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Human Intracranial Atherosclerosis

An Ultrastructural Study of Atheromatous Plaques

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Summary. Morphology of atheromatous plaques from human intracranial arteries was examined by light and electron microscopy. Overall morphology of lesions did not differ from those reported for other arterial beds. In the presence of an intact elastic membrane, lipid cores were localized only to the intima and covered with a fibromuscular cap. Lipid-filled cells at the periphery resembled smooth muscle cells or blood monocytes, but within the atheromatous core took the form of macrophages filled with lipid crystals and variable-density lipid droplets. Calcium crystallized spherules were localized to the surface of translucent lipid crystals which themselves were seen to coalesce with large amorphous lipid droplets. This study together with previous studies on cerebral arteries suggest that fatty streak lesions may undergo transformation to atheromatous plaques. Similarity in structure of atherosclerotic plaques between cerebral and other arterial beds suggests that documented differences in susceptibility to atheroma of various vascular beds in relation to age cannot be resolved by morphological studies alone.

Introduction

Although previous studies have reported on the ultrastructure of cerebral atherosclerosis in animals (Imai and Thomas, 1968), there is little information to date on the fine structural morphology of human cerebral atherosclerosis (Martinez, 1962; Flora *et al.*, 1967). Ultrastructural studies of human cerebral arterial fatty streaks using histochemical techniques were reported in a recent paper from this laboratory (Hoff, 1972b). The present paper describes the morphology of atheromatous plaques taken from the same arteries used for the previously reported study. It was hoped that this study might contribute further morphological information on the possibility of transformation of the fatty streak to the atheromatous plaque. In addition, explanation was sought for the variation in incidence of atherosclerosis at different ages in various arterial beds.

Material and Methods

Specimens of human middle cerebral and basilar arteries were obtained at necropsy from grossly characterized atheromatous plaques within four hours of death from 15 subjects aged 37 to 78 years. Autopsy diagnoses of findings have been reported elsewhere (Hoff, 1972a). Six segments from each bed were excised, prefixed in Karnovsky's glutaraldehyde-paraformaldehyde fixative at 4° C for 12 hours, washed in 0.1 M cacodylate buffer pH 7.4 overnight, post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4 at 4° C for 2 hours, dehydrated in graded ethanol and propylene oxide and embedded in Epon. Semithin sections (0.5 micron) and ultrathin sections were cut on a Reichert UM2 Ultramicrotome. Semithin sections were stained with 1% alkaline toluidine blue and a modified PAS/alcian blue procedure after the epoxy was extracted with a saturated solution of sodium hydroxide

in absolute ethanol. These sections were viewed with a Leitz Orthoplan light microscope equipment with a Nomarski differential interference contrast (DIC) accessory for transmitted light. Micrographs were taken with both conventional and DIC systems. Ultrathin sections were stained with Reynold's lead citrate and viewed with an RCA EM4 electron microscope.

Results

Light Microscopy

Atheromatous plaques in human intracranial arteries consisted of a thickened intima comprised of a predominantly acellular core with abundant extracellular lipid and a fibromuscular or fibrotic cap (Fig. 1a). Lipid accumulation appeared confined to the intima in the absence of a major disruption in internal elastic membrane (Fig. 3a). Lipid droplets were present in numerous spindle and ovoid shaped cells in the thickened intima above the lipid core (Fig. 1a, b), some of these areas resembling fatty streak lesions. Massive lipid droplets with partially extracted areas were found in the atheromatous core, often close to the internal elastic membrane (Fig. 3a). Fragmentation of the lumen side of the internal elastic membrane was the usual finding (Fig. 2). Amorphous lipid droplets were seen embedded in the granular matrix, usually together with lipid crystals the contents of which had been extracted (Fig. 3a, b). Bands of connective tissue interspersed between the lipid accumulations (3b).

Cells containing lipid droplets were found both at the edges of the plaques and dispersed sparsely within the core. Some cells on the plaque borders could be identified as smooth muscle because of their spindle shape and the presence of a PAS-positive basement membrane surrounding them (Fig. 1b, 2a, d). Other cells lacked these characteristics and may be foam cells derived from monocytes (Figs. 1b, 2a, c). Within the plaque core lipid-filled cells had morphological characteristics of macrophages (Fig. 3). There was no clear morphological evidence, however, that these cells were derived from muscle cells or blood monocytes. The shape, size and density of lipid aggregates in macrophages were variable. Some droplets with completely extracted cores (Fig. 1d) were either of uniform spherical shape, or aggregates of such spheres which had coalesced. Other droplets were amorphous spheres of varying size (Fig. 1c). Lipid crystals were seen on occasion within plaque macrophages (Fig. 3d).

Fig. 1a and b. Light micrographs of human cerebral artery plaques. Fig. 1a illustrates the fibromuscular cap above a lipid core. In this case most of the intimal smooth muscle cells are devoid of lipid droplets. Toluidine blue stain. ×180. Fig. 1b also demonstrates part of a fibromuscular cap above a smaller plaque. In this example lipid droplets are found both in smooth muscle cells characterized by the dense basement membrane surrounding them (arrow), and in ovoid-shaped cells possibly derived from blood monocytes (PAS stain). ×450. c and d One half micron thin section of an epoxy-embedded human basilar artery stained with alkaline toluidine blue and viewed with differential interference contrast microscopy. These micrographs depict areas on edges and in center of the atheromatous core of a plaque. ×1100. Fig. 1c demonstrates elongated macrophages containing lipid droplets of different sizes and densities, together with some extracellular debris (arrow). Fig. 1d shows groups of foam cells, some tightly apposed to one another. Most intracellular lipid droplets are of the same size with few exceptions. In one cell (black arrow) the droplets have coalesced into one mass. A cell devoid of lipid (white arrow) can be seen between groups of foam cells

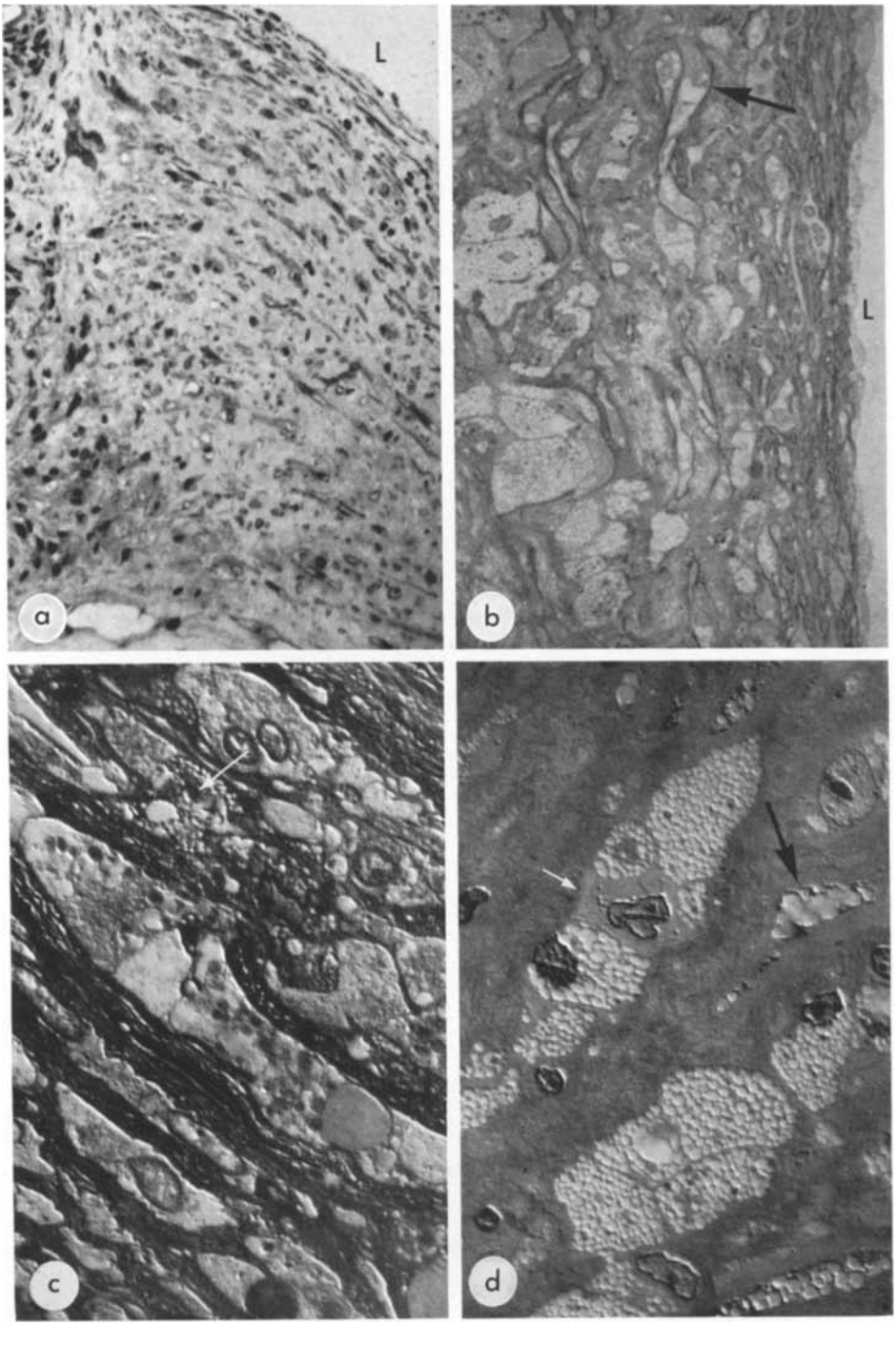

Fig. 1a—d

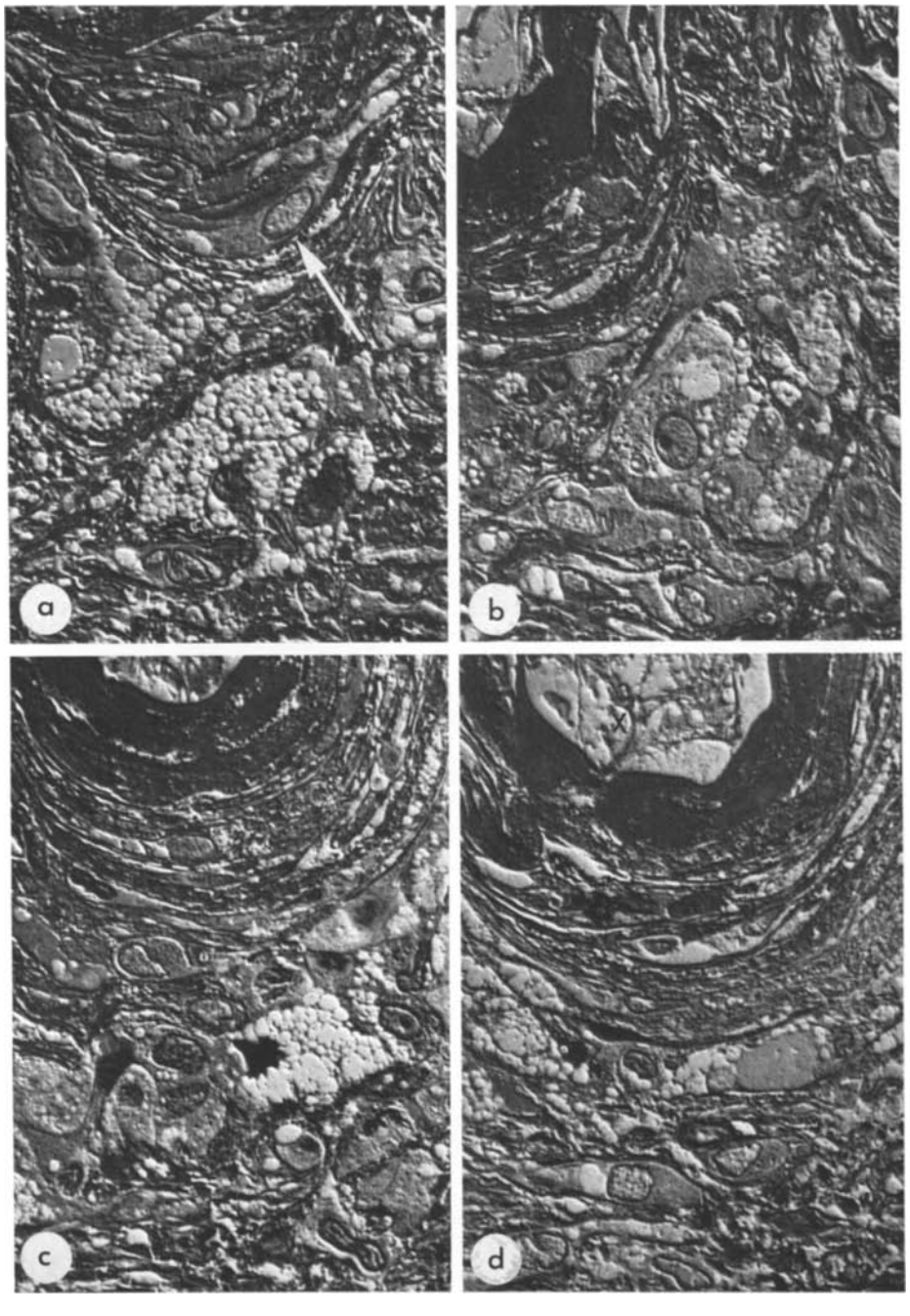

Fig. 2a—d. 0.5 micron thin sections of epoxy-embedded middle cerebral arteries stained with a modified PAS/alcian blue technique and viewed by differential interference contrast microscopy. All micrographs depict areas on the fringe of the atheromatous core just on the lumen side of the elastica interna. × 1100. Fig. 2a demonstrates a group of foam cells between smooth muscle cells, some containing individual lipid droplets. The latter cells have a

Electron Microscopy

At the periphery of the plaque core, elongated cells were seen filled with lipid droplets devoid of a limiting membrane. The centers of these droplets were partially extracted and many coalesced to form larger masses (Fig. 4a). The cell cytoplasm demonstrated numerous organelles, such as mitochondria, primary lysosomes, filaments, whorl bodies, and Golgi Zones, although cell membrane invaginations were sparse (Fig. 4a). These elongated cells were often closely apposed to each other, with occasional microvilli extending into the extracellular space. A number of these elongated cells were surrounded by filamentous electron dense material which possibly represented fragments of basement membrane (Fig. 4a).

Macrophages found within the plaque core contained lipid aggregates of extreme morphological variability (Fig. 4b, 5a). The most common finding was one of lipid droplets with areas of partial extraction. Intracellular lipid crystals were occasionally found together with these spheres (Fig. 5a). On occasion, smaller crystals were found embedded in an electron dense matrix (Fig. 4b). Other devoid shaped macrophages contained not only lipid droplets with extracted centers and edges, but also vacuoles filled with membranous material or with small vesicles (Fig. 4c).

Giant lipid globules were also found in the extracellular space of the atheromatous core. Some had bizarre forms with electron-dense areas and vesiculated cores (Fig. 5b). Vesicles of different sizes were also attached to the outer surface. Calcium, in the form of either nodules or spherules of apatite crystals was often localized to the plaque core usually together with lipid crystals (Fig. 5c), which themselves were embedded in a matrix that appeared to consist of cell debris. Such debris was probably composed of collagen fibers, membrane vesicles of various sizes, and bands of moderately electron dense amorphous material suggestive of fibrin degradation products.

Segments of cerebral arteries uninvolved with atheroma showed only swelling of cytoplasmic organelles, such as mitochondria, and a rough surfaced endoplasmic reticulum. These findings are in accord with previously documented studies of animal arterial beds using perfusion fixation (Hoff and Gottlob, 1968).

PAS-positive basement membrane surrounding them, which, when combined with the Nomarski optical system results in an accentuated relief image of the cell boundary (white arrow). Strands of connective tissue can be seen running between foam and smooth muscle cells. Fig. 2b illustrates a group of ovoid-shaped cells containing lipid droplets of various sizes. The indentations in their cytoplasm indicate the presence of abundant organelles. The internal elastic membrane can be seen on the upper left. Its lumen side appears fragmented. Fig. 2c shows one foam cell between groups of ovoid and spindle-shaped cells, many devoid of lipid droplets. These cells lie closely apposed to the fragmented side of the internal elastic membrane in the intima. Fig. 2d demonstrates numerous spindle-shaped cells filled with lipid droplets, some coalescing together to form larger spheres. Some cells (x) on the medial side of the internal elastic membrane are filled with lipid droplets at a point at which the membrane has a finestra. Note again the smooth contour of the elastica on its medial side while being fragmented on its lumen surface

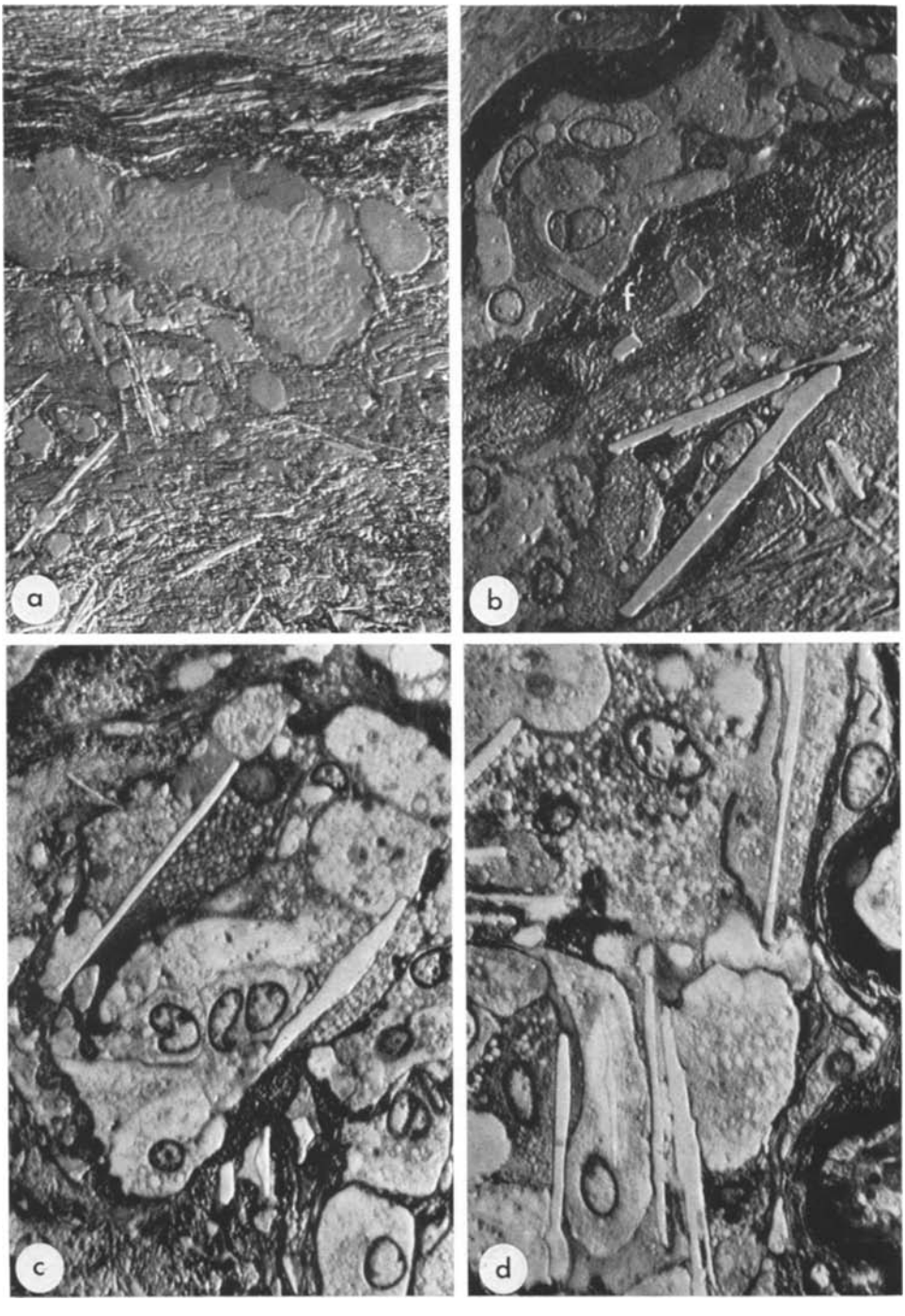

Fig. 3a and b. 0.5 micron thin sections of an epoxy-embedded human cerebral artery stained with alkaline toluidine blue and viewed with differential interference contrast microscopy. These micrographs illustrate the lipid core of an atheromatous plaque. Fig. 3a shows large

Discussion

Atheromatous lesions in human cerebral arteries have been examined for evidence of transition of such lesions from fatty streaks to atheromatous plaques. In a previous report from this laboratory (Hoff, 1972b), it was speculated that two predominant cell types present in the fatty streak lesion were the smooth muscle cells and blood monocytes, many of which contain accumulated lipid. Such cells were identified in the present study at the periphery of the atheromatous lesion and in association with macrophages. A previous histoenzymatic study (Hoff, 1972a) of different human cerebral atherosclerotic lesions which illustrated a gradual shift in enzyme and lipid localization pattern from fatty streak to atheroma, suggests that macrophages seen in this present study may originate from these same monocytes or smooth muscle cells of the fatty streak. Flora *et al.* (1967), in an ultrastructural study of human cerebral atherosclerosis, also illustrated lipid-filled smooth muscle cells in association with another unidentified cell type. Imai and Thomas (1968) also illustrated numerous macrophages in atherosclerotic lesions of the porcine middle cerebral artery that have certain similarities to smooth muscle cells. Macrophages identified in our present study have some characteristics of smooth muscle, such as elongated shape, small foci of myofibrils, and patches of fragmented basement membrane. Others totally lack these characteristics which would support a dual etiology.

The varied morphology of intracellular lipid droplets in atheroma has been frequently described in human aortic lesions (Ghidoni and O'Neal, 1967; Marshall *et al.*, 1967), but the present report is the first to fully describe the bizarre structures of lipid accumulations in human cerebral artery macrophages. For the most part macrophages remotely resembling smooth muscle cells contain lipid droplets without an electron dense limiting membrane. This was in contrast to more ovoid-shaped macrophages which also contain vacuoles filled with membranous material identical to cell debris. Such finding is in keeping with phagocytic activity of the cell. Such vacuoles have also been seen on occasion in cells of fatty streak lesions (Hoff, 1972b).

Intracellular localization of lipid crystals has been reported in foam cells of monkey aorta (Tucker *et al.*, 1971) and in necrotic smooth muscle cells of porcine cerebral artery plaques (Imai and Thomas, 1968). In the present study, whorl bodies were often observed in plaque macrophages, a finding also described by others in corresponding lesions of the human aorta (Weller *et al.*, 1968). Lipid extraction during the dehydration and propylene oxide steps of the electron microscopy embedding procedure cause large parts of intracellular droplets and

accumulations of extracellular lipid with partially extracted areas. Note the numerous lipid crystals at the bottom of the micrograph. ×450. Fig. 3b illustrates several lipid crystals embedded in a granular matrix together with connective tissue fibers (*f*). × 1100. c and d Human basilar arteries prepared in the same manner as Fig. 3a, b. Fig. 3c depicts a group of macrophages together with abundant extracellular lipid in the form of both droplets and crystals. Fig. 3d shows another group of macrophages on the lumen side of the internal elastic membrane. Lipid crystals appear to be present both intra- and extracellularly. Both × 1100

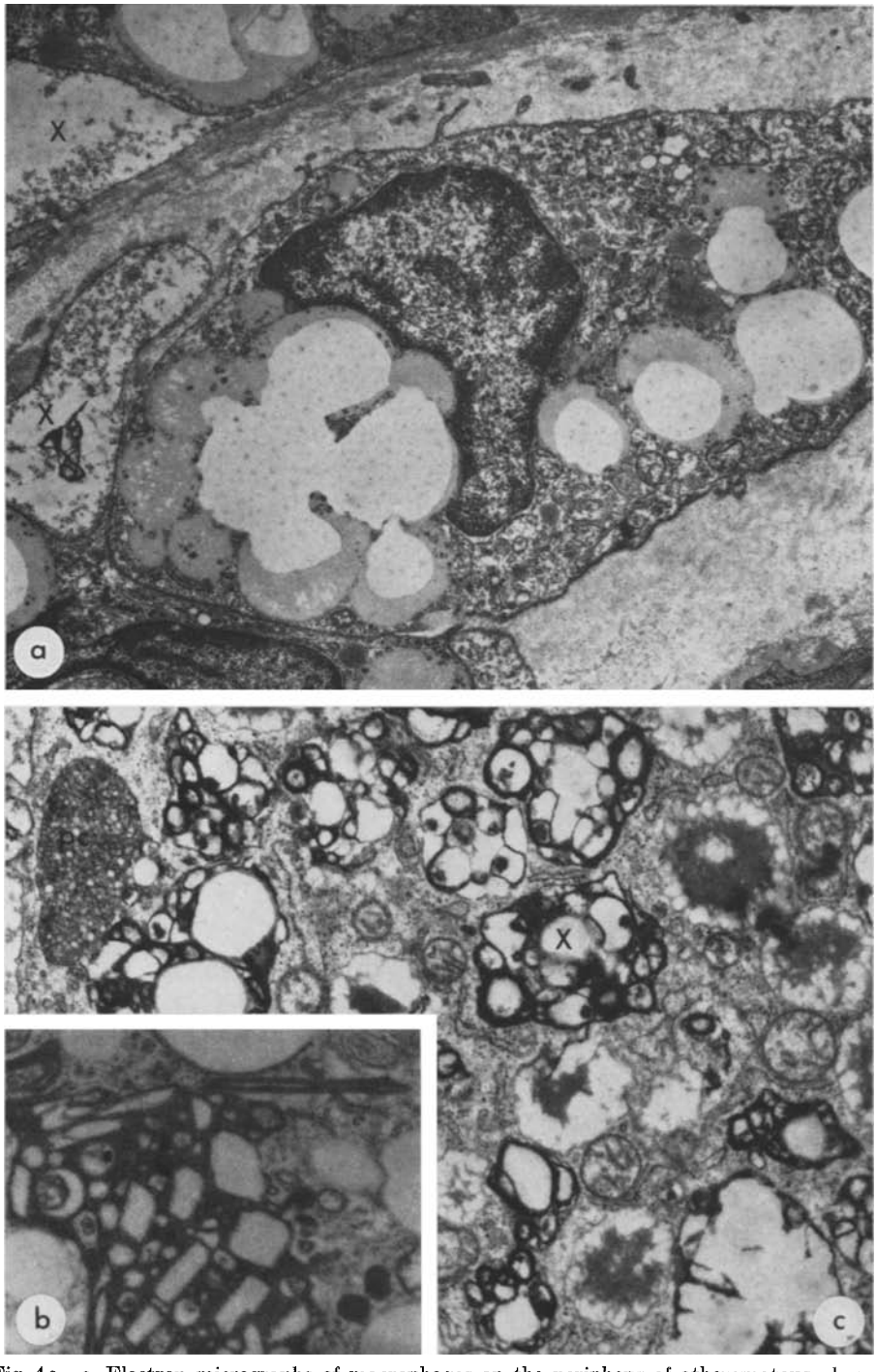

Fig. 4a—c. Electron micrographs of macrophages on the periphery of atheromatous plaque in a human basilar artery. × 9000. Fig. 4a illustrates abundant intracellular lipid droplets many coalescing to form one large droplet. The droplets do not appear to be membrane

whole core of the lipid crystals to appear electron translucent. Atherosclerotic plaques consist primarily of cholesterol esters and phospholipid (Smith *et al.*, 1967). Since fixation with osmium tetroxide is claimed to bind only unsaturated lipid (Bahr, 1954), it appears that saturated lipid is preferentially extracted, hence implying that composition of lipid droplets is not uniform. The opinion is substantiated by the wide variety in patterns of extracted areas in such droplets. In arterial walls, and more specifically in macrophages, phospholipid synthesis is enhanced (Zilversmit and McCandless, 1959; Day, 1964). Whether phospholipid is used to form a limiting membrane around the cholesterol ester droplets and crystals or used in the synthesis of other organelle membranes is still unclear.

Extracellular lipid greatly resembles that found intracellularly, suggesting that its major derivation comes from necrosis of the various lipid-filled cells. Chemical data demonstrating the shift of fatty acid pattern of cholesterol esters from one containing primarily oleic acid in the fatty streak to one containing primarily linoleic acid in the atheromatous plaque (Smith *et al.*, 1967, 1968) suggests that the intracellular lipid of the fatty streak when extruded into the extracellular space is additionally diluted by a further lipid pool. It is tempting to speculate that plasma low-density lipoproteins (LDL) which have high levels of linoleic acid in their cholesterol ester fraction (Smith *et al.*, 1967), are the source of this dilution. Preliminary evidence exists to substantiate this suggestion (Hoff, 1973). Using a fluorescent-labelled antibody preparation to human LDL it has been demonstrated that the antigen of LDL is localized to the lipid core of atheroma. Such a localization pattern corresponds to that for acid mucopolysaccharides (AMP). LDL may form an insoluble complex with AMP thereby being retained at the sites of polysaccharide accumulation (Taylor, 1953; Bihari-Varga and Gerö, 1967; Bernfeld *et al.*, 1957; Cornwell and Druger, 1961). Similar results were obtained in the human aortic plaque (Walton and Williamson, 1968).Whether the LDL antigen has a strong affinity for extracellular lipid accumulations remains to be answered.

Coalescence of amorphous (presumable unextracted), lipid droplets with the always translucent (extracted), lipid crystal has been demonstrated by the use of differential interference contrast (DIC) accessory of the light microscope. By the same rationale used in the discussion of intracellular droplets, such observations suggest that cholesterol ester crystals have a lower degree of unsaturation than many cholesterol ester droplets. Although such crystals have been described in virtually all morphologic studies of human atherosclerotic plaques, few have

bound and their centers have been extracted. The central cell contains numerous cytoplasmic organelles. Note cell surface microvilli. The usual characteristics of smooth muscle cells, myofibrils, cell membrane invaginations and basement membrane are rare or not present. This cell is in close apposition to the cell on the lower left. Note the translucent extensions of neighbouring cells. Some amorphous extracellular material can be observed below the top cells, possibly representing basement membrane material. Fig. 4b illustrates at higher magnification an intracellular accumulation of lipid comprised of crystals embedded in an electron dense amorphous matrix. ×27000. Fig. 4c shows a macrophage filled with numerous vacuoles. Some are lipid droplets (*li*) with extracted edges. Others are presumably phagocytic vacuoles (*pc*) containing membranous material and small vesicles (*x*) suggested to be derived from cell debris. ×18000

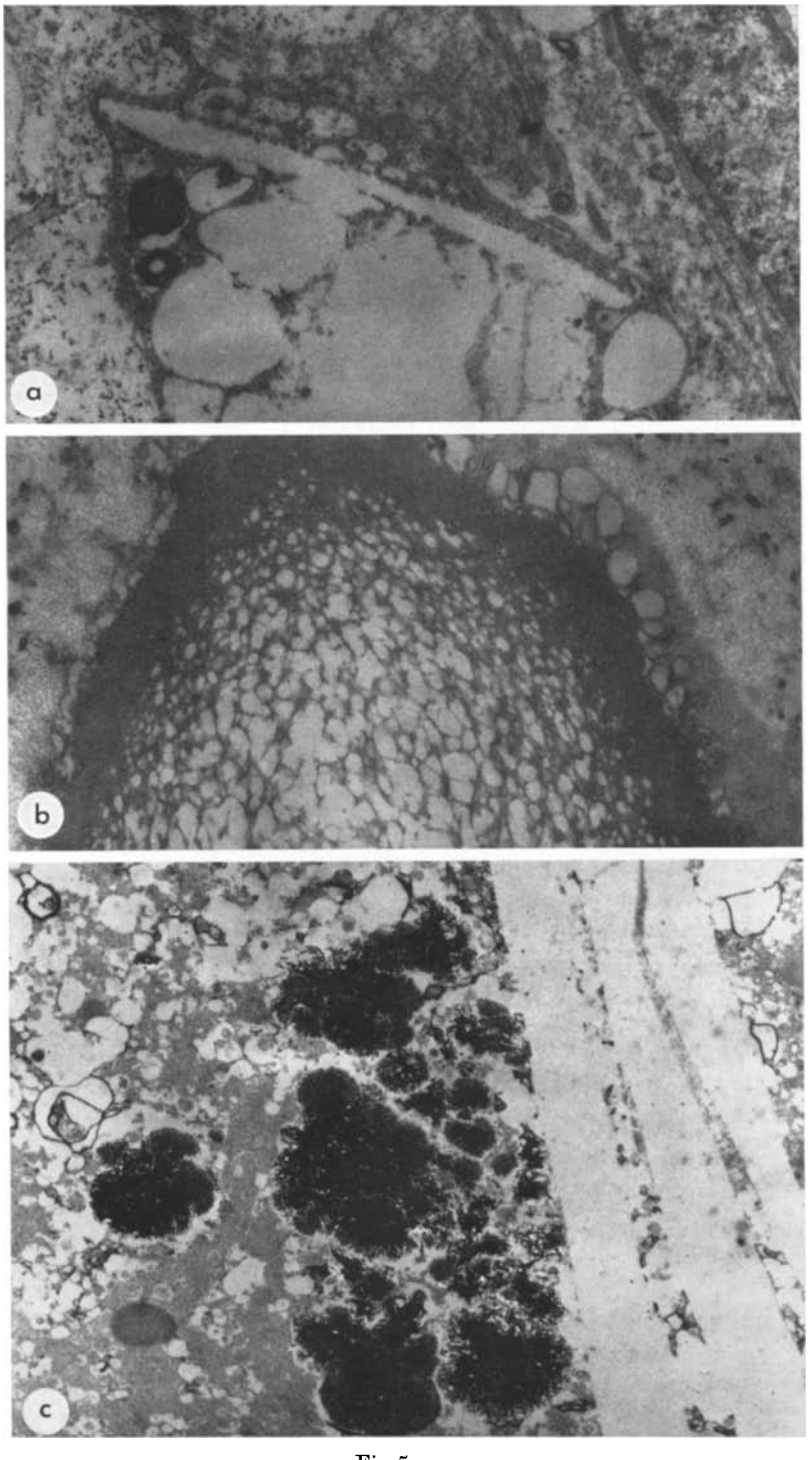

Fig. 5 a—c

illustrated them with the detail afforded by the plasticity obtained when viewing with DIC microscopy. Moreover, corresponding fine structural observations demonstrated close association of these crystals with larger and smaller membrane-bound structures, resembling cell debris and with electron dense crystals presumed to be calcium apatite.

Calcium deposits localized to elastic fibers have been observed in medial calcifications of the rat (Shimamura, 1970) and human aorta (Gardner and Blankenhorn, 1968), and in spontaneous rabbit intimal aortic arteriosclerotic lesions (Haust and Geer, 1970). However, in this present study, as well as in the study on aortic valves by Kim and Huang (1971), calcium spherules composed of radially arranged needle-shaped crystals were localized primarily to lipid crystals.

Some of the extracellular lipid accumulations seen in this report reached giant proportions. Again the nonuniformity of their contents reflects nonuniformity in composition. The vesicular core of one droplet described in this study may correspond to the lipid structures demonstrated by Ruska *et al.* (1972) in the aorta of cholesterol-fed rabbits.

Previous studies on cerebral arteries have emphasized the widening of the endothelial basement membrane during the atherosclerotic process (Suzuki, 1972; Martinez, 1962; Imai and Thomas, 1968). This process appears to be characteristic of early stages of the disease since it is not prominent in the atheromatous plaque. The accumulation of ground substance illustrated by Flora *et al.* (1967) may correspond to the granular matrix of the plaque seen in this study and suggested by us to be acid mucopolysaccharides and LDL localized to such areas (Hoff, 1973).

Based on information obtained from the present study and together with previous studies on enzyme localization and ultrastructure of human cerebral artery fatty streaks, it is concluded that there is no difference in the structure of lesions and progression from fatty streak to atheromatous plaque between major artery beds such as aorta and coronary arteries and the human cerebral artery. This impression is supported by the work of Imai and Thomas (1968) on porcine atherosclerosis. Since there are no structural bases for the varying susceptibilities of each bed, it appears necessary to turn to other approaches, perhaps biochemical in nature, to ascertain possible metabolic differences.

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Fig. 5. a Demonstrates lipid crystals and droplets found within a necrotic macrophage. ×15000. b Shows part of a giant extracellular lipid droplet. It has an electron dense, amorphous surface and a core consisting of numerous vesicular structures. Other vesicles adhere to the surface of this array. Collagen fibers can be seen in close proximity to the vesicles. ×1500. c This micrograph demonstrates an area of calcification in the lipid core of a middle cerebral artery plaque. The calcium crystalline deposits are seen as very electron dense structures embedded in an amorphous granular material together with cell debris. The calcium crystals are usually associated with lipid crystals. Note the association of membrane-bound vacuoles to the crystals. ×11000

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