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Cellular Breakdown within the Arterial Wall

An Ultrastructural Study of the Coronary Artery in Young and Aging Rats

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Summary. The coronary arteries were studied by electron microscopy in normal rats weighing 65 to 535 gm; fixation was accomplished by perfusion for 20 min at 110–130 mm Hg. In rats of all ages (but especially in the oldest) the arterial wall contained deposits of abnormal intercellular material, consisting of granules, vesicles, myelin figures and other debris. These deposits were present in the intima and media, but rare in the adventitia; there was suggestive evidence that medial cells phagocytized some of the material. The adventitia was characterized by 1–4 layers of cells with extremely thin protoplasmic expansions wrapped around the vessel ("veil cells") and containing lysosome-like bodies as well as phagosomes. These findings, taken as a whole, suggest the following sequence of events. During normal life, the media produces cellular debris, by cell death as well as by fragmentation of cellular processes; part of these debris are phagocytized by smooth muscle cells, part diffuse outward to the adventitia where they are taken up by specialized cells (the "veil cells"). However, due to relative inadequacy of phagocytosis in the media, the debris continue to accumulate and form intercellular deposits that increase with age. It is possible that this natural phenomenon may by itself set a maximal limit to the life-span of the arterial wall.

Key words: Electron Microscopy — Coronary Arteries — Atherosclerosis — Aging — Smooth Muscle.

In the course of an electron microscopic study of the coronary artery of the rat, we noticed a peculiar intercellular material, consisting mainly of granules and vesicles, which could not be considered as part of a "normal" arterial wall. Similar observations have been made on other arteries of the rat (Schwartz and Benditt, 1972a, b; Cliff, 1970; Suzuki, 1972; Giacomelli *et al.*, 1972).

The purpose of this paper is to provide a description of this intramural material, and to discuss its possible origin, as well as its pathophysiologic significance.

Materials and Methods

We used 41 male and female Wistar rats weighing 65 to 535 grams, from Jautz, Kisslegg/Allgäu, Germany (fed on Purina Chow) and from Charles River Breeding Laboratories, Wilmington, Massachusetts (fed on Charles River Rat, Mouse and Hamster formula by Country Foods, Agway, Inc., Syracuse, New York). Systolic blood pressure was measured in 8 animals of the same strain, age and weight with an electrosphygmomanometer; it ranged from 100 to 140 mm Hg (Spector, 1956).

All coronary arteries were fixed by retrograde aortic perfusion *in situ* at a pressure of 110–130 mm Hg, using 3% glutaraldehyde in cacodylate buffer. In early experiments, prior to the perfusion with fixative the blood vessels were perfused for three minutes with physiologic solution (Ringer with 5% glucose at pH 7.4, bubbled with oxygen at 95% and carbon dioxide 5%, maintained at 35° C). Later we found that it was possible to perfuse directly with glutaraldehyde, thus minimizing the possibility of artifacts.

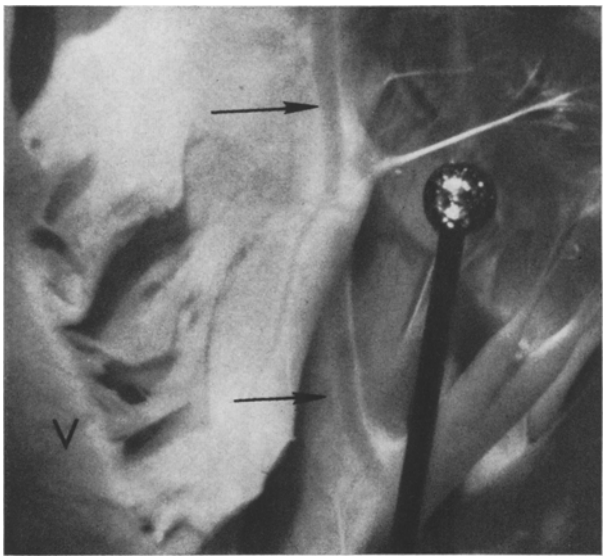

Fig. 1. Inner view of the right ventricle, septal surface, showing the septal artery (arrows), just beneath the endocardium. *V* Cut surface of the myocardium. Diameter of pin head: 1 mm. (Rat heart, fixed by perfusion at 130 mm Hg)

Perfusion Technique. The rat was over-anesthetized with chloroform, and laid on its back on a dissecting board until respiration ceased. Immediately thereupon, the abdomen was opened, the aorta was exposed and an 18-gauge needle (connected with the perfusion bottle) was inserted into the aorta. The perfusion was started, and the jugular veins were then opened; the procedure required 2–3 minutes. Perfusion was continued for 20 minutes and required approximately 600 cc of fixative. Then the heart and the aorta were removed; the aorta was opened longitudinally, pinned out, and small transverse and longitudinal strips were excised. To obtain specimens of coronary artery, the right ventricle of the heart was opened, thereby exposing the artery which runs immediately beneath the endocardium of the interventricular septum ("septal artery", Dbalý, 1973), and is therefore clearly visible over a course of 6 to 8 mm (Fig. 1). Transverse sections were cut from this artery, including a small part of the underlying myocardium.

The aortic strips and blocks of coronary artery were maintained in 3% glutaraldehyde at room temperature (total fixation time including perfusion: 5 hours), then left in cacodylate buffer at 4° C for about 12 hours, post-fixed at 4° C for 2 hours in 2% osmium tetroxide in collidine buffer at pH 7.2–7.4, dehydrated in graded alcohols, and embedded in Epon 812 (Luft, 1961). For light microscopy, sections 1 μ thick were stained with methylene blue and Azur II, or with toluidine blue. Ultrathin sections were cut with a diamond knife on a Reichert OMU-3 ultramicrotome and an LKB Ultrotome III, mounted on uncoated grids, stained with uranyl acetate and lead citrate (Reynolds, 1963; Fahmy, 1967), coated or not with a thin layer of carbon and examined with a Philips EM 300 or a Philips EM 301 electron microscope.

Results

The wall of the septal artery consists of a layer of endothelial cells, an internal elastic lamina, a media with up to 7 layers of smooth muscle cells, and an adventitia (Fig. 2). The features of the endothelial cells will be described in detail elsewhere; especially notable were dense bundles of microfibrils, mostly at

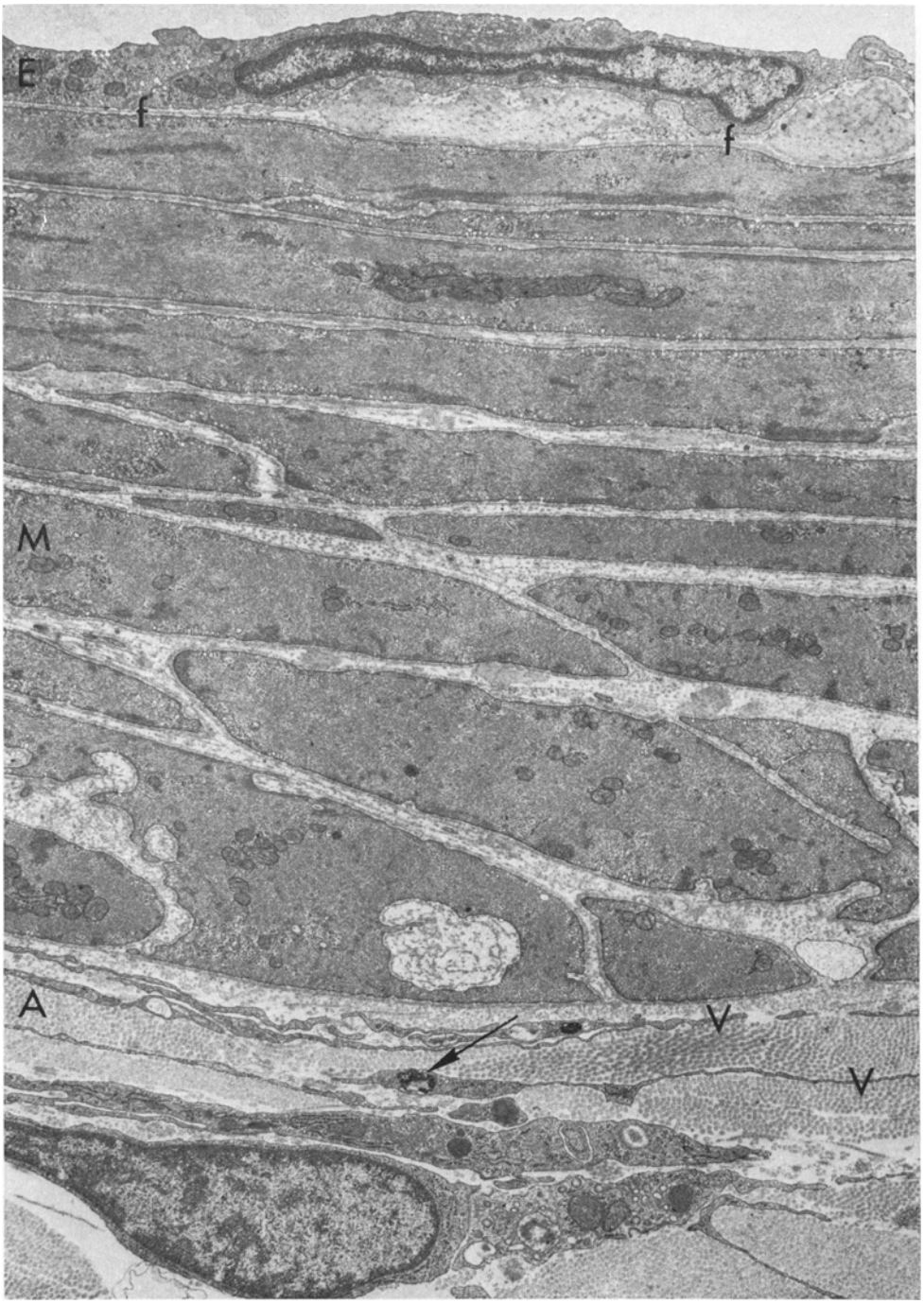

Fig. 2. Topographic view of a rat coronary artery. *E* Endothelium; *f* fenestrae in the elastic lamina; *M* media, becoming somewhat looser toward the adventitia (*A*). In the latter, note veil cells (*V*); one of these contains a phagosome (arrow). No large deposits are present in this area. (Coronary artery: 240 g rat, × 8500)

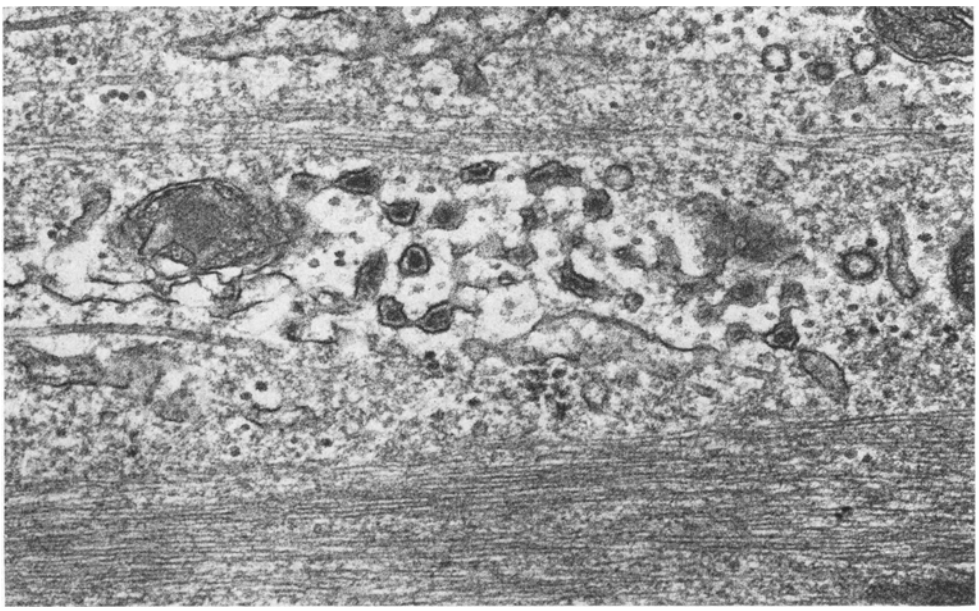

Fig. 3. In the body of a smooth muscle cell, a group of vesicles and tubules containing an electron-dense material: probably part of a Golgi apparatus. (Coronary artery: 535 g rat, × 62500)

the base of the endothelial cells or at their junctions. The first layer of smooth muscle cells was separated from the endothelium by a well-defined internal elastic lamina, somewhat thinner and more electron-dense than the elastic laminae of the aorta of the same animals, and with many fenestrations (Fig. 2). The endothelial sheet appeared to be anchored to the elastic membrane by cellular processes arising from the deep surface of the endothelial cells, and penetrating into niches or full-thickness fenestrae of the elastic membrane. Deeper within the arterial wall, the elastic material was reduced to scattered profiles of fibres, with no definite laminae.

When cut in the direction of their long axis, the smooth muscle cells appeared densely packed, leaving little extracellular space; where they met end to end, the space was usually wider. The texture of the media was somewhat looser in the outer portion of the wall (Fig. 2). In older rats the extracellular spaces were wider, and the ends of the smooth muscle cells had also taken on an irregular, branched or frayed shape. In these areas (between ends of cells) the basement membrane surrounding each cell was often branched or reduplicated; this feature, too, was much more pronounced in old rats. The cytological details of the smooth muscle cells need not be repeated here (Somlyo and Somlyo, 1968). Toward the central part of the cell we often noticed groups of small, rounded profiles of membranes containing a single dense granule (Fig. 3), possibly representing a Golgi apparatus; these clusters were usually present in areas rich in microtubules and endoplasmic reticulum. Dense bodies, presumably lysosomes, were occasionally seen, as well as vacuoles which we interpreted as phagosomes, containing a variety of materials (Figs. 4a, b, c; 5a, b).

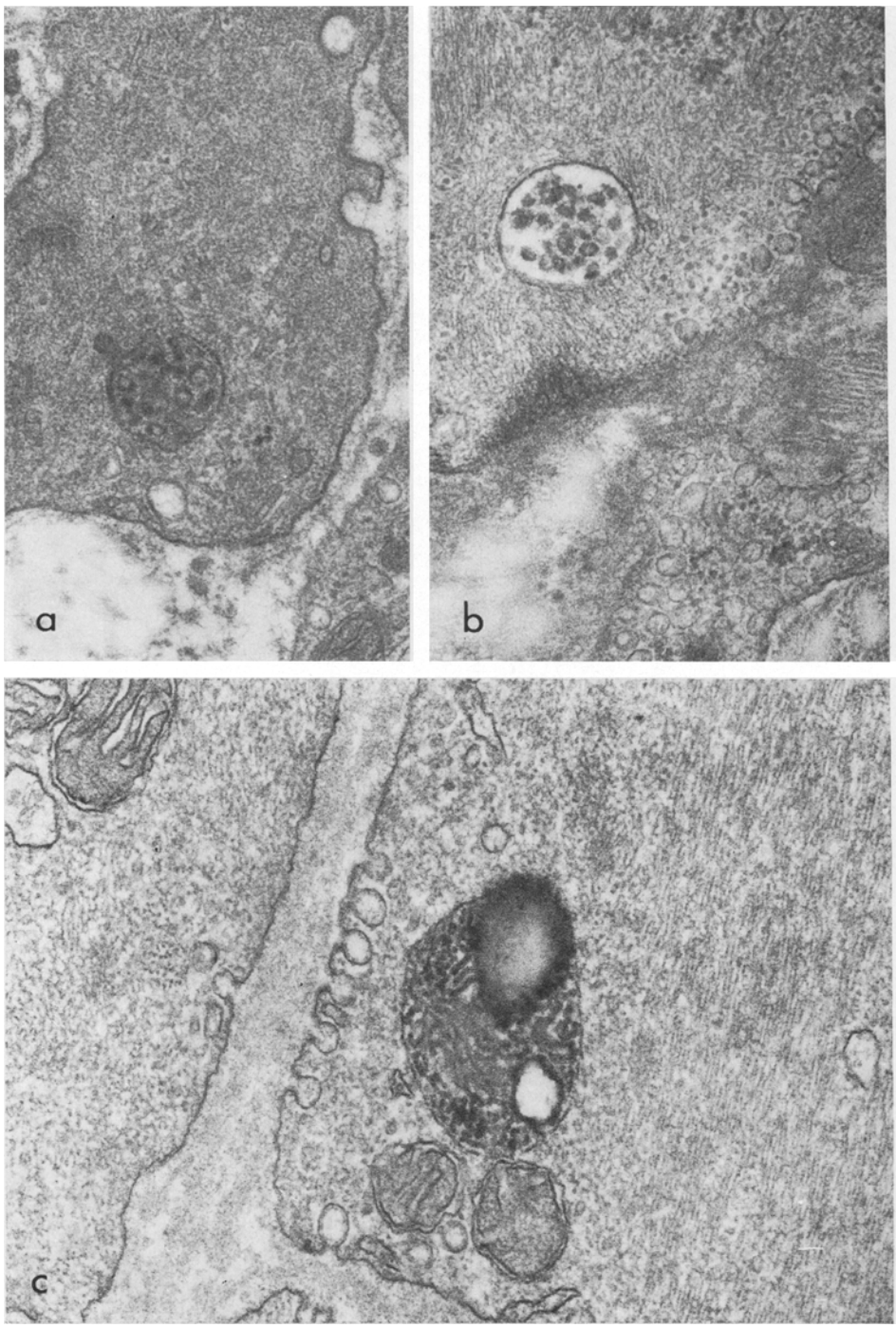

Fig. 4a–c. Rounded bodies within smooth muscle cells in the media; we interpret them as phagosomes containing mostly granular debris. (Coronary artery: (a) 342 g rat, × 51300; (b) 383 g rat, × 31920; (c) 430 g rat, × 85000)

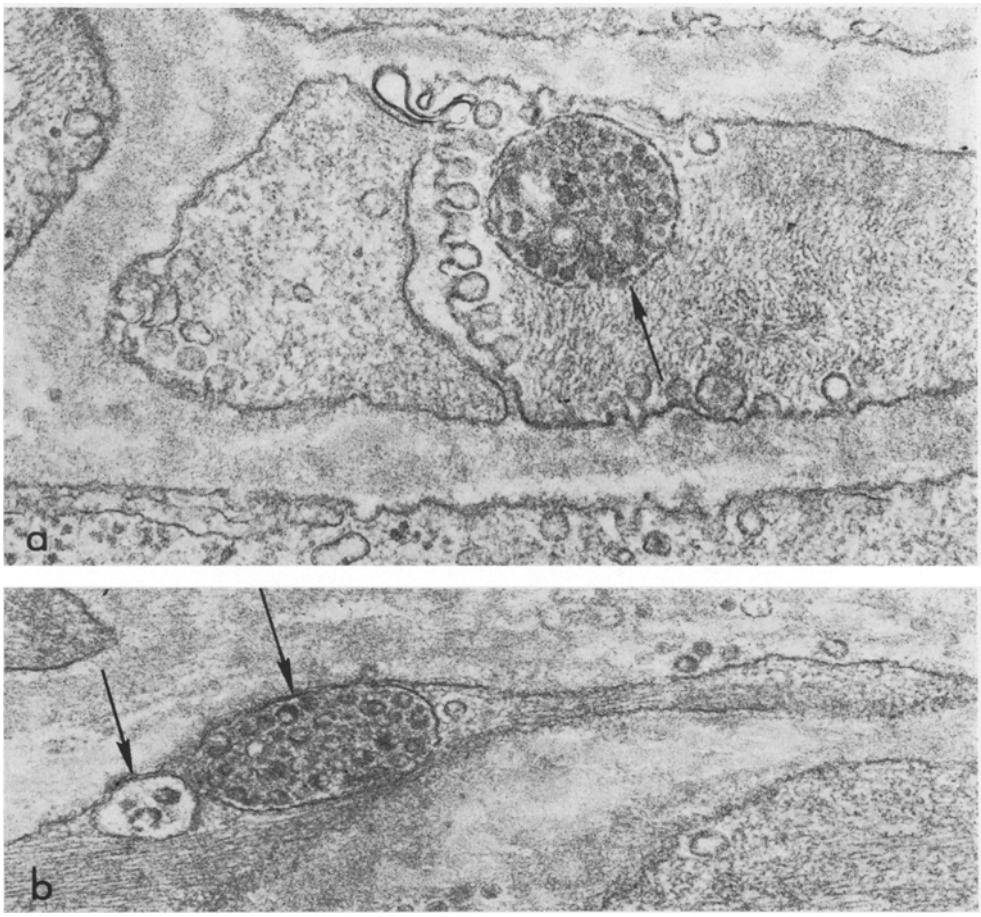

Fig. 5a and b. Details of smooth muscle cells in the media. Arrows point to rounded bodies that we interpret as phagosomes, containing particles such as those that are seen in the extracellular spaces in (b). (Coronary artery: (a) 430 g rat, × 62500; (b) 430 g rat, × 27500)

The adventitia consisted of collagen, with one to four layers of extremely thin cells wrapped around the artery; these have been described earlier as "veil cells" (Majno, 1965; Rhodin, 1968) (Figs. 2, 10, 11).

The Deposits

The abnormal materials found in the arterial wall will be referred to hereafter —for want of a better term—as "deposits". They usually consisted of a mixture of several components (Fig. 6): (a) small vesicles, mostly of the size of pinocytic vesicles, sometimes larger, up to the size of a mitochondrion. Some of these vesicles contained a fibrillar material that made them appear as fragments of smooth muscle cells; others contained smaller vesicles, about the size of those found in smooth muscle; (b) small myelin figures, or small bits of membranes; the largest myelin figure rarely exceeded the size of a mitochondrion; (c) small

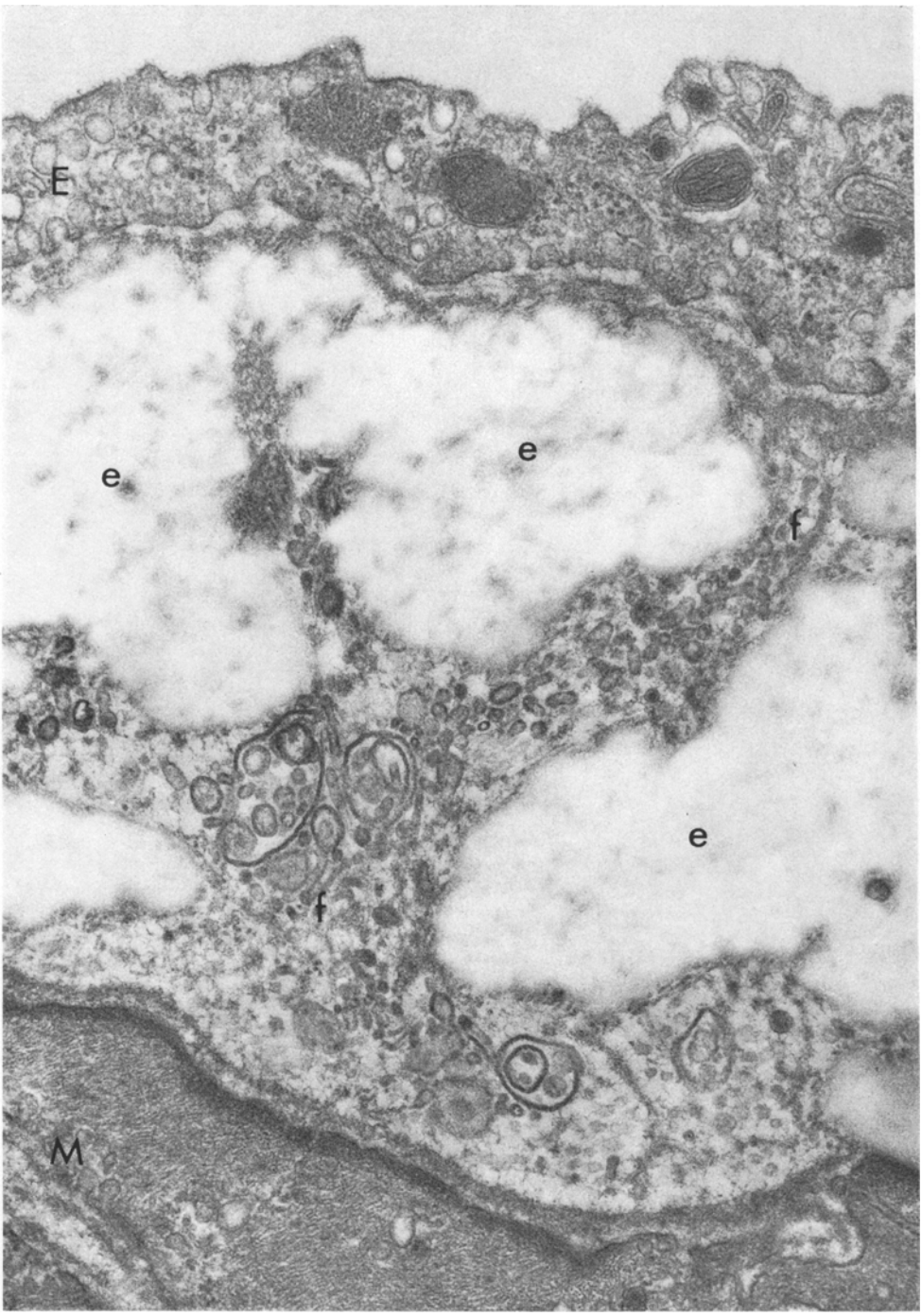

Fig. 6. Intimal part of a coronary artery, showing endothelium (*E*), elastic lamina (*e*), and part of a smooth muscle cell from the media (*M*). Note the cascade of debris filling a fenestra (*f*) and its ramifications in the elastic membrane. (Coronary artery: 367 g rat, $\times$ 47200)

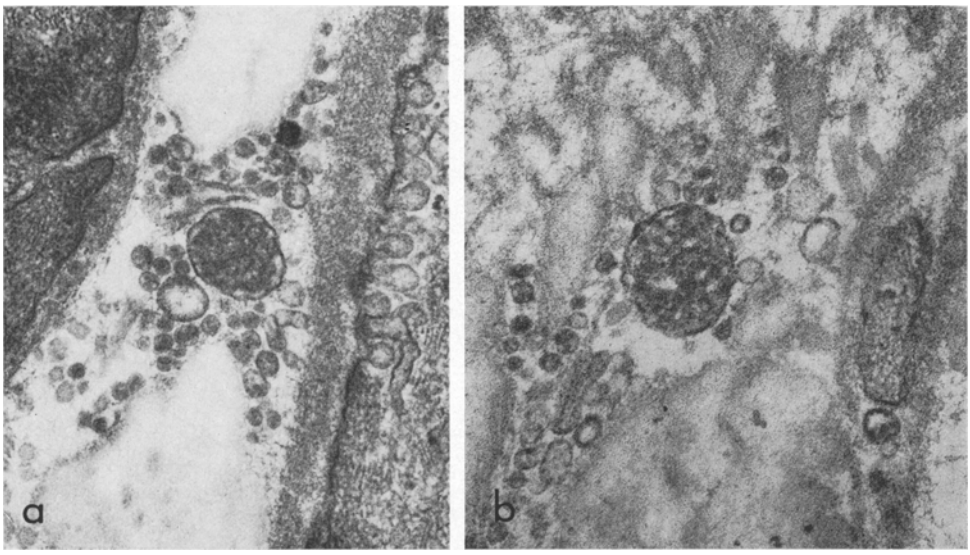

Fig. 7a and b. Extracellular accumulation of debris and rounded bodies that may be phagosomes originating from smooth muscle cells. (Coronary artery: (a) 342 g rat, × 51300; (b) 260 g rat, × 62500)

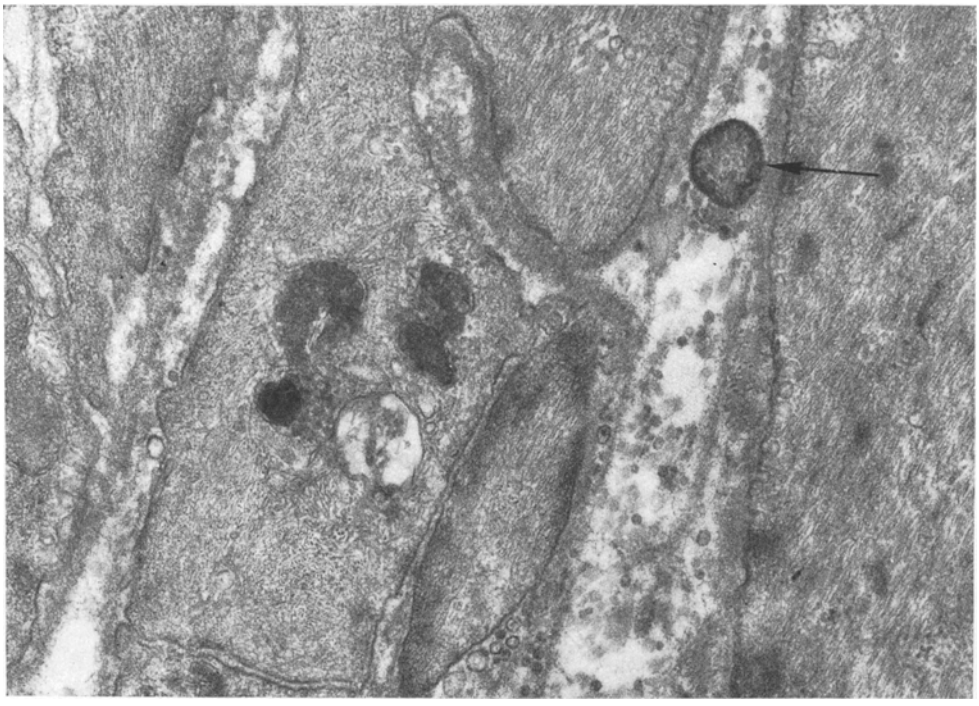

Fig. 8. Extracellular accumulation of debris. Arrow: phagosome-like body, presumably originating from a smooth muscle cell. Note similar bodies in a smooth muscle cell at left. (Coronary artery: 318 g rat, × 31350)

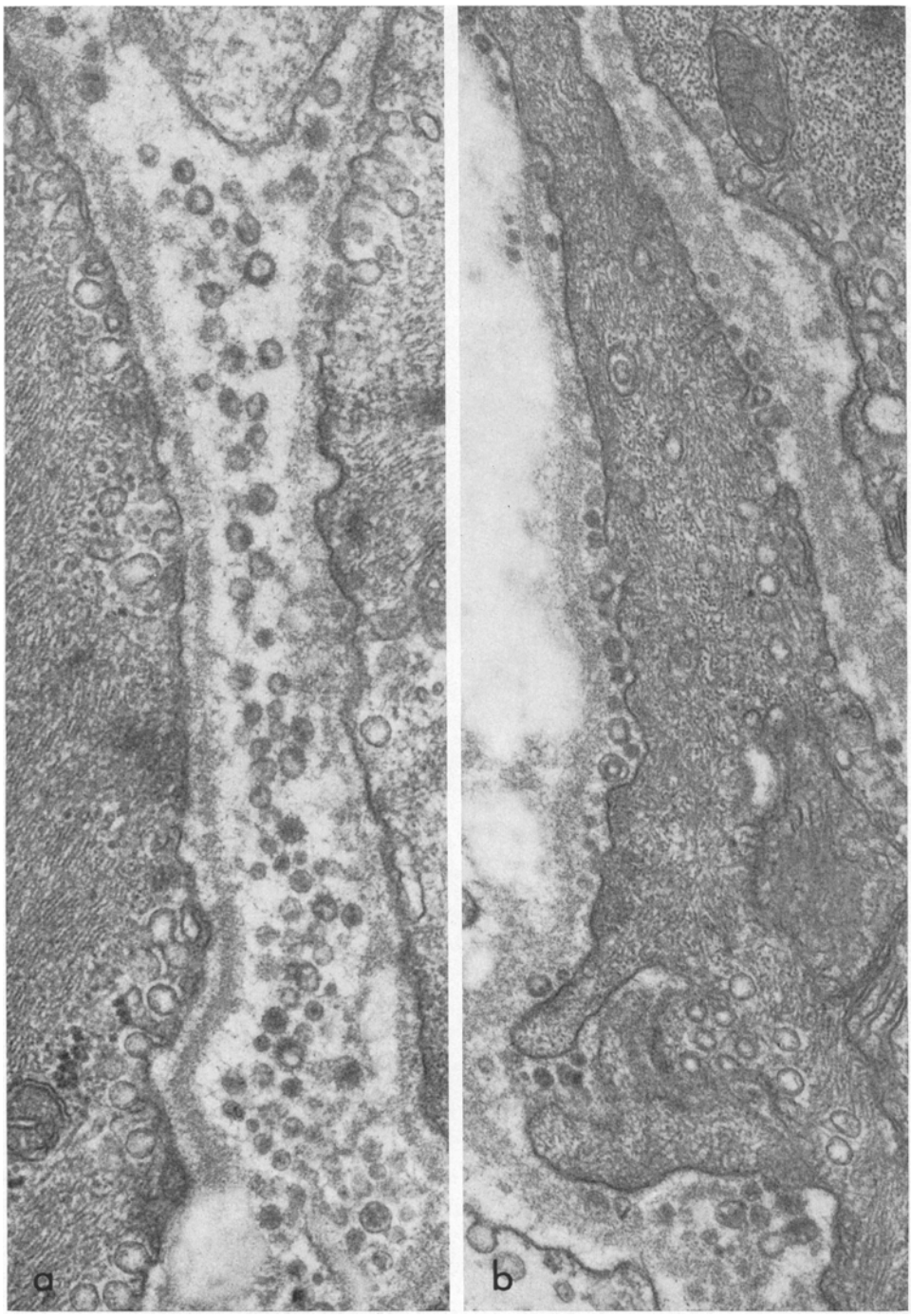

Fig. 9a and b. Two types of topographic relationship between smooth muscle cells and granular deposits: (a) outside the basement membrane; (b) beneath it. (Coronary artery: (a) 342 g rat, × 55400; (b) 342 g rat, × 55400)

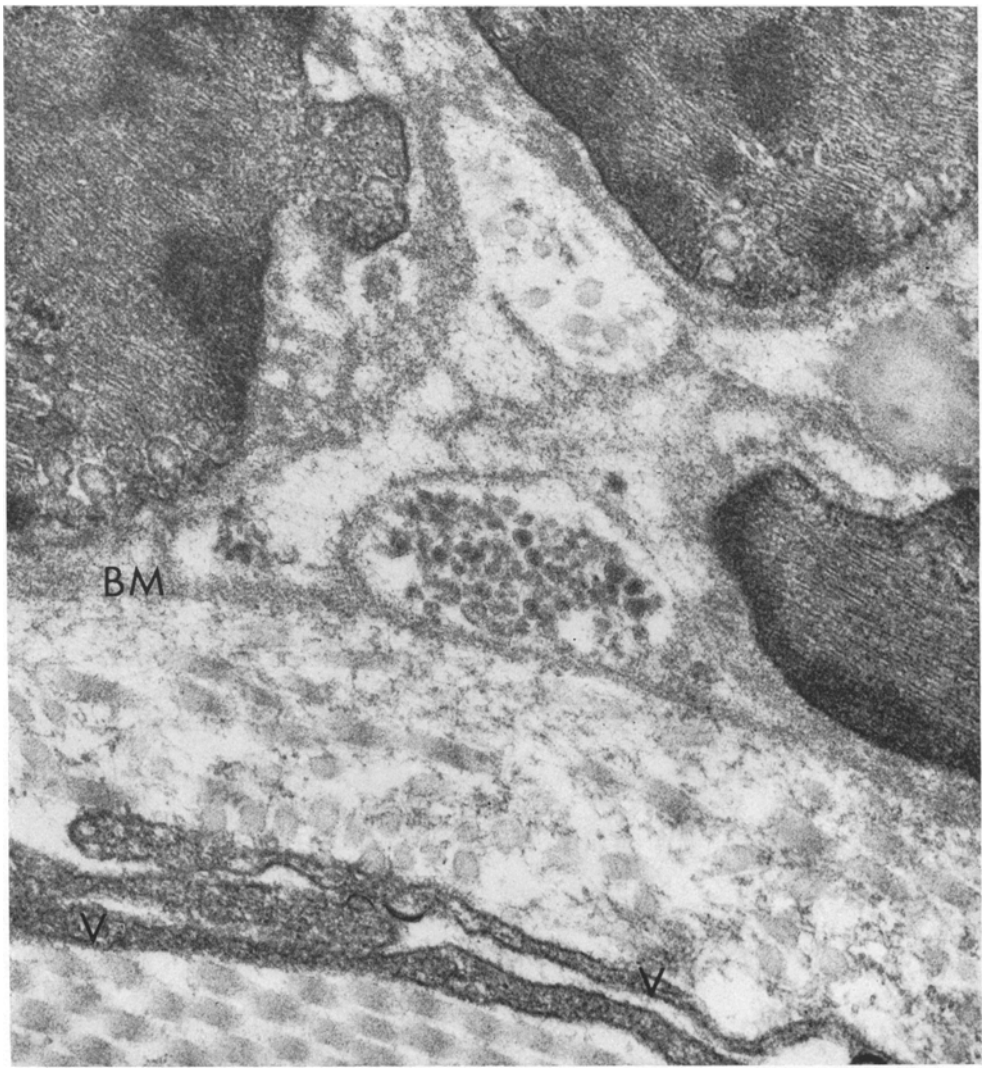

Fig. 10. The top of this figure corresponds to the outermost part of the media; its outer limit is marked by the basement membrane (*BM*). Note cluster of granular deposits held within a "chamber" of basement membrane. In the adventitia, processes of veil cells (*V*). (Coronary artery: 161 g rat, × 55400)

granules, somewhat smaller than pinocytic vesicles. These were of two kinds: some were of even texture, with a fuzzy outline, and recalled the electron density and texture of basement membrane; others were denser, with a sharper outline, and with evidence of a darker core; (d) sundry bodies that appeared to represent parts of necrotic cells, including dense rounded bodies surrounded by a membrane—perhaps remains of phagosomes (Figs. 7a, b; 8). All these types of deposits were found in varying amounts at all levels of the arterial wall, least in the adventitia. We could not convince ourselves that they were more abundant in

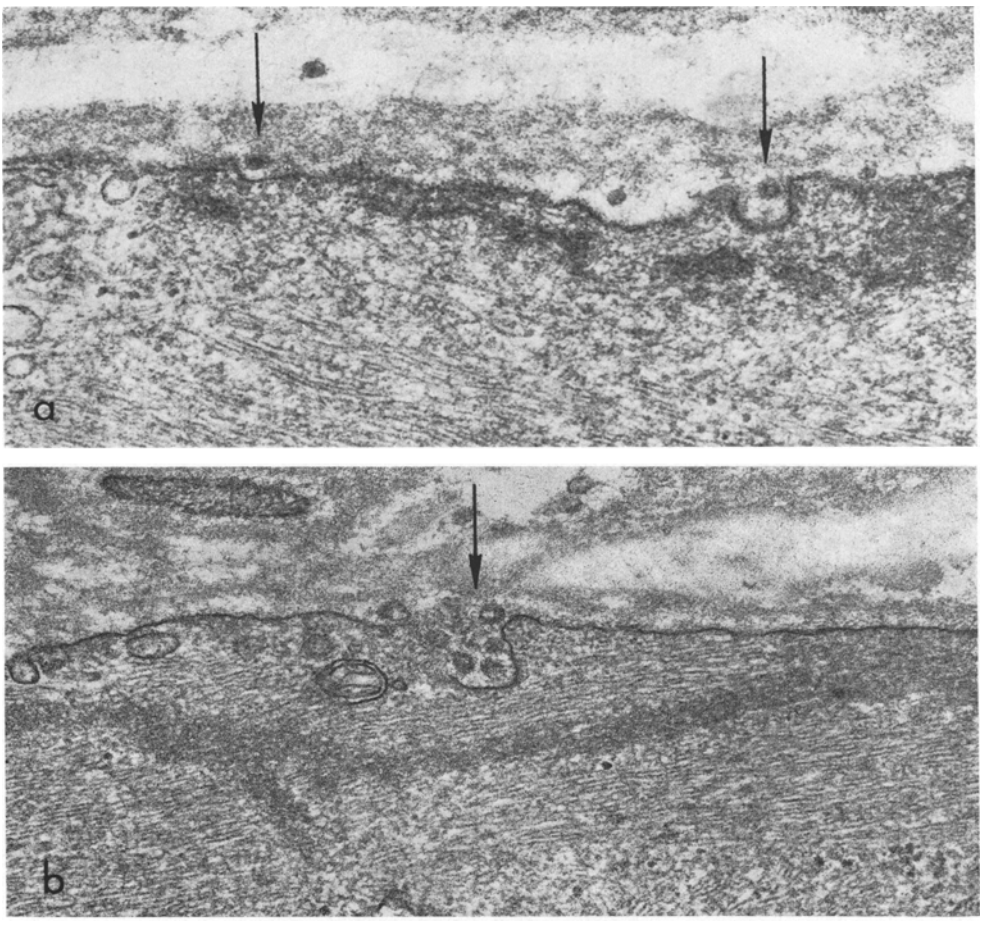

Fig. 11a and b. Details from the surface of smooth muscle cells in the media, showing images (arrows) that might represent phagocytosis of "granules", one of the components of the deposits. In all cases the granules are beneath the basement membrane. (Coronary artery: (a) 342 g rat, ×55400; (b) 260 g rat, ×62500)

any part of the media; moreover, they were focal, though traces could be found in almost any section of the media. They were much more abundant in the free spaces between the tips of smooth muscle cells, quite rare between their tightly juxtaposed bodies.

Beneath the endothelium, the deposits were usually scattered in the thin layer of connective tissue that separated the endothelium from the internal elastica, and more abundant within the fenestrae of the elastica (Fig. 6) as well as around the tips of the endothelial processes that anchor the endothelium to the elastica. Within the media, the deposits formed clusters that tended to streak laterally into all available spaces; sometimes they trickled between smooth muscle cells even where they were tightly apposed. Their relationship to the basement membrane varied. Some were free,—that is, outside the basement membrane (Fig. 9a);

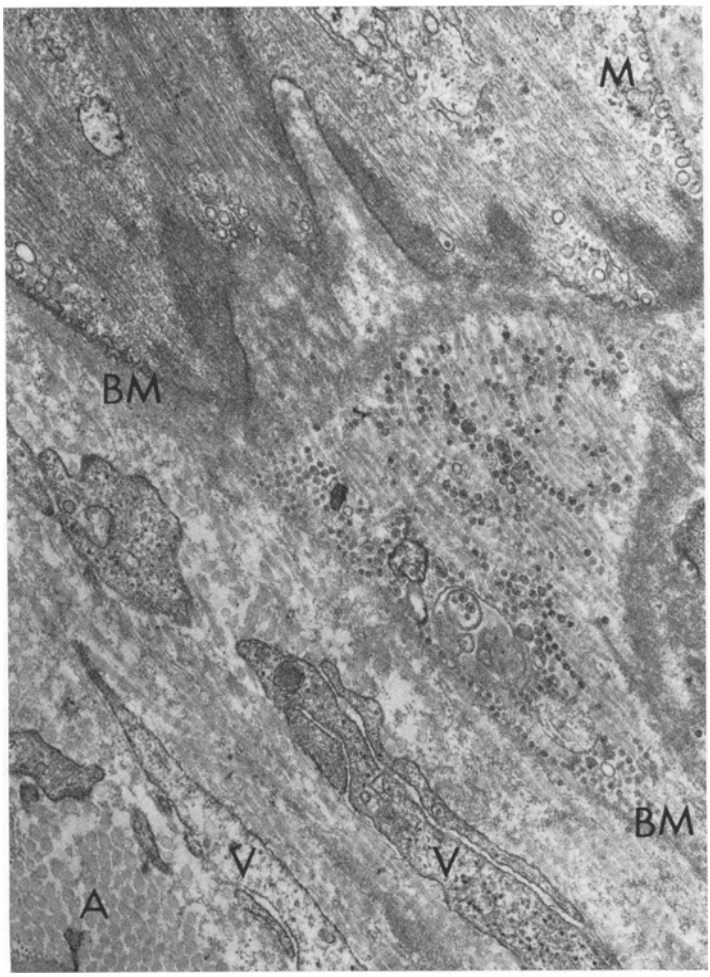

Fig. 12. Boundary between media (*M*) and adventitia (*A*), marked here by a basement membrane (*BM*). Within the media, note wide intercellular space filled with collagen fibrils; trapped between these fibrils are debris, both granular and membranous. Veil cells (*V*) lie in the adventitia just outside; one contains a lysosome-like body. (Coronary artery: 430 g rat, × 27500)

others were caught between the cell body and the basement membrane (Fig. 9b), others yet were clustered inside a ring of basement membrane (Fig. 10).

Occasional granules appeared to be embedded within the substance of the basement membrane itself; others, just beneath it, were neatly fitted into a pinocytic-type vesicle, as if they were in the process of being phagocytized (Fig. 11a, b) (though, of course, it was not possible to decide whether they were being extruded rather than phagocytized).

We searched for possible images of phagocytosis. On the whole, the deposits appeared to be ignored by the surrounding cells; only two or three times did we see a smooth muscle cell that could be interpreted as being in the act of actively

engulfing part of a deposit (Fig. 11a, b). However, a number of smooth muscle cells contained vacuoles that were highly suggestive of phagosomes; many of these contained granules resembling those found in the extracellular spaces, but often somewhat smaller and darker, perhaps suggesting a beginning of digestion (Figs. 4, 5, 8). Phagosomes packed with granules bore a superficial resemblance to multivesicular bodies. These images of phagosomes were definitely more frequent in the older rats.

Between media and adventitia there was no continuous layer of basement membrane. Occasionally, however, a short gap between two muscle cells was bridged by a span of basement membrane; and if deposits were present they seemed to be retained by this diaphragm (Figs. 10, 12); some were enmeshed within the collagen of the media (Fig. 12); very rarely were they found beyond the outer limit of the media. The thin protoplasmic expansions of the veil cells lay just one micron or two beyond this limit. The thicker parts of the veil cells contained rough endoplasmic reticulum as well as dense granules with the appearance of lysosomes (Fig. 2). There seemed to be little doubt that the veil cells were macrophages. Some, indeed, contained phagosomes filled with material that could not be identified. The veil cells were definitely more abundant in the old rats: the profiles of "veils" usually formed a single layer in the younger rats, three or four layers in the older rats.

Discussion

Deposits of abnormal materials, similar to those that we have described in the coronary artery of the normal rat, have been noticed in recent years by others, also in normal animals: in the rat aortic intima (Schwartz and Benditt, 1972a, b), in the aortic media of aging rats (Cliff, 1970), and in the cerebral arteries of aging rats (Kojimahara *et al.*, 1973). The deposits found in these vessels were usually interpreted as "cell debris". It should be noted here that in our experience (and presumably in the other published studies) the abnormal deposits were not identifiable by light microscopy with routine stains, for they blended with the other intercellular materials. However, at the level of electron microscopy, the phenomenon is impressive.

How could cell debris, or debris of any kind, appear within the arterial wall? Three mechanisms seem possible.

1. Origin of Debris by Cell Death. Necrotic cells have been described in the intima of the aging aorta (Gerrity and Cliff, 1972): here the *subendothelial* space contained occasional faint profiles of cells that were obviously dead and were referred to as "ghost bodies". In the coronary artery of our rats, the intima did not contain subendothelial cells (alive or dead) comparable to those described above in the aorta; however, we did see objects that could be interpreted as parts of dead cells. Indirect evidence of cell death *in the media* may be found in certain patterns of basement membranes, which could represent empty spaces left by smooth muscle cells that died and disappeared. Focal reduplication of basement membrane have been described in the pathologic arterial wall of the chicken (Moss and Benditt, 1970) and in many different vessels of hypertensive rats: retinal arteries (Giacomelli *et al.*, 1972), middle cerebral arteries (Kojimahara

and Ooneda, 1970), mesenteric, pancreatic and renal arteries (Wiener *et al.*, 1969) and mesenteric vessels (Wiener and Giacomelli, 1973). Vracko and Benditt (1972) studied the fate of the basement membrane after injuries of skeletal muscle; in that tissue, when the cells (skeletal muscle fibers) died, their debris were temporarily retained within the intact basement membrane; later these debris (together with the old basement membrane) were removed, probably by interstitial cells, while regenerating cells formed a new layer of basement membrane.

In summary, then, it is possible—and likely—that some of the debris arise through cell death within the arterial intima and media.

2. Origin of Debris by Fragmentation of Smooth Muscle Cells. In our material, we believe that many of the deposits represent fragments of smooth muscle cells, broken off and partly degraded. Several pieces of evidence support this mechanism. It is not rare to find images suggesting that a thin branch of a smooth muscle cell, such as in Fig. 5b, breaks up into small pieces. Clusters of deposits are often enclosed within a basement membrane, together with a branch of a smooth muscle cell, suggesting that the debris originated from that cell and remained trapped within its basement membrane (Figs. 10, 11). We should also mention here a peculiar form of cytoplasmic shedding that has been described by a Japanese author. According to Takebayashi (1970), in hypertensive rats (but not in normal rats) smooth muscle cells can lose parts of their cytoplasm by "focal necrosis"; these "dead parts" are then said to be sequestered by the formation of a new layer of cell membrane, and eventually discharged out of the basement membrane by a "micro-apocrine mechanism". The electron micrographs presented as evidence are not entirely convincing; however, the concept of "partial cell necrosis" is interesting and deserves to be studied further.

3. Origin of Debris from the Blood. It is conceivable that some of the components of the deposits may come from the blood stream, possibly as material phagocytized by the endothelium and then emptied into the arterial wall. Endothelial pinocytic vesicles sometimes contain granules resembling the smallest granules that we saw among the deposits. It seems unlikely that this mechanism may account for a significant part of the deposits under normal conditions; it may perhaps play a greater role when the permeability of the endothelial layer is increased, as in atherosclerosis. Deposits have indeed been described in human intracranial arteries with gross fatty streaks (Hoff, 1972), in the middle cerebral arteries of hypertensive rats (Kojimahara and Ooneda, 1970) and in the intracranial arteries of dogs maintained on atherogenic diet (Suzuki, 1972). However, the association with atherosclerosis does not by any means *prove* that the deposits are, or can be, hematogenous.

Fate of the Deposits. Whether they originated mainly through cell fragmentation, cell death, or both, the debris in the intercellular spaces represent "foreign" material. What happens to it? The electron micrographs suggest that, at first, the debris are retained within the basement membranes of the cell from which they derived; thereafter, perhaps favored by the pulsations to which the wall is submitted, they spill out beyond the basement membrane and diffuse into all other available spaces. Phagocytosis of these materials does not seem to be very active: no cellular reaction was observed by either Cliff (1970) or Kojimahara *et al.* (1973).

This may be easily explained by the biology of the surrounding cells. First, smooth muscle cells are not particularly apt to perform phagocytosis, although they may do so under unusual circumstances [in hypertensive rats, Wolinsky *et al.* (1973) noticed an increased number of lysosomes in the aortas, and they believe that these lysosomes may be related to increased "endocytosis"]. Second, in many cases the deposits remain separated from the smooth muscle cells by one or more layers of basement membrane (Figs. 6, 7, 9a, 10, 12). Under these conditions, since the plasma membrane cannot make contact with the foreign material, the process of engulfment cannot be accomplished. Third, there are no macrophages within the arterial wall that could perform this function. Last, it is quite possible, indeed likely, that the deposits do not liberate any chemical materials that may serve as messengers to the surrounding cells, indicating their very presence. We are referring here to the substances, still largely unknown, that are liberated by a focus of necrosis. In the case of small cellular fragments (not equipped with lysosomes), it may well be that no autolysis occurs; hence, no small molecules arise to warn or attract the surrounding cells.

However, *some* phagocytosis seems to be accomplished (Fig. 11a, b). This suggests that the smooth muscle cells are indeed able to engulf foreign material if forced to do so, even though their digestive processes may not be very active: in many of the phagosomes it was possible to recognize granules and even vesicles fairly similar to those described in the deposits (Figs. 4a, b; 5a, b). Vacuoles similar to those that we interpreted as phagosomes have been seen in the cerebral arteries of hypertensive rats, and described as evidence of autophagocytosis (Kojimahara and Ooneda, 1970). We see little reason to invoke a process of autophagocytosis, since the material in the vacuoles is often similar to the granules found in the extracellular spaces, and since possible images of ingestion can also be found (Fig. 11a, b).

We rarely saw deposits within the adventitia, although considerable accumulations were present at the outermost limit of the media (Figs. 10, 12). Since the veil cells in the adventitia contained many lysosome-like bodies, as well as phagosomes (Fig. 2), we concluded that some debris do escape into the adventitia but are quickly phagocytized as soon as they come within reach of the veil cells.

All these images, taken together, suggest the following sequence of events. As the smooth muscle cells of the media grow, they become more and more branched; these branches are susceptible to breaking off (the "microtrauma" may be represented by the traction of the contractile cells themselves, and/or by continuous pulsation to which the cells are submitted). In the meanwhile, cell death occurs both in the subendothelial intima and in the media. The debris arisiug by these different processes spill around and diffuse within the wall, where they are largely ignored—because the smooth muscle cells cannot make contact with them, and also because they are poorly phagocytic. However, a few debris do become phagocytized, while the bulk diffuse toward the adventitia, where the veil cells are prepared to seize and digest them.

In this regard it is interesting to note that the veil cells, because of their shape, are well adapted to catch the escaping deposits wherever they may arise; there is practically no portion of the outer surface of the artery that is left

uncovered by at least one "veil". Thus, the function of the veil cells may be precisely this one: to remove the debris that originate within the arterial wall. This would also be in accordance with the fact that veil cells are more abundant in older animals.

The veil cells have been interpreted by Rhodin (1967) as fibroblasts. Although it is most likely that the adventitia also contains some fibroblasts, the structural details of the veil cells are far more suggestive of macrophages: particularly the presence of lysosome-like bodies, as well as of phagosomes.

Several questions now remain to be answered. (a) *Why are some arteries more susceptible to the accumulation of deposits*? From published evidence, and from our own material, it seems that the wall of the aorta is not affected as much as the coronary artery, the cerebral arteries (Hoff, 1972; Suzuki, 1972) or the retinal arteries (Giacomelli *et al.*, 1972). The reason for this difference is not clear. (b) *Why are the deposits focal*? If it is true that microtrauma play a role in the genesis of the deposits, it is possible that certain parts of the arterial wall (e.g. near the origin of a branch) may be more exposed to this type of insult. (c) *What is the relationship between deposits and hypertension*? Several authors have reported deposits in hypertensive rats (Kojimahara and Ooneda, 1970; Giacomelli *et al.*, 1972; Wiener and Giacomelli, 1973). It may be that the microtrauma of pulsation is increased in this condition; an hypothesis that remains to be proven. (d) *How do the deposits relate to atherosclerosis*? (e) *What are the species differences*, if any, and does the phenomenon apply also to the wall of the *veins*? These problems, too, need further study.

From the point of view of general pathology, the formation of intramural debris in the arterial media is a special case of physiologic cell death, as observed in many other tissues, such as bone and covering epithelia. It is a special case for two reasons: because cellular fragmentation is added to cell death; and because the resulting debris cannot be easily disposed of. This inability is a direct result of the conflicting requirements imposed upon the arterial wall, which must be made of living tissue, yet function as tough impermeable membrane capable of resisting high pressures. The evolutionary response to these requirements has been a compact layer of smooth muscle cells, with no weak spots for penetrating vessels (except in the largest arteries), and no space for extraneous wandering cells, such as macrophages.

Obviously this compromise must lead to difficulties in the "housekeeping" of the intercellular spaces: foreign material is not easily eliminated. Thus, intramural debris accumulate with age; we found that they were definitively more abundant in our older rats, and similar observations have been reported by others (Cliff, 1970; Gerrity and Cliff, 1972; Kojimahara and Ooneda, 1973). It therefore seems that the life span of the artery may have a built-in limit: i.e., the time when it is stuffed with its own debris. Perhaps, in this sense, arteries represent a case of natural "planned obsolescence".

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