Scanning electron microscopy of corneal graft rejection: Epithelial rejection, endothelial rejection, and formation of posterior graft membranes

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Penetrating corneal homografts were performed in rabbits for the purpose of making surface studies with the scanning electron microscope at various stages of rejection. The rejection of graft epithelium was characterized by a disruption and breakdown of its normal structure in an area 60 to 80 nm in width. Desquamated cells, pits, and craters were observed in the area of rejection which moved in a sweeping manner across the cornea. Corneal endothelial rejection began at the host-graft junction. Lymphocytes sighted between destroyed or abnormal endothelial cells appeared to have multiple microvilli and pseudopods which facilitated adherence to each other or to the endothelial cells. In totally opaque grafts, the endothelium had been replaced by flattened cells of various sizes and shapes arranged in multilayered membranes.

Key words: graft rejection, scanning electron microscopy, epithelial rejection, endothelial rejection, retrograft membrane

The several clinical and pathologic studies of corneal graft reaction published during the past ten years have contributed to the knowledge of this condition and facilitated its clinical recognition, particularly during the early postoperative period. Electron microscopic studies of rejecting grafts have also given us additional information about the mechanisms of graft rejection, which may be similar to those occurring in other tissues, but with the advantage that corneal graft rejection can be observed and followed clinically with the biomicroscope.

The scanning electron microscope seems to bridge the gap between the usual histologic preparations and the biomicroscope because it provides a greatly magnified, three-dimensional picture of the anterior and posterior corneal surfaces. This study will demonstrate the alterations of the rejecting graft epithelium, endothelium, and the early formation of retrograft membranes as seen with the scanning electron microscope (SEM).
Materials and methods

Penetrating homografts (6 mm.) were performed between pairs of albino rabbits (3 Kg.). The grafts were secured with interrupted or running No. 7-0 silk sutures which were removed ten days after operation. Skin grafts (5 x 5 cm.), exchanged between each pair, were inserted subcutaneously 12 to 14 days after keratoplasty; rejection of graft epithelium or endothelium (and stroma) began ten to 12 days later. Epithelial rejection preceded or appeared simultaneously with the rejection of stroma or endothelium and was characterized by a line of haziness which would move across the cornea during a period of four to six days. For more precise delineation, this line could be stained with fluorescein, methylene blue, or rose bengal.

For the purposes of studying epithelial rejection, two eyes were used whose epithelium had been rejected over one half of the cornea in one case, and one third of the cornea in the other. Two eyes were studied whose endothelial rejection had just begun or had advanced to the middle of the cornea, respectively. Finally, two additional eyes were examined four to five weeks after the completion of graft rejection and opacification.

In order to examine the specimens via scanning electron microscopy, the following fixation procedure was followed: Before enucleation, buffered four per cent glutaraldehyde at room temperature was poured over the cornea of a rabbit which had been put to death. After the eye was removed, the cornea was excised and immersed in the glutaraldehyde solution overnight at 4° C. The next day, it was rinsed in phosphate buffer, cut into small pieces, and placed in cold one per cent osmium tetroxide for one and one half to two hours. The tissue was then rinsed in distilled water and dehydrated through a graded series of ethyl alcohol. The specimens were lyophilized, mounted on specimen stubs, and coated with gold palladium in a high vacuum. The sections were examined with a Stereoscan microscope (Mark II, Cambridge Instrument Co., Ltd., London, England) at an accelerating voltage of 20 kv. The photographs were recorded on Polaroid sheet film (type 55 P/N).

Results

Epithelial rejection. One or more areas of linear epithelial breakdown appeared at the edge of the graft and gradually developed as a semicircular line ("rejection line") with a peripheral convexity. Low-power examination of the "rejection line" (RL) and adjacent epithelium is shown in Fig. 1. In this instance, the rejection had advanced to less than one third of the graft. The line stained well with methylene blue, and there was scattered punctate staining on either side of the line. On the host side of the line (proximal area), some superficial epithelial cells were detached or absent, and there were many raised areas or lumps. The "rejection line" was characterized by a continuous area of cell alteration and epithelial disorganization with formation of pits or craters and deposition of debris on the surface. The proximal epithelium was uneven, lumpy, and at higher levels (thicker) than the non-rejected graft epithelium, both being separated by the RL. Superficial cells were also absent from the nonrejected graft epithelium in the vicinity of the RL.

The appearance of the "staining" rejection line with several pits which correspond to destroyed epithelial cells can be observed in Fig. 2, while Fig. 3 shows a high-power photograph of one of these craters taken almost perpendicularly. The rough surface of superficial and deep epithelial cells and a deep pit with cell debris is noted.

Fig. 4 shows another specimen with similar alterations in the rejection area. The rough surface of epithelial cells ("microvillae") was evident in normal cells, superficial cells, in cells undergoing rejection (partially detached), and in deeper cells exposed by detachment or destruction (Fig. 5). It was apparent in these specimens that a massive cell destruction occurs in a localized fashion, as was demonstrated in a previous paper with the transmission electron microscope; however, the cell destruction occurs through the whole thickness of the epithelial cell layer. In the specimen in Fig. 6, the cells which appear partially detached on the proximal side of the rejection area are most probably host cells, and their loss could be an artifact. Lymphocytes were not observed on the surface of the specimen studied.

Endothelial rejection. The host-graft endothelial junction shows areas of endo-
Fig. 1 and 2. 1, The area of epithelial rejection is shown at low magnification. Rejection advances in the direction of the arrow. The regenerated epithelium has an uneven surface with several superficial cells detached near the line of rejection. (SEM. Original magnification ×200 at 45 degrees, enlarged 2x.) 2, The rejection line shows thickened partially detached cells, cell debris, and several small craters or pits. Inset shows a cross-section of graft epithelium at the site of rejection. Arrow indicates direction of rejection in both pictures. (SEM. Original magnification ×500 at 65 degrees, enlarged 2x; inset: toluidine blue. Original magnification ×400.)
Figs. 3 and 4. 3. One of the craters or pits in the area of active rejection showing several layers of cells and cystlike formations with amorphous material, possibly cell debris. (SEM. Original magnification x2,000 at 10 degrees, enlarged 2x.) 4. This demonstrates the areas of surface protrusions or "lumps" (arrow) which may be in relation with intraepithelial lymphocytic infiltration or cells with enlarged nuclei. Detached cells are seen in the distal portion of the graft. Normal cells, even though at different levels, blend smoothly, as seen in the lower right cornea. (SEM. Original magnification x500 at 55 degrees, enlarged 2x.)
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Figs. 5 and 6. 5. High-power view of one area of previous picture showing the appearance of a crevice. The microvilli of the surface cells are seen clearly; those of deeper cells are more pronounced. (SEM. Original magnification x2,000 at 55 degrees, enlarged 2x.) 6. Detached epithelial cells in the advancing edge of the rejection area. (SEM. Original magnification x1,000 at 57 degrees, enlarged 2x.)

Epithelial destruction and profuse outpouring of rounded bodies 6 to 12 μ in diameter which spread over the grafted portion of the cornea. In specimens with no clinical evidence of endothelial disease but with vascular ingrowth at the scar, leukocytes were found attached to normal endothelium at the center, near the scar of the graft, or between endothelial cells, as if emerging (or insinuating) between them (Figs. 7 to 10). These cells usually had a fluffy surface, were entirely round or had a rounded surface with a flattened base, and had pseudopodial prolongations extending to-
Figs. 7 to 12. 7, Leukocyte on the endothelial surface of a clear portion of a graft shows irregular, fluffy surface. (SEM. Original magnification x2,000 at 30 degrees, enlarged 1.5x.) 8 and 9, Lymphocytes between endothelial cells. (SEM. Original magnification x5,000 at 55 degrees, enlarged 1.5x.) 10, Lymphocyte over endothelial cell with pits on its surface. (SEM. Original magnification x2,000 at 50 degrees, enlarged 1.5x.) 11, Transmission electron microphotograph of a lymphocyte between two endothelial cells. It shows many microvilli or pseudopods. From A. Kanai and F. M. Polack: Am. J. Ophthalmol. To be published. (Original magnification x7,000.) 12, Another electron microphotograph of a lymphocyte showing many pseudopodial formations. From F. M. Polack and A. Kanai: Am. J. Ophthalmol. To be published.14 (Original magnification x7,000.)

ward adjacent cells or cell residues. The irregular, fluffy surface is due to the microvilli or pseudopods which were frequently observed in EM cross-sections (Figs. 11 and 12).

Pores or pits were also seen in endothelial cells around lymphocytes; however, they could be exaggerations of pores present in normal corneas or artifacts. The white endothelial line seen with the biomicroscope separating the cloudy from the clear cornea is produced by a line of rejecting lymphocytes and other white cells (Fig. 13). With the SEM, the lymphocytes seem to form a barrier of rounded or elongated bodies separating normal from diseased endothelium (Fig. 14). The endothelial mosaic in the rejected area was replaced by abnormal endothelial cells of various shapes arranged at various levels.
Fig. 13 and 14. 13, Flat endothelial preparation showing the rejection line (RL) formed by lymphocytes and the direction of the endothelial rejection (arrow). Many staining lymphocytes are in the subendothelial layer. (Hematoxylin-eosin. Original magnification ×100.) 14, Low-power SEM photograph showing the lymphocytes which form the rejection line. The rejected endothelium is now covered by abnormal endothelial cells or flat unidentified cells. (SEM. Original magnification ×200 at 30 degrees, enlarged 1.5×.)
with leukocytes between or under them (Fig. 15). In areas of very active destruction, separation of cell junctions by leukocytes was observed as well as holes in endothelial cell membranes, cell shrinkage, and craters possibly caused by cell destruction (Fig. 16). Lymphocytes attached themselves by pseudopodial extensions to the cytoplasm of endothelial cells (Figs. 17 and 18). Even though Descemet's membrane seemed to form the bottom of large craters or pits, there was no large area totally devoid of some sort of cells.

**Formation of posterior graft membranes.** Low-power scanning microphotographs of the endothelial surface of one- to six-week-old normal corneal grafts and their host-graft junction show a homogeneous and intact endothelium with clearly defined areas where the still unhealed Descemet's membrane ends at the graft and host sides (Fig. 19). Endothelial cells, however, are lining the scar. In older grafts (four to six months), the cut ends of Descemet's membrane are no longer seen. Fig. 20 shows the posterior surface of an opaque graft six weeks after rejection, which, in contrast to the normal graft endothelium of the last picture, has a rough, irregular surface. When the rejected graft is examined at higher power, there are some areas apparently devoid of cells and others with an array of cells varying from round to oval or hexagonal shapes, as well as cells of elongated shape (Figs. 21 and 22). These cells are arranged in a disorganized manner forming two or more layers in one level, while other places in the level might be devoid of cells (Fig. 23). At times, the cells show well-defined cytoplasmic junctions, but, in other places, parts of the cell body appear to form bridges over the other cells (Fig. 22). The multilayered order of these cells can be observed in cross-section of rejected grafts at this stage (Fig. 24), in which abnormal endothelial cells, fibroblasts, and cell residues, all enmeshed in layers of fibrin, are found.

**Discussion**

Previous studies with the light microscope and the electron microscope had demonstrated the sequence of events, the cell types involved, their origin, and the ultramicroscopic alterations present during rejection.

The SEM photographs shown here illustrate the surface changes of graft epithelium during the stage of rejection, usually localized in an area of 60 to 80 nm. in width. The rejection process starts in the deep epithelial layers in which lymphocytes are found between cells or forming small clumps, usually preceded by, or in the line of, rejection. The lumps seen at the edge of regenerating epithelium shown in several pictures probably represent areas of lymphocytic infiltration or may be related to large basal cells in premiotic stages. Cells destroyed by lymphocytes led to the formation of large pits or craters with amorphous deposits and small round bodies, which are probably cell residues; similar small bodies have been described by others in degenerating epithelial cells. The rough surface or microvilli of the epithelial cell is apparently preserved in most of the altered cells because it can be seen in thickened, partially detached cells in the area of active rejection.

The area of continuous methylene blue staining does not seem to be caused exclusively by the dye pooling in isolated craters or pits, but rather by areas of deep desquamation which leaves exposed cells with a coarser surface or more pronounced "microvilli." In the present study, there was no evidence that lymphocytes were present in the corneal surface, although they could have been present in the tear film.

In studying the rejection of the endothelium, one of the problems encountered was how to differentiate the lymphocyte from cell debris or fibrin clumps which may be seen before the clinical rejection is evident. The morphology and the size of the lymphocyte was studied by spreading cultures of lymphocytes over the endothelium
Figs. 15 and 16. 15. Lymphocytes (Ly) forming the rejection line and destroyed endothelium (En). Some flattened cells with cytoplasmic prolongations (*) lie over endothelial cells. (SEM. Original magnification ×1000 at 35 degrees, enlarged 1.5×.) 16. Endothelial cells (En) immediately before the rejection line of lymphocytes (Ly) show intercellular holes (*). Arrow shows adhesions between lymphocytes and damaged endothelial cells. (SEM. Original magnification ×2,000 at 30 degrees enlarged 1.5×.)
Figs. 17 and 18. 17, Clump of lymphocytes in the area of active rejection showing cytoplasmic junctions by pseudopodial extensions and attachments to damaged endothelial cells (En). Degenerated cells also show thick (denser) bodies (*). (SEM. Original magnification x2,000 at 35 degrees, enlarged 2x.) 18, The pseudopodial extensions of a lymphocyte (Ly) toward an endothelial cell (En) and possibly to Descemet's membrane (*) are shown at high magnification. (SEM. Original magnification x5,000 at 35 degrees, enlarged 2x.)
Figs. 19 and 20. 19, SEM photograph of the endothelial surface of a six-week-old graft at the host-graft junction. The scar (S) is delineated by the cut edge of Descemet's membrane (Δ). Host, H; graft, G. (SEM. Original magnification ×100 at 20 degrees, enlarged 2×.) 20, SEM photograph of the endothelial surface of a host-graft junction in an eye with a six-week-old rejected graft. Note the loss of smooth texture of normal graft endothelium. Host, H; graft, G; scar, S. (SEM. Original magnification ×100 at 20 degrees, enlarged 1.5×.)

of fresh rabbit corneas which were immediately fixed. Various forms of mechanical injuries to the endothelial layer were also done in order to distinguish cell trauma from artifacts. Rounded cells measuring 12 to 18 nm. with discrete surface roughness or with a rough, fluffy surface and prominent pseudopods were the two types of lymphocytes most commonly found. It was assumed that the second type corresponds to the more active lymphocyte, which, in the transmission electron microscope is seen as a cell with a large number of microvilli or pseudopods. In hematoxylin-stained flat endothelial preparations of rejecting grafts, the number of leukocytes is apparently greater than that observed with the SEM in similar preparations, because many of them are in the subendothelial layer, as shown in previous EM studies. The SEM photographs show the superficial leukocytes, but sometimes those insinuating between or under endothelial cells will also be seen as elevations and rounded protrusions of the endothelium. As the endothelial rejection advances, unidentified flattened cells of various types move over
Figs. 21 and 22. 21, The endothelial surface of an opaque graft near the scar (S) shows cells of various shapes at various levels. (SEM. Original magnification ×1,000 at 25 degrees, enlarged 1.5×.) 22, SEM photograph of the central area of an opaque, rejected graft showing cells in multilayered fashion. Large craters are seen between cell layers (arrow). (SEM. Original magnification ×1,000 at 19 degrees, enlarged 1.5×.)
Figs. 23 and 24. 23, Low-power SEM photograph of the mid-periphery of a rejected graft showing well-joined, flattened cells in some areas (a) and membrane clefts in other places (b). A thick membrane is also observed (c). (SEM, Original magnification ×500 at 55 degrees, enlarged 1.5×.) 24, Transmission electron microphotograph showing altered endothelial cells (En*), fibroblasts (F), and fibrin (f) over the Descemet's membrane of a rejected graft. From F. M. Polack and A. Kanai: Am. J. Ophthalmol. To be published. (Original magnification ×12,000.)
Descemet's membrane. With the transmission electron microscope, it is also difficult to identify the severely altered or the regenerating endothelial cell from fibroblasts present in the area of rejected graft endothelium. Fibroblasts originate in the scar of the graft with still unhealed Descemet's membrane spreading along the destroyed endothelium, eventually forming the retrograft membrane.

Histologic studies of failed grafts have shown the high incidence of posterior membranes. Their formation, however, is closely related to healing defects in the posterior aspect of the wound, as well as damage to graft endothelium. Both occur in the early type of graft rejection (before complete healing of Descemet's), but when Descemet's membrane is regenerated (three to four months), the scar as a source of fibroblastic growth is eliminated. The uveal tract then would be the only pathway for rejecting lymphocytes, while fibroblasts may occur by metaplasia of monocytes or endothelial cells.

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REFERENCES