The survival and rejection of epithelium in experimental corneal transplants

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Evidence is presented that the epithelium on a donor corneal allograft is neither sloughed off rapidly in the immediate postoperative period, nor is it even slowly replaced by recipient epithelium in the technically successful corneal transplant. Specific immunologic rejection of the surviving epithelium of a graft may take place long after transplantation, and is characterized by a linear defect consisting of dying epithelial cells and inflammatory cells, stainable by topical methylene blue or fluorescein, which defect migrates slowly across the entire surface of the graft until all donor epithelium has been destroyed.

Key words: lamellar corneal transplants, graft rejection, corneal epithelium, corneal vascularization, corneal stroma, histopathology.

From the very earliest days of experimental and clinical keratoplasty, there has always been a lively interest in the ultimate fate of the cellular elements of the donor tissue. With the growing recognition that the immunologic rejection reaction may lead to failure of the graft, interest in the possible survival of donor cells heightened, since their disappearance and replacement by recipient elements would argue strongly for the role of nonimmunologic factors in the late opacification of corneal allografts. While it has been shown rather convincingly in recent years that donor keratocytes and endothelium may persist in the graft for extended periods of time,1-4 analogous investigations on the fate of donor epithelial cells have yielded conflicting results (see literature review). It seems to be generally believed, however, especially in clinical keratoplasty, that the epithelium of a graft is invariably sloughed off or rapidly replaced by recipient epithelium within a short time after transplantation.

In the course of investigations on the mechanism of corneal allograft rejection in the rabbit, involving the transplantation of large and reproducibly clear lamellar and penetrating corneal grafts, it was noted that the epithelium covering a graft might participate in the rejection reaction even many months after transplantation.5, 6 This observation, suggesting that donor epithelium may survive indefinitely upon a corneal graft and ultimately become involved in the transplantation rejection reaction, was examined more closely by several dif-

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different experimental approaches. The demonstration of the persistence of donor epithelial cells, and of their susceptibility to later allograft rejection, is the subject of this paper.

Review of literature

In the study of the fate of transplanted cells in corneal grafts, the 2 most useful techniques by far have been the sex chromatin marker and radioactive labeling, most notably the tritiated thymidine labeling of nuclear DNA. Using the sex chromatin technique, Basu, Miller, and Ormsby demonstrated the survival of donor keratocytes in the cat for at least 3 months, and Chi, Teng, and Katzin showed survival of donor endothelium in the rabbit for longer than 21 months. With tritiated thymidine, the long-term survival of both keratocytes and endothelial cells has been abundantly verified by the work of Hanna and Irwin and of Polack and co-workers.

Unfortunately, neither of these 2 approaches is satisfactory in the study of the long-term survival of donor corneal epithelium. On the one hand, sex chromatin markers are difficult to demonstrate in corneal epithelial cells; on the other, the rapid proliferation of the corneal epithelium permits detection of the labeled cells for at best 10 days to 2 weeks after transplantation, as Hanna and O'Brien have made clear. Estimates on the fate of transplanted corneal epithelium have therefore been based primarily upon both clinical and histologic examinations of the graft. The results of these studies provide a confusing picture, appearing to depend upon the species employed, upon whether lamellar or penetrating grafts were used, upon whether they were autografts or allografts, and undoubtedly also upon the technical success permitted by the specific transplantation technique that has been employed.

Among the early histologic studies were those of Bonnefon and Lacoste (reviewed more recently by Offret). These investigators performed rectangular nonperforating autografts which were studied carefully at frequent intervals from 12 hours to 5 months after transplantation. They noted fusion of the host and graft epithelium at about the twentieth hour. The epithelial response accompanying this fusion was most active in the recipient cornea, but donor epithelium was not entirely inactive in this respect. Mitotic activity was most prominent on the host side of the wound margin but was observed in the donor epithelium also. In those cases where the graft was successful, the epithelium of the transplant did not show massive replacement, although slow replacement of the donor epithelium by the recipient could not be ruled out. In 1948, Maumenee and Kornblueth studied histologic sections of rabbit corneas after partial penetrating allografts had been performed. These investigators noted that during the first 24 hours after transplantation, the graft became edematous and the epithelium sloughed. This was followed by rapid covering of the graft by recipient epithelium. These observations were confirmed by Dohlman who observed histologically that the corneal epithelium was lost from penetrating grafts during the early postoperative days. This observation was supported further by the finding of a rapid loss of radioactive phosphate label in the corneal epithelial cells following transplantation. By contrast, and more in line with the results of Bonnefon and Lacoste, Kornblueth and Nelken noted that donor epithelium remained intact following transfer of fresh partial lamellar allografts in the rabbit. Again, as early as 24 hours after grafting, mitotic figures were found in the basal layer of the donor epithelium.

In a very careful study of the survival of donor corneal epithelium, using the tritiated thymidine label, Mizukawa and co-workers pointed out that if care is taken not to traumatize the graft epithelium unduly, it does not come off in the immediate postoperative period. These workers indicated that, at least in lamellar
keratoplasty, the epithelial cells from the graft survive for a time, with continued donor basal-cell proliferation and migration to the surface. However, even during the few weeks of postoperative study permitted by the rapid disappearance of the radioactive label, at least part of the corneal graft was covered by epithelial cells which had migrated in from the recipient cornea, leading these workers to conclude that donor epithelium is slowly and inevitably replaced by recipient epithelium.

The fate of epithelial cells in human keratoplasty was studied clinically by de Ocampo and Sunga,\(^5\) using topical methylene blue to stain the transplanted corneas for gross epithelial defects. These authors concluded that the epithelium appeared to survive intact after the corneal autograft but was sloughed as a sheet following the transplantation of human allografts or of chicken xenografts. In both the latter instances the corneal epithelial cells of the recipient began to appear on the surface of the graft on the third postoperative day, and covered the graft completely with host epithelium by the sixth day. The source of human material for the corneal allografts in this study were cadaver eyes, and in this paper the authors make the very important point that especially in human keratoplasty, fresh donor tissue is not generally available. They suggest that it is this factor which accounts for the frequent loss of the entire donor epithelial layer during the early postoperative period.

**Methods and results**

All experiments were carried out on deep 8 mm. lamellar corneal allografts performed on young adult albino rabbits of the New Zealand Giant strain, weighing between 5 and 7 pounds. In a few instances, 8 mm. penetrating allografts were employed. The donor corneal buttons were always fresh, being transplanted to the recipient animal immediately after removal from the donor. The technique of lamellar corneal transplantation employed in this study has been described elsewhere,\(^5\) as has that for penetrating corneal grafts.\(^6\)

**Observations of epithelium in avascular corneal allografts.** In these experiments, vascularization of the corneal transplant was avoided by central insertion of the lamellar graft and removal of sutures on the fifth to sixth postoperative day. As described elsewhere,\(^5\) technically successful and clear grafts can be obtained in some 98 per cent of the transplants performed with this technique. Due to the large size of this graft and the care taken to avoid excessive trauma, only a slight corneal edema is seen in these eyes, limited to the immediate vicinity of the graft margin. In no instance did the donor button come off, and corneal vascularization never extended farther than \(\frac{1}{2}\) to 1 mm. inward from the limbus. Even this minimal vascularization retreated rapidly upon removal of the sutures.

Immediately after corneal transplantation, the graft was stained with 0.5 per cent methylene blue in order to establish the extent of any epithelial defect. Staining at this time was found to be limited to the sutures and the incision line joining the donor and recipient cornea (Fig. 1). Twenty hours later, topical application of methylene blue revealed that the incision line had already been covered by epithelium (Fig. 2). Subsequent daily staining of the transplanted cornea during the first week, and every other day for the succeeding 7 weeks, on no occasion revealed any area of epithelial defect suggestive of a rapid destruction of donor epithelium. In addition, close observation of these stained corneas, especially during the early postoperative period, failed to show even the fine, faintly staining epithelial line that might have been expected if the donor epithelium were slowly being lost in the face of an invasion by recipient epithelium.

Histologic sections taken at 1, 2, 4, 6, and 8 days after transplantation served to confirm these gross observations. At 24 hours, there was a normal thickness of epithelium covering the entire surface of both donor and recipient cornea, except for a thinner layer of epithelium occurring only on either side of the graft margin, indicating that both donor and recipient epithelia were involved in the repair process. During the ensuing days, regeneration on both sides of the wound restored the normal epithelial thickness, and the epithelium in the wound itself gradually filled in the surface defect. At no time in any of these eyes did we see an absence of epithelium such as would have accompanied the rapid destruction of donor elements, in confirmation of our clinical findings. More important, there was never seen any histologic indication of even a gradual replacement of donor by recipient epithelium, such as might have been manifested by a slowly moving microscopic defect over the surface of the donor button, or more especially by signs of a thinning or rapid regeneration of recipient epithelium where it was invading the donor cornea.

**Control experiments.** Both gross and histologic observations were made on a series of 8 mm.
Fig. 1. Methylene blue staining of the cornea immediately after transplantation of an 8 mm. lamellar allograft. The donor epithelium is intact, and the staining is restricted to the donor-recipient border.

Fig. 2. Within 24 hours after transplantation, the epithelial defect outlined in Fig. 1 has been covered over, so that methylene blue staining fails to reveal any interruption in epithelial continuity at this time (or, in the absence of specific rejection, subsequently).
lamellar autografts performed by the same technique as that employed previously for allografts. The response of the host to the autograft was in no way different from that described for the allograft, suggesting that in the avascular rabbit cornea, the one is no more favored than the other.

To establish the validity of the histologic observations described, it was necessary to ascertain the minimum time required for an 8 mm. diameter corneal button to completely lose its epithelium and regain a normal thickness of recipient epithelium. To accomplish this, the donor epithelium was intentionally removed by scraping, prior to transplantation of 8 mm. lamellar buttons, and the process of regeneration of the host epithelium over the donor stroma was studied. Twenty-four hours after transplantation, the recipient epithelium had just crossed over the graft margin onto the donor stroma, as revealed by methylene blue staining. Within 2 to 3 days, the epithelium was found to cover the peripheral one third of the graft, and within 4 to 5 days only the central area of the graft, some 2 to 3 mm. in diameter, was seen to take the stain. Only at the sixth to seventh day after transplantation was the graft completely covered by host epithelium. Histologic sections of this process taken at regular intervals showed a zone of flat and thin recipient epithelial cells sliding over the graft until closure of the defect was complete. Restoration of the full thickness of the epithelium over the donor stroma required an additional 2 to 3 weeks. It would thus appear that even at its most rapid pace, complete replacement of the epithelium on an 8 mm. diameter graft would require no less than a month, and certainly much longer if the process were so gradual as to escape histologic detection.

Radioactive labeling of donor epithelium. As indicated in the previous section, any replacement of the donor epithelium on a corneal allograft by recipient epithelial cells, if it occurs at all, must be an extremely slow process. Despite the demonstration that the radioactive thymidine labeling of the nuclei of epithelial cells survives usefully for only some 10 to 14 days, this approach was employed in the present study on the premise that the invasion by recipient epithelium of even a small fraction of a millimeter during the first weeks after transplantation should be demonstrable by this technique.

Fig. 3. Radioautograph of a section of corneal epithelium at the donor-recipient border 10 days after transplantation of an allograft whose epithelium (on the left) had been highly labeled with tritiated thymidine. The dark specks over the cell nuclei represent labeled DNA. The unlabeled recipient epithelial cells have, during this period, shown no tendency to cross the graft border which is indicated by arrows. (Hematoxylin and eosin-radioautograph, ×500.)
For this purpose, the epithelium of the prospective donor was intensively labeled with tritiated thymidine prior to transplantation. This was accomplished by first scraping the donor cornea centrally over an area of about 10 mm. in diameter. Several drops of a solution of tritiated thymidine (100 µc per milliliter) were applied topically twice a day for 2 weeks, during the process of epithelial regeneration. Corneal transplantation was performed 3 days after discontinuation of the drops, and the grafts were removed 10 to 14 days after operation. The eyes were fixed, embedded, sectioned, and radioautographs prepared according to well-established procedures for the localization of isotopically labeled cells. These radioautographs (Fig. 3) clearly demonstrate that not only does donor epithelium heal and survive during this 2 week period, but also that it shares in epithelial wound healing. Even after 2 weeks, one half of the graft scar is covered by donor epithelium and the other half by host cells which show no tendency to invade the graft and replace the donor epithelium during this period.

Control experiment. In a parallel experiment, the cornea of the recipient was labeled by scraping its epithelium over a wide area, followed by topical application of tritiated thymidine as described. Two weeks later, an 8 mm. lamellar graft with nonlabeled epithelium was placed centrally upon this cornea. The grafted corneas were removed 10 days to 2 weeks after transplantation, and radioautographs prepared as described previously. In this instance, labeled epithelium could be seen covering only the host stroma and a portion of the adjacent graft scar. During this 2 week period, there was no tendency on the part of labeled recipient epithelial cells to invade the surface of the donor button.

Observations of epithelium in vascularized corneal allografts. In studies to be reported elsewhere it was observed that the epithelium covering a donor allograft is apparently able to participate in a specific manner in the rejection process. To recapitulate these observations briefly, epithelial rejection induced during the early period after transplantation presents as a linear defect, stainable by methylene blue, which slowly proceeds across the surface of the donor graft, until the entire donor surface has been traversed. The "epithelial rejection line" invariably starts precisely at the donor-recipient graft junction, and always in close approximation to the blood vessels which

Fig. 4. Methylene blue staining of the epithelial rejection line on an eccentrically placed lamellar allograft. Vascularization of the superior quadrant has stimulated graft rejection which is manifested by an arcuate epithelial defect extending from one margin of the graft to the other. During the course of the rejection process, this defect is seen to traverse the entire donor corneal surface, starting always in the zone adjacent to the invading capillaries.
have invaded from the limbus to the graft border. The epithelial rejection line is characterized histologically by a dense infiltrate of inflammatory cells and dead and dying epithelium; in front of this advancing line lies a normal thickness of healthy donor epithelium, while behind it is found a thin layer of recipient epithelium rapidly sliding in to heal the advancing defect. It was felt that the demonstration of the specific nature of this rejection process, and the ability to elicit it long after the graft had been put into place, would constitute the best proof that allogeneic donor epithelium can survive indefinitely on the recipient eye.

The most efficient way to achieve sensitization by and rejection of the lamellar allograft is to stimulate vascular ingrowth from the limbus to the graft margin. This may readily be accomplished by placing the graft eccentrically in the recipient cornea, within a millimeter or two of the limbus. In this circumstance, that quadrant of the graft nearest the limbus becomes vascularized. In a series of 50 such eccentrically placed 8 mm. lamellar allografts performed in connection with this and another study, spontaneous rejection of the graft was observed to start within 2 to 6 weeks after transplantation in 50 per cent of the cases. In most instances, a discrete epithelial rejection line was observed. Whether early or late after transplantation, the line invariably was first seen precisely at the graft margin in apposition to the zone of vascularization. As the stainable rejection line proceeded across the cornea in the direction of the avascular area (Fig. 4), it always extended as an areolate line from one margin of the graft to the other and never invaded the recipient cornea.

Another way to induce rejection of the lamellar allograft is to incite vascular ingrowth toward a centrally placed graft by postponing removal of the irritating sutures employed to fix the graft in place. Should only a single suture be left in place, then frequently only one or a few capillary loops will grow in toward the graft margin. We have observed in a number of such instances the onset of graft rejection only in the immediate vicinity of these capillaries (Fig. 5), with subsequent extension of the methylene blue-stainable epithelial defect from its initial location at one border all the way across the graft to its opposite side. When all the sutures are left in the centrally placed lamellar graft, vascular ingrowth occurs from the entire circumference of the limbus to-
Fig. 6. Delayed removal of the sutures in a centrally placed lamellar allograft has led to vascularization of the entire cornea. The earliest sign of epithelial rejection was the appearance of a positively staining linear defect (retouched) extending around the full periphery of the graft, whence it moved in toward the center.

ward the graft. When such vascularization is heavy, a large proportion of these grafts will spontaneously reject within 2 to 8 weeks. Again, in almost every instance, a stainable epithelial defect can be seen. This defect is first seen over the entire 360 degrees of the graft margin and, starting at the donor-recipient border, it migrates centrally (Fig. 6) until the last donor-epithelial island is seen at the center of the graft.

Whereas the heavily vascularized graft will often succumb to a spontaneous rejection process, this occurs far less frequently in grafts that are only mildly vascularized. In this case, however, rejection may be induced with high frequency by the subsequent transplantation of an orthotopic skin allograft derived from the same donor that provided the original corneal transplant. With this approach, we have been able to induce epithelial rejection as late as 6 months after corneal transplantation. Even at that late date, the phenomenon is identical to that observed much earlier; i.e., initial appearance of the epithelial defect precisely at the donor-recipient border and in close relationship to corneal vascularization, its migration across the entire surface of the donor cornea, and its persistent failure to encroach upon the surface of the recipient cornea. The severity of epithelial rejection as determined by the rate of migration of the epithelial defect line is by far greater and is accompanied by a more severe anterior chamber response, when rejection has been initiated by skin grafting. Such rejection, which usually starts within 2 to 4 days after rejection of the skin graft, is more often also associated with stromal rejection. In this case, the completion of epithelial rejection (the migration of the defect line across the donor cornea) takes only 24 to 72 hours, whereas in spontaneous rejection the epithelial rejection often precedes the rejection of stroma unless the graft is heavily vascularized, and frequently takes from several days to as long as 2 weeks to complete its migratory course.

Control experiments. A series of 8 mm. lamellar autografts was performed and permitted to become highly vascularized, as outlined. These grafts were observed periodically by methylene blue staining, and in no instance was a linear epithelial defect observed. In another series of control experiments, donor epithelium was completely scraped off the cornea prior to transplantation, so that following the procedure the graft was covered completely by recipient epithelium. When, at a later time, allograft rejection was induced, only the typical stromal rejection process could be seen, with no involvement of the overlying recipient epithelium.
**Discussion**

The long-standing controversy on whether late clouding of the corneal allograft could properly be ascribed to specific immunologic rejection processes stimulated an active interest in the ultimate fate of donor cellular elements, since their disappearance and replacement by host elements would be incompatible with the immunologic hypothesis of graft destruction. This question has been partly resolved in recent years by the demonstration by several groups of investigators that donor endothelium and keratocytes may survive, apparently indefinitely, in the recipient eye.

The situation with respect to the ultimate fate of allogeneic epithelium on the corneal graft has still not been fully resolved, and a large and somewhat contradictory literature exists on this subject. Donor epithelium is variously reported to be sloughed rapidly in the immediate postoperative period, to be replaced by the slow incursion of recipient epithelium, or to offer no detectable alteration on which to base a decision. Especially in clinical keratoplasty, in which frequently the epithelium is removed prior to transplantation, it appears to be the general impression that donor epithelium is invariably replaced in the early postoperative period. To be replaced by the slow incursion of recipient epithelium, or to offer no detectable alteration on which to base a decision. Especially in clinical keratoplasty, in which frequently the epithelium is removed prior to transplantation, it appears to be the general impression that donor epithelium is invariably replaced in the early postoperative period. However, as de Ocampo and Sunga point out, these observations may be due to the use of donor tissue which has been stored for varying periods of time, with consequent deterioration of the epithelial layer.

In the present experiments, with fresh donor material in the rabbit, we have used a new approach to the transplantation of deep 8 mm. lamellar allografts, which involves only minimal trauma to the donor epithelium and which results in technically successful clear transplants in 98 per cent of the cases. A large group of transplants was performed with this technique, and observed clinically with methylene blue staining of the cornea and histologically at various intervals after transplantation. In no instance was a massive loss of epithelium seen during the period immediately following the grafting procedure. Further, during the succeeding 8 weeks, no epithelial defect was observed indicative of the slow replacement of donor by host cells, nor was there seen histologically any thinning of recipient epithelium or hyperplasia of its basal cells, such as might have been expected to accompany an epithelial invasion over the donor graft surface. In another experiment, the possibility of the slow replacement of donor by recipient epithelial cells was studied using tritiated thymidine as a radioactive label of cell nuclei. Despite the fact that the usefulness of this approach is limited to the first 2 weeks after transplantation, it was found, in confirmation of the observations of Kornblueth and Nelken, that both donor and recipient epithelium participate jointly in filling in the defect at the margin of the graft. Studying either radioactively labeled donor or recipient epithelial cells, no evidence of retreat by donor cells from the immediate graft border, nor any sign of incursion by host cells onto donor territory appeared during the 2 weeks after grafting. The viability of donor epithelial cells and their participation in the healing process is further supported by our observation that donor epithelium will grow out over the recipient's stroma when it has been demuced of epithelium prior to transplantation.

Perhaps the most convincing evidence that allogeneic donor epithelium normally survives intact and is apparently not even slowly replaced by recipient epithelium emerges from the demonstration that the host may engage it in a specific immunologic rejection process even 6 months (and probably longer) after corneal transplantation. This epithelial rejection process is characterized by a methylene blue-staining linear defect which, once started, traverses the entire surface of the donor button. Histologically, the defect presents as a narrow, discrete zone of dead and dying epithelial cells surrounded by leukocytic
infiltrate, in front of which lies apparently normal donor epithelium, and behind which is found a thin layer of recipient epithelium sliding in to heal the defect. Most significant in the present discussion is the fact that whenever this epithelial rejection commences, even 6 months after transplantation, it always starts precisely at the margin of the graft, indicating quite clearly that there has been no substitution of host for donor epithelial cells across the graft border.

The characteristic migrating epithelial line described is considered to be a specific allograft rejection reaction to donor epithelium based upon the following observations:

1. It occurs only on allografts when the donor epithelium is intact; on no occasion was a similar phenomenon seen involving autograft epithelium, or on the surface of allografts when the donor epithelium was removed prior to transplantation and permitted to be replaced by recipient epithelium.

2. It is not a nonspecific response to the rejection of underlying stroma, since it has been observed to precede, to follow, or to progress in concert with the rejection of stroma. Furthermore, rejection of the pure stromal allograft covered by host epithelium is unaccompanied by a similar epithelial defect. Finally, preliminary work with pure epithelial allografts indicates that donor epithelial rejection may take place over the surface of the host's own stroma and endothelium.

3. The starting point, extension, and direction of migration of the epithelial rejection line are invariably related closely to the extent and distribution of corneal vascularization near the graft margin, and the moving epithelial defect is never seen to extend beyond the boundary of the donor graft onto host cornea.

4. In the mildly vascularized lamellar graft in which rejection does not occur spontaneously, epithelial rejection may be induced by orthotopic skin transplantation only from the same donor which provided the corneal graft.

5. In the heavily vascularized lamellar allograft, the minimal time of epithelial rejection (14 days) is the same as that observed by Billingham and Boswell in their study of the rejection of corneal grafts transplanted ectopically onto a vascularized bed formed on the skin. Moreover, the rejection of epithelium on the corneal graft has been found to be accompanied by sensitization of the host, as evidenced by second-set rejection of a skin graft from the same donor.

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REFERENCES
histologiques sur la greffe cornéenne auto-plastique, Arch. f. Ophth. 33: 206; 267; 326, 1913.


