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Transplantation of cultured bovine corneal endothelial cells to rabbit cornea: Clinical implications for human studies

(keratoplasty/corneal endothelium/fibroblast growth factor)

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ABSTRACT **Rabbit corneas denuded of their endothelium were coated with bovine corneal endothelial cells (from steers) previously maintained in tissue culture for short (20 generations) or prolonged (200 generations) periods. When grafted back into female rabbits, the corneal buttons remained clear and showed no edema. In contrast, denuded corneas coated with bovine keratocytes and grafted into rabbits became opaque and edematous within 7 days and remained so thereafter. Bovine corneal endothelial cells of the grafted corneas, which had remained clear for over 100 days, proliferated actively when put back into tissue culture. The corneal endothelial cells of the graft had a chromosome number of $2n = 60$. The sex chromosomes were characteristic of the male (XY). The chromosome number of the endothelium of the recipient rabbit was $2n = 44$ with sex chromosomes characteristic of the female (XX). Results of the karyotype analysis show that there was no invasion of the corneal button by the recipient endothelium and, conversely, no invasion of the recipient endothelium by the endothelium on the corneal button. These results demonstrate that cultured corneal endothelial cells remain functional *in vitro* and can replace a damaged or nonfunctional endothelium *in vivo*.**

The cornea owes its transparency to its uniform structure, avascularity, and deturgescence. The relative dehydration of the corneal tissue is determined primarily by the active transport pump of the corneal endothelium. The ability of most corneal endothelia to repair themselves *in vivo* is severely limited by their lack of proliferative capacity (1, 2). Although small endothelial wounds can be repaired through the process of endomitosis and cell enlargement (3), extensive wounds may result in swelling of the corneal stroma and loss of transparency. Chronic edema may result in vascularization of the stroma and a further loss of transparency. An intact endothelium is therefore essential for normal vision.

Corneal transplantation (keratoplasty) is one of the most important ophthalmic surgical procedures yielding a high degree of restoration of vision. However, it has an intrinsic limitation in the availability of corneas with an endothelium suitable for keratoplasty. If a method could be found to replace the damaged endothelium with a viable one that would retain its capacity to proliferate, this would dramatically increase the use and success of keratoplasty. That such an approach is feasible has been shown by Maurice *et al.* (4), who reported in an abstract the transplantation into rabbit eyes of early-passage cultures of rabbit corneal endothelial cells (4). We therefore investigated the possibility of replacing damaged corneal endothelium with a new, vigorously growing endothelium that had been maintained in tissue culture for short (20 generations) or prolonged (200 generations) periods and that originated from bovine eyes (5, 6).

Our results demonstrate that rabbit cornea coated with bovine corneal endothelial cells previously maintained in tissue culture remained clear when grafted into a recipient. This demonstrates that cultured bovine corneal endothelial cells remain functional and can replace a damaged or nonfunctional endothelium *in vivo*. New methods for transplanting corneal endothelia are foreseen as a further result.

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MATERIALS AND METHODS

Tissue Culture of Bovine Corneal Endothelial Cells and Keratocytes. Cultures of bovine corneal endothelial cells derived from steer eyes were established as described (5). The cells were maintained on gelatinized dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 5% dextran, 50 μg of gentamycin per ml, and 2.5 μg of Fungizone per ml. Fibroblast growth factor (100 ng/ml) was added every other day (5, 6). The cultures were passaged every week with a split ratio of 1:64. The morphological and biochemical characteristics of these cultures have been described (5–10). Cultures of bovine corneal keratocytes were established as described (7).

Coating of Denuded Descemet's Membrane of Rabbit Cornea with Bovine Corneal Endothelial Cells or Keratocytes. Female Dutch rabbits were used throughout this study. The procedure is summarized in Fig. 1. After being anesthetized, the animal was sacrificed and the eye was enucleated. The cornea and a rim of sclera were then excised. The cornea was placed in a petri dish so that the endothelial layer faced upward. A cotton swab was used to sweep the surface of the cornea (6). [Control studies with an alizarin red staining technique have shown that the entire endothelial cell layer is removed and that no damage is done to Descemet's membrane (6, 9).] A corneal button was cut with an 11-mm trephine and placed in a Teflon container fashioned to fit the contour of the cornea. Concurrently with the dissection and preparation of the cornea, confluent endothelial cell and keratocyte cultures were trypsinized with 0.005% trypsin in phosphate-buffered saline/0.01 M EDTA. (The process of trypsinization was followed with an inverted phase-contrast microscope.) As soon as the cells had rounded up (5 min at room temperature), they were collected in medium containing 0.1% calf serum, and the cells in an aliquot of the suspension were counted in a Coulter Counter. The cell suspension was then centrifuged, the supernatant was aspirated, and the cell pellet was resuspended in medium/0.1% calf serum at a final concentration of 10^5 cells per 70 μl; 120 μl was added to each cornea. The corneas were incubated in a water-jacketed humidified CO_2 incubator for 2–12 hr. Evaluation of the plating efficiency and of the cellular morphology was done by using alizarin red staining of the cornea (6–11). The cells can also be plated in unsupplemented medium or in aqueous humor. Under these conditions, the plating efficiency and the morphology of the cells are similar to those of cells plated in the presence of medium containing 0.1% serum (6). Serumless medium or aqueous humor is pref-

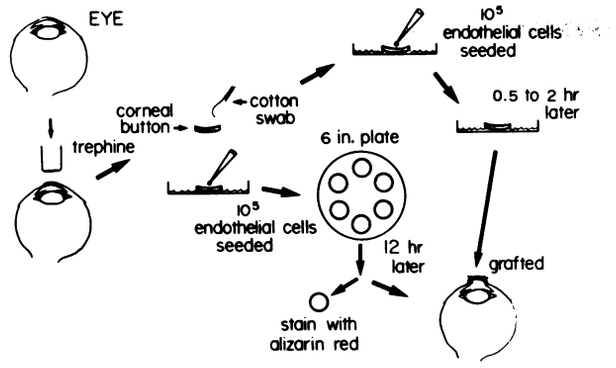

FIG. 1. Schematic representation of procedure for seeding corneal endothelial cells on corneal button. A corneal button is removed from the eye with an 11-mm trephine and placed in a tissue culture dish with its endothelium facing upward. The endothelium is scraped off with a cotton swab. New endothelial cells are seeded onto the corneal button which is then incubated for 30 min to 2 hr prior to being grafted back into the same animal or kept overnight in a humidified CO_2 incubator to be grafted the following day(s) into another animal.

erable in order to minimize immunological reactions that might be elicited by the nonspecific adsorption of serum proteins onto the stroma of the cornea.

Keratoplasty Technique. Rigorous dilatation of the rabbit eye was carried out for 1 hr prior to commencing the surgical procedure. Aspirin (300 mg) was administered rectally to inhibit prostanglandin release in the anterior chamber. The rabbit was then anesthetized with 2.5% Surital intravenously, and 0.25 ml of atropine sulfate was given subcutaneously. Superior and inferior rectus bridal-stay sutures were passed; in the host, partial trephination was carried out to a depth of two-thirds of the corneal thickness. The donor's cornea was then brought from the incubator to the operating table, and a 7-mm corneal button was trephined out on a Teflon block, with the endothelium facing upward. The donor tissues were covered with balanced salt solution until sutured onto the host. Attention was then directed toward the host's cornea. The anterior chamber was entered with a razor blade, and tissue was excised with curved corneoscleral scissors. One drop of heparin (1000 units/ml) was introduced into the chamber; a freshly prepared solution of epinephrine (without preservatives) occasionally was needed as a mydriatic. The donor tissue was sutured with 10-0 nylon in either a running or interrupted manner. Rigorous dilation was carried out postoperatively. Sphincterectomies occasionally were required.

During and after the operation, photographs were taken to evaluate the degree of edema present in the cornea. After the operation, the cornea was examined for clarity at 2-day intervals and photographs were taken. After 2 weeks, which we consider to be the critical period, photographs were taken at weekly intervals. Dexamethasone was administered topically daily for the first 10 days to prevent the invasion of blood vessels into the cornea. After 31 days, all the sutures were removed.

Karyotyping. The animals that had clear corneas were sacrificed at various intervals after keratoplasty, and the transplanted corneas were excised. The grafted corneal button was dissected out from the recipient cornea. Each piece of tissue was then placed, endothelial side up, in a petri dish so that its endothelium could be lightly scraped with a grooved director (5, 6). The tissue fragments were maintained on gelatinized dishes in medium supplemented as described for the bovine corneal endothelial cell cultures (5). Fibroblast growth factor (100 ng/ml) was added every other day. Within 2 days, the tissue fragments had adhered to the bottom of the dishes and begun to proliferate. At day 5, the cultures were karyotyped as described by Hsu (12). Between 30 and 50 Giemsa-band metaphase cells were counted in each case. Five hundred metaphase cells were rapidly scanned for the presence of bovine cells in recipient tissue and of rabbit cells in the grafted endothelium. Because the recipients were female whereas the transplanted corneal buttons were coated with endothelium originating from male animals, special attention was given to the identification of the sex chromosomes.

RESULTS

Seeding or Coating of "New" Corneal Endothelium. When rabbit cornea denuded of its endothelium was seeded with bovine corneal cells previously maintained in tissue culture, most of the cells attached within 15 min and in 2 hr a new endothelium had settled upon the denuded Descemet's membrane (Fig. 2). For an 11-mm corneal button and a plating efficiency of 80%, the ideal inoculum is 1.5×10^6 cells (6). One can therefore envision two possible approaches (Fig. 1). One can take the cornea, plate the cells on it, and transplant it 6–12 hr later into other recipients (homologous transplant, as far as the corneal epithelium and stroma are concerned). Alternatively, one can use the same animal as both donor and recipient. A corneal button is removed, the cells are plated on it, and the same cornea is grafted 2 hr later into the same animal (autologous transplant). The tissue of foreign origin being transplanted will in this case be the bovine corneal endothelium coated on an autologous cornea. In view of our success, the second approach has several drawbacks. The cornea thus transplanted is not edematous and is completely clear at the time of grafting. Because it remains clear after transplantation, evaluation of the graft thereafter is difficult. We therefore chose the first approach, which consisted of keeping the transplant in organ culture for 6–12 hr after coating. During this time the corneas that do not have a rim of sclera become edematous. When grafted into the host, the homologous transplant coated with bovine endothelial cells achieves a dramatic degree of clarity during the operation, and 1 day later it is completely clear (Fig. 3). The longest time a graft has remained clear has been more than 8 months and prospects remain bright for these animals (Fig. 4 *A* and *B*; Table 1).

Unlike corneal buttons coated with bovine corneal endothelial cells, corneal buttons coated with bovine keratocytes became completely opaque within 7 days postoperatively (Fig. 4*C*) and remained opaque thereafter (Table 1).

These results demonstrate that bovine corneal endothelial cells maintained in tissue culture are functional when coated

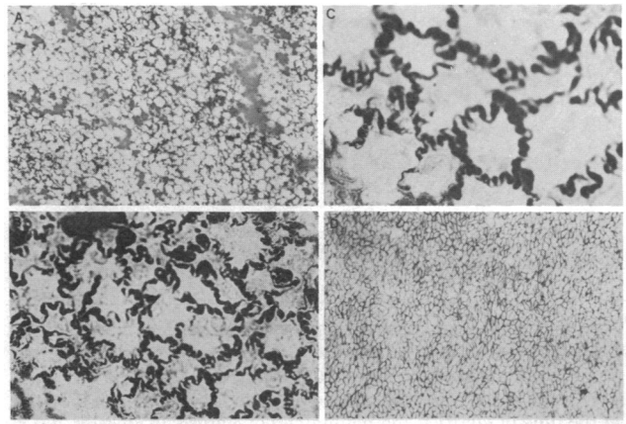

FIG. 2. Bovine corneal endothelial cells (2×10^5) seeded on an 11-mm corneal button. The corneas were then stained 30 min later (*A–C*) or 24 hr later (*D*) with alizarin red. (*A* and *D*, ×20; *B*, ×100; *C*, ×200.)

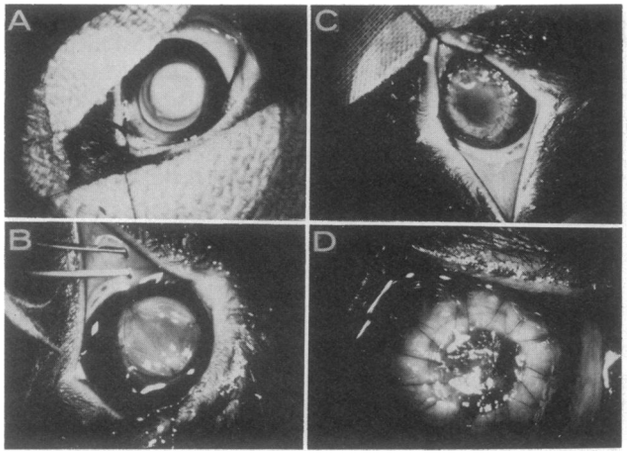

FIG. 3. Stages in keratoplasty. (*A*) Placement of opaque donor corneal button over trephined host's cornea. (*B*) Placement of the first temporary interrupted sutures. (*C*) Completion of suturing with 10-0 nylon. (*D*) At 24 hr after operation.

on rabbit corneas previously denuded of their endothelium. They performed the same function as that of the native endothelium—namely, that of an active pump which maintains the state of deturgescence of the cornea and will therefore keep the graft clear and transparent. Early- (20 generations) and late-passage (200 generations) corneal endothelial cells were equally functional (Table 1).

The rate of failure was low: of nine transplants, seven were successful. Two transplants failed because of an excessive number of cells plated onto the cornea. These cells later became deposited in the aqueous humor channels and caused a secondary glaucoma. Since this effect of an excessive number of cells was recognized, washing of the endothelial side of the transplant prior to keratoplasty has completely resolved the problem.

Evaluation of the Graft. Because rabbit corneal endothelial cells are known to keep their ability to divide and to regenerate after trauma, it is of extreme importance to establish the nature and origin of the cells present in the graft after long-term transplantation. Because these studies were performed with recipient and donor tissues from different species as well as from different sexes, the endothelial cells present in recipient and grafted tissues can be identified easily by karyotyping. The karyotype of the endothelium present in the graft always had male sex chromosomes (XY) and a diploid number of chromosomes ($2n = 60$) with a chromosomal structure like that described by Hsu and Benirschke (13) for *Bos taurus* (Table 1; Fig. 5). In contrast, the karyotype of the corneal endothelial

Table 1. Comparison of clarity of rabbit corneal buttons coated with either bovine corneal endothelial cells or keratocytes and grafted into rabbit recipients considered as a function of time

Case	Days*	Corneal clarity†	Karyotype‡
		Corneal endothelial cells	
1	102	++++	XY
2	101	++++	XY
3	73	++++	XY
4	62	++++	XY
5	57	++++	XY
6	50	++++	XY
7	29	++++	XY
8	13§	++++	XY
9	10§	++++	XY
		Keratocytes	
1	30	− −	XY
2	25	− −	XY
3	16	− −	XY
4	12	− −	XY
5	12	− −	XY

* Number of days between keratoplasty and sacrifice.

† Clarity of the cornea: −, opaque; +, cloudy; ++, slight haziness; +++, slight haziness; ++++, transparent.

‡ Karyotype of the cells obtained from the endothelial side of the graft at the time of sacrifice and maintained in culture. In all cases, $2n = 60$.

§ Terminated early because an excessive number of cells were plated onto the cornea. These cells later became deposited in the aqueous humor channels and caused a secondary glaucoma.

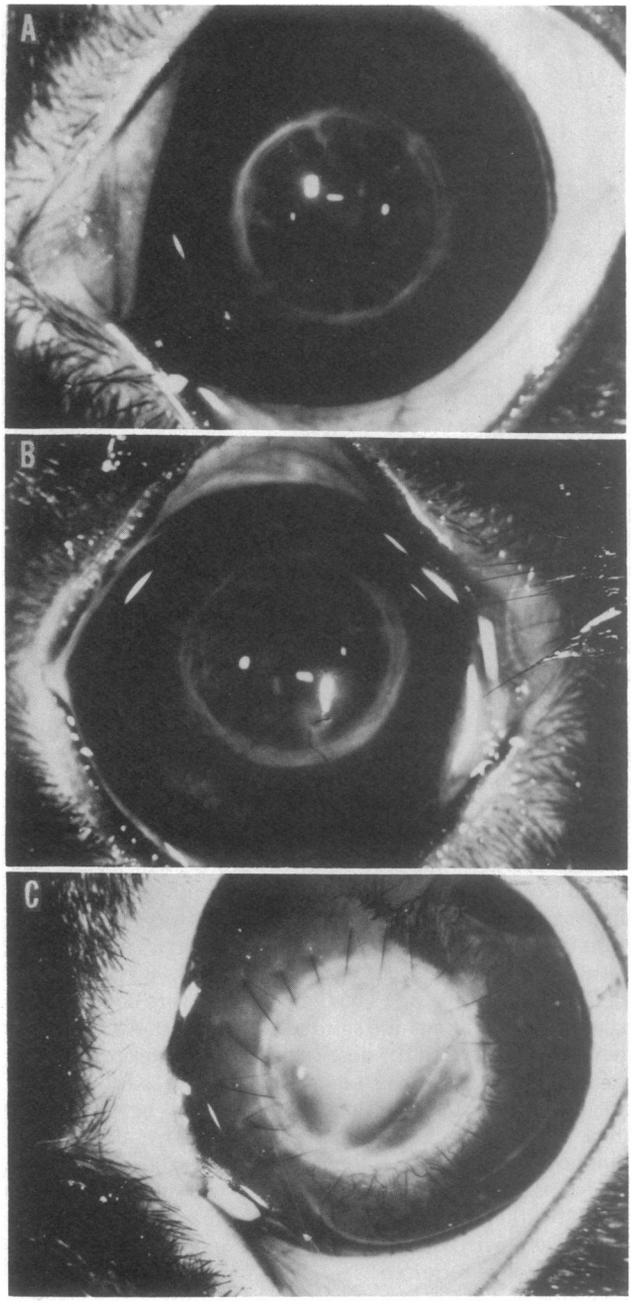

FIG. 4. Comparison of clarity *in vivo* of corneal buttons coated with either corneal endothelial cells or keratocytes. (*A* and *B*) Corneal buttons coated with bovine corneal endothelial cells became transparent within 48 hr after keratoplasty and remained transparent thereafter. Pictures were taken after 90 days. (*C*) Corneal button coated with bovine keratocytes. The graft became completely opaque within 48 hr postoperatively and remained so thereafter. Picture taken 20 days after keratoplasty.

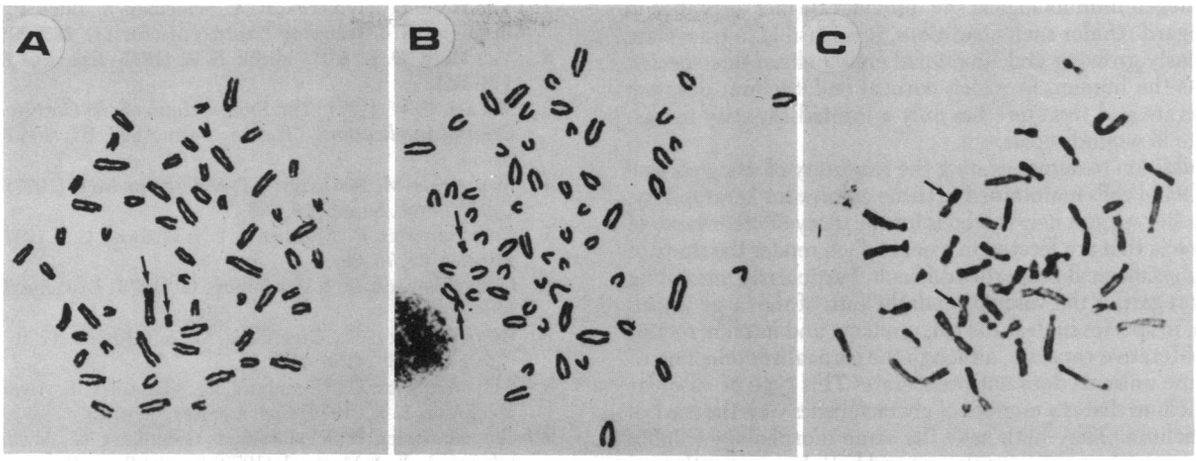

FIG. 5. Karyotypes of corneal buttons coated with bovine corneal endothelial cells, bovine corneal keratocytes, or rabbit corneal endothelium. Bovine corneal endothelial cells (*A*) and bovine corneal keratocytes (*B*) were put back in tissue culture after being maintained *in vivo* for 42 and 16 days, respectively. The cultures were exposed to colchicine for 12 hr. The cells were then karyotyped and their karyotype was compared to that of the corneal endothelial cells (*C*) originating from the recipient, put in tissue culture at the same time, and exposed to colchicine for 12 hr. The arrows indicate sex chromosomes.

cells derived from the recipient endothelium always had female sex chromosomes (XX) and a diploid number of chromosomes ($2n = 44$) with a chromosomal structure like that described by Nichols *et al.* (14). Similar results were obtained when the karyotype of corneal buttons coated with keratocytes was compared to that of recipient corneal endothelium.

It is therefore demonstrated that no invasion of the bovine endothelium coating the corneal button by the recipient endothelium (rabbit) took place. Conversely, no invasion of the recipient endothelium by the endothelium or by keratocytes coated on the corneal button could be observed.

DISCUSSION

Rabbits were used in these studies, for reasons of economy and availability. Because it is known that the corneal endothelium of the rabbit, unlike that of other species such as felines or primates, retains its ability to divide and to regenerate after trauma *in vivo* (2, 15–17), it is imperative to establish that the clarity of the graft was not due to a recolonization of the graft by the recipient rabbit corneal endothelial cells. That this was not the case can be concluded from four lines of evidence: (*i*) After keratoplasty, the graft containing bovine corneal endothelial cells remained clear from the outset. If the corneal endothelial cells migrating into the corneal button had been responsible for this clarity, they would have had to migrate and cover the graft within 24 hr, obviously impossible when it requires covering a corneal button 7 mm in diameter. (*ii*) Corneal endothelial buttons denuded of their endothelium and implanted *in vivo* do not become clear until 3 weeks later. Therefore, if transplanted corneal endothelium had failed during that period, the corneal button should have become cloudy. This was never observed and the cornea remained clear at all times. (*iii*) Corneal buttons coated with keratocytes became opaque after 7 days and remained so until autopsy. If the recipient endothelial cells had migrated into the graft, the cloudiness of the corneal button should have progressively disappeared as the invasion of the corneal button by the recipient endothelium proceeded. This was never observed. (*iv*) Karyotyping of the cells covering the endothelial side of the corneal button demonstrated irrefutably that they were of bovine origin.

Although the failure of rabbit endothelial cells to invade the corneal button might seem surprising at first, it is not so surprising if one takes the following observations into consideration. (*i*) Invasion by rabbit corneal endothelial cells has been reported by others to take place but only in denuded areas (3, 15–17). In the experiments described here, we juxtaposed *in vivo* two similar surfaces covered by the same tissue (corneal endothelium) but from different species. Therefore, there was no denuded area. (*ii*) Invasion could have occurred if the bovine endothelial cells had died. Because they survived quite well, there was no denuded area in the graft into which the recipient endothelium could migrate. (*iii*) Corneal and vascular endothelia share the property of growing as a highly contact-inhibited cell monolayer with a nonthrombogenic surface exposed either to the bloodstream or to the aqueous humor (7–9). Therefore, when two corneal endothelia from different species are put in close contact with each other, it is virtually impossible for them to overgrow each other. Similarly, the possibility that the keratocytes will grow over the recipient endothelium is decreased because of the latter's nonthrombogenic surface which does not permit cell attachment.

Our results therefore demonstrate that corneal endothelial cells maintained in tissue culture remain functional. As soon as they are transplanted *in vivo*, they resume their main function—maintaining the state of deturgescence of the cornea—and thereby prevent the formation of edema. In this respect, early-passage corneal endothelial cells (20 generations) are as functional as late-passage corneal endothelial cell cultures (200 generations).

The capacity of the corneal endothelium grown in tissue culture to be transplanted is considerably enhanced by the rapidity with which the cells plated and reorganized themselves into a monolayer when placed on denuded heterologous corneas. This allowed new methods of transplantation, because one would have to wait only 1–2 hr between the removal of a damaged cornea and its transplantation back into the same recipient. Although this technique was used infrequently in the present study, because we wanted to operate under the worst possible conditions (edematous cornea), when cats were used as experimental animals instead of rabbits, the donor and recipient corneas were the same (9). This latter approach has evident advantages. The corneal button thus prepared will be in perfect condition and free of edema from the outset. The possibility that the blood vessels will grow in the stroma of the cornea is minimized. The rejection phenomenon, which could only involve the endothelium (since it is the only tissue of foreign origin) becomes, in turn, exceedingly unlikely. The barrier between blood and aqueous humor, which makes the cornea

a privileged immunological site, provides further assistance in this regard. Under such conditions, it is feasible to transplant vigorously growing and functional endothelium into species, such as the human, in which corneal endothelium does not proliferate and therefore has only a limited capacity to participate in wound repair.

In addition to demonstrating the feasibility of using corneal endothelial cells maintained in tissue culture for keratoplasty, our studies suggest new methods for the study of other types of endothelia that are located in places which render the study of their physiological properties difficult. Particularly interesting in this regard is the vascular endothelium of the large vessels which, in species such as the cat, monkey, and human, retains its proliferative capacity, although the corneal endothelium of the same animals does not proliferate. This type of vascular endothelium shares a number of characteristics with the corneal endothelium. They both have the same morphology (highly contact-inhibited cell monolayer) and both have a nonthrombogenic apical cell surface. Both function as a selective barrier (8, 9, 18) and manufacture basement membranes that are similar in composition (type IV collagen and fibronectin) (8, 9, 18, 19). Although the principal differences between vascular and corneal endothelia resides in their transport polarities, this difference may not hold for the vascular endothelium of the large vessels, because denudation of the intima will result in local edema in the aorta. Recent corneal transplantation studies using cat and rabbit as recipient suggest that cultured bovine vascular endothelial cells derived from large vessels can substitute for the corneal endothelium when transplanted *in vivo* (9). Keratoplasty can therefore be further simplified because, instead of using a heterologous corneal endothelium, one could use the vascular endothelium of the recipient, grown in tissue culture, to coat its own cornea and to replace the deficient corneal endothelium.

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