

## HISTOPATHOLOGICAL AND HISTOCHEMICAL ALTERATIONS IN THE EARLY STAGES OF CORNEAL GRAFT REJECTION\*

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The high percentage of acceptance of corneal homografts, which is in marked contrast to the almost invariable rejection of other tissue grafts, may be due to corneal avascularity rather than to its lack of antigenicity. This has been demonstrated in a series of experiments by Medawar (1), Maumenee (2), Mueller and Maumenee (3), Billingham and Boswell (4) Greaves (5), and Basu and Ormsby (6) in interlamellar grafts. The corneal graft rejection has been produced experimentally, only if vessels reach the transplant. In man, corneal grafts made in vascular corneas often become opaque, and the mechanism of rejection may be analogous. Graft sickness in avascular corneas requires an additional explanation.

The histopathology of experimental graft rejection, based on hematoxylin- and eosin-stained sections cut vertically to the corneal surface, has been described by Maumenee (2). It appeared that application of other methods, including flat endothelial preparations, metachromatic staining, S<sup>35</sup> sulfate uptake (synthesis of sulfated mucopolysaccharides), and study of cell types in flat or tangential sections, which are advantageous in the study of the stromal cells, might add information of value, if alterations at the earliest stages were investigated. The following is a report of such a study.

### *Materials and Methods*

*Surgical Technique.*—Albino rabbits obtained from one dealer and weighing 2 to 3 kg were used as donors and recipients of corneal grafts. They were operated on in pairs, each serving as host for the corneal transplant removed from the other. The animals were anesthetized with intravenous sodium pentothal (38 mg/kg). Neosporin (Burroughs Wellcome & Co. Tuckahoe, New York), proparacaine HCl, 0.5 per cent (ophthaine, E. R. Squibb & Sons, Brooklyn), and 10 per cent neosynephrine were used locally. The operations were performed under a Zeiss operating microscope. A 4.5 mm graft, trephined from the right eye of one

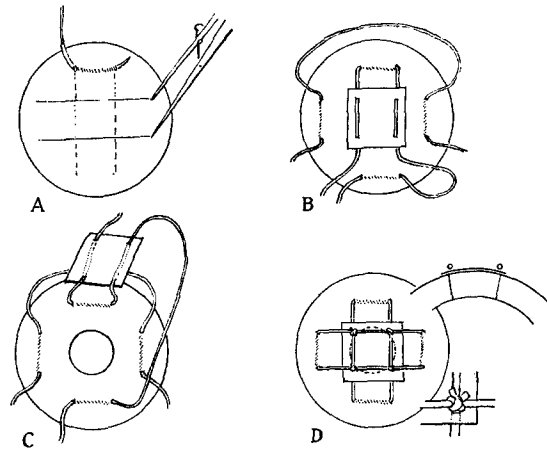
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rabbit, was interchanged with a graft of the same size obtained from the left eye of a second animal. A polyethylene film, used as a splint to support the graft, was secured by 2 double cross sutures, as shown in Text-fig. 1.

*Clinical Appearance.*—Two weeks after corneal transplantation, one or two vessels were present near the suture site in the host cornea, but extended only 2 to 3 mm, from the limbus (Fig. 1). At this time one square inch of skin was exchanged and implanted in the panniculus carnosus of the abdomen of each host-donor corneal pair.

One week after the skin transplantation, the eyes were examined daily with the slit lamp. Haziness of the graft usually developed 12 to 14 days after the skin graft had been made. In



TEXT-FIG. 1. Schema showing the operative technique. A: Two double cross lines are marked on the cornea with a caliper set at 4 mm. B: Two "U" sutures (6.0 silk) are placed as shown. One set of them perforates a small polyethylene splint; the other set overlies the plastic. C: The suture and the splint are moved aside to perforate the cornea and place the graft. D: The sutures are tied and four security knots are placed at the angles of the central square.

4 cases this process was permitted to continue 5 to 7 days longer until the graft became opaque and vascularized. Alterations of the endothelium were investigated in whole flat mounts of the graft and surrounding host cornea. The scar tissue, the collagen fibers, the metachromatic reaction, and stroma cells of the graft were studied in frozen sections cut tangentially to the corneal surface. A total of 30 grafts were studied.

*Histology.*—Flat endothelial mounts were prepared of corneas fixed in Carnoy's solution for 24 hours and then changed to 70 per cent alcohol for 2 to 4 hours. The Descemet's membrane and endothelium of the host and graft were removed in one layer under the operating microscope, mounted on a gelatin-coated slide and stained with hematoxylin. The remaining stroma was washed and sections made parallel with the corneal surface, which were stained with Harris' hematoxylin and eosin. Frozen sections cut tangentially to the surface were also made from corneas fixed in 8 per cent formalin for 5 days or longer, and stained with the silver carbonate technique of del Rio Hortega (7). Those used to study the metachromatic reaction were also fixed in 8 per cent formalin and stained with toluidine blue (pH 4.5), cleared and mounted.

*Uptake of S<sup>35</sup>.*—In order to determine the comparative ability of the graft and host to synthesize sulfated mucopolysaccharides, 1.0 mc/kg of carrier-free S<sup>35</sup>-labeled Na<sub>2</sub>SO<sub>4</sub> was injected intravenously into rabbits: (a) at the very beginning of the graft rejection process, (b) after the grafts had become completely opaque, and (c) with clear grafts (control cases). Twenty-four hours after sulfate injection the rabbits were killed and both eyes removed. The unoperated eye served as control. The corneas were excised with a rim of sclera, and washed with agitation for 6 hours in several changes of 1.4 per cent non-radioactive Na<sub>2</sub>SO<sub>4</sub> solution to remove the S<sup>35</sup> not organically bound in the cornea. Radial cuts were made in the corneas so that they could be mounted flatly between two sheets of saran wrap (8) and dried at 50°C for 72 hours. Appositional radioautographs were made on Kodak A-1 radioautographic plates which were exposed for 4 weeks.

#### RESULTS

Approximately 12 ( $\pm 3$ ) days after skin transplantation, vessels from the upper, and occasionally from the lower, corneal limbus became engorged and began to grow rapidly towards the graft. They reached and were distributed along the scar tissue, at times encircling the transplanted cornea before macroscopic changes were observed in the graft; however, if examined with the slit lamp, edema of the endothelium, and of the posterior stromal layers, was observed in the periphery of the graft at the point where the ingrowing vessels reached the scar. There was no flare in the aqueous humor in these cases, although in two instances the iris was moderately congested. Shortly after the first changes were observed, if only one set of vessels was present above the graft, a crescent-shaped area of corneal haziness started to invade the graft from the region of these vessels (Fig. 2). Most of the cases were studied at this, or at a slightly earlier stage.

In another group in which vessels grew towards the graft from more than one point and invaded the scar, edema of the posterior layers appeared simultaneously all around the graft. In addition to the peripheral haziness, in two eyes a horizontal area of haziness was observed in the center, midway between two large sets of vessels (Fig. 3). Four corneal grafts, which were allowed to become completely opaque and vascularized, were studied 5 to 7 days after opacification first appeared (Fig. 4).

In two eyes, the vascular reaction in the host cornea was very slight. Thin vessels from one or two areas had reached the scar, and a zone of endothelial edema was observed with the slit lamp near the periphery of the graft, without any concomitant stromal opacification. Gradually a retrocorneal membrane started to grow centripetally from the scar. Finally, in two cases in which no vessels entered the host cornea, no reaction was obtained at all. Cases have been reported by Medawar (1), Maumenee (2), and Billingham and Boswell (4), showing that vascularization is a requisite for the "immune" reaction.

The skin introduced in the panniculus carnosus of the abdomen was reduced to a granulomatous mass composed of lymphocytes, monocytes, and plasma cells when the corneal rejection started.

The histologic and histochemical changes in the grafted cornea will be described with respect to (1) scar, (2) endothelium, (3) stroma cells, (4) stromal fibers, (5) metachromatic reaction, and (6) uptake of  $S^{35}$ -labeled sulfate.

1. *Alterations in the Scar Tissue.*—Although the scar is presumably more a component of the host than of the graft, it does play a part of considerable importance in the graft rejection. Histologic studies of healing of scars of normal corneal grafts demonstrated that this is a most active area of regeneration in the first few days following surgery. Several weeks after the operation, the proliferation of connective tissue in the normal scar decreases gradually, and several months later the scar is reduced to a thin line, in which the fibers of the scar tend to fuse with the pattern of normal corneal fibers (Castroviejo (9)). After blood vessels reached the wound, the scar tissue was found to swell posteriorly, as observed with the dissecting microscope in freshly removed corneas. Histologic observations of cross-sections showed the scar tissue to be infiltrated by large and small lymphocytes, plasma cells, and, in some cases, by polymorphonuclear leucocytes. The vessels which reach the scar do so usually through the anterior third of the cornea as shown in (Fig. 5). This infiltration reached Descemet's endothelium at the same time or before invading the graft stroma. The sequence of infiltration of the scar tissue, and its relation to the stroma and endothelium is shown in (Fig. 6).

Flat frozen sections demonstrate clearly the severe infiltration of the scar (Fig. 7); in all cases the infiltration was more severe at the point where the incoming vessels reached the scar.

2. *Alterations of the Endothelium.*—The earliest change observed in the endothelial layer was a loss of cells adjacent to the scar, and at the periphery of the graft (Fig. 8), always accompanied by infiltration by round cells and fibroblasts which advanced towards the center of the graft. This condition correlated well with the biomicroscopic observations that haziness of the graft started near the scar. When endothelial destruction and infiltration was present in one pole of the graft, it corresponded to the crescent-shaped opacity shown in (Fig. 2). Endothelial cells were of normal appearance, but round cells were found scattered amongst them in the center of the graft. In two cases, endothelial cells in the vicinity of the infiltrating cells showed several small, or large, vacuoles (Fig. 9). Few polymorphonuclear leucocytes were observed in the peripheral areas of infiltration. In cases where haziness was allowed to progress until complete opacification of the graft, the whole endothelial layer was lost (Fig. 10).

3. *Alterations of the Stroma Cells.*—Judging the alterations of the keratocytes was somewhat more difficult than judging those of the endothelial cells, because the alterations varied in different lamellae. Morphologic changes in the connective tissue cells were not specific; but seemed to be similar to those observed after physical injuries (trauma, acid burns, cold, etc.) (Wolter (10)), and con-

sisted in retraction of processes, elongation of the nucleus and cytoplasm, and proliferation (Fig. 11). It seems that the stroma cell changes follow the endothelial alterations: however, keratocytes from the anterior layers of the cornea, which are in a more intimate relationship with the newly formed vessels, may be destroyed or become converted to fibroblast-like cells simultaneously with or before the endothelial damage. Monocytes, lymphocytes, plasma cells, and fibroblasts invade the stroma in later stages of graft rejection.

4. *Alterations of the Stromal Fibers.*—Alterations of the stromal fibers were observed only in the late stages of graft sickness. Changes in both the width of the fibers, and their arrangement within the bundles are caused by edema, and perhaps by liberation of proteolytic enzymes after cell destruction. Invasion of the stroma by blood vessels and fibroblasts occurs simultaneously with or following the edema.

5. *Alterations in the Metachromatic Reaction.*—The amount of mucopolysaccharides present in the host and grafted cornea was judged by the degree of metachromasia present in flat corneal sections stained with toluidine blue. In that group where the haziness began in one pole of the graft, the metachromasia was diminished or absent in the affected area. Metachromatic alterations in grafts where the haziness appeared peripherally were difficult to judge; however, it seems that metachromasia was retained in the central area, and was absent in completely opaque grafts.

6. *Uptake of Radioactive Sulfate  $\text{Na}_2\text{S}^{35}\text{O}_4$ .*—Clear, 4-week-old, corneal grafts incorporated  $\text{S}^{35}$  sulfate to the same degree as did the host and the non-grafted control cornea (Figs. 12 *a* and 12 *b*). Nine grafts labeled at the beginning of the rejection phenomenon showing a definite haziness, revealed a decrease in the uptake of  $\text{S}^{35}$  sulfate in the hazy area (Fig. 12 *c* to *g*); however, this was not observed in one case where the amount of  $\text{S}^{35}$  still remained comparable in degree to that of the host cornea. Grafts allowed to become partially opaque and vascularized showed a decreased amount of  $\text{S}^{35}$  in the hazy, non-infiltrated area; but in the part of the graft where invasion by vessels and fibroblasts was present, increased uptake of sulfate was observed, as would be expected (29) in a rapidly growing scar tissue (Fig. 12 *h*).

#### COMMENT

The data presented here are in agreement with the concept that the immune reaction to transplanted tissue depends on vascularization near, or in, the grafted tissue as a prerequisite for its rejection. In the present cases, the graft rejection phenomenon did not occur if vessels did not reach the graft. In an earlier series of ten corneal grafts (not reported in this paper) skin from the corneal donor which had been frozen and stored for 2 weeks was grafted subcutaneously. This did not produce a vascular reaction in the host corneas, even when two separate transplantations of skin were made.

Graft sickness occurring in avascular corneas theoretically could be caused by antibodies present in the aqueous humor. With serologic techniques, Smith and Woodin (11) demonstrated that antibodies could be evoked by corneal extracts and Tsutsui and Watanabe (12) have found antibodies to grafted corneas in the serum of the host.

In our experiments we have observed that destruction of endothelial cells occur over the scar and at the periphery of the graft. It is tempting to relate the destruction of these cells with the absence of Descemet's membrane at this level. The gap in Descemet's membrane made by the original incision had not regenerated in these cases. This would permit blood-borne cells, and fibroblasts, to enter the endothelium of the graft and proceed centripetally. The fact that destruction of cells proceeds in the direction of the donor tissue agrees with the observations of Medawar (1), who reported that in the rejection phenomena there is a precise limit where the destruction of tissue ends and is limited to that of the donor. This fact may explain the success of lamellar or intralamellar heterografts (Babel and Bourquin (13), Choyce (14), Basu and Ormsby (15), Payrau, Pouliquen, and Faure (16)) and the absence of homograft rejection in this type of operation (Kornblueth and Nelken (17)) in contrast to the repeated failures of perforating heterografts (Ortin (18)).

The alterations present in the endothelium of two cases, edema and vacuolization of the cell body, resemble the changes observed in cells treated with antibodies in tissue cultures (Mountain (19), Ellem (20), and Goldberg and Green (21)), and changes in corneal epithelial cells treated with anticornea serum by Ehrlich and Halbert (22). These changes are considered to be alterations of the cell membrane, and are reversible in tissue cultures, according to Goldberg and Green (21). However, the most frequent finding was that of cell destruction associated with leucocytic infiltration of the endothelial layer.

Alterations in the keratocytes, similar to those observed in the endothelial cells, were not observed, but non-specific alterations were common. Death of keratocytes was not observed in early stages of graft rejection. The stromal edema could explain the decreased metachromatic reaction, Ashton (23), or possibly a halt occurred in the synthesis of metachromatic material due to functional damage of the corneal cells.

The rejection of grafts placed interlamellarly (Basu and Ormsby (6)) may occur as if they had been placed in a vascular bed elsewhere in the body, since the graft became surrounded by host vessels and no scar tissue formed between. Basu and Ormsby (24), in another paper, reported that grafts placed in this way can be removed without difficulty after 3 months.

The metachromatic reaction of toluidine blue indicates the quantity of mucopolysaccharides present in the graft, but the amount of the sulfated forms being synthesized at a specific time, is shown by the degree of incorporation of radioactive sulfate. Most of this sulfate will be incorporated in the chondroitin

sulfate or keratosulfate fraction of the ground substance (Wortman and Strominger (25)). 10 to 14 days after grafting, the rate of synthesis of these compounds by the graft was found to be similar to that of the host cornea (Dohlman (26)) and was confirmed by these experiments.

A net loss of sulfated compounds has been described at the edges of corneal wounds by Smelser and Ozanics (27), and at the periphery of corneal grafts by Espiritu, Kara, and Tabowitz (28); however, in the first stages of regeneration of wounds there is an increase of these compounds at the edges of the wound (Dunnington and Smelser (29)). Since a massive destruction of keratocytes has not been observed at the beginning of the graft sickness, some other mechanism is needed to explain the decreased uptake of radioactive sulfate. A failure of the incorporation mechanism of  $S^{35}$ , or perhaps an altered exchange of sulfate ions from the anterior chamber into the stroma, because of the damaged endothelium of the graft, could account for this observation. Our findings of a definite decrease in uptake of  $S^{35}$  in grafts with an altered endothelium may be explained by the report of Smelser (30) that, in the absence of Descemet's endothelium, synthesis of sulfated mucopolysaccharides in the cornea was greatly reduced. We must assume that enough radioactive sulfate was available to the graft, because, if present in the aqueous humor, it should have diffused into the cornea in the presence of an altered or absent endothelium. Maurice (31) reported that electrolytes enter the cornea mostly from the aqueous, and Dohlman (32) mentioned that the incorporation of  $S^{35}$  into the cornea seems to be primarily from the aqueous and secondarily from the periphery. It is tempting to assume that if a small amount of radioactive sulfate enters the cornea from the periphery, it could be trapped by the very active connective tissue of the scar. The increased uptake of  $S^{35}$  by the turbid graft in the later stages of the disease could be accounted for by the proliferation and activity of the fibroblasts there (Smelser and Ozanics (27)).

#### SUMMARY

Rejection of corneal grafts was produced in rabbits after skin from the corneal donor was grafted subcutaneously. Clinical observations showed that the graft sickness started at the periphery of the graft after blood vessels from the host cornea reached the scar. Histologic studies demonstrated that the scar tissue was first invaded by vessels and infiltrated by lymphocytes, monocytes, and plasma cells. This infiltrate reached the endothelium through the gap in Descemet's membrane. The early histological picture of the graft sickness was characterized by endothelial destruction and infiltration at its junction with the scar. Keratocytes changed in shape and apparently became active fibroblasts.

Decreased amounts of ground substance in the early stages of rejection, as indicated by appearance of haziness in the graft, were indicated in sections by

a decrease in metachromatic staining. Diminished uptake of radioactive sulfate also occurred in the early stages of the graft sickness; which was followed by an elevated uptake of sulfate as the graft became opaque and filled with active fibroblasts.

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## EXPLANATION OF PLATES

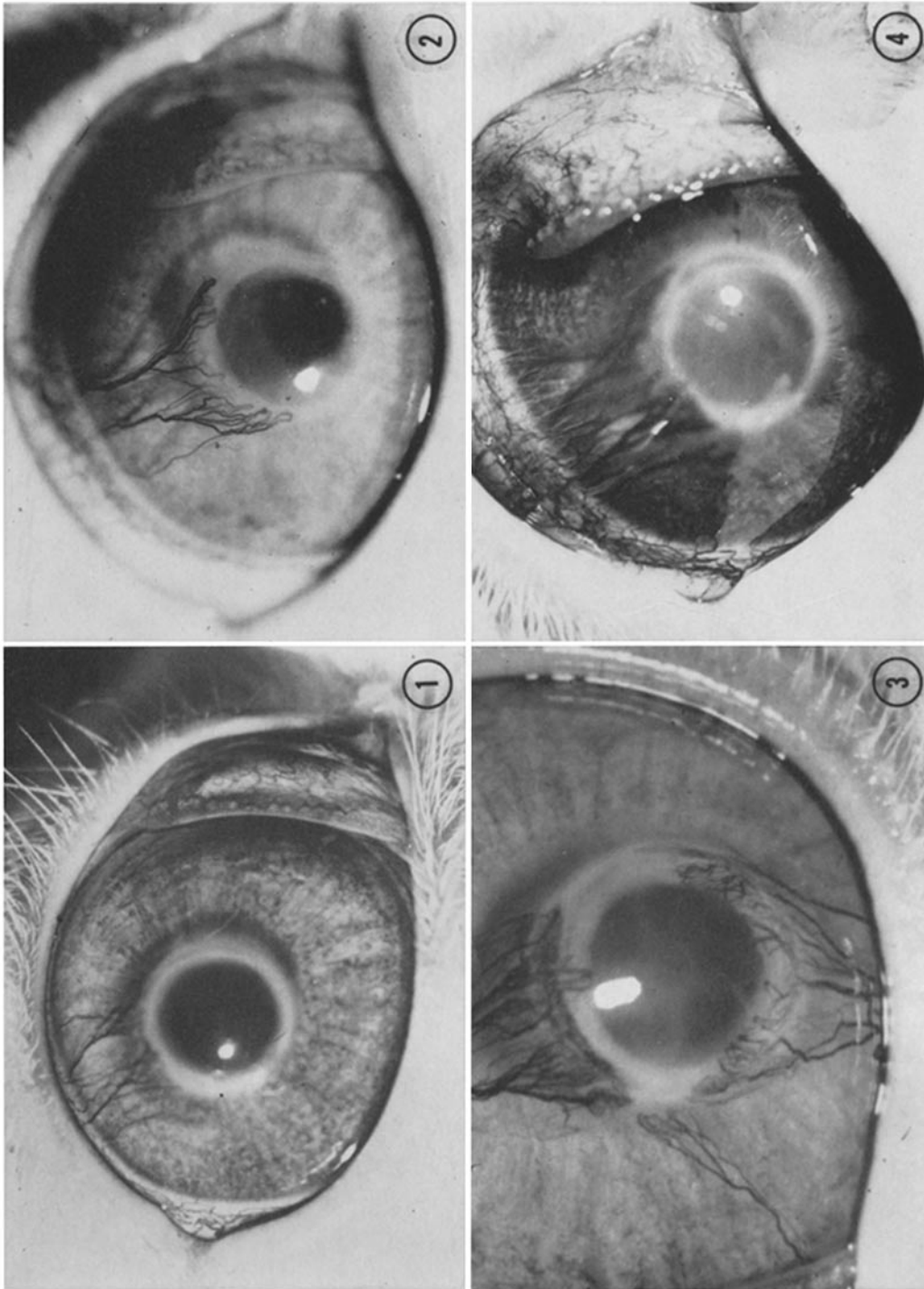
## PLATE 89

FIG. 1. A photograph of a clear rabbit homograft 2 weeks after operation.

FIG. 2. Crescent-shaped opacity of the graft adjacent to the vessels in the scar at the beginning of the rejection process.

FIG. 3. A photograph of a corneal homograft on the 2nd day of the rejection phenomenon. The graft shows peripheral edema and a horizontal hazy band midway between two sets of vessels.

FIG. 4. Graft rejection 1 week after the onset of opacification. Same eye as shown in Fig. 1.

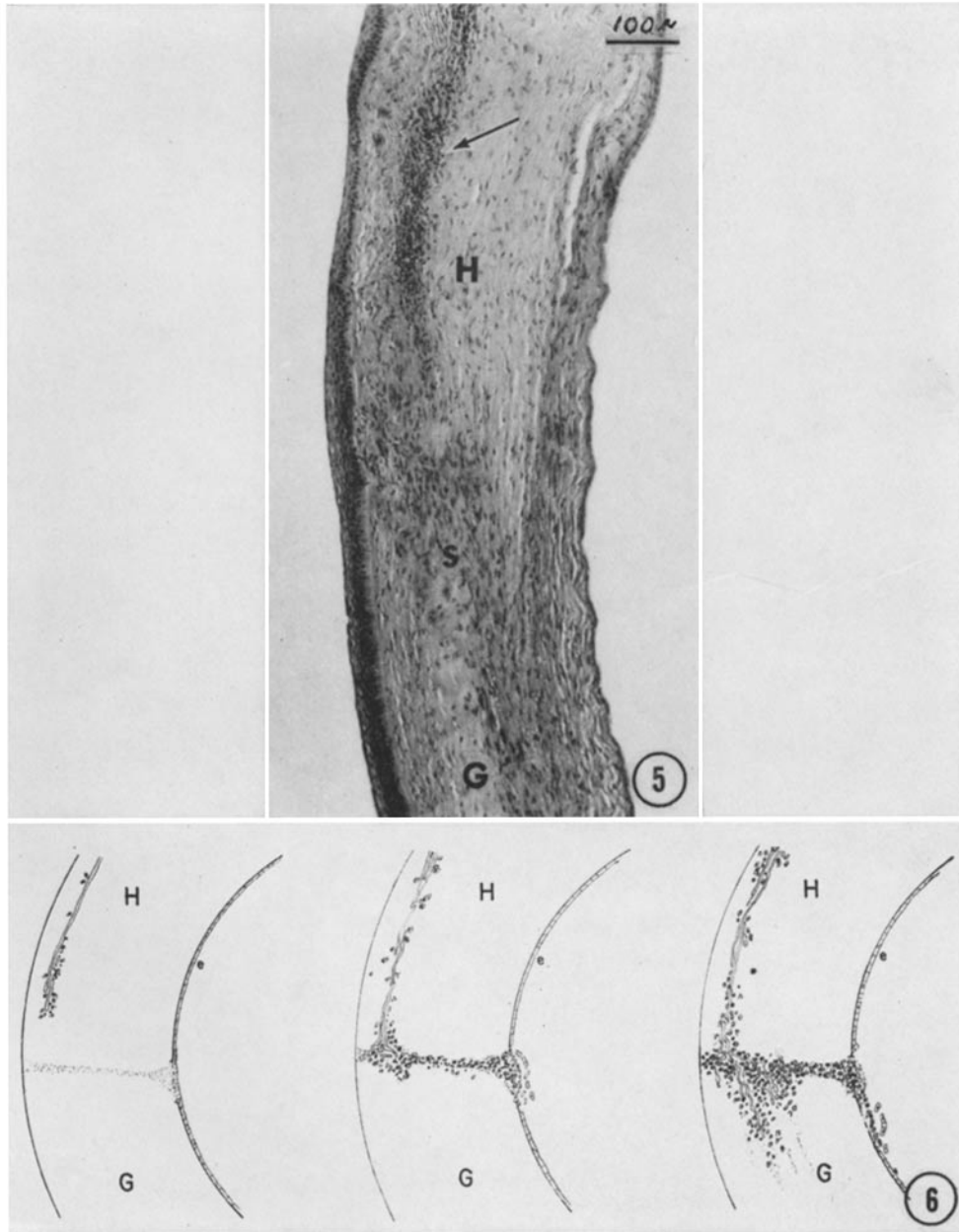


(Polack: Corneal graft rejection)

PLATE 90

FIG. 5. Histologic cross-section of a rabbit cornea 2 weeks after corneal grafting. Thin vessels accompanied by round cells (arrow) travel in the anterior third of the host cornea (*H*) and reach the scar tissue (*S*). Graft (*G*). Hematoxylin and eosin stain.

FIG. 6. Schema showing the progression of newformed vessels from the host (*H*) towards the graft (*G*). Round cells infiltrate the connective tissue of the scar and invade Descemet's endothelium (*e*).

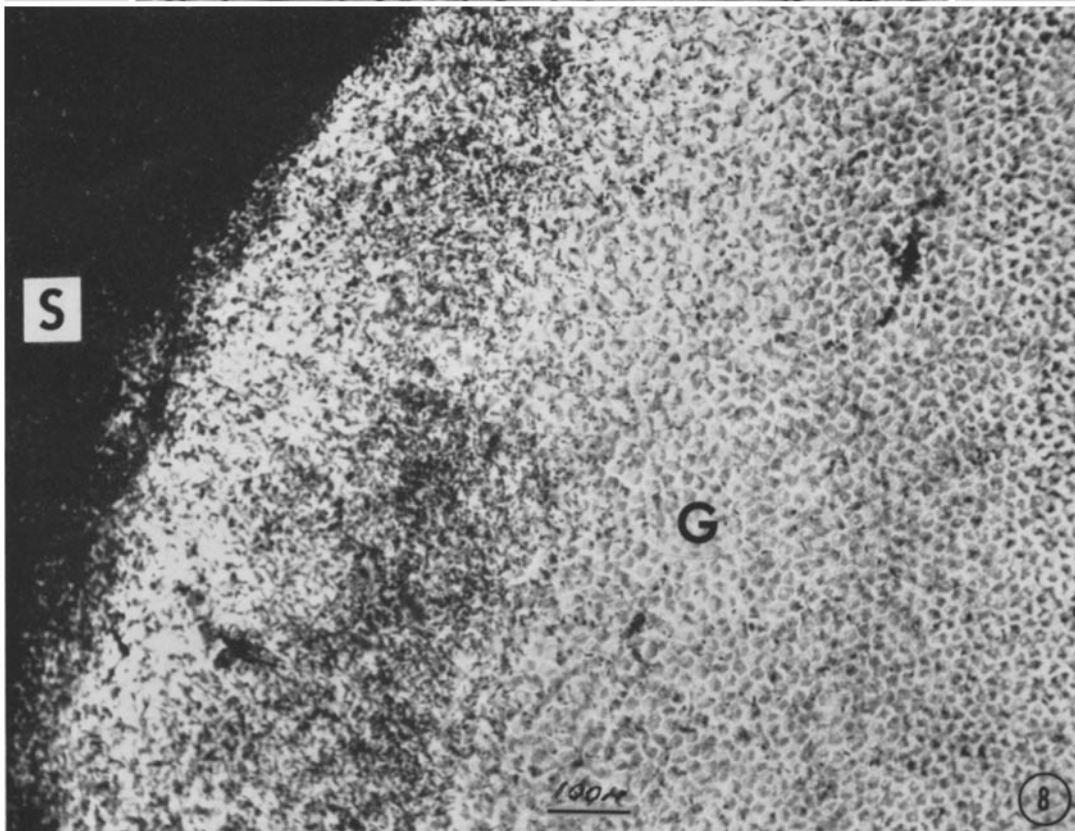
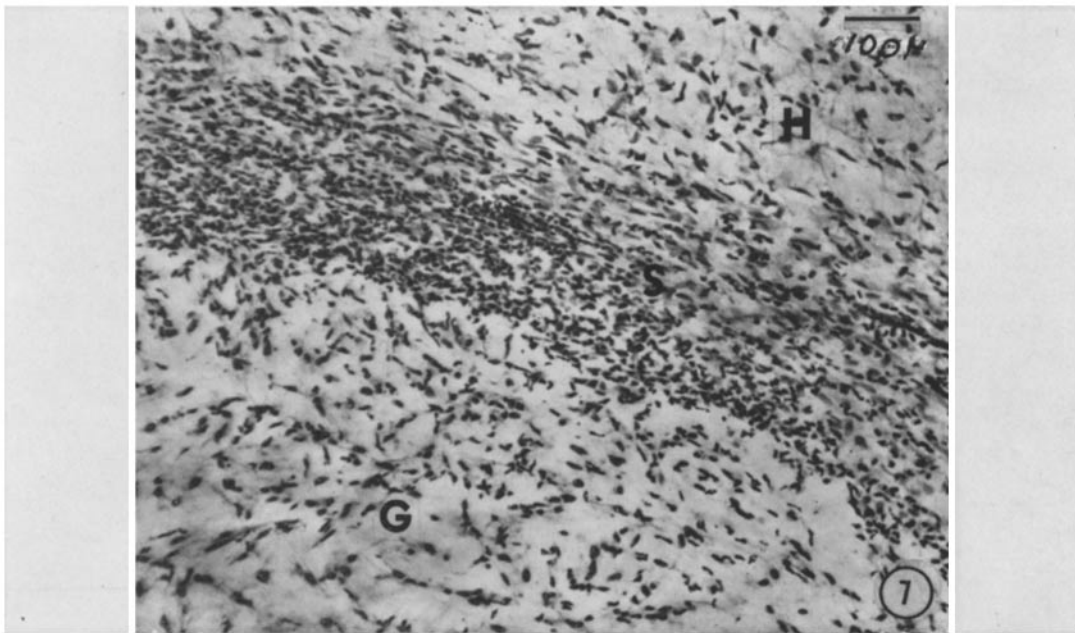


(Polack: Corneal graft rejection)

PLATE 91

FIG. 7. Flat frozen section showing the infiltrative process along the scar (*S*), graft (*G*), host (*H*). Silver carbonate of del Rio Hortega.

FIG. 8. Endothelial preparation showing destruction of cells at the periphery of the graft (*G*). A band of round cells proceeding from the scar (*S*) has invaded the graft. Hematoxylin and eosin stain.



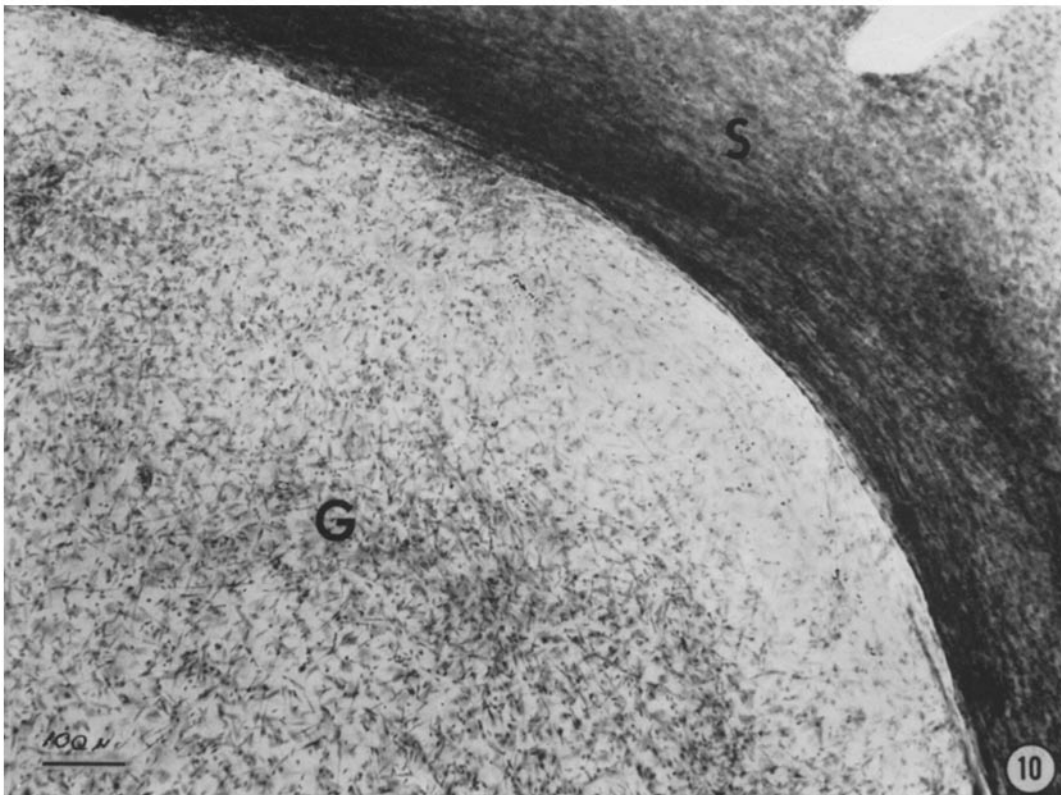
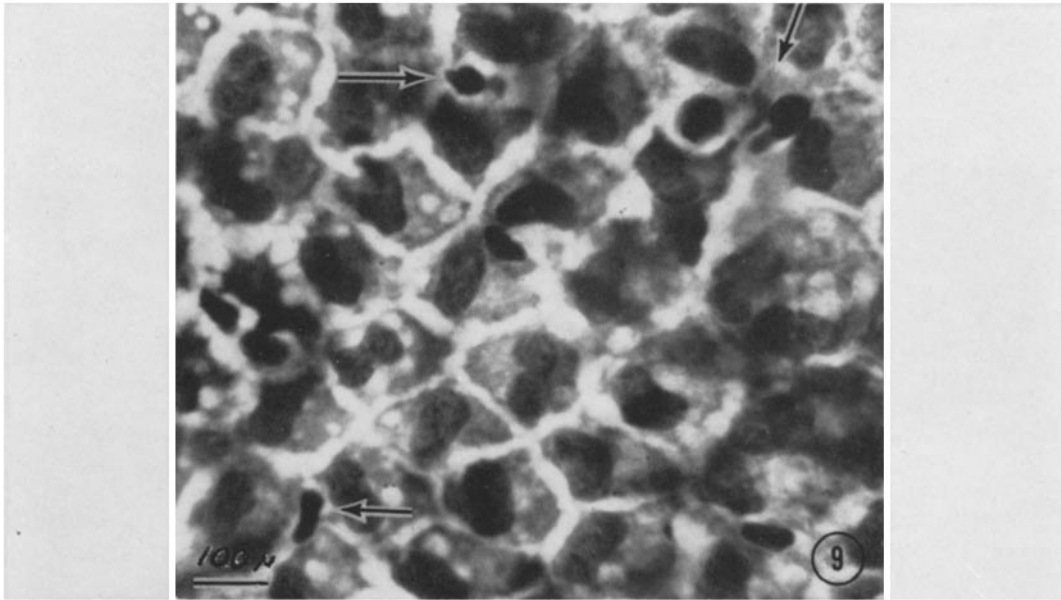
(Polack: Corneal graft rejection)

PLATE 92

FIG. 9. Endothelial cells showing vacuolization of their cytoplasm. Plasma cells and lymphocytes (arrows) have infiltrated the endothelial layer. Hematoxylin and eosin stain.

FIG. 10. Whole mount of Descemet's membrane showing total destruction of the endothelium of the graft (*G*). Only few round cells and fibroblasts remain on Descemet's membrane. Cells from the stroma are seen through this glass membrane. Hematoxylin and eosin stain.



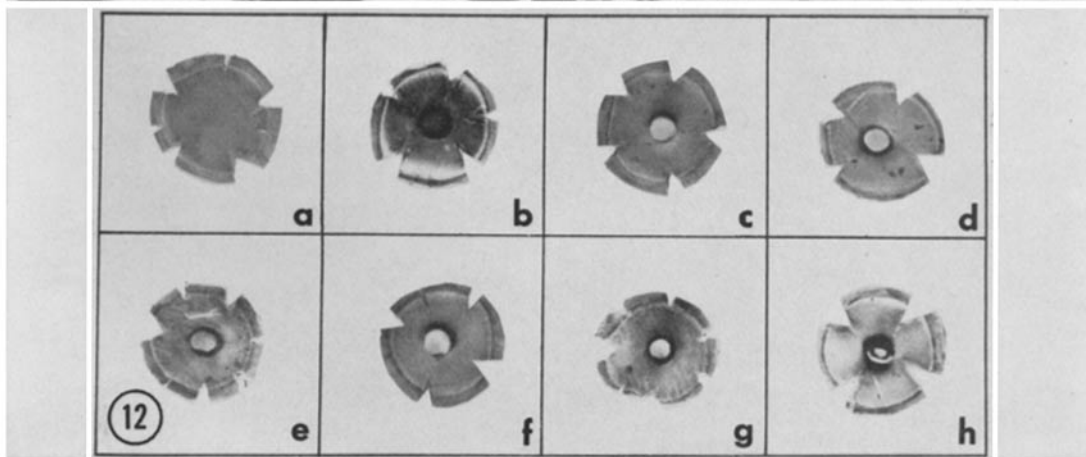
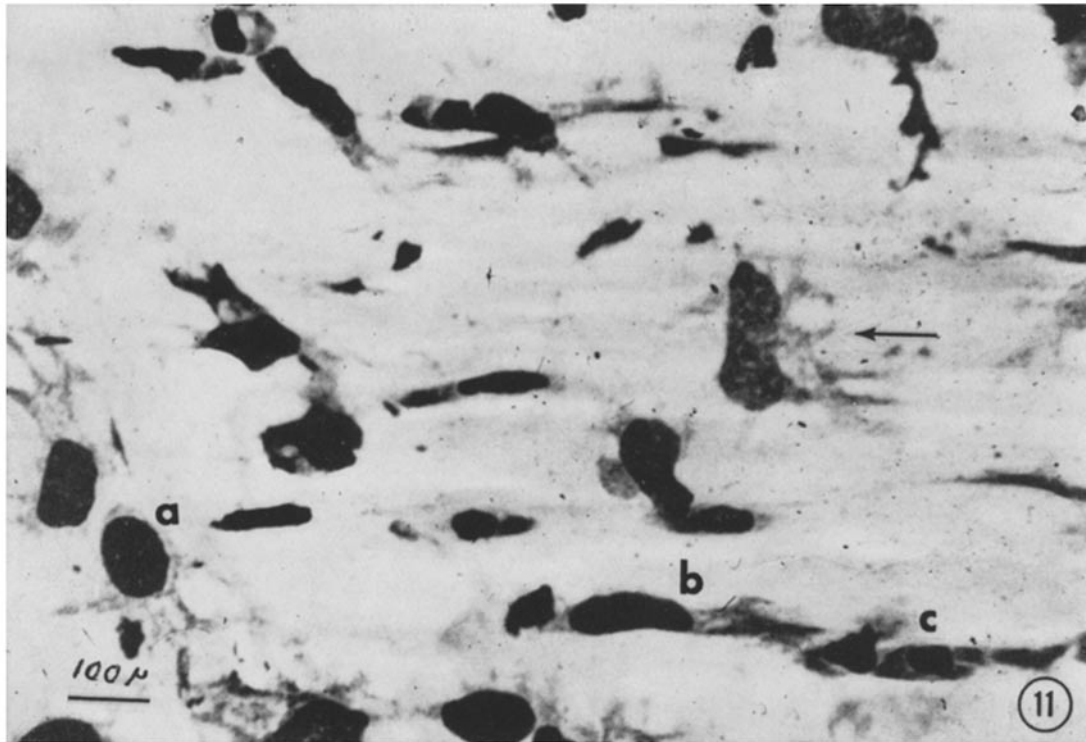


(Polack: Corneal graft rejection)

PLATE 93

FIG. 11. Flat frozen section of a hazy graft showing keratocytes with hypertrophic changes (*a*). Others show elongated bodies and loss of processes (*b*). Some leucocytes have invaded the stroma (*c*). Arrow points to a normal stroma cell. Silver carbonate of del Rio Hortega.

FIG. 12. *a*, Radioautograph of a non-grafted cornea 24 hours after the injection of  $S^{35}$ -labeled  $Na_2SO_4$ . *b*, Radioautograph showing the incorporation of  $S^{35}$  by a 4-week-old corneal graft. The density of the autograph of the graft is similar to that of the host cornea, and the control non-grafted cornea. *c* to *g*, Radioautographs showing a decreased  $S^{35}$  uptake by the graft at the onset of the rejection. *h*, Radioautograph of a partially opaque and vascularized graft in more advanced stages of graft rejection. The dark area corresponds to increased uptake of  $S^{35}$  by the scar tissue. A small area of the graft which was hazy shows a decreased amount of radioactivity. Dark areas in the host cornea correspond to the zone of cellular infiltration and fibroblast activity.



(Polack: Corneal graft rejection)