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Ultrastructural Localization of Plasma Lipoproteins in Human Intracranial Arteries*

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Summary. The fine-structural localization of apoB, the major protein constituent of both the low and very low density plasma lipoprotein fractions, was described in human middle cerebral and basilar arteries. Using an immunoperoxidase technique together with electron microscopy, apoB was localized only in arteries with atherosclerotic involvement and to the following regions in these arteries: 1. on the outer aspects of extracellular spherical structures with diameters of 250 to 700 Å found predominantly in lipid cores and between bands of collagen fibers of advanced atherosclerotic lesions; 2. on the surface of reduplicated elastica; 3. along collagen fibers and; 4. on aggregates of extracellular spherical lipid globules. These results suggest that the extracellular spheres may represent the fine-structural morphology of deposited low and very low density lipoproteins and that free apoB may be bound to lipid globules, elastica, and collagen fibers.

Key words: Atherosclerosis — apoB — LDL — VLDL — Immunoperoxidase — Electron microscopy.

Introduction

ApoB represents the major protein and antigenic constituent of both the low (LDL) and very low (VLDL) density fractions of human plasma lipoproteins (Morrisett *et al.*, 1975). Elevated values of these fractions, either combined or alone, have been implicated in the pathogenesis of atherosclerosis. Elevations of both plasma LDL and VLDL as found in type II und IV hyperlipoproteinemias (Fredrickson and Levy, 1972) are accompanied by a greater incidence of coronary atherosclerosis leading to myocardial infarction (Kannel *et al.*, 1962). Similarly, increases in plasma VLDL as in type IV hyperlipoproteinemia have been shown to correlate with increased incidence of intra- and extracranial atherosclerosis leading to stroke (Randrup and Pakkenberg, 1967; Farid, 1973; Kannel *et al.*, 1974; Mathew *et al.*, 1975).

Recently evidence has emerged suggesting that not only cholesterol but also apoB, the major protein in LDL and VLDL, may play a role in atherogenesis (Ross and Glomset, 1973; Fischer-Dzoga *et al.*, 1974). Although the localization of apoB in human atherosclerotic arteries has been well documented on the light microscopic level employing immunofluorescence procedures (Kao and Wissler, 1965; Walton and Williamson, 1968; Knieriem, 1970; Hoff *et al.*, 1974; Hoff *et al.*, 1975a), no extensive study has been performed on the electron microscopic level. We have therefore utilized an immunoperoxidase procedure in conjunction

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with the electron microscope (Avrameas, 1970; Nakane, 1970) to determine the ultrastructural localization of apoB in human intracranial arteries with and without atherosclerotic involvement.

Material and Methods

1. Immunochemical Procedures

ApoB was prepared from plasma LDL from normolipoproteinemics by delipidation and subsequent solubilization with sodium decyl sulfate as described in detail previously (Gotto, 1970). Monospecific antibodies against apoB were prepared by immunizing goats and a globulin fraction was isolated by ammonium sulfate fractionation (Hoff *et al.*, 1974). Horseradish peroxidase (Type VI Sigma Chemical Co., St. Louis, Mo.) was conjugated to anti-apoB employing the bifunctional acylating reagent tolylene, 2,4-diisocyanate which forms intermolecular crosslinks between the peroxidase and globulins (Modesto and Pesce, 1973). The following procedure is a modification of that of Modesto and Pesce (1973). To 5 ml of a peroxidase solution in 0.04 M sodium phosphate buffer pH 7.5 was added 0.05 ml Hylene TM (Dupont Chemical Co., Wilmington, Del.) at 0° C for 15 minutes with stirring. The mixture was then centrifuged and the organic phase discarded. The remaining solution was allowed to stand at 0° C for 1 hour. Five ml. of a 1.5% globulin fraction of antibody in 0.025 M Na borate buffer pH 9.5 was then added and the mixture was stirred for one hour at 37° C. The resulting conjugate was first dialyzed overnight against 0.01 M $(NH_4)_2CO_3$ in order to inactivate any unreacted active sites on the Hylene TM-peroxidase complex, and then dialyzed against several changes of 0.1 M Tris-HCl buffer pH 7.4. The conjugate was then purified by affinity chromatography on a column of Sepharose, to which LDL had been covalently linked, which functioned as a solid phase immunoadsorbent. All immunologically inactive dimers of IgG remained in the void volume, together with free peroxidase and unreacted Hylene TM-peroxidase complex. The specific antibody-peroxidase conjugate was eluted with 0.15 M NaCl titrated to pH 11 with ammonium hydroxide and then stabilized with 1% bovine serum albumin (Hoff *et al.*, 1974).

2. Histochemistry and Electron Microscopy

A total of 125 specimens of middle cerebral and basilar arteries both with and without atherosclerotic involvement were obtained from nine subjects at autopsy. In a preliminary study the effects on the antigenicity of tissue apoB of two fixatives at different concentrations and times were examined in cryostat sections of arterial lesions. The results of this study as shown in Table 1 indicated that paraformaldehyde concentrations up to 4% for 30 minutes did not appreciably affect the localization of apoB in lesions, although the intensity of reaction product was less than in unfixed serial sections. Glutaraldehyde even at 0.25% for 5 minutes completely destroyed antigenic activity as determined by the presence of reaction product for peroxidase. Karnovsky's fixative (Karnovsky, 1965) containing both glutaraldehyde and paraformaldehyde also destroyed antigenic activity at all times and concentrations shown in Table 1. Therefore, the arterial specimens (5 mm length) were fixed for 30 minutes at 4° C in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2, following a brief rinse in 0.1 M phosphate buffered saline pH 7.0. This was followed by three 10 minute washes in 0.1 M phosphate buffer pH 7.2. Specimens were then embedded in 5% agar and 50 to 100 micron-thick sections were cut with a Sorvall TC-2 tissue sectioner and washed three times for 10 minutes on 0.1 M phosphate buffer pH 7.2. The sections were then incubated at 23° C for 24 hours with constant agitation with one of the following:

1. Purified peroxidase-conjugated anti-apoB.
2. Globulin fraction of non-immune serum conjugated with peroxidase (control 1).
3. Free peroxidase (0.05 mg/ml) (control 2).
4. 0.1 M phosphate buffered saline pH 7.0 (PBS) (control 3).
5. Purified peroxidase-conjugated anti-apoB absorbed with apoB (control 4).
6. Initial incubation of sections with non-conjugated anti-apoB for 24 hr, three one hour washes in PBS, followed by treatment of sections with peroxidase-conjugated anti-apoB (control 5).

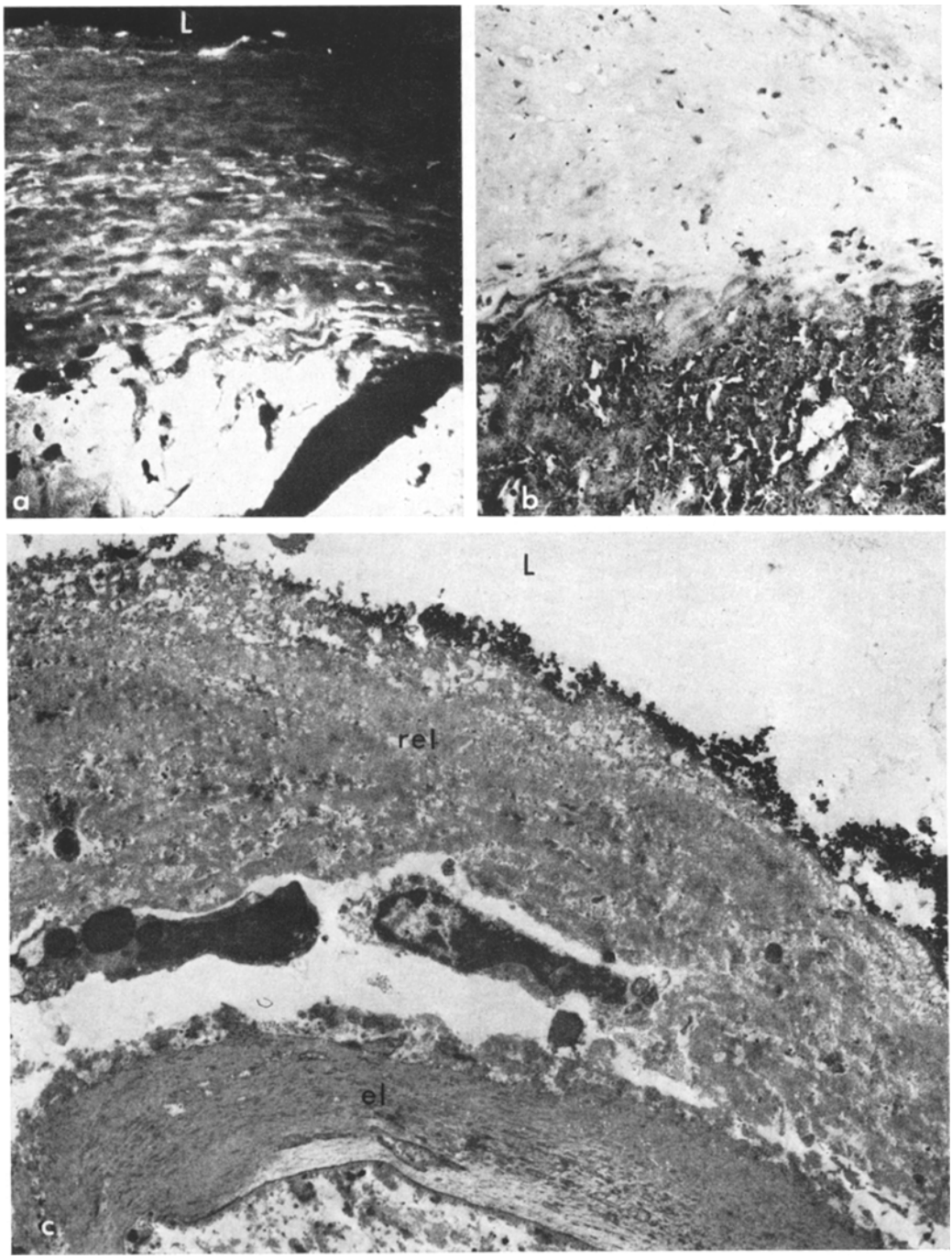


Fig. 1a—c. Localization of apoB by immunofluorescence techniques (a) (white areas) and by immunoperoxidase procedures (b) (dark areas) in similar areas of a plaque lipid core. Note the similar localization pattern as seen in cryostat sections on the light microscopic level. (a) and (b) ×80. (c) Electron micrograph of a fatty streak lesion illustrating the localization of electron dense reaction product on the lumen side of reduplicated elastic fibers (*r e l*). Note the granular appearance of this lamina. The internal elastic membrane (*e l*) can be seen in the lower part of the micrograph. ×6,000

Table 1. Effect of fixation with paraformaldehyde on apoB immunological activity in cryostat sections of atherosclerotic lesions

Fixative concentration	Fixation time (min)			
	5	15	30	60
0.25%	+++++[a]	+++	+++	++
0.50%	+++	+++	++	+
1%	+++	+++	++	+
4%	+++	++	+	−

(−) = negative.

When glutaraldehyde was substituted for paraformaldehyde, all immunological activity was lost at each concentration and fixation time shown above.

[a] Based on +++++ for unfixed sections.

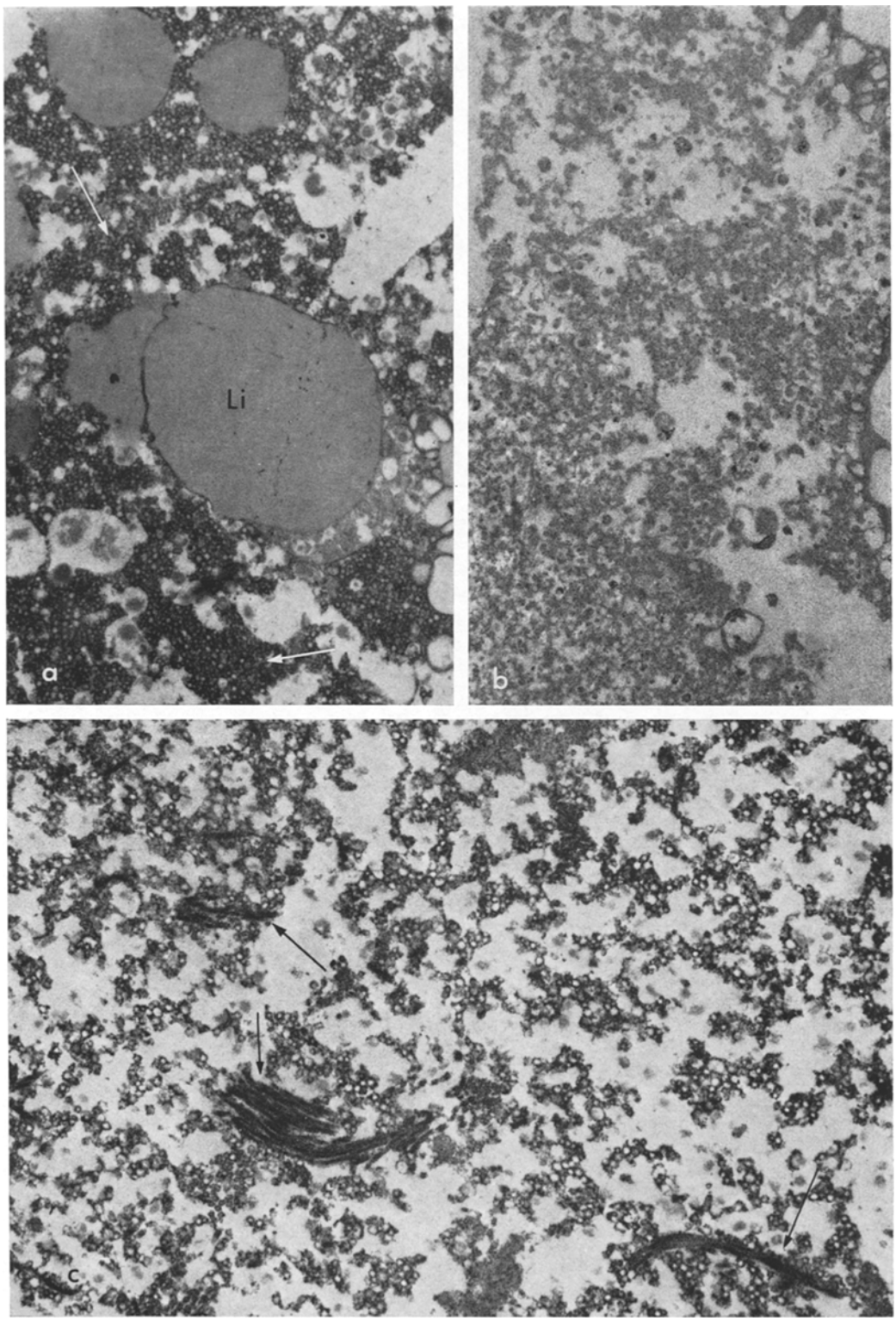
Following the incubation sections were washed three times for 10 minutes in PBS and then with the same buffer for 24 hours at 4° C. Sections were then fixed in 3% glutaraldehyde in 0.1 M cacodylate pH 7.2 for 10 minutes. This brief fixation period was chosen because glutaraldehyde has been shown to reduce peroxidase activity (Yokota, 1973). Following three more 10 minute washes in 0.1 M cacodylate buffer, the sections were incubated in the peroxidase localization medium consisting of 3-3′ diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide for 30 minutes at 23° C (Weir *et al.*, 1974). Following three 5 minute washes and one 15 minute wash in 0.1 M cacodylate pH 7.2, the sections were fixed in 1% OsO_4 in 0.1 M cacodylate buffer pH 7.2 at 4° C for 1 hour, followed by dehydration in graded ethanols and embedding in Spurr's Medium (Spurr, 1969). One micron and ultra-thin sections were cut and viewed for light and electron microscopy respectively.

Negatively stained extracts of arteries were used to confirm the presence of intact lipoproteins in arteries. Minces of both atherosclerotic lesions and uninvolved intracranial arteries were placed in PBS for 12 hours. The material extracted by the PBS was briefly centrifuged at 1,000 g and an aliquot of the supernatant mixed 1/100 v./v. with Na phosphotungstate pH 7.0. This negatively stained preparation was placed on Formvar-coated grids and viewed with the electron microscope. The presence in the extract of apoproteins from plasma LDL and VLDL (apoB) was determined by reacting an aliquot of the extract with anti-apoB on a double gel diffusion plate.

Results

Localization of apoB was observed only in human intracranial arteries with atherosclerotic involvement. The apoB localization pattern in cryostat sections was the same whether immunoperoxidase or immunofluorescence procedures were used (Fig. 1a, b). Using the electron microscope at low magnification, apoB was localized as a diffuse array around lipid crystals and droplets in lipid cores. ApoB was also associated with large bundles of collagen in advanced lesions, as well as

Fig. 2. (a) Electron micrograph of a plaque lipid core following immunoperoxidase treatment. Note the presence of electron dense reaction product for peroxidase on the outer surface of numerous small spheres (arrows) between large lipid droplets (*Li*) and cell debris. ×12,000. (b) Similar areas as in (a) but treated only with the peroxidase localization procedure. Note the lack of reaction product depicting apoB. ×16,000. (c) Electron micrograph of a plaque lipid core illustrating the occasional association of these extracellular spheres surrounded by reaction product with bands of collagen fibers (arrows) ×18,000


Li
a
b
c

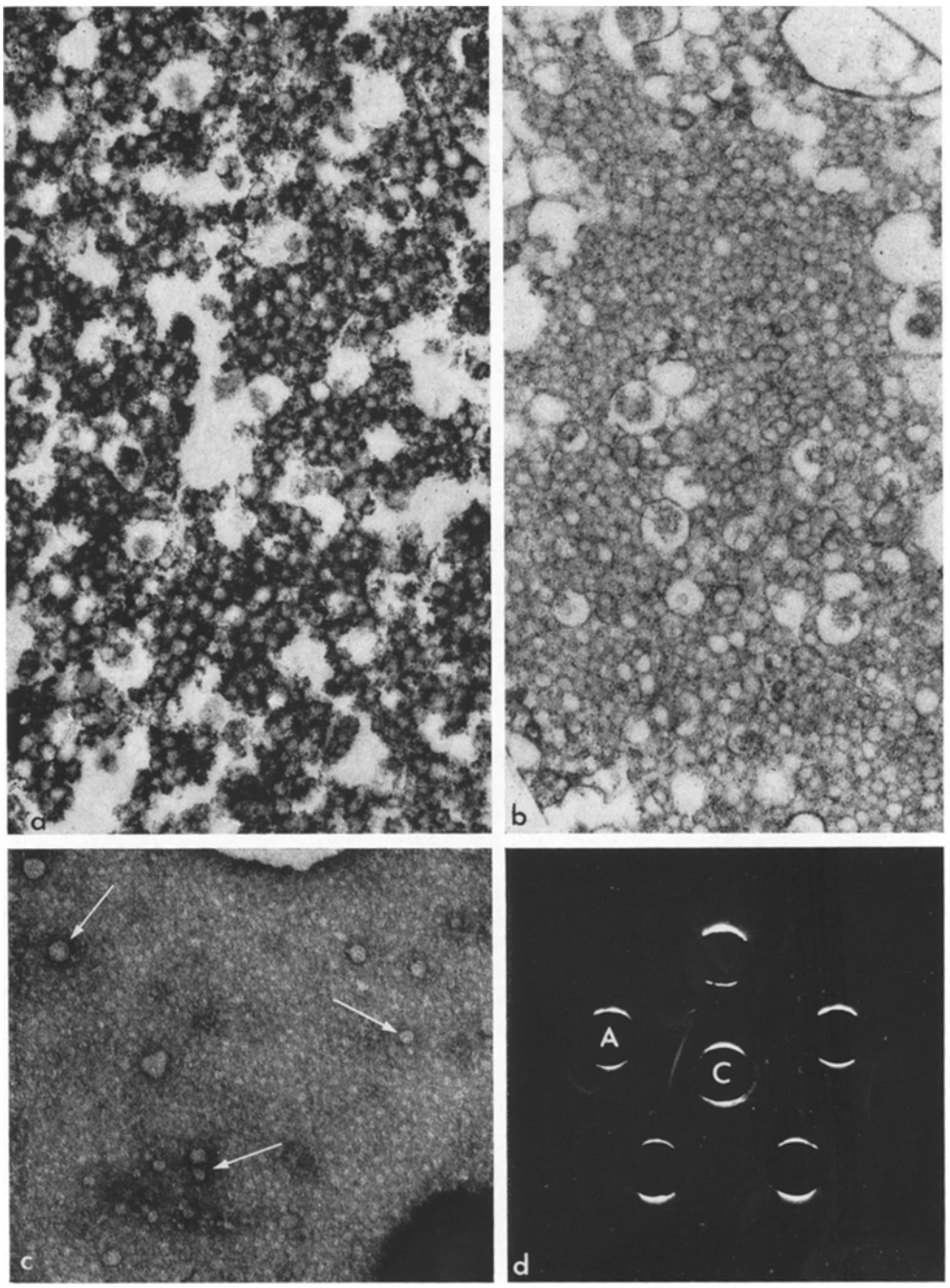


Fig. 3. (a) Electron micrograph of the extracellular spheres surrounded by immunoperoxidase reaction product in which the diameters of the spheres ranged from 250 to 700 Å. Note that in areas in which, the spheres are closely packed, reaction product completely fills the spaces between spheres. ×40,000. (b) Electron micrograph of a plaque lipid core from a type II

on fragmented or newly formed elastic fibrils in both fatty streaks and fibrous plaques (Fig. 1c). All five control incubations failed to demonstrate any extracellular localization of reaction product. The blocking studies (controls 4 and 5) inhibited essentially all positive reactivity.

In lipid cores of advanced lesions, reaction product representing the presence of apoB could be seen at higher magnification on the outer aspect of vesicular structures. The structures were determined to be spherical (Fig. 2a). Similar areas not incubated with the specific conjugated antibody failed to demonstrate such localization (Fig. 2b). These apoB positive spheres were occasionally associated with collagen fibers (Fig. 2c). Reaction product completely filled the spaces between these spheres (ranging in size from 250 to 700 Å diameters) when they were tightly packed (Fig. 3a). Sections of similar areas from arterial specimens not treated by the immunoperoxidase procedure (only stained with lead) could be observed not surrounded by dense reaction product. Fig. 3b shows such an area from a type II hyperlipoproteinemic.

Spherical particles ranging in diameters from 250 to 700 Å could be visualized in negatively stained preparations of saline extracts of advanced lesions (Fig. 3c). These extracts also demonstrated immunological reactivity for apoB (Fig. 3d).

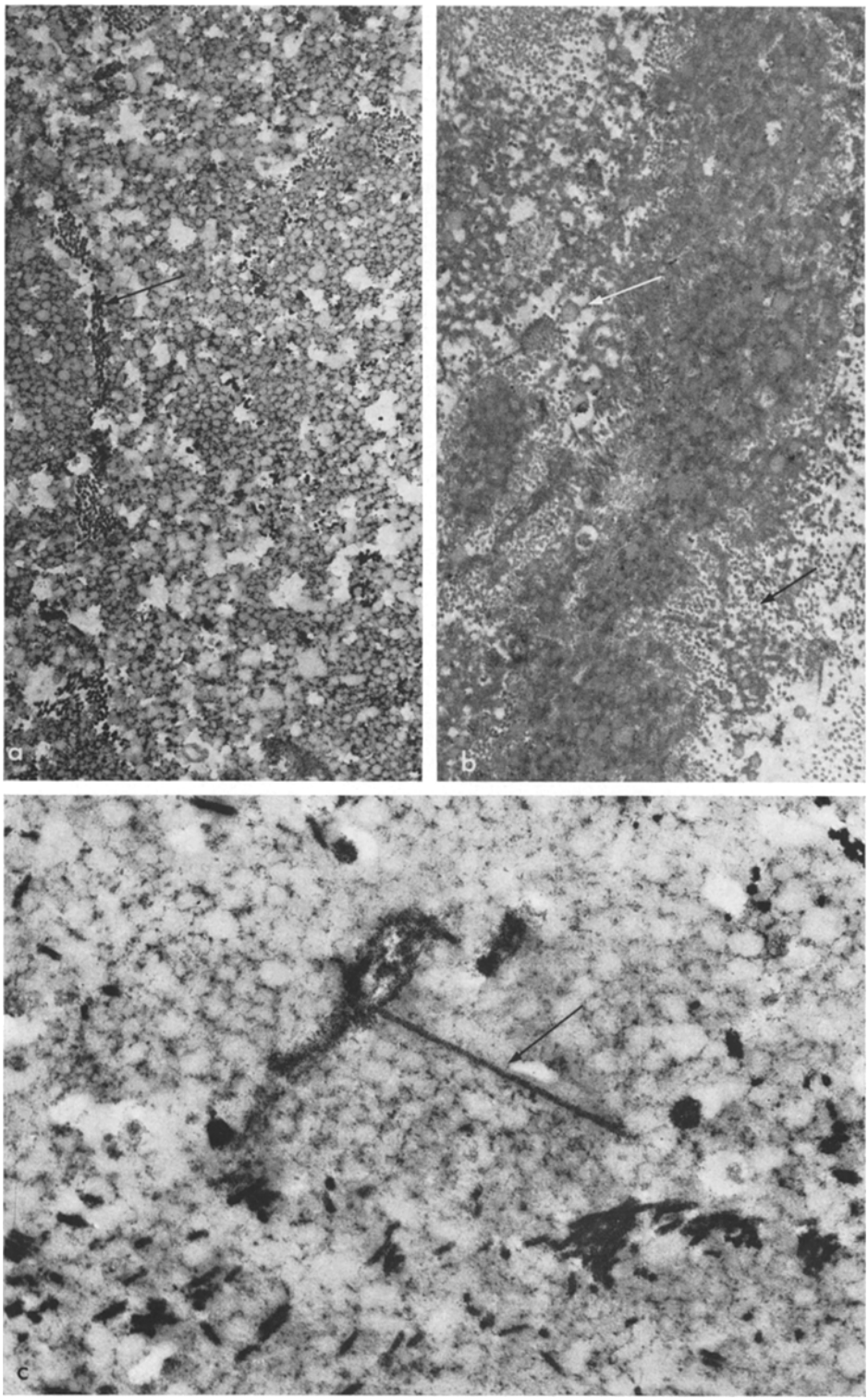
Reaction product for peroxidase depicting the localization of apoB was also occasionally associated with the surface of extracellular lipid droplets of 1,000 to 3,000 Å diameters and along collagen fibers giving the latter greater electron density (Fig. 4a) in unstained ultrathin sections. Control tissue (incubations without anti-apoB, with peroxidase or with DAB only) failed to demonstrate these findings (Fig. 4b), exhibiting a morphology of very low contrast in which these lipid droplets and collagen fibers were hardly discernible. At higher magnification banding along collagen fibers could be seen in areas from tissue treated with the immunoperoxidase procedure even without lead staining (Fig. 4c).

Discussion

The fine-structural localization of apoB has been determined in human atherosclerotic lesions employing an immunoperoxidase technique. Watts (1971) has also described the localization of apoB within intimal cells of human atherosclerotic plaques using ferritin-labeled antibodies, but not enough data was given to assess the specificity of the ferritin localization. Although not previously performed in whole arteries, the peroxidase-antibody technique has been utilized to obtain the ultrastructural localization of apoB within arterial smooth muscle cells grown in culture (Fischer-Dzoga *et al.*, 1973) and reaction product was found around intracellular lipid droplets.

ApoB could not be detected in univolved segments of human intracranial arteries either in these present ultrastructural studies employing immunoper-

hyperlipoproteinemic untreated by the immunoperoxidase procedure but stained with lead citrate. Note the presence of spheres of a similar size range as those seen in (a). ×36,000. (c) Electron micrograph of a plaque saline extract negatively stained with Na phosphotungstate pH 7.0. Note the presence of spherical particles (arrows) ranging in size from 250 to 700 Å diameters. ×68,000. (d) Double gel diffusion plate illustrating the single precipitin line obtained between a plaque saline extract (center well C) and anti-apoB (outer well A)


a
b
c

oxidase techniques or in a previous study using immunofluorescence procedures (Hoff *et al.*, 1975a). These results are in contrast to those of Smith (1974) who detected significant amounts of apoB in the intimal layer of uninvolved human aortas. One possible reason for this discrepancy is the difference in arterial beds studied. The smaller intracranial arteries rarely demonstrated intimal thickening without atherosclerotic involvement, whereas aortic intimas show a progressive thickening with age. Smith (1974) in fact, showed that the very thin aortic intimas of children demonstrated negligible amounts of apoB, whereas in adults the amounts of intimal apoB correlated with intimal thickness. A further possible reason for the inconsistency could be the difference in techniques employed. The electro-immunodiffusion technique used by Smith (1974) measures the amount of apoB extracted from an intimal mince where the apoB antigen is apparently distributed diffusely throughout the intima. On the other hand the immunohistochemical procedures using peroxidase and fluorescein labeled anti-apoB (Hoff *et al.*, 1975a) detects a focal localization of the apoB in a tissue section.

The localization of peroxidase around spherical particles in arterial lesions suggests that these spherical structures may represent low and very low density lipoproteins. This is consistent with our data in which saline extracts of arterial lesions possessed immunological reactivity for apoB from LDL and/or VLDL and contained particles with the approximate size range of these lipoproteins. By contast, extracts of uninvolved arteries failed to demonstrate these properties. Moreover, the extracellular spheres, as well as apoB as detected by immunofluorescence techniques were more extensive in intracranial arterial lesions from type II cases than from type IV cases or from normolipoproteinemics (Hoff *et al.*, 1975a b). These published results are consistent with a greater initial concentration gradient present between plasma and arterial wall in the former group than in the latter two groups due to the elevated plasma LDL levels in type II cases (Hoff *et al.*, 1975a). These spheres observed in this study differ from those derived from cell debris since the latter are more heterogeneous in size and frequently have electron dense cores (Joris and Majno, 1974). The localization of reaction product only on the surface of the extracellular spheres would imply that the antigenic determinants of apoB, the protein moiety of LDL reside on the surface of the lipoprotein. It cannot, however, be ruled out that some apoB might have been extracted with the particle's core material, presumed to be lipid during the electron microscopy procedure.

In our hands, ultrastructural tissue preservation was not optimal after 4% paraformaldehyde fixation for 30 minutes followed by extensive incubation times

Fig. 4. (a) Electron micrograph of a plaque lipid core illustrating the presence of an aggregate of numerous extracellular lipid droplets (1,000 to 3,000 Å diameter) surrounded by a fine layer of immunoperoxidase reaction product. Collagen fibers (arrow) are abnormally electron dense. Immunoperoxidase procedure. ×11,000. (b) Electron micrograph of a similar area but treated only with the enzyme localization medium. Note the overall loss of contrast relative to Fig. 4a maling visualization of the lipid droplets (white arrow) and collagen (black arrow) difficult. ×12,000. (c) At higher magnification the localization of reaction product around extracellular lipid droplets and finely distributed along collagen fibers (arrow) can be more readily discerned. ×48,000

with conjugated antibody. In particular, the poor preservation of smooth muscle cells suggested excessive autolysis, making any assessment of intracellular apoB localization in this present study questionable. Moreover, it has been suggested that the peroxidase-conjugated antibody complex would be too large to penetrate the cell membrane during incubation, unless conjugated Fab fragments of the immunoglobulin were utilized (Kraehenbuhl *et al.*, 1971).

The increase in electron density of collagen following the immunoperoxidase procedure appears to result from the binding of enzyme reaction product to the collagen fiber, and may represent the adhesion of free apoB to certain fibers. Control incubations did not demonstrate collagen fibers with increased density. Reduplicated elastic fibers and extracellular lipid droplets (possibly representing accumulated chylomicrons) were also associated with greater or lesser accumulations of peroxidase reaction product, possibly representing the binding of free apoB to these regions. Positive immunofluorescence for apoB was still observed on plaque collagen fibers and lipid cores after extraction of small blocks of lesions with phosphate buffered saline for 24 hours (unpublished observation). This result suggests that part of the LDL or VLDL is delipidated rendering the antigen insoluble, or that the lipoprotein is bound so tightly to certain tissue components that its extraction from the tissue is prevented. More extensive studies on the interaction of plasma lipoproteins with such lesion components as connective tissue and extracellular lipid are needed before this binding mechanism can be elucidated.

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