THE ELECTRON MICROSCOPIC FEATURES AND FIBRINOLYTIC PROPERTIES OF " NEO-INTIMA "

B. A. WARREN* AND L. G. BROCK

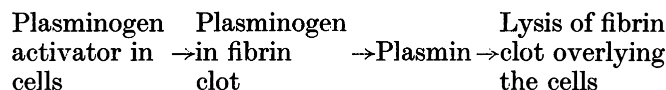
From the Sir William Dunn School of Pathology and the Nuffield Department of Surgery, Oxford

Received for publication March 5, 1964

A NUMBER of reports (Meijne, 1959; Mackenzie and Loewenthal, 1960; Florey, Greer, Poole and Werthessen, 1961; Florey, Greer, Kiser, Poole, Telander and Werthessen, 1962) have shown that the cells lining fabric grafts of the aorta are indistinguishable morphologically by either light or electron microscopy from endothelial cells in the normal aorta. In a study of the fibrinolytic activity of vascular endothelium it was found that aortic endothelial cells will induce lysis of fibrin under suitable conditions whereas other cells of the arterial wall do not show this reaction (Warren, 1963, 1964). This system provides a functional test of the identity of the cells lining aortic grafts with normal endothelium.

The results of testing dog aortic graft endothelium for fibrinolytic activity by the fibrinolytic autograph technique of Todd (1958, 1959), together with some electron microscope studies of dog aortic graft endothelium are reported.

The histological technique for the localisation of plasminogen activator described by Todd is based on the reaction:



The method consists of incorporating the section or cells in a fibrin clot and incubating at 37° in a moist atmosphere. The presence of activator is indicated by lysis of the fibrin clot.

MATERIALS AND METHODS

Fibrinogen.—i. Bovine Fibrinogen. Commercial fibrinogen (Bovine plasma fraction 1, Armour) was used in 2 per cent and 4 per cent solutions in isotonic saline buffered with veronal acetate buffer (Michaelis modified by Biggs and Macfarlane, 1962) pH 7·2 to 7·4 and ionic strength 0·16. ii. Dog Fibrinogen. This was prepared by the method of Jaques (1943) and the method is fully described in a previous paper (Warren, 1963). *Thrombin.* One ampoule of Maw's thrombin containing 5,000 N.I.H. units was dissolved in 50 per cent glycerol to give a stock solution of concentration 1,000 units per ml. The stock solution was diluted with veronal acetate buffered saline to give a working solution of 25 units per ml.

Animals.—Four adult mongrel dogs were used weighing 10–13 kg. each.

Operation.—The animal was anaesthetised with Nembutal (Abbott) and its trachea intubated. A paramedian incision was made in the lower abdominal wall and the abdominal contents packed upwards and downwards so as to display the aorta from the origin of the renal vessels to the posterior mesenteric artery. The peritoneum overlying this area was incised and the aorta cleaned of surrounding tissue. Glover's or similar aortic clamps were

* Present address: Division of Oncology, The Chicago Medical School, Institute for Medical Research, 2020 West Ogden Avenue, Chicago 12, Illinois, U.S.A.

applied to the aorta below the origins of the renal vessels and above the caudal mesenteric artery. The isolated segment (4–5 cm.) was then excised. A knitted polyethylene glycol terephthalate (" Dacron ") graft was selected which had a similar diameter and length to the excised portion of the aorta. About 5 ml. of blood were aspirated from the animal's inferior vena cava and emptied into the graft where it was allowed to clot in the interstices of the fabric. An end-to-end anastomosis of the graft to the aorta was made starting at the proximal end using over and over polyester (" Mersilene ") sutures. The proximal clamp was released for a few seconds ; then the distal, then both and a pad held firmly over the area. After an initial copious oozing through the fabric the flow rapidly diminished and when the area was dry the peritoneum was sewn over the graft and the abdominal wound closed. A collodion dressing was applied.

Preparation of specimens.—The dogs were killed one each at 4 and 5 weeks and 2 at 6 weeks after the insertion of the graft, by anaesthetising with Nembutal and exsanguination *via* a carotid cannula. The abdomen was opened and the graft together with a portion of the aorta above and below the suture lines (Fig. 1) was pinned on to a narrow polythene sheet and removed from the body of the animal. The grafted vessel was opened longitudinally and pinned out flat so that the lining of the graft could be washed with warm veronal acetate buffered saline to remove the blood contained in its lumen. Material was taken for electron microscopy at this stage. The remaining portion of the graft was divided into small pieces approximately 5 mm. square and frozen Häutchen preparations made from them.

Frozen Häutchen preparations.—These were prepared by allowing the endothelial layer of vessel or graft to become frozen to a glass slide and then rolling the deeper layers back with forceps. This left only the surface layer on the slide. A chuck, standing in a plastic beaker half-filled with a freezing mixture of alcohol and dry ice, was used as a flat freezing source.

A clotting mixture of fibrinogen and thrombin was then spread evenly over the slide or preformed fibrin sheets were placed on the preparation. The preparations were put in a slide rack which was stood on end in a one litre beaker containing a little water, so as to keep the slides level and the fibrin clots moist. The beaker was put in an incubator at 37° and the slides examined at intervals of approximately half an hour and removed when areas of fibrinolysis were seen.

Fixation and staining.—After incubation the Häutchen preparations were fixed in formol saline for 15 min., washed in distilled water, stained with Harris's haematoxylin and eosin and mounted in a glycerol mixture.

Preparation of specimens for electron microscopy.—Thin slivers of graft and aorta were removed as soon as practicable after opening the vessel and placed in Caulfield's medium for electron microscopy (Caulfield, 1957). The fixed material was embedded in araldite with and without treatment with phosphotungstic acid. The tissue blocks were re-orientated and after additional hardening were cut with glass knives on a Huxley microtome and the sections floated on distilled water. They were mounted on either formvar-coated or plain copper grids. These preparations were observed and photographed in a Philips EM 100 B electron microscope using 60 kv.

RESULTS

The grafts from the dogs killed 4 and 5 weeks after operation were unsuitable for testing for fibrinolytic activity because of a recent blood clot in the former and narrowing with partial obstruction in the latter. Both of these defects prevented the making of adequate frozen Häutchen preparations. The last 2 grafts (each 6 weeks old) were patent and material from each of these was suitable.

The macroscopic appearance of the grafted aortic segment is shown in Fig. 1. The smooth glistening surface of the normal aortic endothelium continues uninterrupted over both suture lines. There is a much thicker layer of tissue over the troughs in the crimped fabric graft than over the ridges, that in the former being 2–3 times thicker than the latter (Fig. 3). The arrow in Fig. 2 indicates a trough which is shown in transverse section in Fig. 3. The tissue lining the inner aspect of the graft was continuous with that on the outer aspect, but both stripped readily from the fabric graft.

EXPLANATION OF PLATES

FIG. 1.—Inner surface of graft and adjoining segments of aorta taken from dog 6 weeks after insertion. The pattern of the dacron weave can be seen through the semi-translucent tissue lining the graft. Three-quarters natural size.

FIG. 2.—Inner surface of graft to show the greater thickness of tissue in the troughs of the crimped material obliterating the pattern of the dacron. ×2·7.

FIG. 3.—Transverse section of a " trough " in the region of the arrow in Fig. 2. The tissue has been fixed with osmium tetroxide. N—neointima ; D—dacron graft ; A—neoadventitia on the outer surface of the graft. ×23.

FIG. 4.—Frozen Häutchen preparation of dog aorta covered with 1 per cent bovine fibrin clot. Sheets of endothelial cells (E) are surrounded by pale areas of lysis. (L) is the edge of one such area of lysis. Incubation 1 hr. Haematoxylin and eosin. ×49.

FIG. 5.—Frozen Häutchen preparation of dog inferior vena cava covered with 1 per cent bovine fibrin clot. A sheet of endothelial cells (E) is surrounded by an area of lysis the edge of which is marked by arrows. The fibrin film is intact over a group of sub-endothelial cells (SEC). Incubation 45 min. Haematoxylin and eosin. ×145. (Warren, 1964.)

FIG. 6.—Frozen Häutchen preparation of dog aortic graft endothelium covered with 2 per cent dog fibrin clot. (*a*) A sheet of endothelial cells (E) is covered and surrounded by an area of lysis as demarcated by arrows. The fibrin film is intact over a group of sub-endothelial cells (SEC). ×49. (*b*) Detail of the sheet of endothelial cells from the graft shown in (*a*) within a square. The nuclei are oval in shape and appear to be all similarly orientated. ×145. Incubation 8 hr. 45 min. 2 per cent dog fibrin clot. Haematoxylin.

FIG. 7.—Frozen Häutchen preparation of dog aortic graft surface cells. An island of endothelial cells (E) is surrounded by an area of lysis (L) whereas the fibrin film is intact over the remainder of the preparation comprising sub-endothelial cells (SEC) and possibly some other surface cells. Incubation 8 hr. 2 per cent dog fibrin clot. Haematoxylin. ×49.

FIG. 8.—Frozen Häutchen preparation of dog aortic graft surface cells. There is lysis (L) over endothelial cells (E) but not over sub-endothelial cells (SEC). Incubation 5 hr. 15 min. 2 per cent dog fibrin clot. Haematoxylin. ×49.

FIG. 9.—This figure demonstrates the differences in appearance in these preparations between graft endothelial cells shown in (*a*) and sub-endothelial cells shown in (*b*) in the same frozen Häutchen preparation of dog aortic graft tissue. In (*a*) the cell nuclei are oval whereas in (*b*) a long thin nucleus predominates. Incubation 8 hr. 45 min. 2 per cent dog fibrin film. Haematoxylin. ×640.

FIG. 10.—Electron micrograph of an endothelial cell from a normal region of dog aorta. Vesicles (V) are present throughout the cytoplasm and are numerous in some areas, and caveolae intracellulares are seen on the luminal surface of the cell (CI). Portion of the nucleus of the endothelial cell is seen (N) and there are a number of mitochondria (M). Fenestrations occur in the internal elastic lamina (IEL). ×8,400.

FIG. 11.—Electron micrograph of a section through the neointima showing graft endothelial cell with underlying modified smooth muscle cells. Two nuclei are present (N). There are stacks of vesicles in one area of the endothelial cell probably representing part of the Golgi complex (S). Mitochondria are quite numerous (M). Fibrillary regions of the cytoplasm of several of the modified smooth muscle cells are present (SM). All electron micrographs shown are from tissues treated with phosphotungstic acid before embedding in araldite. ×19,650.

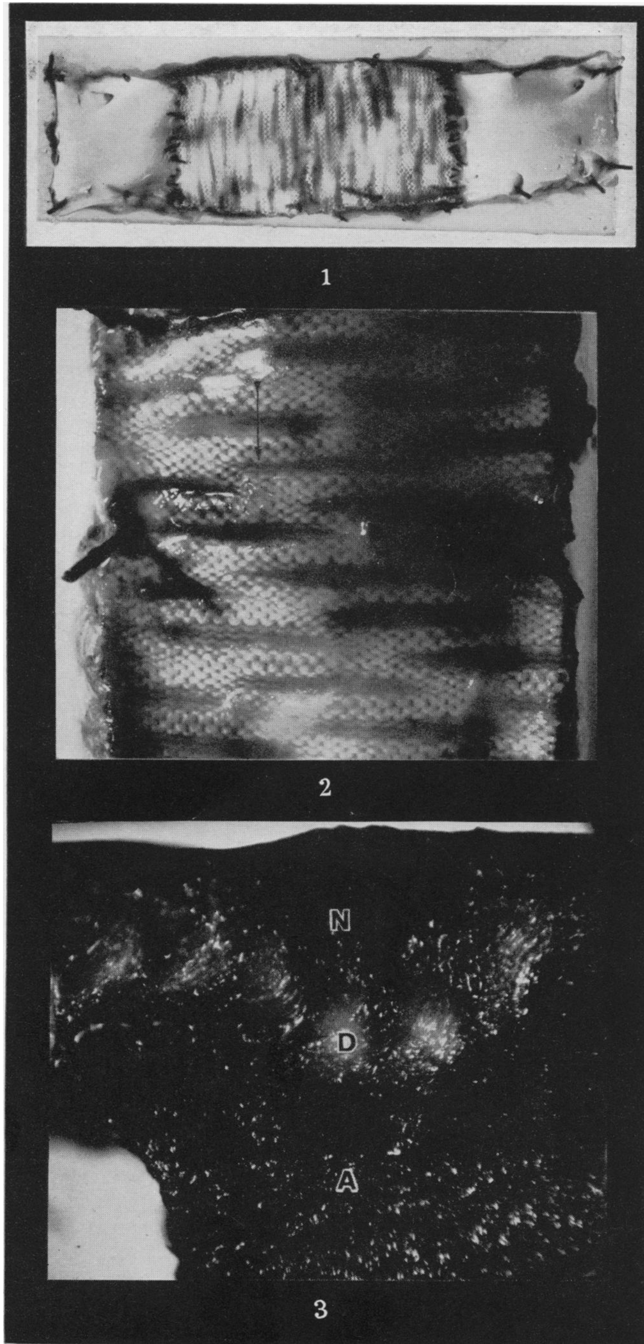
FIG. 12.—Electron micrograph of a cell junction in graft endothelium. (*a*) Regions similar to adhesion plates in endothelium in normal situations are shown (AP). A structure resembling a centriole is present near the junction (CT). ×24,700. (*b*) Lower power view of the area of the cell junction (CJ) shown in (*a*). Two endothelial nuclei (N) are seen one of which has a nucleolus (Nc). The underlying layer contains modified smooth muscle cells (SM), collagen (COL) and elastin fibres (EL). ×4,500.

FIG. 13.—Electron micrograph of a part of a graft endothelial cell showing caveolae intracellulares (CI), mitochondria (M), rough-surfaced endoplasmic reticulum (ER), stacks of vesicles (S), Golgi apparatus (GA), and a centriole (CT) in the cytoplasm. One edge of the nucleus (N) is present and this contains a nucleolus (Nc). Modified smooth muscle cells (SM) containing fibrils lie beneath this endothelial cell. ×9,500.

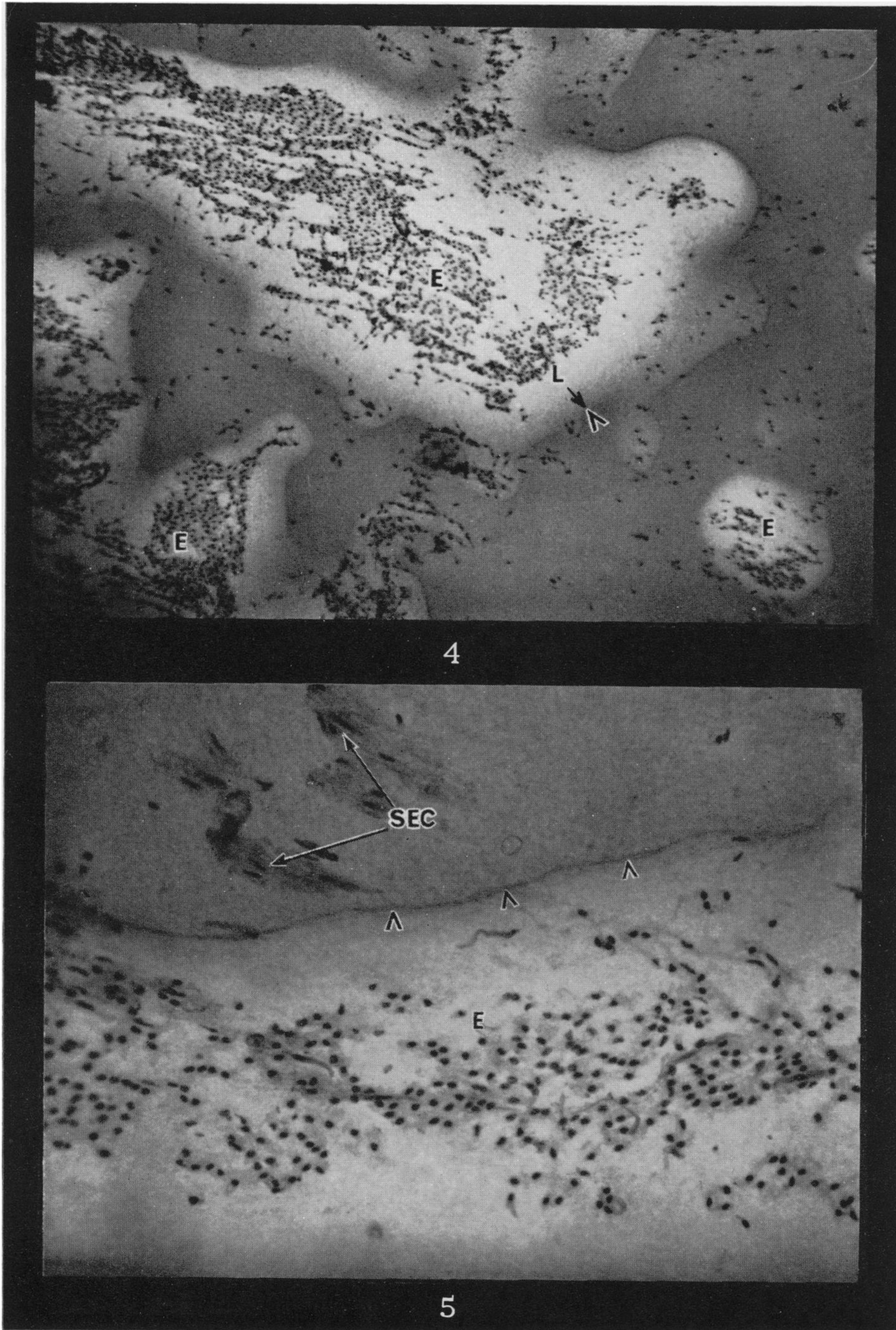
FIG. 14.—Electron micrograph of surface cells of dog aortic graft neointima which are dissimilar to normal dog aortic endothelium. A macrophage (M) is present with a typical " ruffle " border (R). ×4,800.

FIG. 15.—Montage of electron micrographs to show a longitudinal section through a sheet of graft endothelial cells. Sections of three endothelial cells with their nuclei (N) are present, one of which contains a nucleolus (Nc). The endoplasmic reticulum (ER) is prominent in these cells and there are numerous mitochondria (M). The Golgi apparatus is represented by a series of vesicles and paired membranes (S). Two cell junctions showing considerable overlap and interdigitation occur at CJ and CJ′. Modified smooth muscle cells (SM) are present in the sub-endothelial layers of the graft. (Reduced from 6,000.) ×1,100.

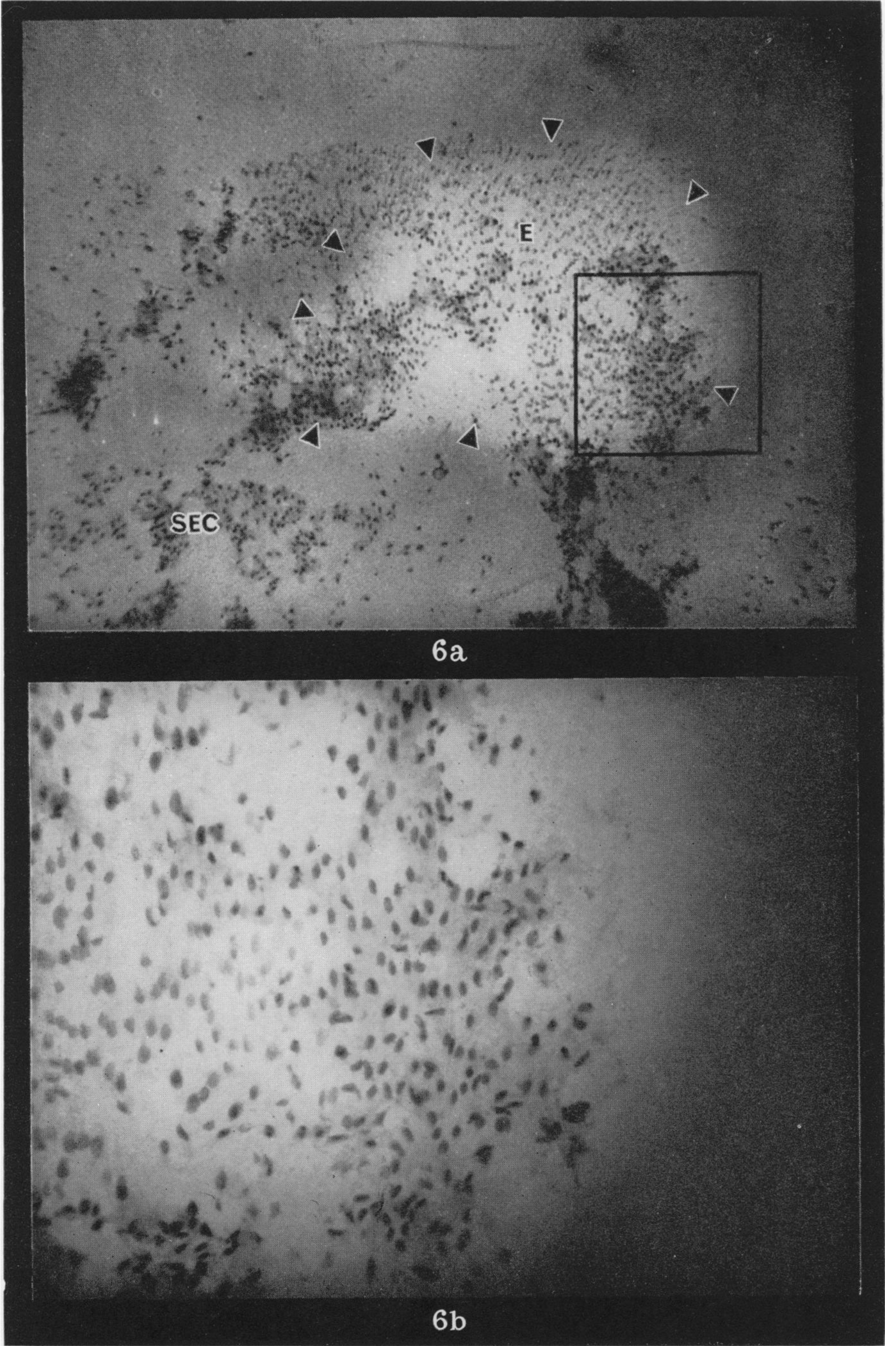
FIG. 16.—Composite photomicrograph of four electron micrographs showing a cross-section of the neointima in the region of a trough. Three layers are present ; a superficial unicellular endothelial layer, a deep densely fibrous layer (COL) with few cells (F) and an intermediate layer containing modified smooth muscle cells (SM), collagen and elastin fibres. In the endothelial layer part of a cell nucleus (N) and cell junction (CJ) are present. (Reduced from 7,850.) ×2,900.



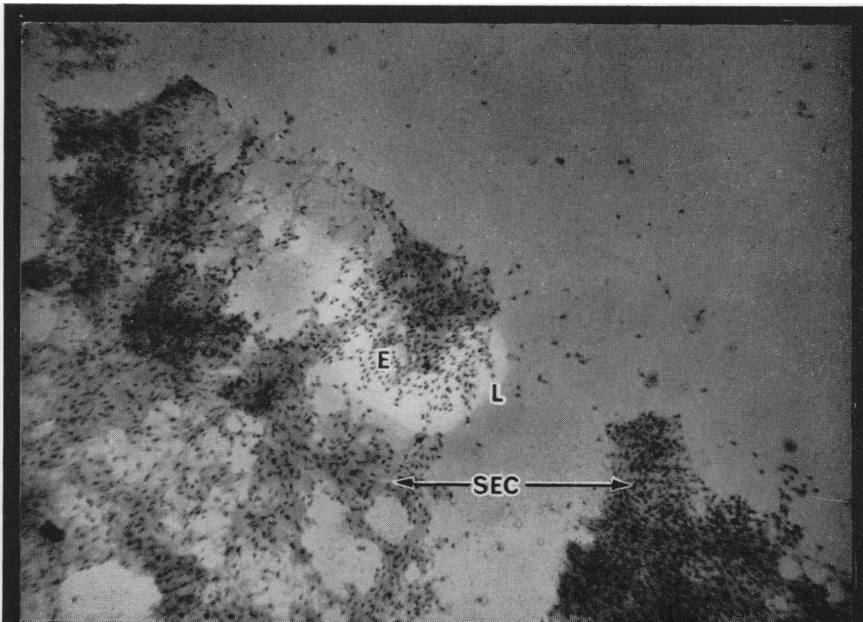
Warren and Brock.



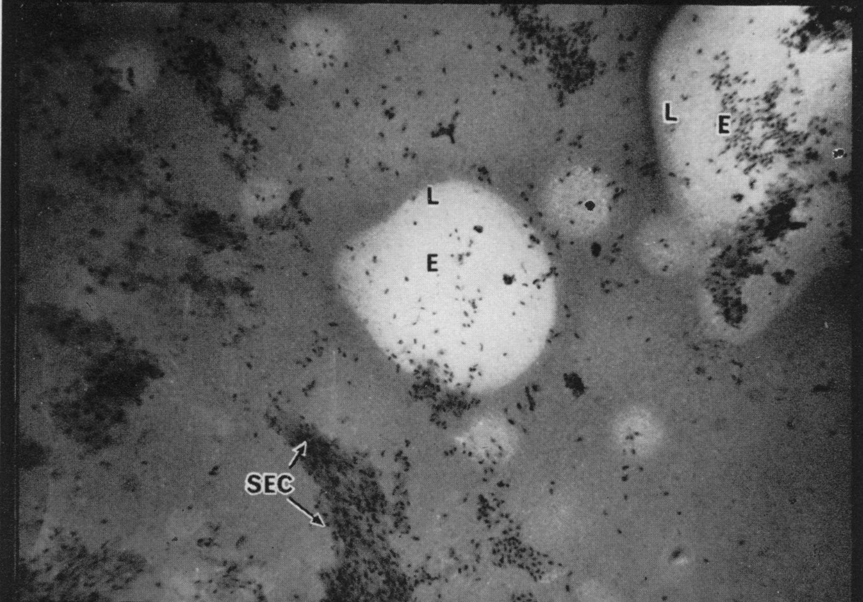
Warren and Brock.



Warren and Brock.

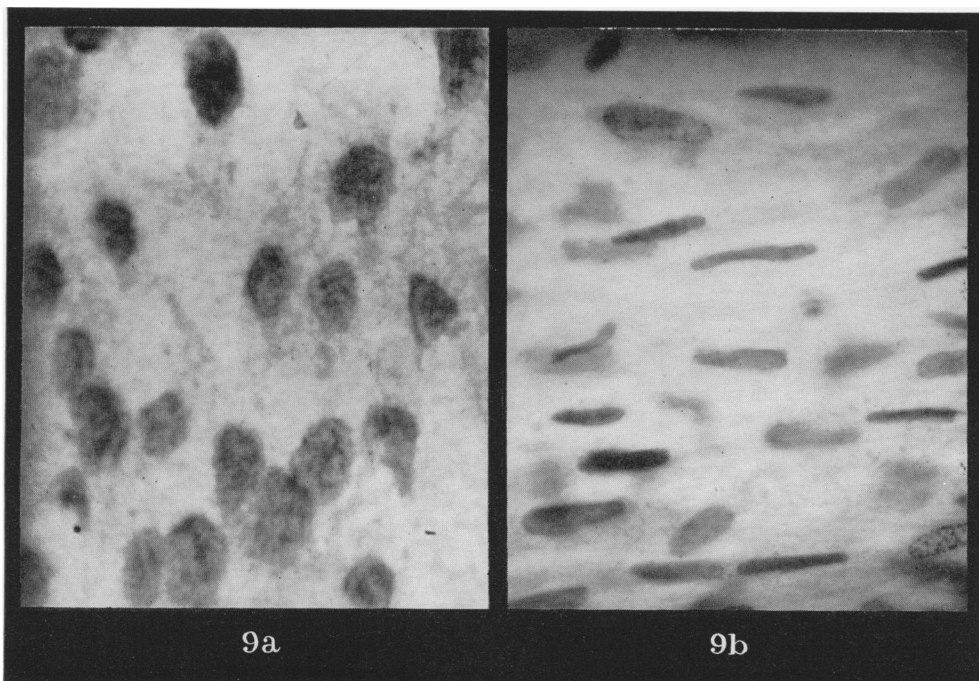


7

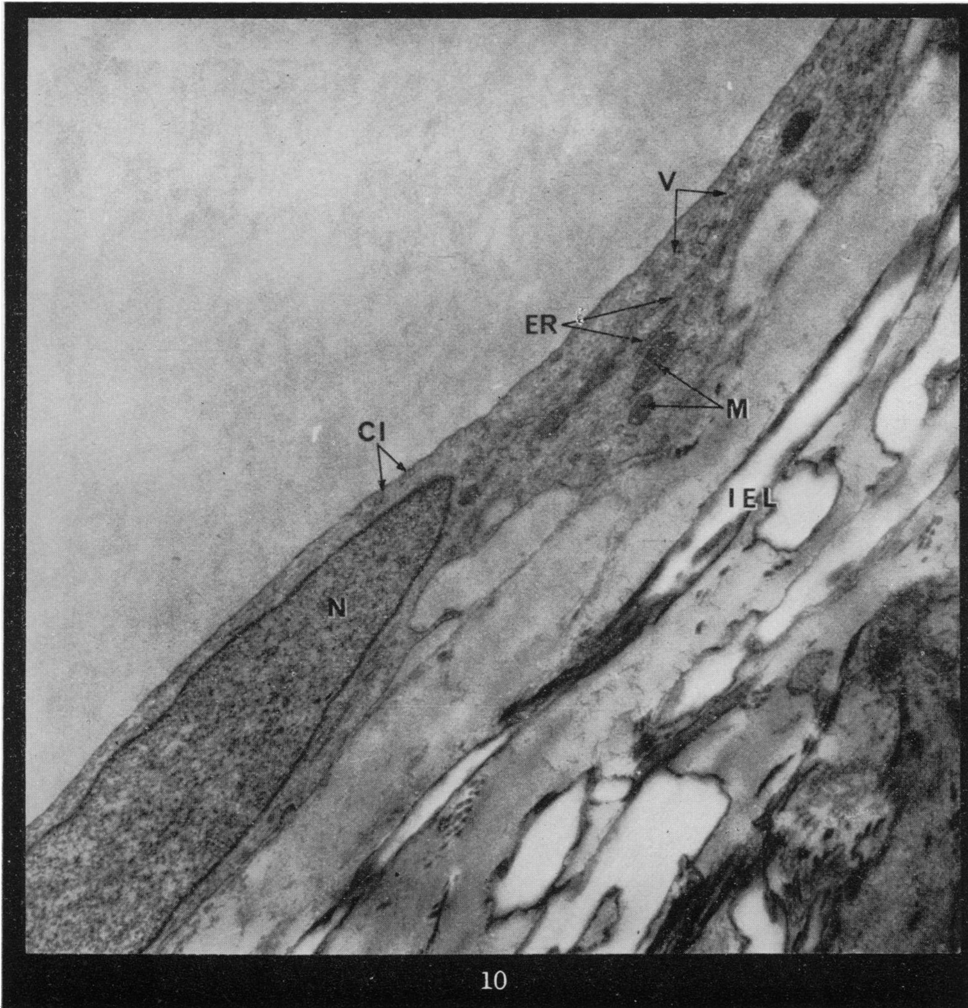


8

Warren and Brock.



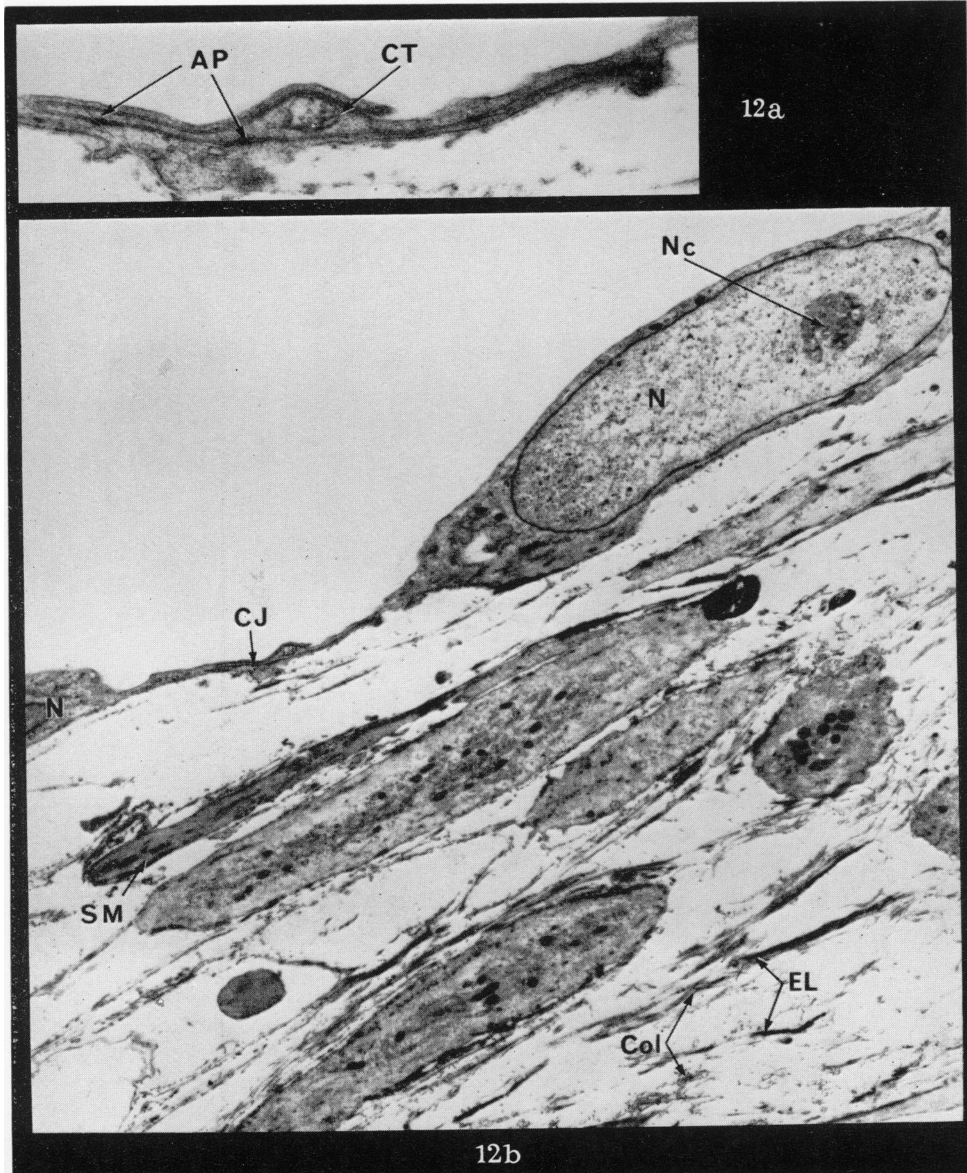
Warren and Brock.

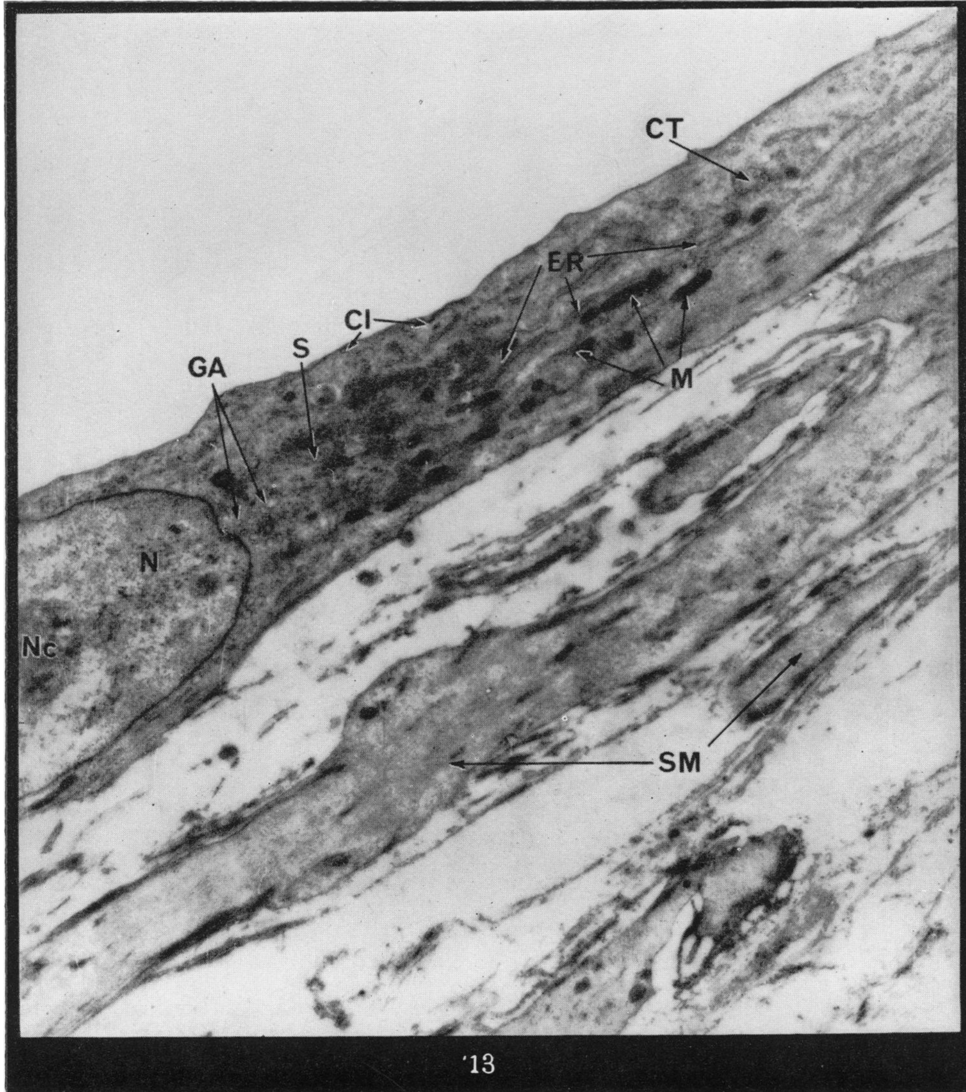


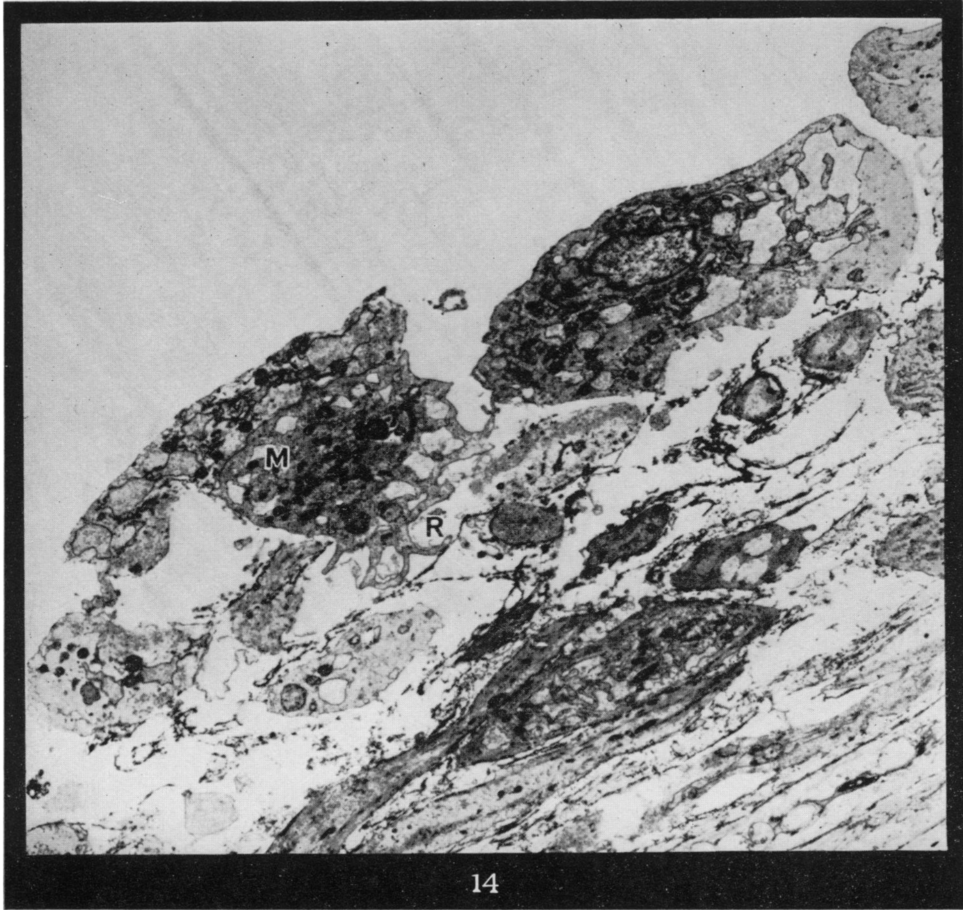
Warren and Brock.

11

Warren and Brock.

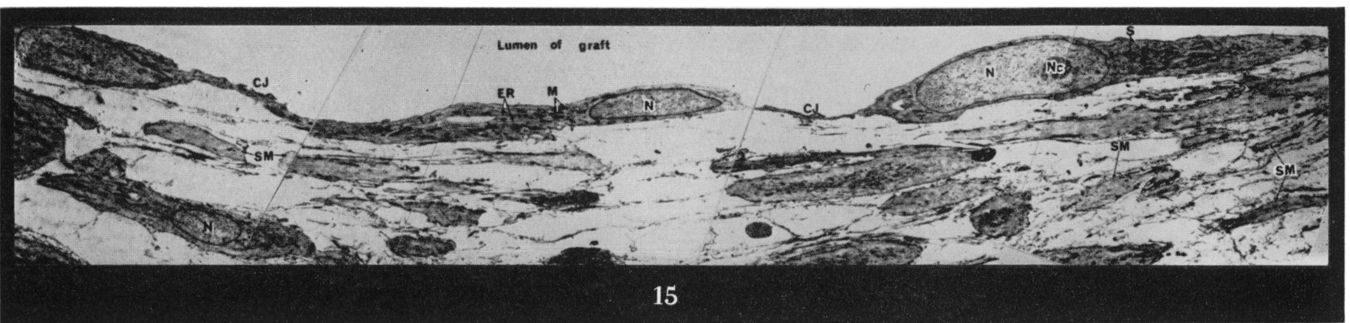

AP
CT
12a
Nc
N
CJ
N
SM
EL
Col
12b


CT
ER
CI
S
GA
M
N
Nc
SM
13

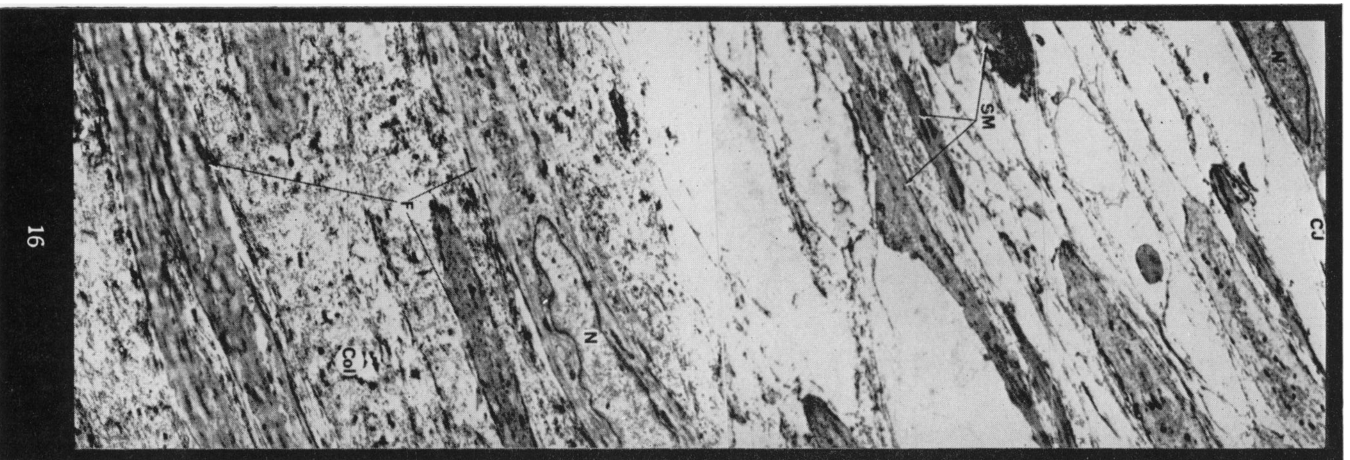


14

Warren and Brock.



15



16

Warren and Brock.

Normal dog endothelial cells were found to induce lysis in overlying bovine and dog fibrin clot within 1–3 hr (Figs. 4 and 5). Subendothelial cells were inactive (Fig. 5). The frozen Häutchen preparations yielded islands of surface cells from the graft morphologically identical to endothelial cells (Figs. 6, 7, 8 and 9) with round or oval plump nuclei. Sheets of cells of this type induced lysis in dog fibrin clot but at a much slower rate than normal aortic endothelial cells taking 5 to 9 hr to induce lysis to a comparable extent. They occurred with and were often surrounded by smooth muscle cells in the preparations, and these cells were inactive (Figs. 6, 7 and 8). As well as preparations, from the central graft area preparations were also made from strips of tissue which included the suture line and contained segments both of aorta and of the lining tissue of the graft. In these preparations the aortic endothelial cells induced lysis much earlier (within 3–4 hr) than the endothelial cells on the graft side of the suture line.

Figs. 11 to 16 show the characteristics of graft endothelial cells by electron microscopy. They have ovoid nuclei, the usual cell organelles, fairly numerous mitochondria and vesicles. The endoplasmic reticulum was prominent in all graft endothelial cells observed. In some cells structures resembling centrioles were seen (Figs. 12 and 13). Centrioles are associated with the astral spindle of the dividing cell and with all regions where a fibrillary appearance of the cytoplasm is found (Causey, 1962). Here they probably indicate immaturity of the endothelial cell. Caveolae intracellulares and numerous vesicles were seen in all graft endothelial cells (Figs. 11, 12 and 13). Cell junctions were often serpiginous and had areas similar to adhesion plates (Fig. 12). The similarity in morphology of graft endothelial cells and endothelial cells in normal situation in the dog aorta can be seen by a comparison of Figs. 11 and 12 with Fig. 10. The appearance of the vesicles, mitochondria, and nuclei and the general form of the component cells are very similar in the 2 tissues. The only striking difference is the prominence of the endoplasmic reticulum in the graft cells.

The tissue which comes to line an aortic graft prosthesis in the dog six weeks after insertion consists of three principal layers. There is an inner layer in contact with the blood stream composed in some areas of cells which appear to be endothelial in nature (Figs. 11 to 13). In other regions, there are cells dissimilar to endothelial cells together with obvious macrophages (Fig. 14). The deepest layers of the lining tissue are composed of dense fibrous tissue, few cells and giant cells which surround the dacron threads. Between the weave small vessels enter the neointima to act as its vasa vasorum. Between these two layers there is an intermediate layer consisting of modified smooth muscle cells, collagen and occasional elastin fibres (Figs. 15 and 16).

DISCUSSION

Todd (1959) showed, using the fibrinolysis autograph technique, that the plasminogen activator found in tissue sections was concentrated around veins, venules and pulmonary arteries in man, and appeared to be closely associated with their endothelium. Endothelial cells from systemic arteries in man evoked lysis only if streptokinase was incorporated in the bovine fibrin clot (Todd, 1961). However, in ox, pig and rat frozen Häutchen preparations from the aorta will induce lysis in bovine fibrin clots without the addition of streptokinase (Warren, 1963).

Dog aortic endothelium will induce lysis in 2 per cent bovine or dog fibrin in 1 to 3 hr incubation at 37° whereas the other cells of the vessel wall are inactive. The fibrinolysis autograph technique provides, therefore, a method of distinguishing between endothelial cells and other cells of the arterial wall.

A number of workers have suggested that in fabric grafts the lining is formed by fibroblasts (Griffith, Eade, Zech and Harkins, 1955; Wesolowski and Sauvage, 1956; Harrison, 1957, 1959). However, Meijne (1959) and Mackenzie and Loewenthal (1960), using *en face* preparations, showed that these grafts were eventually lined by cells whose morphological characteristics were identical by light microscopy with those of normal endothelium. Florey, Greer, Poole and Werthessen (1961) and Florey, Greer, Kiser, Poole, Telander and Werthessen (1962) and Macpherson and Muir (1963) confirmed by examination with both light and electron microscope that the cells lining the fabric grafts were indistinguishable in appearance from true endothelium. In the present functional test of this identity graft endothelial cells were found to induce lysis in dog fibrin in a similar way to true endothelium though at about half the rate.

Electron microscopy of graft endothelial cells and endothelium from a normal region of aorta confirmed the work of Florey *et al.* (1961; 1962). The nuclei of graft and aortic endothelium were of similar ovoid shape. The mitochondria were of like size and shape though apparently more numerous in the graft cells, and numbers of vesicles were noted in both the aortic and graft endothelium. The cell junctions of the graft endothelium were frequently of a sinuous shape and adhesion plates were observed in many junctions (Fig. 12).

The prominence of the endoplasmic reticulum in immature endothelial cells has been reported by Donahue and Pappas (1961), Hogan and Feeney (1963) and Cliff (1963). Donahue and Pappas reported a study of the fine structure of capillaries in the rat from a 20-day foetus to the adult. In the 20-day foetus they noted dilated endoplasmic reticulum composed of canaliculae and vesicles, together with unattached ribonucleoprotein particles scattered through the cytoplasm. Hogan and Feeney (1963) noted dilated cisternae of the endoplasmic reticulum in the vessels of 5-month human foetus. Cliff (1963) described prominent endoplasmic reticulum in the capillary sprouts in healing tissue in the rabbit ear chamber and considered that this was one of the characteristics of immature endothelium. It has been suggested that this prominence is related to greater synthetic activity on the part of the cells (Hogan and Feeney, 1963). The prominence of endoplasmic reticulum in some surface cells lining dog aortic graft prostheses is therefore consistent with the supposition that these cells represent immature endothelium.

While there was great similarity between the surface cells in many areas of the graft with aortic endothelium in normal situations in other regions the lining cells were dissimilar and macrophages were present with them (Fig. 14). The surface of the aortic grafts examined were thus only partly covered by cells morphologically similar to endothelial cells in normal situations.

SUMMARY

The fibrinolytic activity of aortic graft endothelium from dogs killed six weeks after operation was examined by the fibrinolysis autograph technique. Frozen Häutchen preparations of dog aortic graft endothelium induced lysis in

dog fibrin similar to that seen over sheets of normal endothelium. Subendothelial cells were inactive.

The cells lining dog aortic grafts at 6 weeks were similar in morphology to aortic endothelial cells. They differed from mature endothelium principally in having a more prominent endoplasmic reticulum.

At 6 weeks the tissue lining aortic grafts in the dogs consisted of three layers; islands of endothelium, a layer containing modified smooth muscle cells with collagen and elastin and a dense layer containing collagen with few cells.

We would like to thank Professor P. R. Allison who inserted one of the aortic graft prostheses and made available the resources of the Nuffield Department of Surgery; Professor Sir Howard Florey for his constant interest and help; Dr. M. A. Jennings for help in preparing this paper; Mr. J. H. D. Kent and Miss J. Candy for technical assistance and Mr. S. A. Buckingham for the photography. The work was done while one of us (B.A.W.) was in receipt of a British Commonwealth Scholarship. We are grateful to the *British Medical Bulletin* for permission to reproduce Figs. 4 and 5.

REFERENCES

BIGGS, R. AND MACFARLANE, R. G.—(1962) 'Human Blood Coagulation,' Third Edition, Oxford (Blackwell). Appendix II (8), 370.
CAULFIELD, J. B.—(1957) *J. biophys. biochem. Cytol.*, **3**, 827.
CAUSEY, G.—(1962) (Electron Microscopy), Livingstone, Edinburgh Chapter 3, p. 39.
CLIFF, W. J.—(1963) *Phil. Trans.*, **246**, 305.
DONAHUE, S. AND PAPPAS, G. D.—(1961) *Amer. J. Anat.*, **108**, 331.
FLOREY, H. W., GREER, S. J., POOLE, J. C. F. AND WERTHESSEN, N. T.—(1961) *Brit. J. exp. Path.*, **42**, 236.
Idem, GREER, S. J., KISER, J., POOLE, J. C. F., TELANDER, R. AND WERTHESSEN, N. T.—(1962) *Ibid*, **43**, 655.
GRIFFITH, C. A., EADE, C. G., ZECH, R. K. AND HARKINS, H. N.—(1955) *Surg. Gynec. Obstet.*, **101**, 225.
HARRISON, J. H.—(1957) *Ibid.*, **104**, 81.—(1959) *Ibid.*, **108**, 433.
HOGAN, M. J. AND FEENEY, L.—(1963) *J. Ultrast. Res.*, **9**, 10.
JAQUES, L. B.—(1943) *Biochem. J.*, **37**, 344.
MACKENZIE, D. C. AND LOEWENTHAL, J.—(1960) *Brit. J. Surg.*, **48**, 212.
MACPHERSON, A. I. S. AND MUIR, A. R.—(1963) *Scot. med. surg. J.*, **8**, 262.
MEIJNE, N. G.—(1959) *Arch. Chir. Neerl.*, **11**, 41.
TODD, A. S.—(1958) *Nature, Lond.*, **181**, 495.—(1959) *J. Path. Bact.*, **78**, 281.—(1961) Thesis for the degree of Doctor of Medicine in the University of Durham.
WARREN, B. A.—(1963) *Brit. J. exp. Path.*, **44**, 365.—(1964) *Brit. med. Bull.*, **20**, 213.
WESOLOWSKI, S. A. AND SAUVAGE, L. R.—(1956) *Ann. Surg.*, **143**, 65.