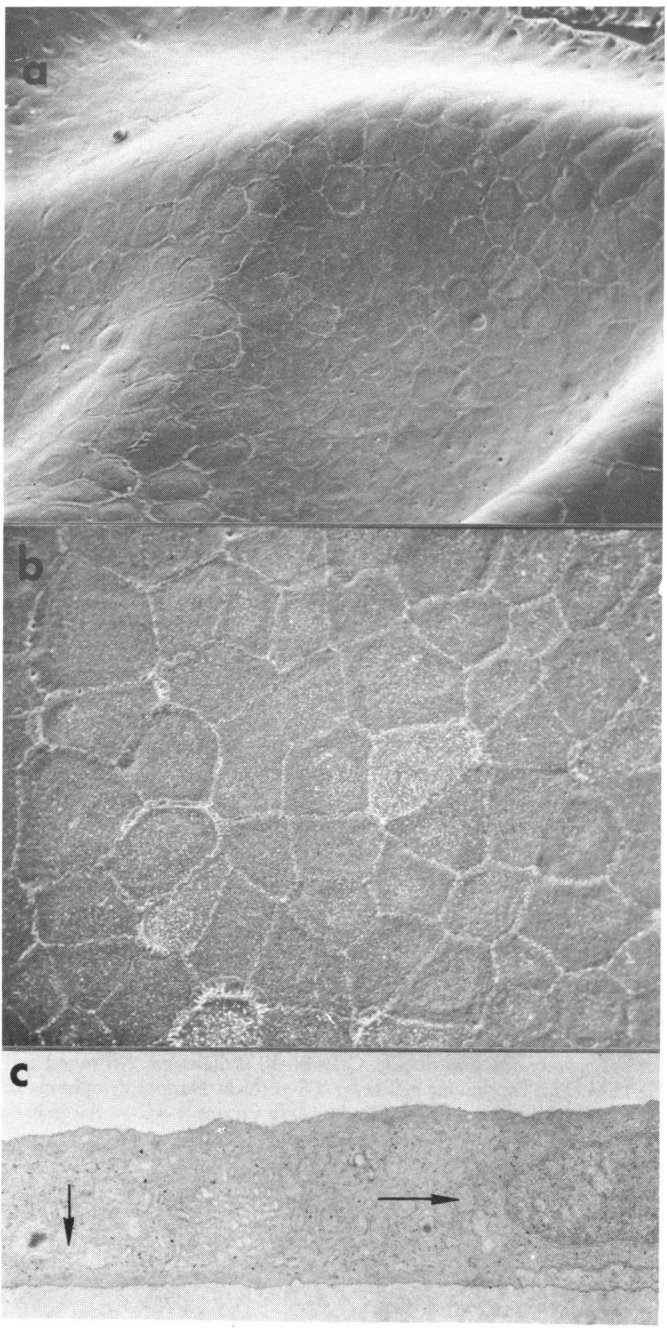

a
b
c

FIGURE 11 *(opposite)*

Donor #5. Postmortem time 12 hours, organ culture 21 days. a. SEM: Intact endothelial cell layer. Cells 30-40 μ diameter (same as 10 days). Rolling contour to Descemet's. No round cells. (× 205) b. SEM: Endothelial cells irregular in size and shape as at day 10. (× 530) c. TEM: Cell 1.8 μ thick. Mitochondria abundant (arrows). Giant mitochondrion at far left (× 22,000).

In the latter group, SEM demonstrated normal cell diameters and an intact layer completely covering Descemet's membrane at both 10 and 20 days. We can presume from previous work[24] that because the postmortem time was less than 12 hours, there were fewer areas of cell lysis. Therefore, the viable endothelial cells did not have to spread to the extent that was necessary in the group with postmortem times exceeding 24 hours, the group that demonstrated large flat cells. Age of the donor could not account for this difference since both groups were approximately the same age distribution (Table 1). The group with postmortem time under 12 hours also showed that up to 21 days, there was no increase in size of endothelial cells after being placed in organ culture since cell size was the same at 10 and 20 days. The phenomena of endothelial thinning and spreading to completely cover Descemets is a fascinating process with many implications. For instance, it may give us the first in vitro model of wound healing, a model already being studied in skin. However, more relevant to this study is the question of how functional are these large flat endothelial cells. TEM demonstrated that although they were very thin, they appeared to be viable because they contained a normal posterior plasma membrane, nucleus, cytoplasm, and except for decreased numbers of mitochondria, relatively normal organelles. The mitochondria were swollen and reduced in number especially in cells with attenuated cytoplasm. The answer to the question of how functional these cells are cannot be answered until in vitro physiologic studies and in vivo grafting have been done. We have shown, however, that the cells maintain ultrastructural integrity up to 21 days in organ culture and that the endothelial layer completely covers Descemet's membrane. This is in marked contrast to corneas stored in moist chambers where the endothelial cells are disrupted or lost by seven days.[24]

SEM demonstrated contour changes (Figures 7a, 11a) that would not have been discovered by other methods of examination. We feel this is due to placing the corneal button with the epithelial side down, causing a wrinkling of the central cornea. Since this could damage endothelium, we are developing a special organ culture container for the cornea in order to avoid this problem.

SEM demonstrated round cell formation (Figures 2a, 6a, 6b, 7a) after organ culture storage in three of the corneas, one at 10 days (donor #1) and two at 21 days (donors #50, 52). Each of the organ cultures in which these round cells were noted were under some form of "stress". Donor #1 culture was probably infected and donors #50 and #52 were in moist chamber storage for a prolonged period of time. TEM of what we believe to be one of these cells (Figure 7b) demonstrates an ultrastructurally "healthy" cell. These same cells have been observed in other organ systems (#30, 31 and 32) and in these other systems were considered to be dividing cells. Dividing cells cannot be identified ultrastructually and only other studies, such as radioautography using labeled thymidine, will resolve this question.

Descemet's membrane appeared normal in all of our material. We wondered if any new Descemet's membrane was formed by the spreading and thinned endothelial cells. None was seen.

Dense bodies such as those seen in figures 7b and 7c, have also been seen by us in cat endothelium in organ culture and have been seen by others during organ culture of human fetal symovium.[33] In the human fetal synovium, these dense bodies are thought to represent lipid that has been taken up by the cells from the serum in the media. Our organ culture media contains calf serum and it is likely that these dense bodies are also lipid accumulations in the cells.

CONCLUSION

The ultrastructure examination (SEM and TEM) of paired human corneal endothelium in moist chamber and after varying lengths of time in organ culture has demonstrated that the cells form a complete covering of Descemet's membrane and maintain their ultrastructural integrity. A dynamic process of endothelial cell enlargement and thinning to cover Descemet's membrane was noted in those corneas with prolonged postmortem times before being placed in organ culture. This seems to be a repair process by which the remaining viable endothelial cells spread out to replace the dead cells that have been lysed. The functional ability of these cells as well as the definition of the nature of rounded cells seen on the surface of some of these corneas await further studies.

SUMMARY

Utilizing transmission electron microscopy (TEM) and scanning electron microscopy (SEM) six pairs of human corneas were studied as to the ef-

fect of moist chamber storage vs varying lengths of time in organ culture (up to 3 weeks) on the ultrastructure and surface morphology of the endothelial cells. The length of moist chamber storage time was proportional to the area and severity of cell damage. The fellow cornea in organ culture 21 days demonstrated covering of the defect left by these damaged cells by a dynamic process of endothelial cell enlargement and thinning, thereby spreading to cover these defects. Although these cells appeared to maintain ultrastructural integrity, they were extremely thin ($<1\mu$) and contained few mitochondria. Corneas kept in most chamber no longer than 12 hours and stored from 10 to 21 days did not demonstrate this endothelial cell thinning and enlargement. These cells also maintained ultrastructural integrity although in some the mitochondrial population was decreased. Round cells, that may represent dividing cells, were seen on the endothelial surface of organ cultured corneas where moist chamber storage was prolonged or the culture medium may have been infected.

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DISCUSSION

DR A. RAY IRVINE. The authors have demonstrated that the corneal endothelium is not only better preserved in organ culture than it is in moist chamber storage, but that it is improved by storage in culture media. This correlates with our experience with pure endothelial cell culture in which cell death occurs after about seven days when protein (fetal bovine calf serum) is excluded from the culture media.

I was impressed with the uniformity of cell size and pattern in donors with postmortem times less than twelve hours and believe that their corneas would do better in transplant than those with postmortem times of 24 to 74 hours in which large flat cells were demonstrated.

Although the spreading and thinning of cells in an attempt to cover defects caused by lysed cells and their desquamation is certainly evidence of viability of the cultured endothelium, it cannot be concluded that the structural integrity of the cells ensures their normal physiologic behavior. It has been our experience from clinical-pathologic correlations that the ability of the endothelium to dehydrate the cornea is related to the concentration of endothelial cells and that as the population of these cells decreases, focally or generally, from whatever reason, that the cells become increasingly pleomorphic and that concommitantly they become increasingly less able to dehydrate the cornea. Therefore, unless the endothelial cell population is actually maintained, presumably as evidenced by mitoses, it may be that transplants of corneas with a large portion of their endothelium comprised of pleomorphic flattened cells will become edematous.

The dense bodies within the cytoplasm demonstrated in figures 7b and 7c were thought to be lipid in nature. However, they somewhat resemble the intracytoplasmic precursors of Descemet's membrane described by Chi, Teng, and Katzin in their work on endothelial gutatta and also resemble the periodic acid Schiff positive bodies that we have observed in tissue culture of corneal endothelium. We know that in conditions deleterious to the endothelium, Descemet's membrane often thickens, and we wonder whether the dense bodies represent material that will be incorporated into that layer.

Unfortunately, scanning electron microscopy does not differentiate dividing cells from those that are dying. Both become round and are about the same size. I suspect that confluent rounded cells in patches represent cell death. At least in cell culture, mitoses seem to occur in a random spotty manner throughout the monolayer; rather than in large numbers of adjacent cells.

Perhaps the greatest contribution of this research is that an in vitro model has been provided to study endothelial cell response to injury. The authors are to be congratulated on their excellent work, and I thank them for the privilege of preview.

DR JOHN H. KING, JR. The clinical implications of this research study may be far-reaching. It could help extend the applications of keratoplasty in the lesser developed countries, where the need is often greater than in the developed nations.

In 1956, we first introduced to this Society a method for the preservation of human corneas by dehydration in glycerine. The endothelium was not viable and such tissues were useful for lamellar grafting and patch grafts only. They do restore vision when corneal scarring involves the superficial layers only. They cannot be applied for penetrating grafts.

Despite its limitation to some 20 per cent of those patients requiring a graft, this method stimulated the establishment of eye banks in many developing countries.

The viability of the fresh donor cornea can be extended to 10 to 14 days by replacing the aqueous with 25 per cent PVP (polyvinylpyrroline) solution. I used such a cornea 10 years ago while abroad, and obtained a clear graft.

Long-term "fresh" preservation is successful by the Kaufman-Capella method of freezing corneas in liquid nitrogen. Its use demands a trained technician and the present container cannot be shipped on commercial airlines.

A reliable method of medium-term preservation of fresh corneas is a definite need. If viability can be maintained for 10 to 14 days, with shipments in a small container, the International Eye Bank would be able to send "fresh" corneas to any area of the world where they are desperately needed.

I would like to encourage these researchers to continue their efforts and to develop a practical method which will benefit not only the "have" nations but also to assist our fellow surgeons world-wide.

DR CLEMENT MCCULLOCH. I have enjoyed this presentation. The authors have allowed me to see their manuscript. I have a couple of points to make.

The authors' work is particularly interesting on at least two scores. Firstly, they advance the thesis that after organ culture for several weeks the cornea has not changed structurally and therefore the corneal cells have not lost viability. Possibly the microscopic appearance of an intact structure is not sufficient evidence that the endothelial cells are viable. The finding of viable cells by tissue culture would be helpful. In the practical world of grafting, the deciding finding would be ability of the donor to be accepted and to continue viable as a clear graft. Chromosome or Barr body studies to show that the cells of the donor are the true inhabitants would be confirmatory evidence. Donor cells after organ culture may be of decreased virility and may be less capable of active adjustment and growth when implanted in a recipient.

Secondly, is the decreased antigenicity in organ cultured corneas, which they have demonstrated, applicable to humans? Even though we do not know which components in the donor cornea are potentially antigenic, it would seem not improbable that storage and washing in organ culture medium would leach out some of the donor's antigen.

In 1957 and 1959, Ormsby and Basu showed that by submerging cornea in fluid medium, or by storage in vivo, antigenic strength of heterografts was decreased. Species specific antigens were mostly affected. The effect was more prominent with intralamellar than with full thickness grafts. Finally, the heterograft chicken to rabbit has a low immune expression. Therefore, a decrease of

species specific antigens in a weakly antigenic model, chicken to rabbit, may not apply to other laboratory models and may not apply in the homograft situation, as between humans.

It is interesting that in 1964 Wine and Basu described in vitro wound healing, migration of epithelium, and its effect on proliferation of stroma cells. The ocular in vitro model of wound healing is not new.

In tissue culture when a cell "rounds up" it is (a) loosening from the underlying tissue and (b) in a state preceding death. It seems reasonable to assume the "round cells" seen by the authors were in such a state and were not preceding cell division.

Large and atypical cells are regularly seen in the normal human and animal endothelium, and their number varies from eye to eye, even in different regions of the same eye. Therefore, in this study one must be guarded in reading significance into their presence.

The authors are to be congratulated on, with modern techniques, reconsidering some old studies. I am sure they will agree that much must be done before the work could be applicable to human corneal grafting.

Dr John E. Harris. I would like very much to thank the discussants for their encouraging words.

In answer to the question of whether these bodies are lipid, of course we don't know. An interpretation has been made, and your explanation is equally good.

As far as the appearance of the organ cultured endothelium as compared to what Dr Stocker found in his technique is concerned, this has been studied by one of our co-authors, Dr Van Horne, and she finds the endothelial cells completely lysed in 7 days; so the organ culture technique does by this measure at least show some cellular integrity.

I would like to point out, however, that this is just one measure of the viability of the cornea and of the endothelium. This whole project has obviously two aspects. First of all, how viable is this cornea after it has been stored for 21 or 30 days? Secondly, there is the question Dr McCulloch particularly alluded to, as to whether it is antigenistic. We have embarked on this, not as a vertical study but as a horizontal study, starting off in all directions at once. There is one measure of viability, namely, that we do show relative integrity of the endothelium. We have demonstrated this, incidentally, in light microscopy as well.

However, we have done other studies. We have shown physiologically that these corneas digest very well after storage periods of 21 days and longer. We have done allografts of stored rabbit cornea. We have also transplanted human cornea (after storage) into the cat. We use the cat because it's cornea more closely approximates the thickness of human cornea.

After organ culture — and I am not making any suggestions that we have a success, necessarily, or that we have been able to cross species — if you put this human cornea in the cat, the human cornea will digest it. The human cornea will remain clear for several weeks. I am just saying this indicates that the organ-cultured cornea after several days is a viable cornea. I am not claiming — and

I want this very clear — that this necessarily means we have successfully, at this juncture, crossed species barriers, although we feel this might be feasible.

As to the alterations of the antigenicity, we do not know at this time whether we do modify the antigenicity of the human cornea when we place it in another human — in other words, in doing an allograft. The data of Summerlin and Good and their associates suggest that the antigens remain in the skin but somehow do not express themselves. This is an alteration of antigenicity in a manner still unclear as far as skin in concerned.

We do feel in the final analysis that, as far as we know, there should be some alteration of the antigenicity. We have demonstrated this clearly in studies reported elsewhere; that using allografts from one species to another, the organ culture does reduce the antigenicity, or shall we say, improves the "take" when we cross species barriers.

I am sure you all realize that we have a long way to go. I appreciate Dr King's remark that this might well be the method in the future for intermediate storage time of cornea. We are working on this ourselves at this time. We are interested in developing shipping techniques. What is the future of these corneas after they have been organ cultured for a period of time? Can we take them out and ship them to Dr King's many places?

The antigenicity studies are basic and these are things we are doing. We find this very exciting work, but I would like to make it fairly low-profile. We haven't done human-human yet on these cultured corneas. We have a number of patients who we feel can justifiably use this, and we hope by this time next year we will have some successes to report to you.