AN ELECTRON MICROSCOPIC STUDY OF THE HOMOGRAFT REACTION

JOSEPH WIENER, M.D., DAVID SPIRO, M.D., PH.D., AND PAUL S. RUSSELL, M.D.*

From the Departments of Pathology and Surgery, College of Physicians and Surgeons of Columbia University, New York, N.Y.

The rejection of living homologous tissues transplanted to genetically dissimilar hosts is accomplished by an immunologic mechanism initiated in the recipient by the introduction of the foreign graft.[1-5] Though there is general agreement that the homograft reaction is an immunologic phenomenon, there is considerable disagreement about the role of the specific cellular inflammatory response evoked in the grafts. The majority opinion holds that the cellular infiltration accompanying graft destruction is the primary event and that death of the graft results from the interaction of antibody closely associated with these cells and the transplantation antigens.[6-14] Another view contends that the most important feature of this immune reaction is the production of classic serum antibody by the host, and that the cellular phenomena that occur in the reaction are of secondary importance.[15-21] There is no doubt that homografts provoke the formation of serum antibodies which in some circumstances can exert a strong cytotoxic effect *in vivo,* especially against homografts which are inoculated as suspensions of dissociated cells.[22-31] The evidence favoring each of these hypotheses has been recently reviewed by Kretschmer and Pérez-Tamayo.[32]

This electron microscopic study was undertaken to gain further information concerning the intimate relationship between the cells of graft and host. It re-emphasizes the apparent importance of certain cells, newly characterized at this morphologic level, in graft rejection.

In this study, the morphologic events associated with the rejection of full thickness skin homografts in rabbits were compared to the same process using pure epidermal sheets as grafts and the nonspecific inflammatory process which follows the local injection of turpentine. Pure

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* Present address: Massachusetts General Hospital, Boston, Mass.

epidermal grafts offered the special advantage of excluding the necessity for graft vascularization and providing an easy distinction between cells of the host as distinguished from those of graft origin.

Material and Methods

Genetically heterogeneous adult male New Zealand rabbits were used as donors and recipients in these experiments. In each case, 6 full thickness disks of skin were removed from the dorsal aspect of the donor's ear. These were then placed, in open style, after trimming excess dermal connective tissue from the undersurface, on a rectangular full-thickness bed of exposed panniculus carnosus on the lateral thoracic wall of the recipient. The grafts were covered with a fitted vaseline gauze square surmounted by a gauze bandage and encased in a light plaster sheath, according to the method of Billingham and Medawar.[33] Sterile technique was used throughout. Alternatively, in some experiments pure epidermal sheets measuring some 15 by 15 mm. were prepared by a tryptic digestion process from aural Thiersch grafts by methods also described by Billingham and Reynolds.[34] These were likewise transplanted in a similar fashion as homografts to beds prepared on unrelated recipients.[34] Autografts were similarly prepared and transplanted to the homograft recipients.

The homografts and portions of immediately underlying graft beds were excised in one piece, immediately fixed in cold buffered 1 per cent osmium tetroxide with added sucrose,[35] and cut into thin longitudinal strips containing both graft and graft bed. These strips were then transferred to fresh osmium tetroxide and further fixed in the cold for 4 hours. The specimens were dehydrated in acetone and embedded in Araldite according to the method of Stenger and Spiro.[36] The tissue segments were placed longitudinally in the embedding capsules so that the entire thickness of the grafts presented. Full thickness grafts were removed at intervals of 3, 5, 7 and 9 days following transplantation. Samples of pure epidermal grafts were excised at 5, 7, 9 and 11 days following their transfer.

In separate experiments, turpentine, diluted 1:20 in olive oil, was injected intradermally into the lateral thoracic wall of rabbits. Segments of epidermis and dermis from the resultant inflammatory lesions were excised 1, 3 and 7 days after the injections, and fixed and processed in a similar manner.

Thin cross sections of all tissues were stained with uranyl acetate and examined with an RCA EMU-3F and Siemens Elmiskop I electron microscopes. To facilitate orientation and in order to decrease the sampling error, ancillary light microscopic studies were performed. Sections of the Araldite-embedded material cut at 2 μ were examined with the phase microscope, and suitably stained paraffin-embedded material was examined by ordinary light microscopy.

Results

Light microscopic observations on the two types of grafts were in accord with those previously described by other investigators. The sequence of events occurring in the full thickness and the pure epidermal grafts were essentially the same, except for the following differences. Complete rejection, as evidenced by complete epidermal necrosis, of the full thickness grafts generally occurred within 9 days, while the same process required 11 days in the case of the pure epidermal grafts.[34] Similar pathologic changes occurred in the transplanted dermis of full thickness grafts and in the host granulation tissue of pure epidermal grafts.

Homograft rejection is most conveniently described as occurring in 3 stages which merge sequentially with one another. The first stage was characterized by proliferation of the graft epithelium and fibro-vascular tissue in the graft bed. The second stage was characterized by a striking infiltration of mononuclear cells into the dermis and epidermis. The graft epidermis rapidly died during the third stage and was eventually sloughed with the rest of the graft.

The duration of the first stage was about 5 days in the case of full thickness grafts and 7 days in the case of pure epidermal grafts. During this period the grafts became firmly anchored to the host tissues. The epithelial cells increased in number, with resultant increase in thickness of the epidermis. Binucleated epidermal cells, as well as mitotic figures within the epithelial cells, were occasionally present. The epidermal cells were well preserved and exhibited the usual fine structural features described by others for cells of this type.[37–44] Their nuclei were large and oval and exhibited prominent nucleoli (Figs. 1 and 2). The cytoplasm of these cells contained small amounts of rough-surfaced endoplasmic reticulum, large numbers of non-membrane-associated ribosomes as well as vesicles and tonofibrils (Figs. 1, 2 and 7 to 14). The tonofibrils coursed through the cytoplasm of the epidermal cells and were most conspicuous in the peripheral portions of the cells (Figs. 1, 2 and 8 to 14). The tonofibrils, which were composed of tonofilaments, 60 Å in diameter, terminated on the inner face of the attachment plaques of the desmosomes (Figs. 2, 8 and 12 to 14). Neighboring epidermal cells were separated by an intercellular space of variable size into which projected microvilli (Figs. 1, 2 and 8). A continuous basement membrane, which measured 200 to 300 Å in thickness, separated the basal layer of the epidermis from the dermis proper.

In this initial period there was striking fibro-vascular proliferation in the graft beds, resulting in the formation of abundant granulation tissue consisting of fibroblasts, newly formed capillaries and collagen fibers (Fig. 3). Scattered through the connective tissue were numerous polymorphonuclear leukocytes (Fig. 4). Some of these cells were degenerating and exhibited extrusion of their nuclei, disruption of cell surface membranes and swelling of cytoplasmic organelles (Fig. 4). Numerous free nuclei, disrupted cells and cell fragments were also scattered through the graft bed (Fig. 4).

The second stage in the series of events that culminated in graft rejection occurred from the fifth to seventh days following transplantation of full thickness grafts and was delayed until the seventh through ninth days for pure epidermal transplants. The epidermal cells through this period remained essentially normal and showed no degenerative changes. The leukocytes and cellular debris previously described in the graft beds

were no longer seen. Light microscopic studies during this stage revealed another cell type both in the dermis and epidermis (Fig. 5). These cells had centrally located nuclei which were surrounded by a thin rim of basophilic cytoplasm. The nuclei exhibited variably shaped profiles which might be circular, somewhat elongated or, in some cases, irregularly indented (Fig. 5). These nuclei consisted of a number of dense aggregates of intensely basophilic chromatin and were easily differentiated from the larger vesicular epidermal cell nuclei. These cells were usually 5 to 7.5 μ in diameter but occasionally measured up to about 10 μ.

By electron microscopy, the invading cells were first noted within and adjacent to dermal capillaries in small numbers (Fig. 6). Subsequently, they were found in large numbers in the dermis as well as the epidermis. The infiltrating cells were first observed in the basal layer of the epidermis (Fig. 7) and later were found in the more superficial layers (Figs. 8 and 9), extending almost to the stratum corneum. They occupied greatly expanded intercellular spaces within the epidermis (Figs. 7 to 14) and often surrounded and isolated individual epidermal cells (Fig. 9).

The nucleus of the invading cells was large, irregular and presented numerous small peripheral indentations (Figs. 6, 10 and 11). The nucleoplasm formed a definite and irregular electron-dense band adjacent to the nuclear envelope (Figs. 6 to 11). Nucleoli were prominent (Figs. 6 to 9 and 11). A Golgi complex, consisting of parallel lamellas and vesicles, was prominent on occasion (Fig. 10). The cytoplasm contained a few cisternae of smooth-surfaced endoplasmic reticulum, large numbers of non-membrane-associated ribosomes, moderate numbers of vesicles, electron-dense droplets and multivesicular bodies (Figs. 6 to 14). The mitochondria varied from 2 to 8 in number and tended to collect at one pole of the cell (Figs. 6, 9, 10, 12 and 14). The mitochondria averaged 0.5 μ in diameter and about 0.8 μ in length. These cells were all essentially similar in structure.

The apposing cell surfaces between the invading mononuclear and epidermal cells were intimately related, often being separated by extracellular spaces as small as 40 to 60 Å (Figs. 7 to 14). Numerous cytoplasmic projections and infoldings were present and accounted for a complicated interdigitated arrangement at the adjacent cell surfaces (Figs. 8 to 13). In addition, there were frequent areas at these interfaces where one or both cell surface membranes were poorly defined and perhaps discontinuous (Figs. 12 and 14).

The third interval was characterized by necrosis of the graft epidermis. This stage occupied the seventh to ninth days for full thickness

and the ninth to eleventh days for pure epidermal homografts. Degeneration of the epithelium first appeared in the basal cell layer and then extended to involve the more superficial layers. Epidermal changes were first manifested by swelling of the cells and their cytoplasmic organelles and by clumping of the tonofibrils (Fig. 15). Numerous cytoplasmic vesicles, vacuoles and electron-dense droplets were present, and the nuclear chromatin showed striking margination (Fig. 15). More advanced necrobiotic changes consisted of a generalized decrease in the electron density of the cytoplasm, first apparent in the perinuclear regions, with loss of ribosomes and tonofibrils (Fig. 15). In such cells, there were frequent disruptions of the nuclear envelope and loss of nucleoplasm (Fig. 15). The end stage of this process resulted in a shrunken epidermal cell with generally intact surface membranes devoid of cytoplasmic constituents except for scattered tonofibrils. Well-preserved mononuclear cells appeared among the degenerating epidermal cells throughout the last stage of graft rejection. Polymorphonuclear leukocytes were observed both in the dermis and between the epidermal cells when the latter revealed marked degenerative changes. Small numbers of plasma cells, easily recognized by their eccentric nuclei and well-developed rough-surfaced endoplasmic reticulum, were noted in the dermis at this time (Fig. 16).

The process of rejection was terminated by dissolution of the epidermal cell surface membranes and desquamation of the necrotic epidermis. No hemorrhage was observed at any time, and the collagenous connective tissue appeared unaffected throughout.

Examination of the tissue 24 hours after the turpentine injection revealed necrosis of the dermis and overlying epidermis, extravasation of red cells and deposition of fibrin. Thereafter, large numbers of heterophils were seen in the necrotic areas. No mononuclear cells of the type described above in the homografts were present. The autograft controls healed without incident, and were not rejected.

Discussion

The evolution of the homograft reaction occurred in 3 sequential stages cytologically. The first phase of the homograft reaction, as described previously, was characterized by proliferation of the graft epithelium and the formation of granulation tissue in the graft bed. This was indicated by an increase in the thickness of the epidermis, occasional mitotic figures and binucleated epidermal cells in the basal layer, as well as the large numbers of ribosomes present in these cells. These observations correlated well with the cytoplasmic basophilia of the epidermal cells seen by light microscopy,[45,46] as well as the increased

RNA content of these cells following grafting, demonstrated histochemically by Scothorne and Tough.[47] The deposition of collagen fibers and the formation of new capillaries in the graft bed was a form of wound healing.[48–50] The process of capillary maturation was similar to that previously described in organizing thrombi.[51] The polymorphonuclear leukocytes and cellular debris evoked in the graft bed were consistent with the surgical trauma and wound repair initiated at the sites of transplantation.

The most prominent cytologic feature in the second stage of the homograft reaction was the rapid and massive invasion of the graft tissues by cells of host origin. These cells first appeared in the capillaries of the dermis, then in perivascular aggregates in the dermis and finally in the epidermis. They occurred in the basal layer of epidermis initially, and then progressively infiltrated the more superficial layers. Their intravascular location suggested that these cells reach the graft by the bloodstream, in accord with a generally held belief.[52] Their perivascular localization suggested that they migrated through the intact endothelium of the graft vessels, probably the small venules,[53] in a manner previously described by Florey and Grant [54] and Williamson and Grisham.[55] After leaving the vessels and entering the tissues, they invaded the epidermis. Though intimately related to the epidermal cells, they were confined to the extracellular space throughout and never directly invaded the epidermal cells, as has been suggested by previous light microscopic studies.[23,56,57]

In the final stage of graft rejection there was rapid and progressive necrosis of the epidermis. The connective tissue appeared unaffected. The necrosis first appeared in the basal layer, and then spread in a wave-like fashion to engulf successively more superficial portions of the epithelium. This progression directly paralleled the manner in which the mononuclear cells invaded the epidermis. Necrosis of epidermal cells remote from the areas of infiltration occurred very infrequently and might have been a manifestation of an oblique plane of sectioning not revealing the invading cells. In addition, the necrosis supervened without the appearance of other cells, the invading cell population remaining entirely homogeneous until the late stages when polymorphonuclear leukocytes appeared. The heterophils were clearly secondary invaders, responding to the necrosis previously incited.

Several observations indicated that the invading mononuclear cells were of prime importance in homograft rejection. The first was the intimate association of these cells with intact epidermal cells for a finite interval prior to the onset of necrosis. The second was the virtual absence of necrosis in portions of the epidermis devoid of these cells.

Third, other types of inflammatory cells did not appear in the epidermis in appreciable numbers until rejection was well advanced. However, the structurally intact invading mononuclear cells were present throughout the final as well as the second stage of graft rejection. Finally, the progression of necrobiotic changes from the stratum basalis to the stratum corneum reflected the order in which the mononuclear cells first appeared in different layers of the epidermis. The apparent dependence of graft rejection on the presence of the infiltrating cells strongly suggested that the latter were the immunologically competent elements. One cannot conclude, of course, from these observations that the infiltrating cells were alone sufficient as vectors of the immune response. Waksman has previously emphasized the importance of infiltrating mononuclear cells in mediating various types of delayed hypersensitivity.[56]

What then is the nature and origin of the immunologically competent cells? These cells undoubtedly correspond to the lymphoid cells seen in homografts with light microscopy by previous workers.[1,48,56-59] Unfortunately, there are no striking ultrastructural differences between lymphocytes and monocytes.[60-64] However, certain cytologic characteristics are more prominent in one or the other of these cell types. The peripheral distribution of chromatin, the relatively large amount of cytoplasm, the prominence of the Golgi apparatus and cytoplasmic vesicles are features of monocytes. The size of the cells, the dimensions of the mitochondria and the low density of the cytoplasm are more characteristic of lymphocytes. Irregular nuclear profiles and dispersed ribosomes are present in both cell types. The cells associated with graft rejection, therefore, possess some structural details which are common to both immature and mature lymphocytes as well as monocytes.

As discussed previously, the presumed immunologically competent cells all exhibited essentially similar fine structural characteristics. No transitional forms indicating an origin from another cell type were identified. This suggested that the competent cells were end stage cells on arrival and did not arise at the graft site. Despite numerous light microscopic investigations, as well as the present study, the precise definition and nomenclature of the cell type associated with graft rejection remains undefined. These cells were not found in the turpentine-induced inflammatory lesions and could be specific for the process of graft destruction. For these reasons, we have avoided specific terminology and believe that the more descriptive term, graft rejection cells, is appropriate at present. In an electron microscope study of the graft versus host reaction, Binet and Mathe[65] described the proliferation of similar cells in lymph nodes.

Gowans, McGregor, Cowen and Ford,[66] using tracer techniques, showed in rats that small lymphocytes probably initiated the reaction against first-set skin homografts. They also showed that in the regional lymph nodes draining skin homografts, some of the small lymphocytes in the nodes reacted to the graft antigens and gave rise to large pyroninophilic cells, undoubtedly similar to the pyroninophilic cells previously described by Scothorne[67] and Scothorne and McGregor[68] in similar lymph nodes. These latter cells in turn gave rise to new groups of small lymphocytes which left the nodes, entered the bloodstream and invaded the graft. The small lymphocytes that Gowans and co-workers[66] described were probably represented by the graft rejection cells described here. Impairment of the homograft response and depletion primarily of small lymphocytes in thymectomized animals further confirms the role of lymphoid tissue in graft rejection.[69–73]

The mechanism by which the immunologically competent cells destroy the antigen-containing cells of the homograft remains in doubt. The extracellular location of the competent cells both prior to and during the process of rejection is especially noteworthy. The precise nature of the transplantation antigens remains unknown. Kandutsch and Reinert-Wenck[74] have shown that they contain both carbohydrate and protein, and are chemically similar to the blood group antigens. Snell[29] previously suggested that the isoantigens which are determined by histocompatibility genes are probably present in the cell surface and intracellular membranes. The close association between graft rejection and epidermal cells may provide for the interaction of antigen and antibody located on or within the donor and recipient cells respectively. The apparent discontinuities of the adjacent cell surface membranes of the epidermal and invading cells appeared to be far too frequent to be accounted for by a tangential plane of section or by artifacts. In general, the preservation of the various cells, including their surface membranes, was excellent. These interruptions in the surface membranes of the two types of cells might play a role in the immunologic phenomena leading to graft rejection.

The numerous non-membrane-associated ribosomes account for the cytoplasmic basophilia of the graft rejection cells[45,46,75,76] and may be involved in the synthesis of the appropriate antibodies.[77] Cells actively engaged in the synthesis of secretory proteins, such as plasma cells which elaborate humoral antibody, have abundant formations of rough-surfaced endoplasmic reticulum, consisting of both cisternal membranes and ribonucleoprotein particles.[62,64,78,79] The paucity of such structures, despite the profusion of scattered ribosomes within the graft rejection cells may be related to the fact that these elements produce antibodies

which are not secreted but rather remain bound to the cells.[4,6-14] Differentiating and neoplastic cells, which are presumably synthesizing structural and cytoplasmic protein, also exhibit numerous non-membrane-associated ribosomes and very little rough-surfaced endoplasmic reticulum.[80]

Other cell types, including histiocytes, monocytes and eosinophils, which have been observed by others in graft rejection[1,49,66,81-88] were not observed in this study. Plasma cells, however, appeared in small numbers in the dermis after graft rejection was far advanced. The late occurrence of humoral antibodies in the homograft reaction is probably a secondary or parallel event, and may be related to the presence of these cells.

Summary

The skin homograft reaction has been studied by light and electron microscopy. The homografts were progressively infiltrated by mononuclear cells which were present for about two days prior to as well as during the phase of graft necrosis. These mononuclear cells had fine structural features which were not consistent with those of known hematopoietic cells and have been termed graft rejection cells. Observations are cited which indicate that these cells are of prime importance in the initiation of homograft rejection.

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[*Illustrations follow*]

Legends for Figures

Key:

T = Tonofibrils
G = Golgi complex
GRC = Mononuclear (graft rejection) cell
B = Multivesicular body

Figures 1 to 4 represent the first stage of the homograft reaction. Figures 5 to 14 are light and electron micrographs from the second stage, and Figures 15 and 16 illustrate the final stage of graft rejection.

Fig. 1. Portion of homograft epidermis, 3 days after transplantation. The cells are well preserved and contain numerous non-membrane-associated ribosomes, mitochondria and tonofibrils. × 15,000.

Fig. 2. Similar to Figure 1. A desmosome with associated tonofibrils is noted in the upper central portion of the figure (arrow). Numerous cytoplasmic projections (microvilli) are seen at the cell surfaces. The membranes and vesicles of the Golgi complex are adjacent to the nucleus. × 14,000.

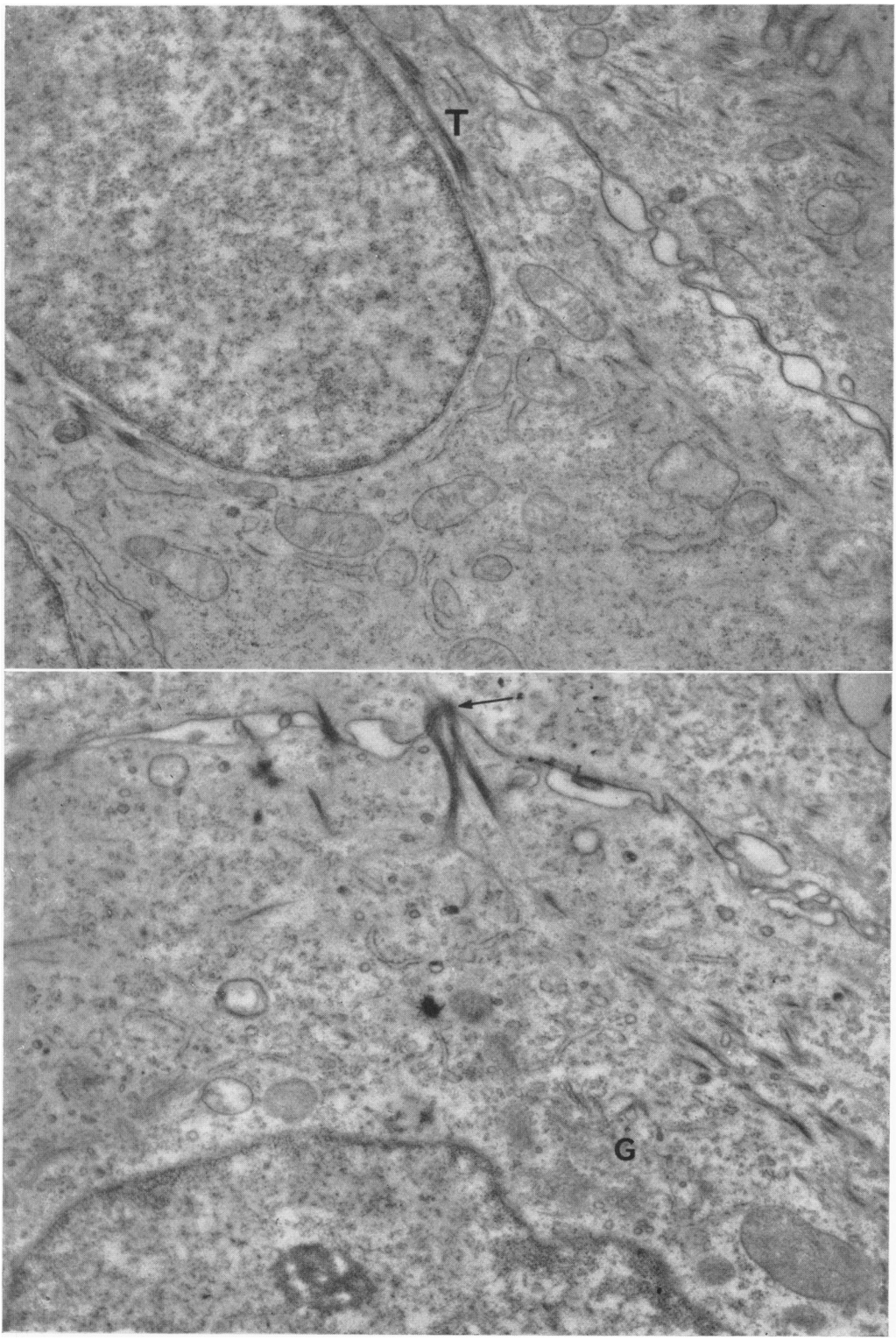

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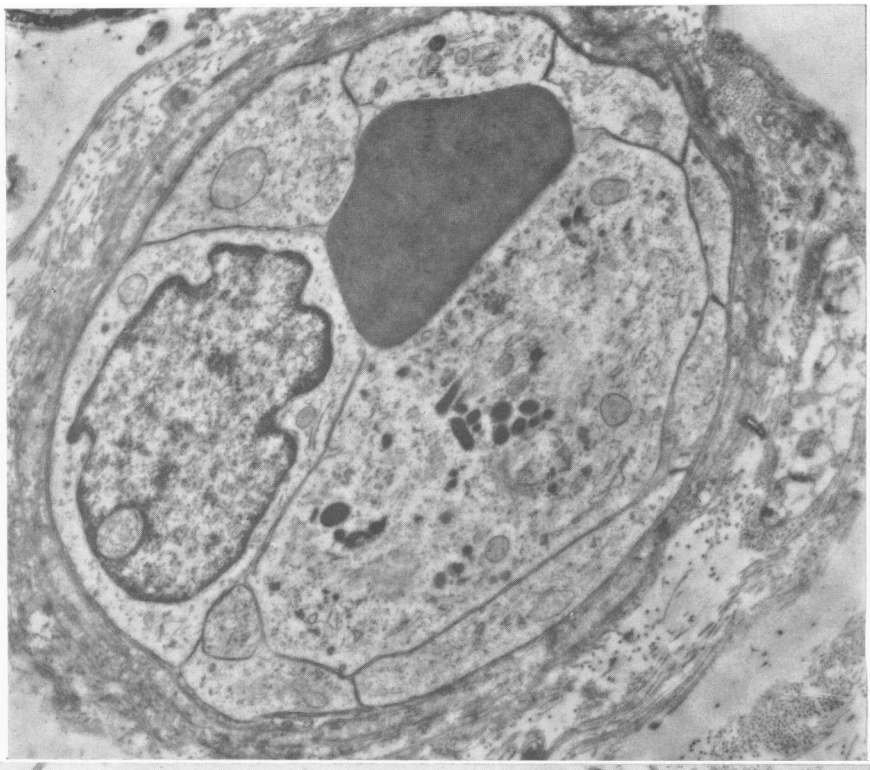
FIG. 3. A transverse section of an immature capillary from the graft bed. The small lumen is completely filled by a red cell. × 7,000.

FIG. 4. The dermis demonstrates a polymorphonuclear leukocyte with a partially extruded nucleus. In the upper right there is a fragment of swollen cytoplasm from a degenerating cell. × 19,000.



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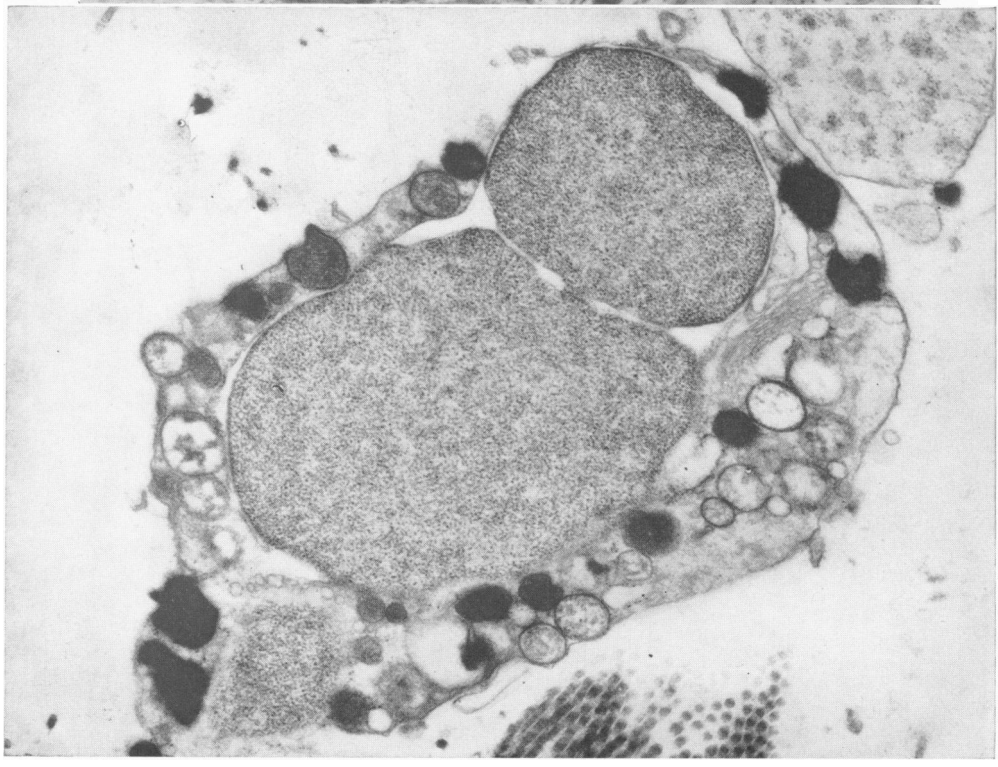

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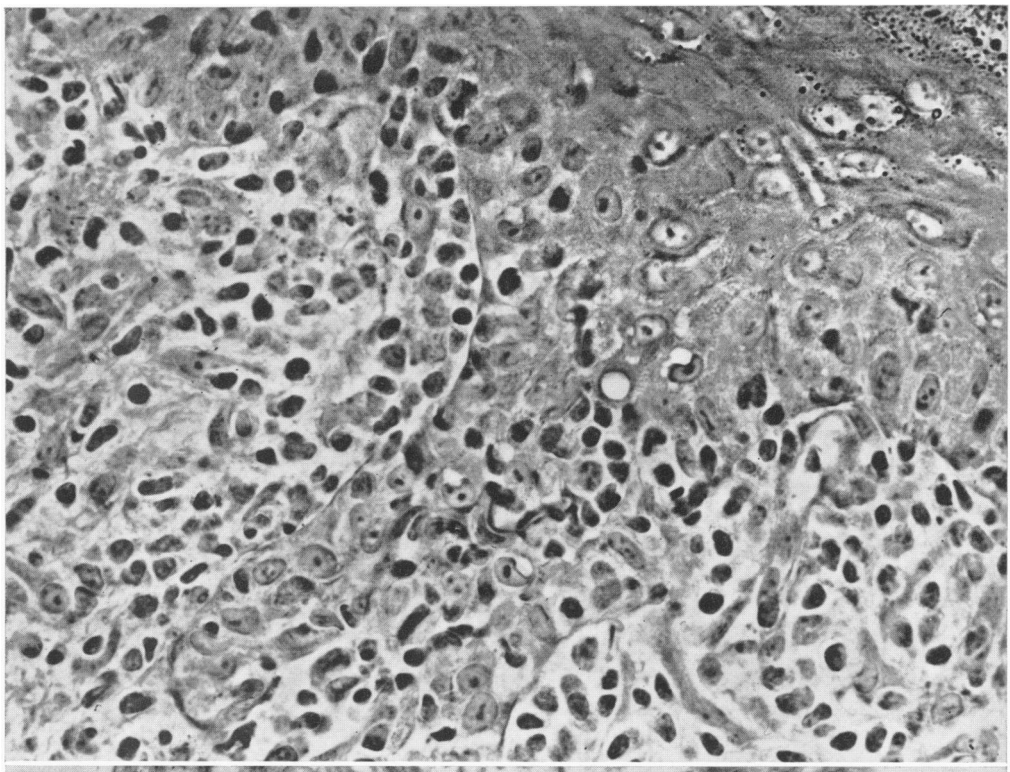
FIG. 5. Phase contrast, taken during the second stage of graft rejection. A portion of stratum granulosum (upper right) and a rete peg are included. Large numbers of invading cells are present both within the dermis and epidermis. The nuclei of these cells vary in shape, are extremely dense and are easily differentiated from the vesicular nuclei of epidermal cells. × 400.

FIG. 6. Graft bed in the second stage of the homograft reaction. Several mononuclear cells showing peripheral clumping of their chromatin are seen in the vicinity of a dermal capillary at the upper left of the illustration. × 10,000.



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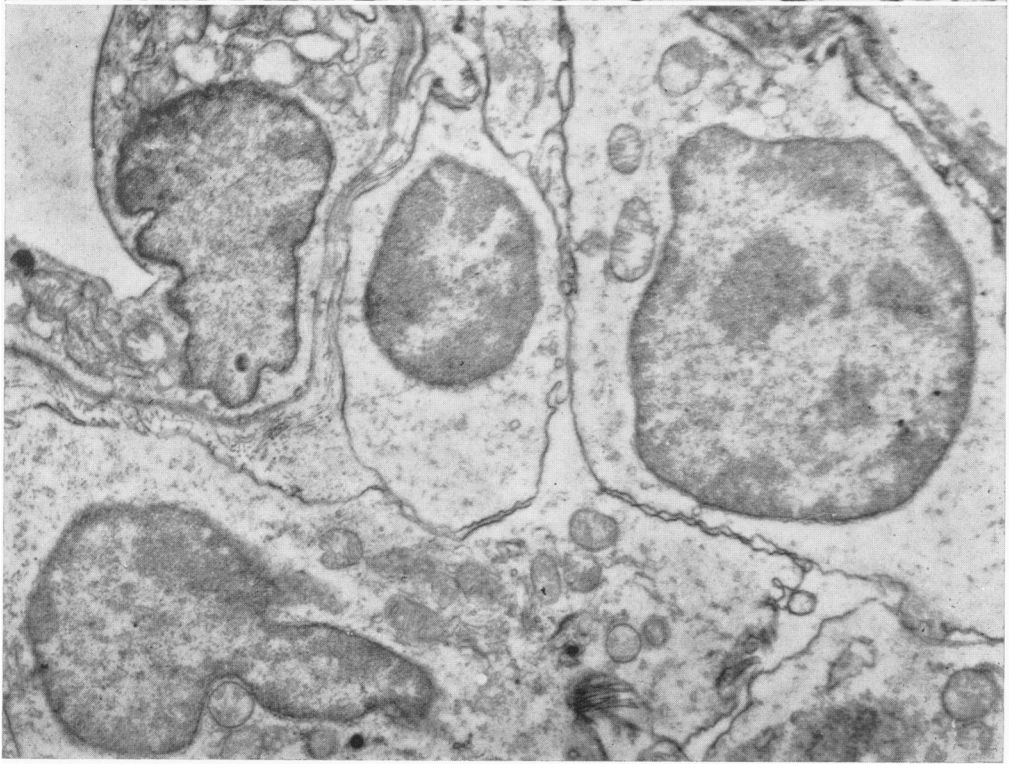

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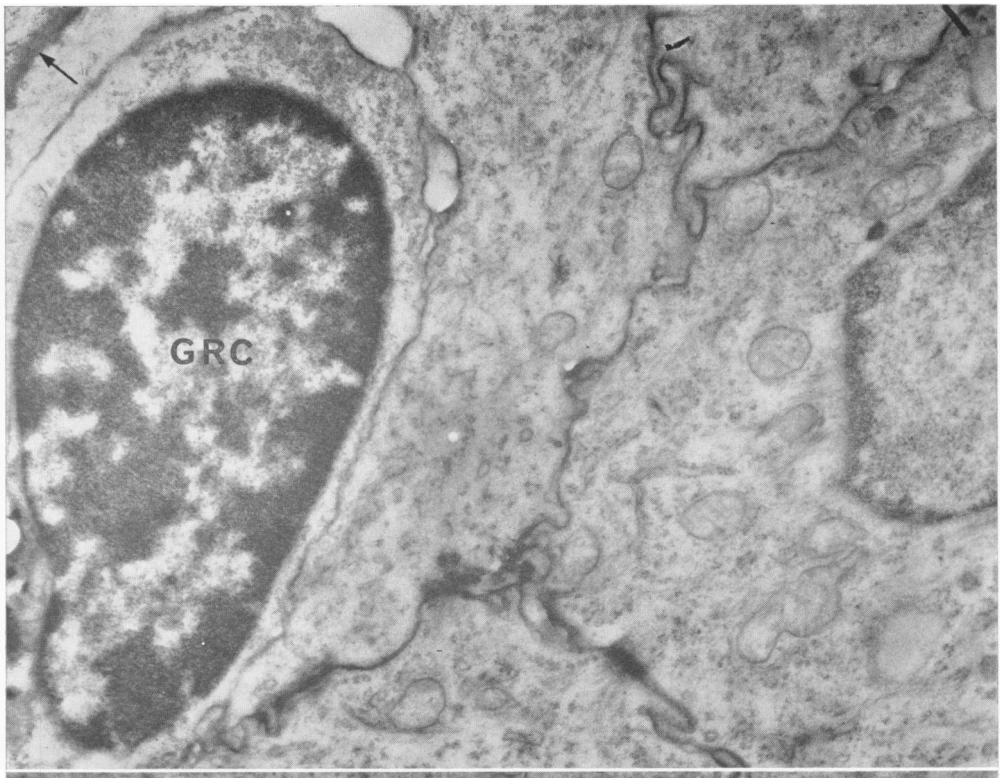
FIG. 7. An invading mononuclear cell is seen within the basal layer of the epidermis. A small portion of the epidermal basement membrane (arrow) is present on the upper left. The epithelial cells are well preserved. × 17,000.

FIG. 8. A mononuclear cell appears within the malpighian layer of the epidermis, which is otherwise unaltered. The chromatin of this mononuclear (graft rejection) cell is marginated. × 10,000.



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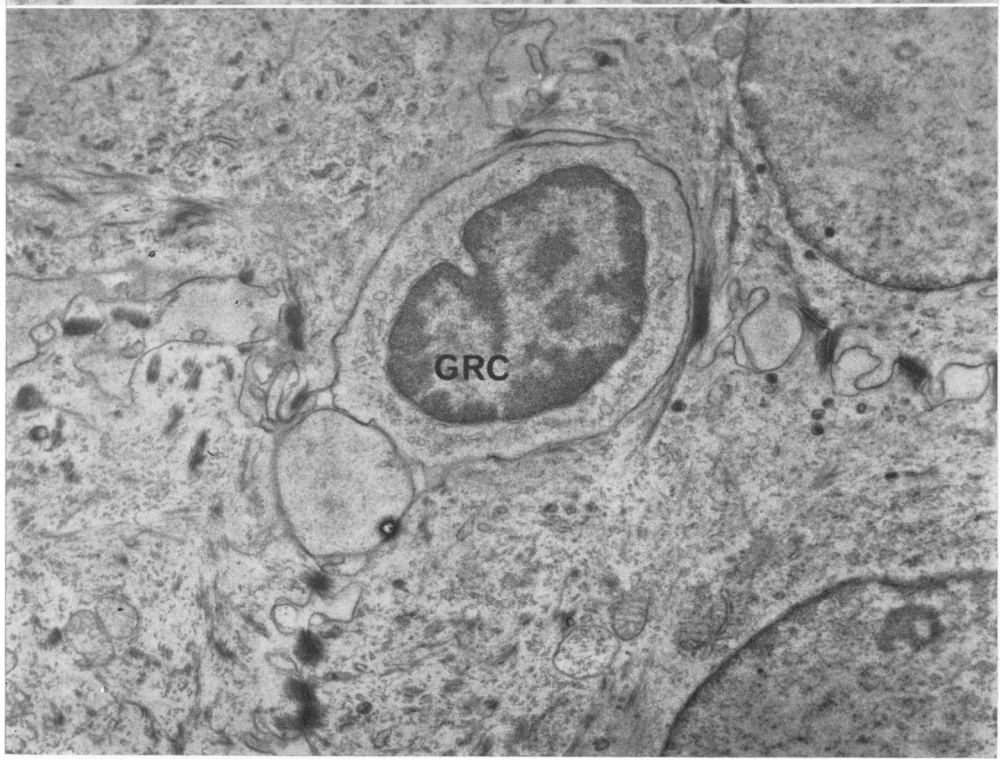

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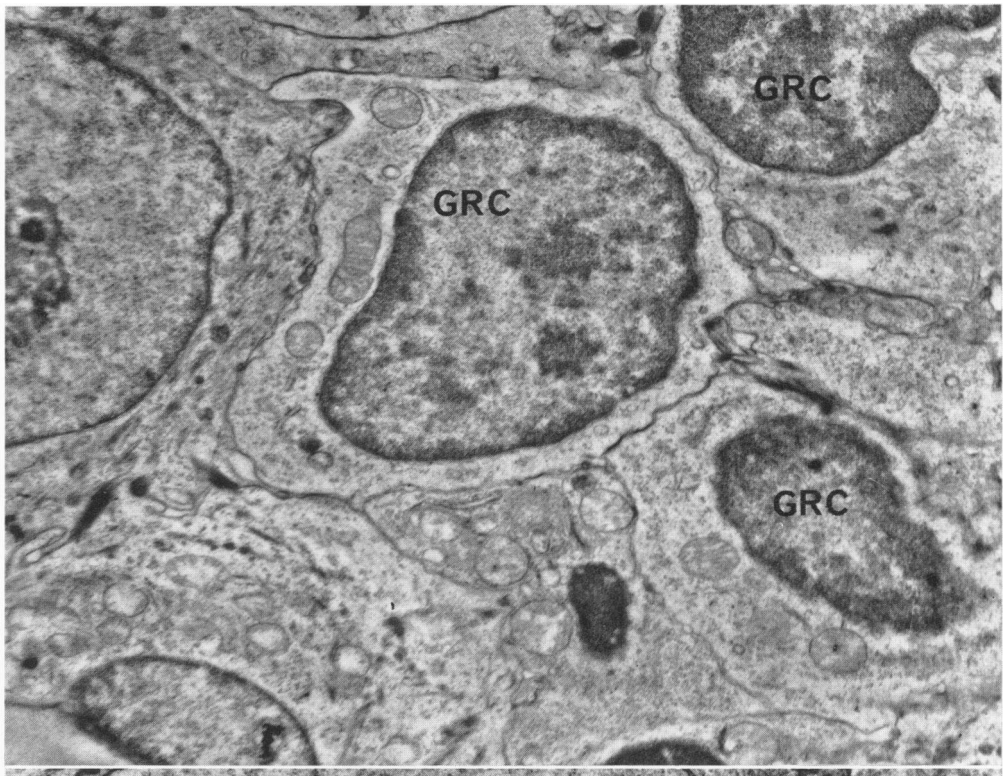

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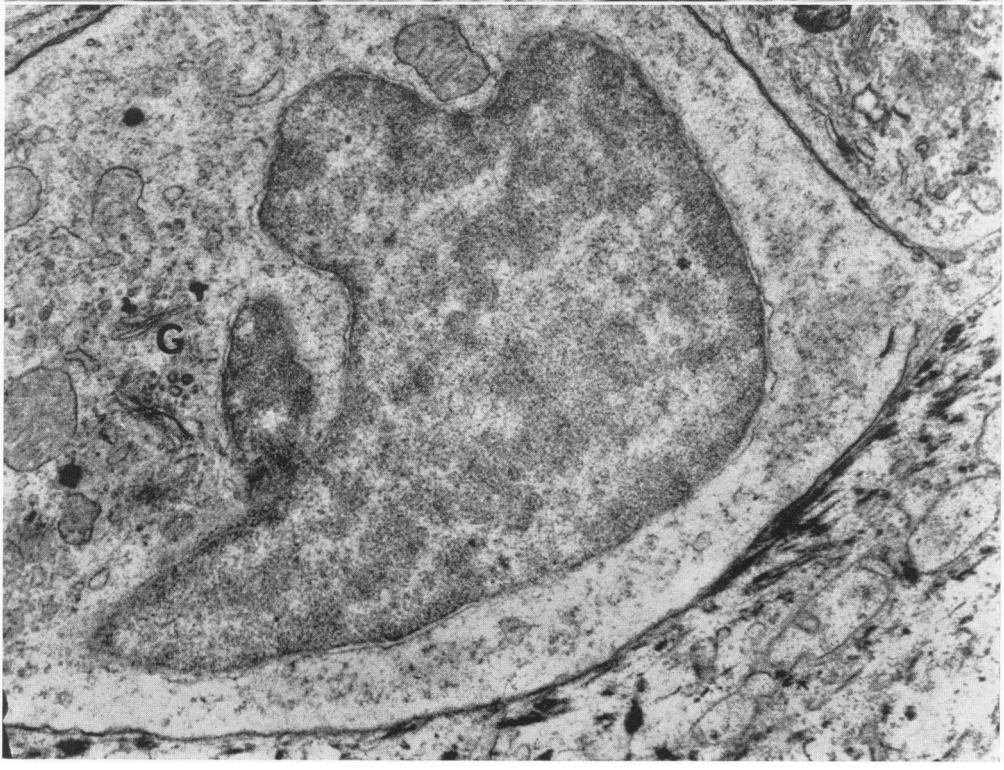

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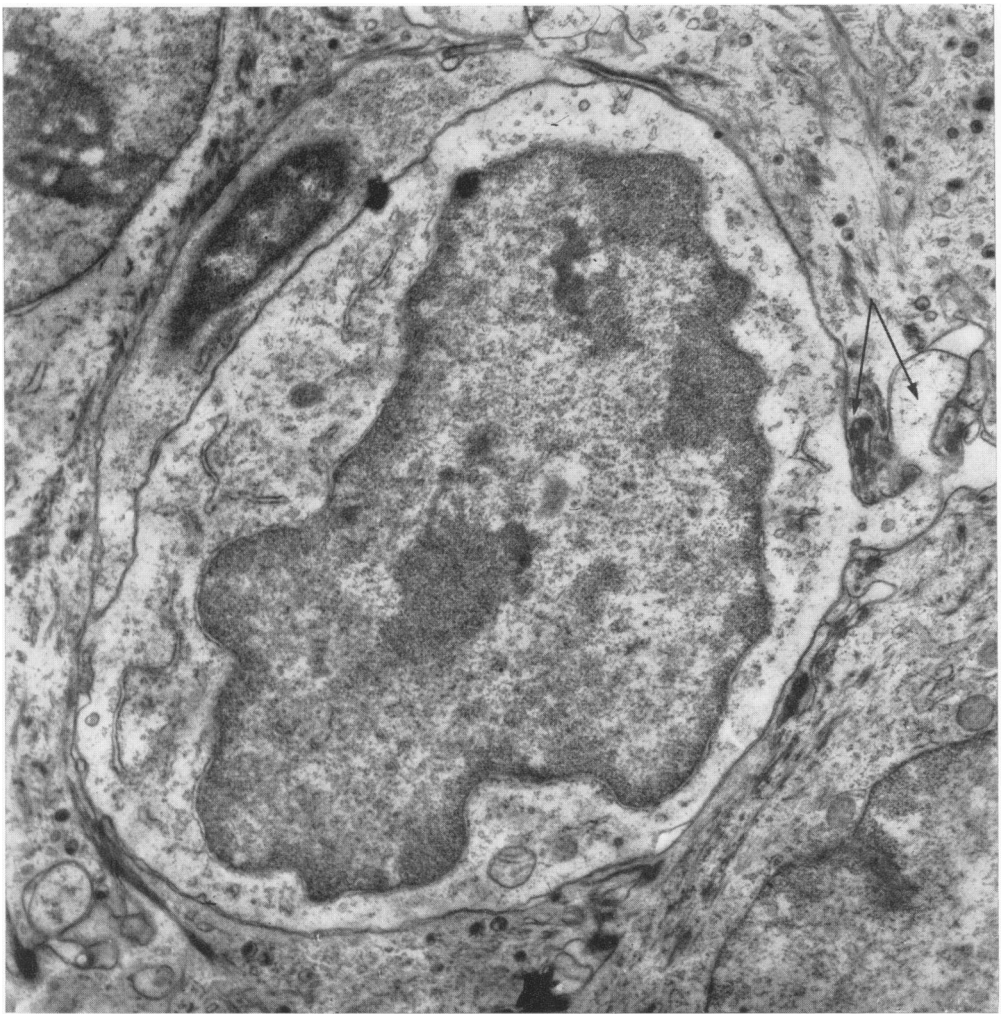

FIG. 9. Similar to Figure 8. Several graft rejection cells are seen within the epidermis. × 10,000.

FIG. 10. An invading mononuclear cell lies between several epithelial cells. The graft rejection cell contains several mitochondria and a Golgi zone. × 14,000.

FIG. 11. The close proximity of a graft rejection cell to the surrounding epidermal elements is shown. Interdigitation of cytoplasmic processes of both types of cells is evident (arrows). The irregular nuclear profile, peripheral concentration of the chromatin and paucity of rough-surfaced endoplasmic reticulum in the graft rejection cell are manifest. × 17,000.

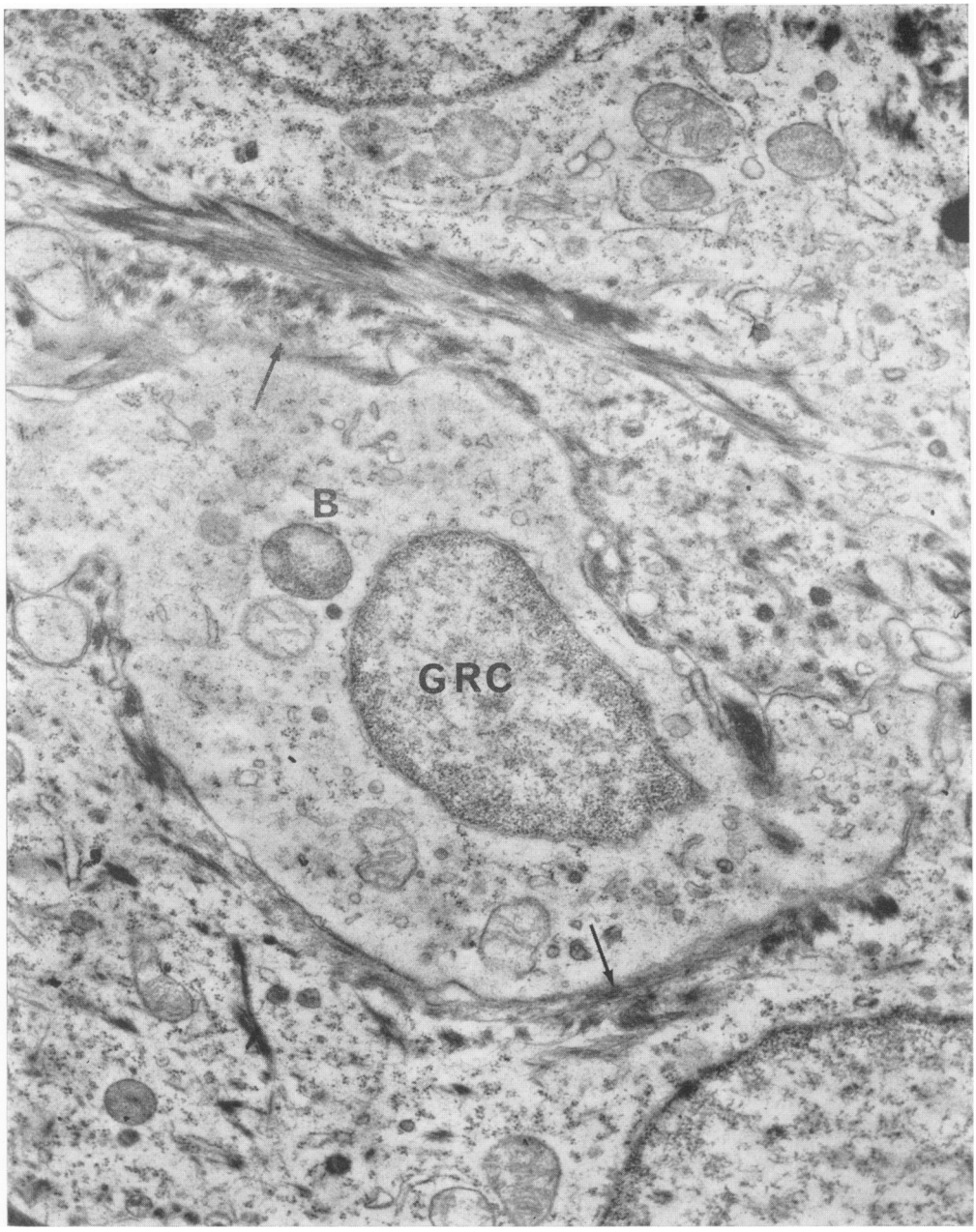

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FIG. 12. An infiltrating cell contains moderate numbers of dispersed ribosomes and vesicles as well as a multivesicular body. The tonofibrils, mitochondria and other constituents of the epidermal cells are intact. There is intimate relationship of the apposing cell surface membranes which are poorly defined in areas (arrows). × 22,000.

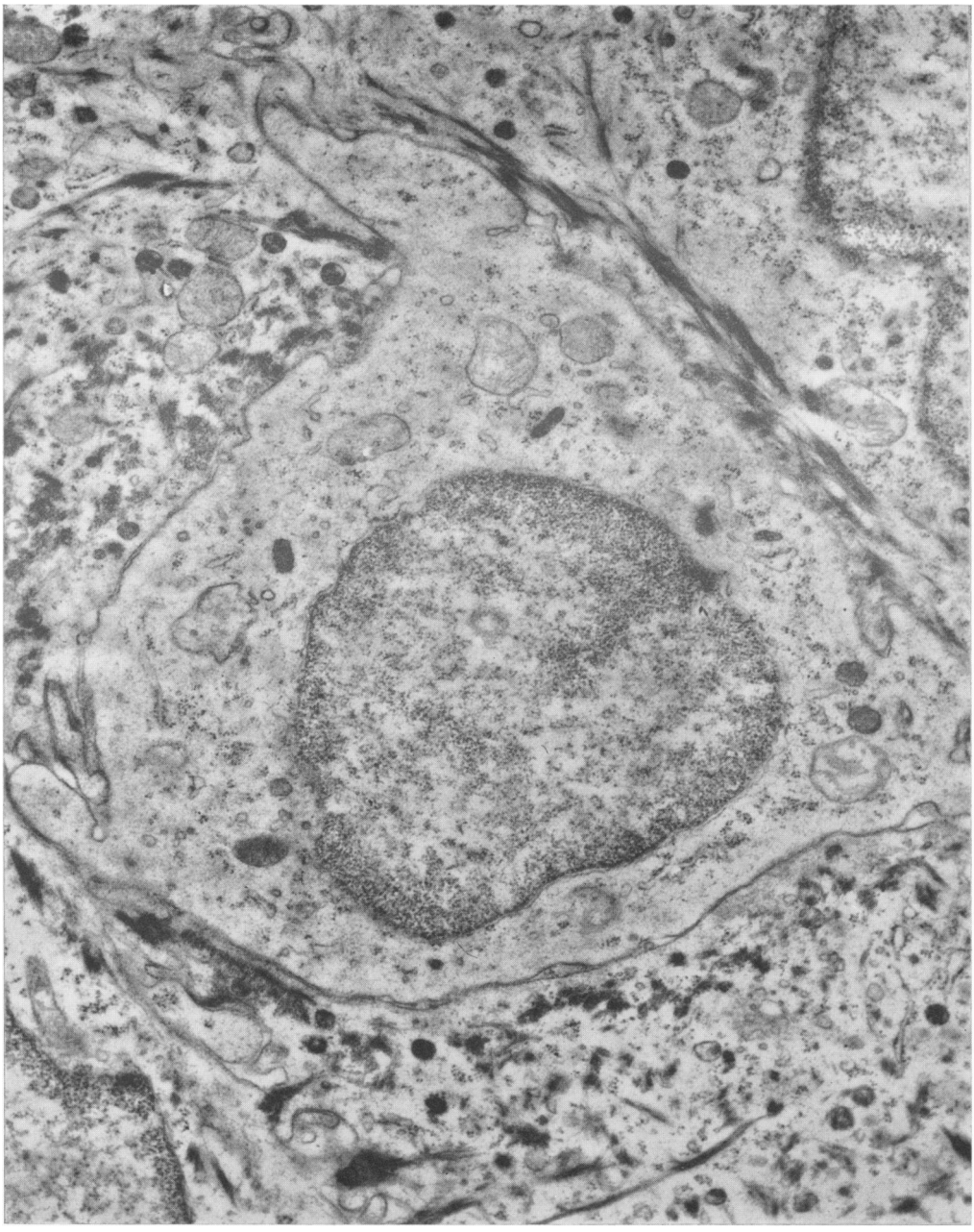

FIG. 13. A graft rejection cell in intimate contact with apparently normal epithelial cells exhibits several cytoplasmic processes. × 20,000.

FIG. 14. A portion of an interface between a graft rejection cell on the left and epidermal cells on the right. The cytoplasmic constituents of both cell types are well preserved and are similar to those described in previous figures. Individual tonofilaments, which comprise the tonofibril, are seen within the epidermal cells. There are several apparent discontinuities of the surface membranes of the two cells (arrows). × 65,000.

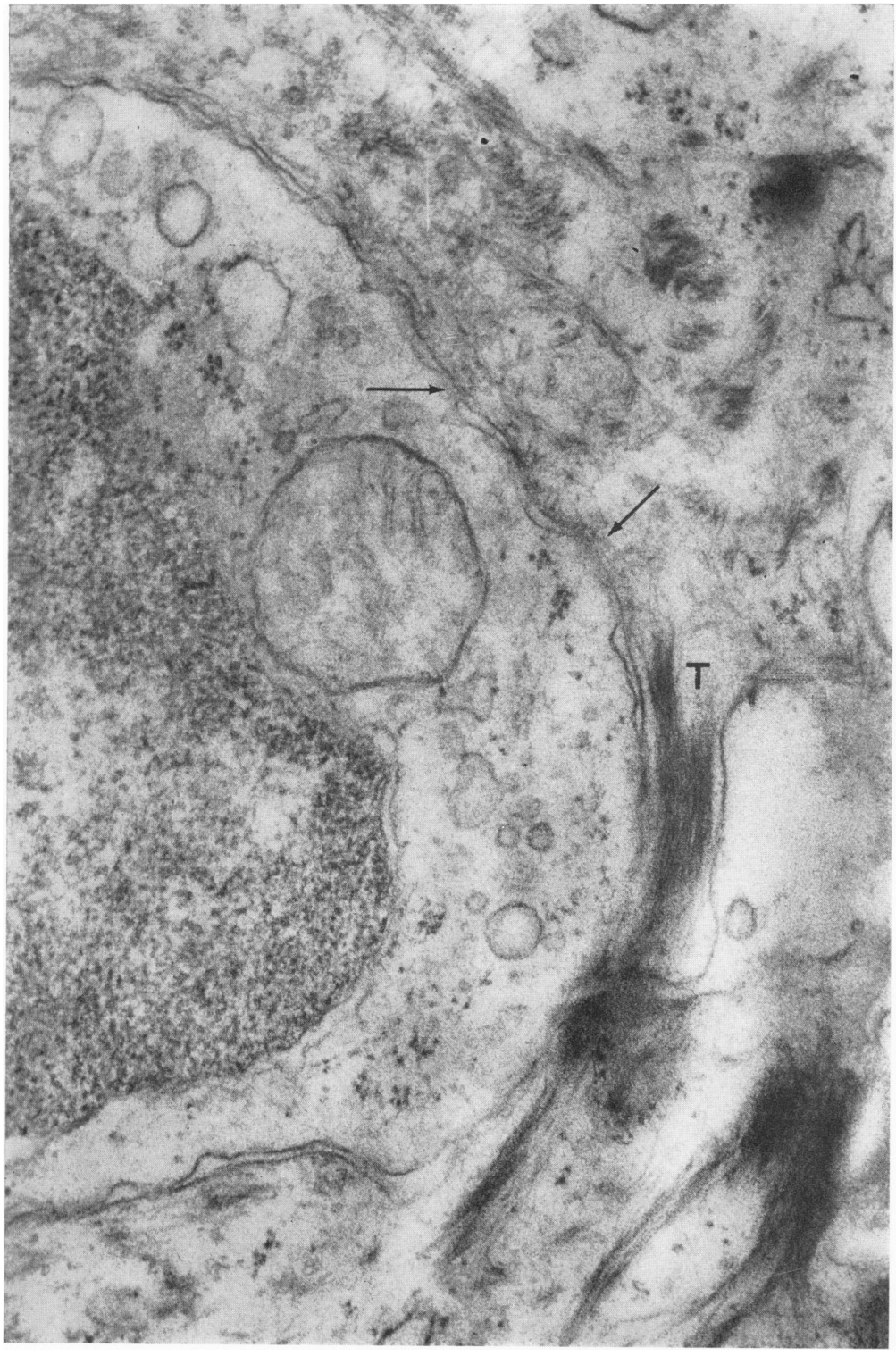

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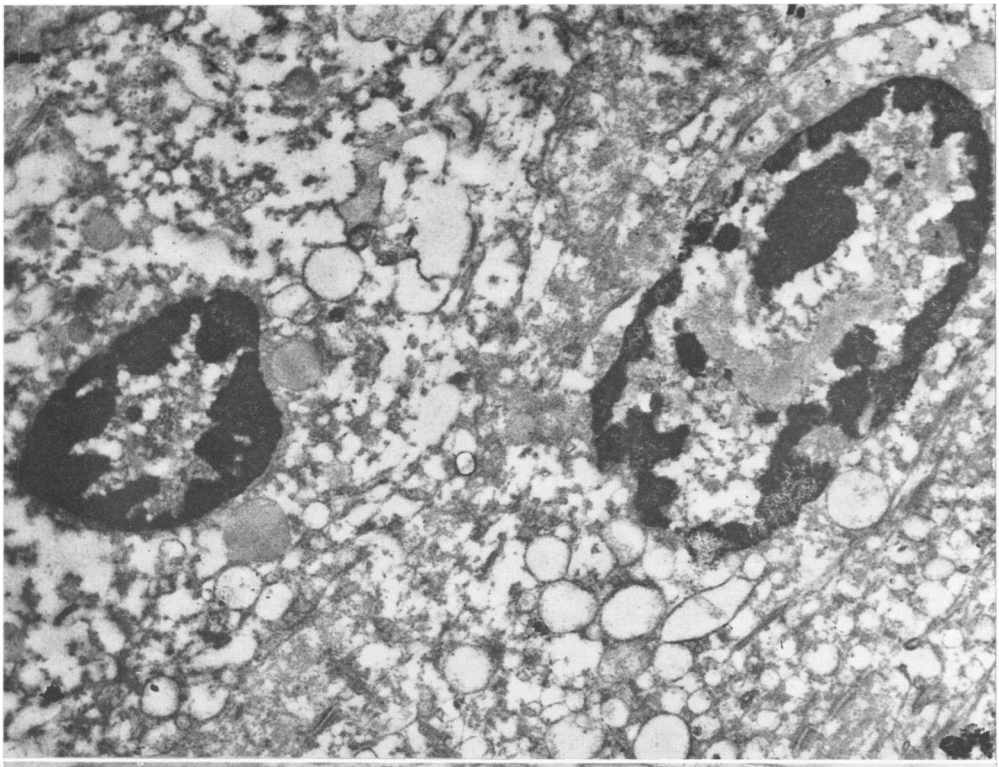
FIG. 15. Final stage of homograft rejection. The necrotic epidermal cells show marked clumping of chromatin as well as swelling and disorganization of cytoplasmic organelles. × 15,000.

FIG. 16. A plasma cell in the dermis exhibits numerous dilated cisternae of rough-surfaced endoplasmic reticulum which contain electron-dense material. × 17,000.



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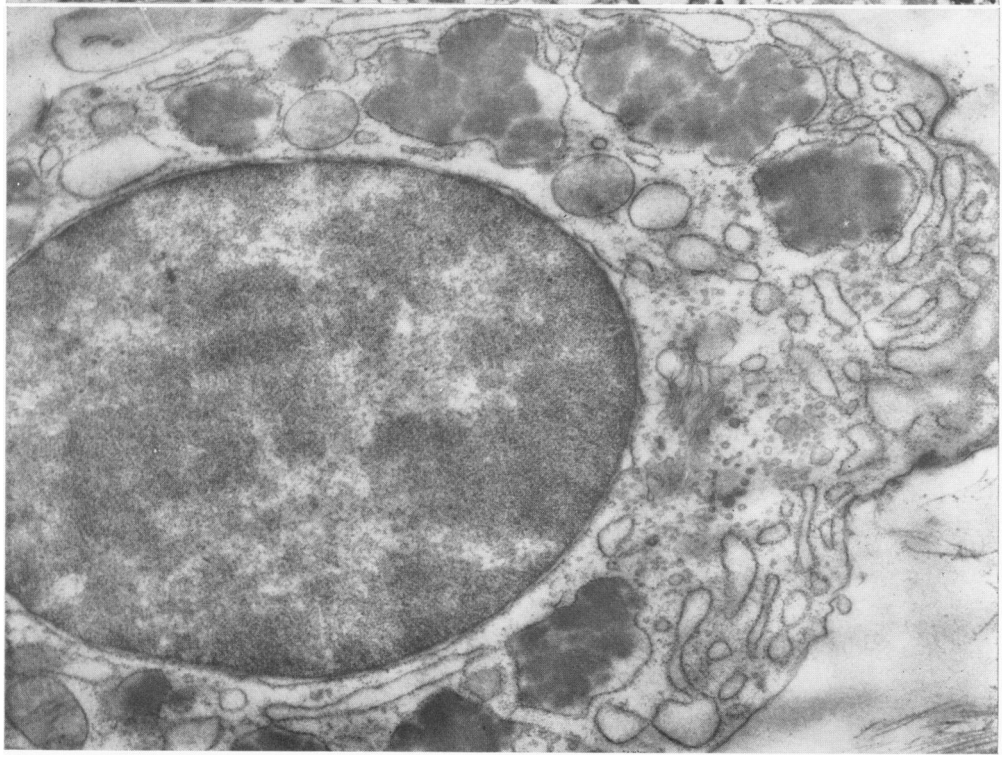

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