Virchows Arch. Abt. A Path. Anat. 352, 99–110 (1971)

Studies on the Pathogenesis of Atherosclerosis with Experimental Model Systems

IV. Ultrastructural and Lipid-Histochemical Changes in the Lipoprotein-Filled Doubly Ligated Carotid Artery

H. F. Hoff

Max-Planck-Institute for Psychiatry, Munich, Germany

Received September 28, 1970

Summary. The doubly ligated common carotid artery of the rabbit was filled with either human serum beta or pre-beta lipoproteins and the morphological alterations were studied from one to twenty days post-ligation using electron microscopy and lipid histochemistry and compared with the contralateral carotid. Of interest was the general mode of cholesterol accumulation and its fate, and the effect on the artery of varying the cholesterol concentration within the injected lipoprotein (beta vs. pre-beta). The percentage of arterial cells with intracellular lipid droplets (mainly containing triglycerides) increased to a maximum of 85% four days post-ligation and gradually fell to 18% seventeen days after ligation. Cholesterol accumulated mostly in the intima and inner media following injections of beta lipoproteins and persisted up to the seventeenth day. Ultrastructurally exogenous cholesterol was thought to exist as vacuoles with transparent centers from which material had partially been extracted by the technical procedures. Such cholesterol was also seen as large lamellated bodies and occasionally as intracellular clefts. These findings were compared to the morphology of early human atherosclerosis, and to results of previous double-ligation studies.

Introduction

Double-ligation of the rabbit common carotid artery has been used as an experimental model for studies on the pathogenesis of atherosclerosis (Hackensellner *et al.*, 1965; Hoff, 1970), particularly after filling the ligated segment with lipids or lipoproteins (Friedman *et al.*, 1966; Hoff and Gottlob, 1969a, b). The assumption of this model's applicability to the disease is based on the similarity in morphology between the changes that develop in the ligated artery and those observed in human atherosclerotic arteries, particularly with respect to the formation of a thickened intima comprised of modified smooth muscle cells. In the present study, the lumen of the doubly ligated rabbit carotid artery was filled with human beta or pre-beta serum lipoproteins. These macromolecules contain cholesterol (Lindgren *et al.*, 1964; Fredrickson *et al.*, 1967), which has been shown to be intimately associated with atherosclerosis and coronary heart disease (Gofman *et al.*, 1950; Brown, 1969; Frantz and Moore, 1969; Adams, 1967; Friedman and Byers, 1965). It was decided to test: (1) whether high cholesterol-containing macromolecules (beta lipoproteins) are more atherogenic than lower cholesterol-containing ones (pre-beta lipoproteins) (Adams, 1967; Kritchesvsky,

1967); (2) whether accumulations of cholesterol remain within the arterial wall for longer periods than other lipid moieties, as was shown in human atherosclerotic arteries (Adams, 1967) and in experimental animals (Friedman and Byers, 1965; Courtice, Schmidt-Diedrichs, 1962); and (3) to compare the morphologic changes, both light and electron microscopic, following ligation and injections of cholesterol-containing lipoproteins, with those following ligation and injections of triglyceride-containing lipoproteins (Hoff and Gottlob, 1969b; Friedman *et al.*, 1966) or lipid emulsions (Hoff and Gottlob, 1969a), a physiologic salt solution (Hoff, 1970), and finally to establish comparisons with human and animal atherosclerotic vessels.

Material and Methods

Rabbits of mixed strain and of either sex, averaging three kg, were anesthetized with pentobarbital. The right common carotid artery was exposed and a double-ligature was applied as previously described (Hoff and Gottlob, 1969a). Prior to application of the distal ligature, up to 0.5 ml of a 5% solution of either human beta or pre-beta serum lipoproteins was injected into the lumen of the ligated artery. This was accomplished by first flushing out the remaining blood out of the lumen and then filling it, under pressure, with lipoprotein. Both the human beta and pre-beta lipoproteins were isolated by ultracentrifugation and their concentrations were measured refractometrically according to the methods of Lindgren *et al.* (1964). At periods of 1, 2, 3, 7, 11, 17, and 20 days following operation, the ligated segment and the contralateral carotid were excised and prepared for lipid histochemistry and electron microscopy. Two or three animals were used for each time sequence studied. Several segments were cut from each artery and were immersed either in Baker's Ca-formol for lipid histochemistry or in 1% osmium tetroxide—0.23 m sucrose in 0.1 m veronal-acetate buffer pH 7.4 for two hours at 4° C for electron microscopy (E.M). Some arteries were also pre-fixed in 5% glutaraldehyde in 0.1 m cacodylate buffer pH 7.4 before immersion in osmium tetroxide. Frozen sections were cut from the formol-fixed segments and stained with OTAN (osmium tetroxide-alpha naphthylamine) (Adams, 1967) in order to differentiate between neutral and polar unsaturated lipids), and PAN (perchloric acid-alpha naphthoquinone) (Pearse, 1968) for demonstrating cholesterol. Some of these sections were viewed under polarized light in order to localize cholesterol by its birefringent characteristics within the tissue. The segments for electron microscopy (E.M.) were then dehydrated in acetone and embedded in Araldite. Sections were cut on a Reichert ultramicrotome; sections one-half micron in thickness were stained with alkaline toluidine blue and viewed with the light microscope for survey purposes. Ultra-thin sections stained with 1% lead citrate were viewed with a Siemens Elmiskop 1 electron microscope. The percentage of cells containing lipid droplets was tabulated as described previously (Hoff, 1970).

Results

Fig. 1 summarizes the results of counting the number of arterial cells containing at least one lipid droplet as averaged over all the experiments using human lipoproteins for a specific time sequence. One day after ligation, 14% of the cells contained lipid droplets, after three days the figure was 81%, after four days, 85%, after seven days 81%, after eleven days 28%, and after seventeen days 18%. OTAN staining (Fig. 2a) showed that these droplets were almost entirely hydrophobic while PAN staining demonstrated some accumulations of cholesterol, especially when beta lipoproteins were injected into the artery (Fig. 2e). The presence of cholesterol was substantiated by examinations with polarized light (Fig. 2f) in which anisotropic material was localized in the intima and inner

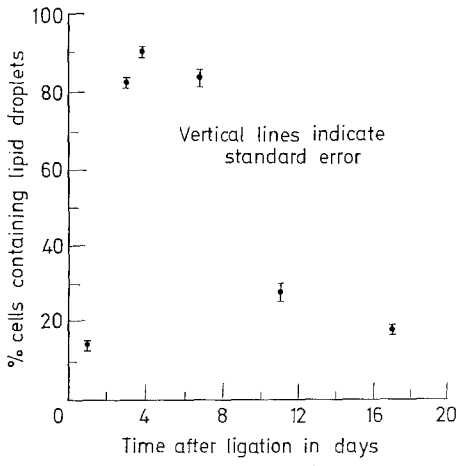

Fig. 1. Percentage of arterial cells containing lipid droplets at various times following double-ligation

media. It can, therefore, be assumed that the majority of the diffuse pattern of intracellular lipid consists of triglyceride, while cholesterol is localized in large quantities only in the intima and inner media and particularly following injections of the cholesterol-rich beta lipoprotein fraction. At later time intervals (17 days) most of the triglyceride had disappeared as demonstrated with OTAN (Fig. 2b). Even at eleven days post-ligation, parallel staining with OTAN, PAN, and viewing with polarized light (Fig. 2c–f) demonstrated that almost all the lipid-staining material was localized in the intima and inner media and contained cholesterol.

The major differences between the type and localization of lipid could be demonstrated only with electron microscopy. Following pre-beta lipoprotein injections, lipid was found either as amorphous droplets or as concentric structures, both regular with a periodicity of about 100 A (Fig. 3a) and irregular (Fig. 4). Often these lamellated bodies appeared to be in various stages of disorganization (Fig. 4a–c). They were found in all cells of the intima and were particularly prominent in the endothelial cells. On rare occasions, an electron-translucent, crystalline structure, presumed to be a cholesterol cleft, was found in the intimal cell, usually attached to a lamellated body (Fig. 3b).

The ultrastructure of the lipid in the intimal area following injections of beta lipoproteins consisted primarily of electron translucent vacuoles with fuzzy halos. The limiting membrane was smooth following prefixation in buffered glutaraldehyde (Fig. 5b), but it was ruffled when fixation in buffered OsO_4 alone was employed (Fig. 5a). Often, only part of the contents of the vacuole was translucent, suggesting extraction of cholesterol during the E.M. procedure. Very often, vacuoles of various sizes with electron-dense limiting membranes could be seen aggregated together, both within endothelial (Fig. 6) and subendothelial cells (Fig. 7) but usually in the latter. Many vacuoles appear to have coalesced to-

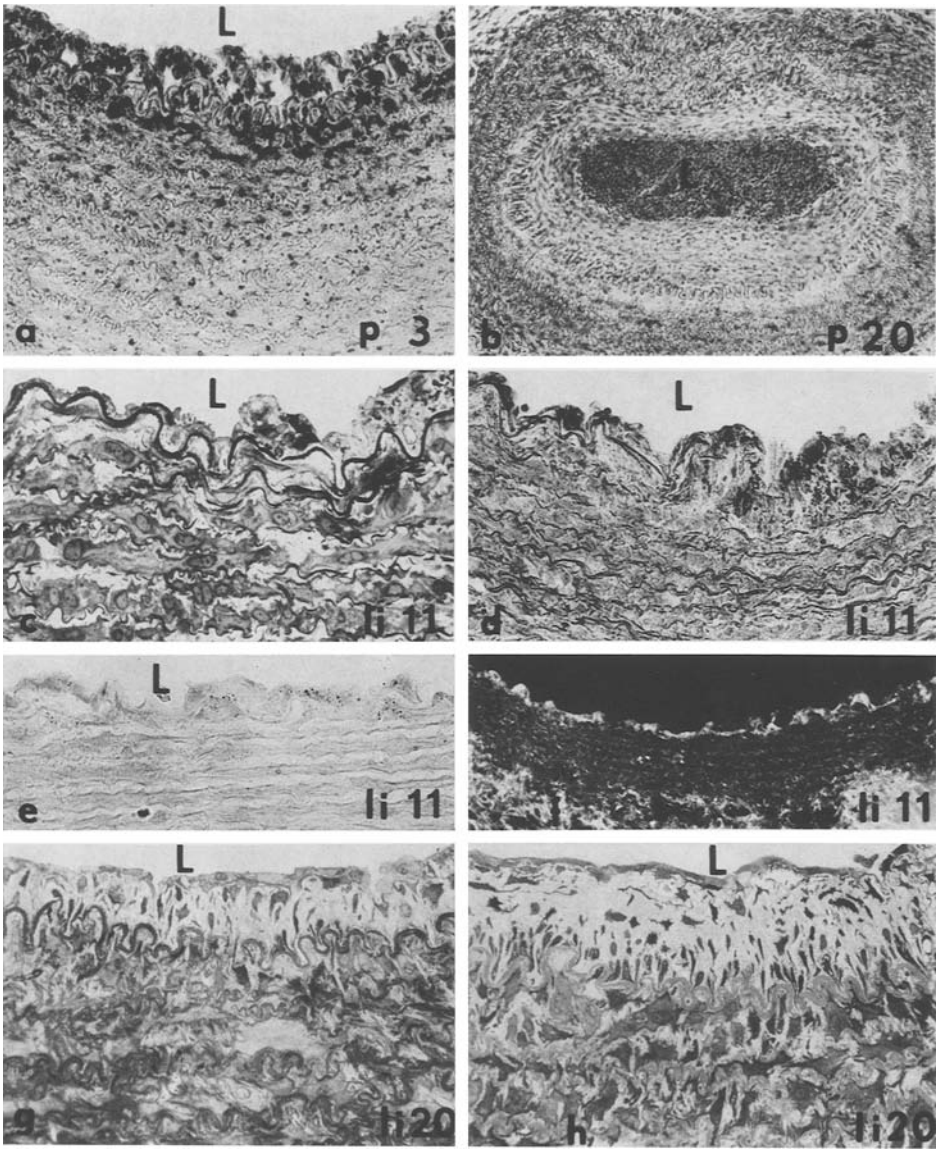

Fig. 2a–h. Light micrographs of the doubly ligated rabbit common carotid artery filled with human serum lipoproteins. The subscripts on the lower right of each micrographs have the following connotations: *p* indicates injection with pre-beta, *li* indicates injection with beta-lipoproteins; while the number indicates the days following ligation. One-half micron-thick sections stained with alkaline toluidine blue were cut from Araldite-embedded material, while OTAN and PAN stains were cut from ca-formol-fixed frozen blocks. *L* lumen. a OTAN stained artery shows intense accumulation of mainly unsaturated hydrophobic lipid around the intima and inner media, but more diffusely in the remaining media. ×200. b At this late time concentric intimal thickening narrows the lumen which is now filled with erythrocytes, probably derived from capillary sprouts. Almost no lipid can be seen. ×80. c Toluidine blue stained artery demonstrating numerous vacuoles in the intima. ×300. d OTAN stained artery from adjacent area to that shown in Fig. 2c demonstrating accumulation of unsaturated hydrophobic lipid only in the intima-inner media area. ×200. e PAN staining for free and esterified cholesterol with localization only in the intima and inner media. ×80. f Same section as Fig. 2e, but under polarizing filters demonstrating birefringent material in the intimal area, presumably cholesterol. ×80. g and h Adjacent areas demonstrating intimal thickening (toluidine blue stained) and a patent endothelial lining. Many spindle-shaped cells can be seen, but almost no lipid droplets. ×300

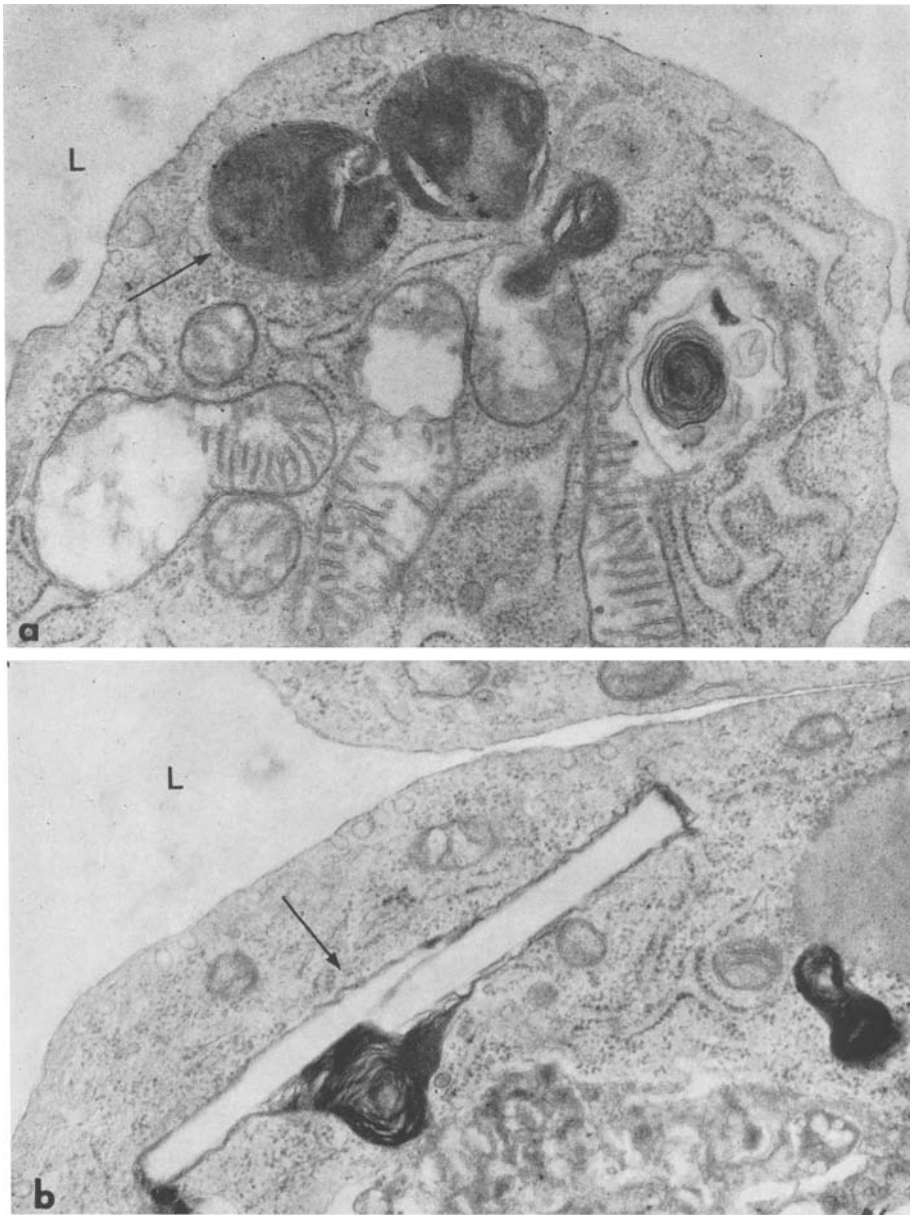

Fig. 3a and b. The endothelial lining of the rabbit common carotid artery five days after double-ligation and injection with human serum pre-beta lipoproteins. a Note the membrane-bound lamellated bodies (arrow) which are suggested to contain exogenously-derived cholesterol. In some cases they may be derived from degenerating mitochondria as seen on the right. The membranous sheaths are fairly well oriented with a periodicity of about 100 A. b Note the large cleft attached to a lamellated body (arrow). This is assumed to be a cholesterol crystal which has been extracted during the electron microscopy procedure. *L* lumen. a and b $\times 30000$

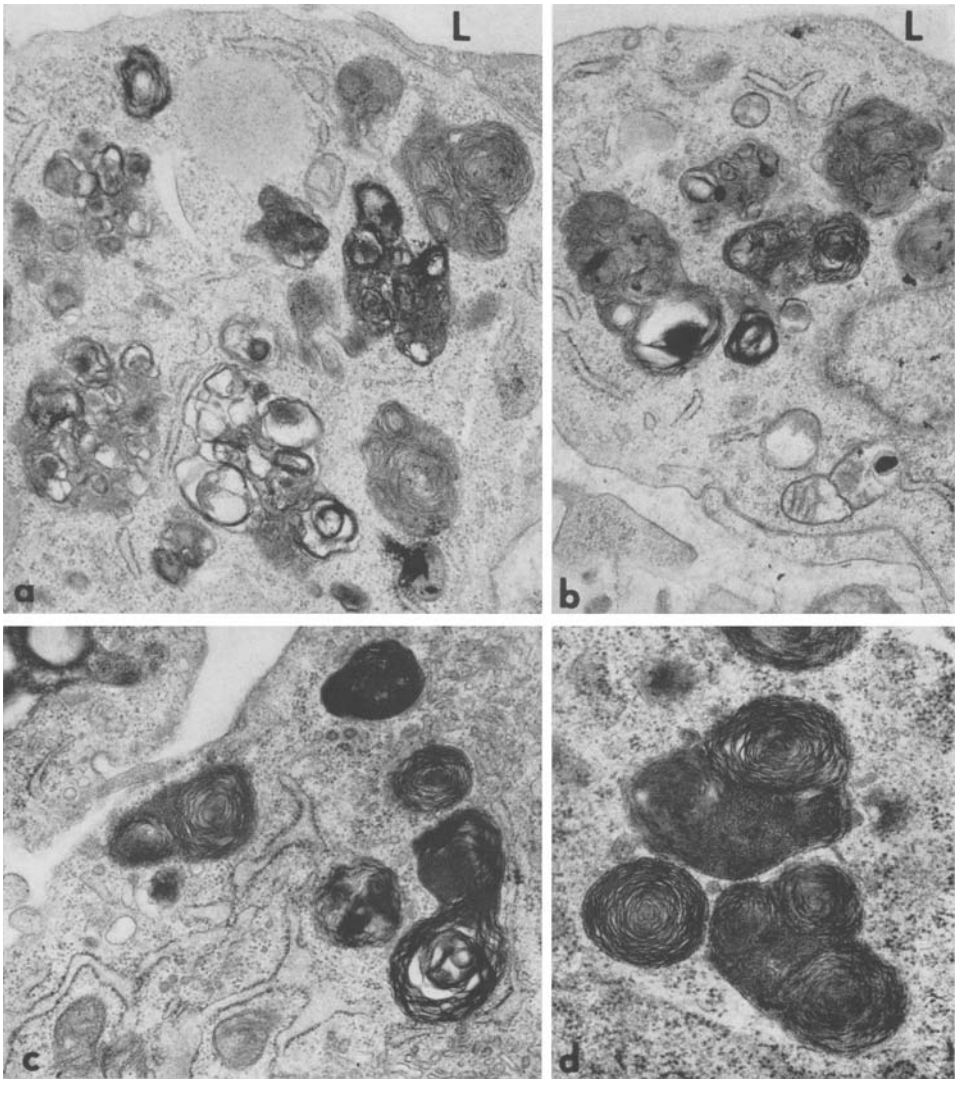

Fig. 4a–d. Lamellated bodies with disoriented sheaths in endothelial (a and b) and smooth muscle cells (c and d) of the rabbit common carotid artery five days after double-ligation and injection with human serum pre-beta lipoproteins. Note the variety in density and periodicity of the membranes in these organelles. a–c ×20000; d ×26000

gether to form an amorphous mass. The localization of these aggregates coincides with the sites of PAN staining (Fig. 2e). Occasionally it appeared that the entire mass was membrane-bound (Fig. 7). From one to three days, amorphous droplets presumably containing triglycerides were also seen in abundance, especially in the media, but from eleven to seventeen days they were rarely present.

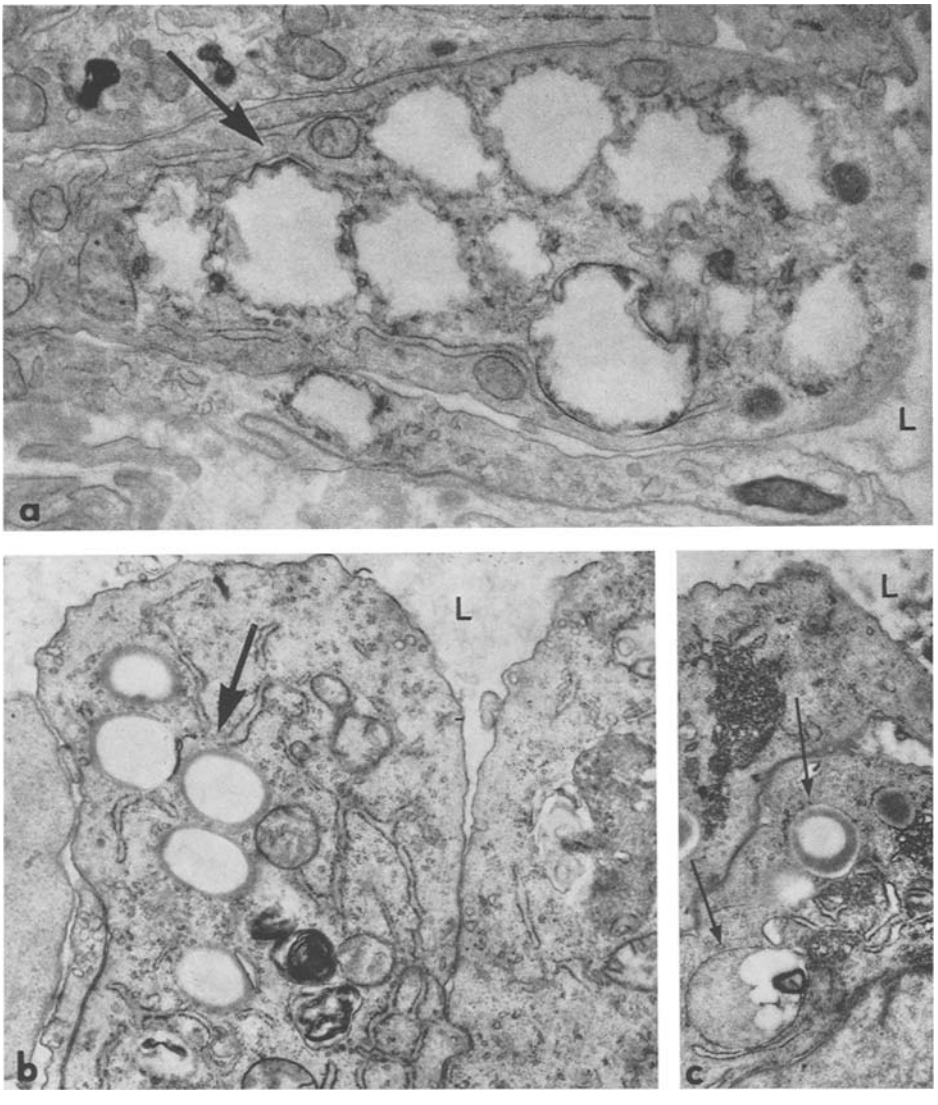

Fig. 5a–c. Endothelial lining of the rabbit common carotid artery after double-ligation and injection with human serum beta lipoproteins. Note the round vacuoles (arrows) assumed to have contained cholesterol which has been extracted. a (Fixation in buffered OsO_4) the vacuoles have a crenated surface while in b and c (prefixation in buffered glutaraldehyde) their surfaces are round. (Although some of the vacuoles in (a) look like degenerating mitochondria, it should be pointed out that neighboring mitochondria appear intact.) In (c) the material in the vacuoles appears only partially extracted. *L* lumen. a eleven days post-ligation ×20000; b four days post-ligation ×18000; c two days post-ligation ×18000

Discussion

The purpose of this experiment has been to study the sequential changes in the ligated artery following injections of lipoproteins with varying cholesterol concentrations. Human beta lipoproteins contain almost 60% total cholesterol per

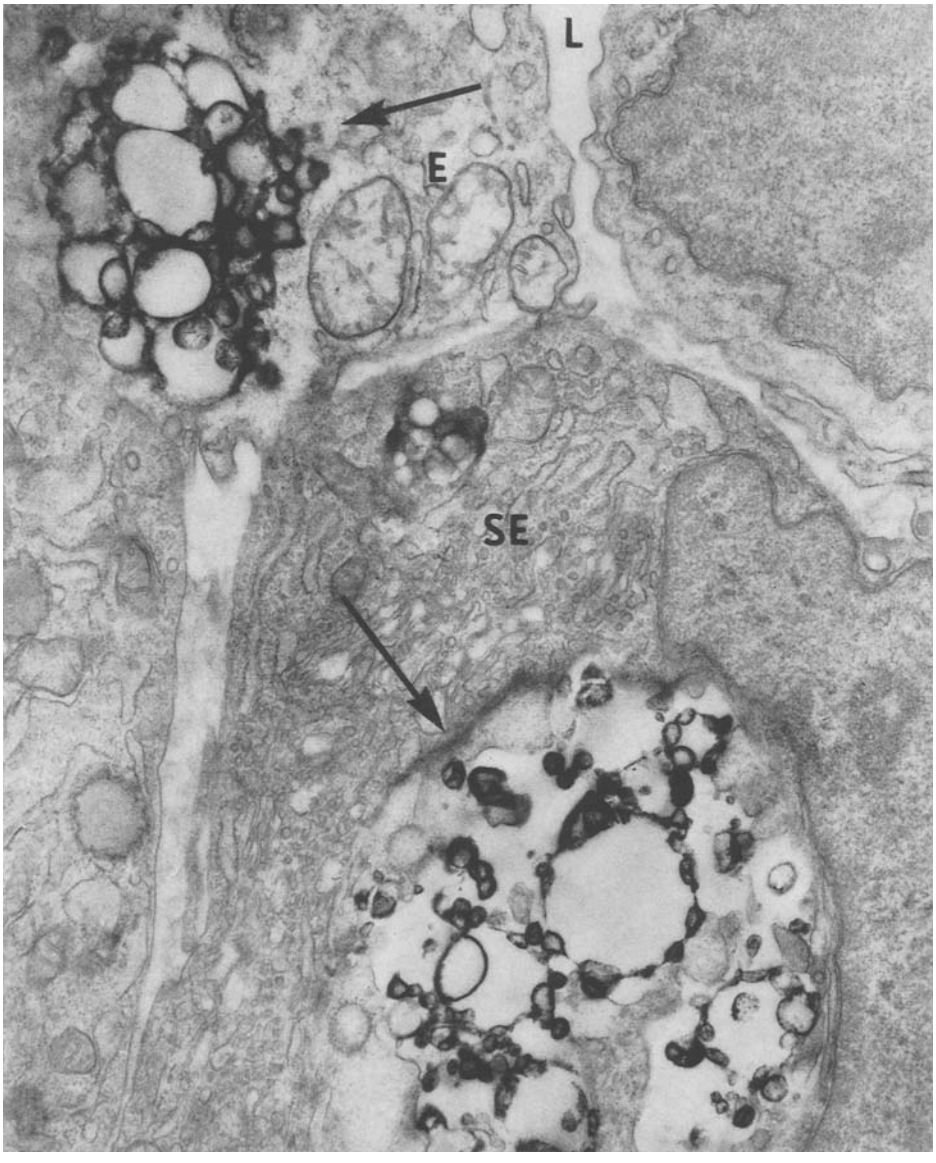

Fig. 6. The rabbit common carotid artery eleven days post-ligation and injection with human serum beta lipoproteins. Note the large aggregate of electron translucent vacuoles with dense surfaces in endothelial (*E*) and subendothelial cells (*SE*). They are assumed to have contained cholesterol which has been extracted, and correspond to the PAN positive material shown in Fig. 2e. *L* lumen. ×25000

total lipid, while human pre-beta lipoproteins contain only between 10 and 25% total cholesterol per total lipid (Frederickson *et al.*, 1967). It is therefore not surprising that accumulations of cholesterol following injections of the former lipoprotein are greater than following the latter. Intriguing was the difference in the

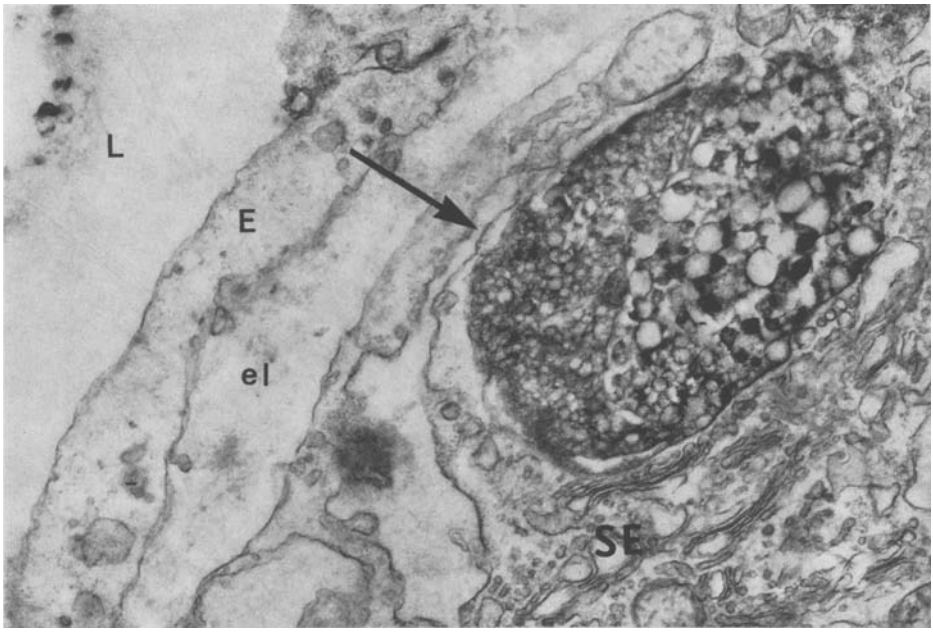

Fig. 7. Same as Fig. 6. Note the cholesterol-containing vacuole in the sub-endothelial cell (*SE*) which is closely packed and membrane-bound. *E* endothelial cell, *el* elastic membrane, *L* lumen. a ×16000, b ×24000

ultrastructural features of cholesterol. They were seen mainly as lamellated bodies following pre-beta lipoprotein injections and as vacuoles with halos or electron-dense limiting membranes following injections of beta lipoproteins. Lamellated droplets have also been seen in atherosclerotic arteries (Weller *et al.*, 1968; Imai and Thomas, 1968; Parker and Odland, 1966). Weller *et al.* (1968), suggested that this lamellar structure consisted of exogenously-derived free cholesterol within the arterial wall, together with endogenous phospholipids. They further suggested that the less-organized type of lamellated bodies represented a gradual shift of the cholesterol to an esterified form, which becomes amorphous. Using polarized light, Weller (1967) showed that as these bodies shifted from lamellated structures to amorphous droplets, they also changed from anisotropic to isotropic droplets. This corresponded to esterification of cholesterol, catalyzed by a tissue acyl-transferase which transfers fatty acids from phospholipids to cholesterol (Weller, *et al.*, 1968; Adams, 1967). Why such bodies were seen only in the pre-beta lipoprotein portion of this study is still unclear. They differ in morphology from the lysosomal-like bodies which appear to have both phagocytotic and autophagic properties. As expected, these latter bodies are rich in acid phosphatase, as seen following double-ligation and injection with physiologic saline (Hoff, 1970a, b). One cannot however, rule out that some of the concentric structures are derived from degenerating mitochondria, as is perhaps suggested by some of the structures in Fig. 3a.

The cholesterol clefts, seen on rare occasions in these studies, corresponded to those observed in human and animal atherosclerotic arteries (Marshall *et al.*, 1966; Parker and Odland, 1966; Still, 1963; Ghidoni and O'Neal, 1967; Simpson and Harms, 1969), as were numerous vacuoles with presumably extracted centers and assumed to contain cholesterol (Geer, 1965; Marshall *et al.*, 1966; Ghidoni and O'Neal, 1967; Parker and Odland, 1966; Still, 1963). Clear vacuoles with very electron-dense borders resembling those seen in large aggregates in this study have also been observed in human atheroma, though localized extracellularly (Geer, 1965). The vacuoles with ruffled borders were also seen in atherosclerotic arteries following fixation in buffered OsO_4 alone (Geer, 1965; Knierem, 1967; Parker and Odland, 1966).

From this study alone it is impossible to ascertain the mode of cholesterol uptake by the arterial cells. It is assumed that the lipoprotein molecule is broken up at the cell surface (Robertson, 1967) and that free cholesterol enters passively, governed only by physical-chemical principles (Newman and Zilversmit, 1966; Dayton and Hashimoto, 1966; Hashimoto and Dayton, 1966). Material that might correspond to lipoproteins was not seen in the cell cytoplasm. However, the large aggregates of cholesterol-containing vacuoles (Figs. 6 and 7), often surrounded by a limiting membrane may represent a coalescence of phagocytozed lipoproteins.

Some interesting findings have emerged from the semi-quantitative determination of lipid within the artery by counting cells containing at least one lipid droplet. Up to about two days post-ligation, the increase in cells with lipid droplets parallelled that found in similar studies following injections with physiologic saline (presumed to represent the effect of double-ligation alone). For periods ranging from two to eleven days post-ligation, however, the percentage of cells with lipid droplets increased to levels as high as 85% following lipoprotein injections, compared to maximum values of lower than 50% in the physiologic saline injected arteries (Hoff, 1970a). At later times (after eleven days) the values in both series converged again. The results suggest that the initial increase in lipid droplets represents endogenously-derived lipid, perhaps the result of "fatty degeneration" due to the reduction in oxygen and nutrients to the tissue (Hoff, 1970a), while the massive accumulations at times between two and eleven days represent exogenous lipoprotein-derived lipid, localized predominantly in the intima and inner media. The gradual decrease of intracellular droplets at later times in both series of experiment may signify that the artery is becoming better able to remove the lipid accumulations, perhaps owing to the return of nutrients and oxygen by collateral circulation (Hoff, 1970a). Very little lipoprotein was found in the lumen at these stages. Cholesterol, however, did not appear to be removed from the artery as is suggested by its persistence at later times even after most of the triglyceride has been removed. This is in agreement with studies of the lipid composition of human atherosclerotic arteries in which triglyceride levels were low (Abdulla *et al.*, 1969), while cholesterol, especially in its free form was present in high concentrations (Adams, 1967). Apparently the artery is unable to entirely remove cholesterol (Adams, 1967; Kritchevsky, 1970). This was also demonstrated in other experimental models combining injury with hypercholesterolemia (Courtice and Schmidt-Diedrichs, 1962; Friedman and Byers, 1964).

In conclusion, it has been shown that when combining double-ligation of the rabbit common carotid artery with injections into the arterial lumen of human beta or pre-beta lipoproteins, one obtains a series of morphologic changes similar to those found in human atherosclerosis, such as the massive accumulation of cholesterol-rich lipid in the intima-inner media area. The presence of abundant cholesterol within the artery at times when triglyceride accumulations had been removed appears to represent a still very early stage in the development of atherosclerosis, and suggests that this accumulation of cholesterol becomes the main sclerogenic agent, eventually leading to the formation of an atheromatous plaque (Adams, 1967).

Acknowledgments. The author wishes to thank Mrs. Falkenberg for technical assistance. This work was performed while Dr. Hoff was an Advanced Research Fellow of the American Heart Association.

References

Abdulla, Y., Adams, D., Bayliss, O.: Relative absence of triglyceride in coronary atherosclerotic lesions. J. Atheroscler. Res. **12**, 149–152 (1969).

Adams, C.: Vascular histochemistry. London: Lloyd-Luke Ltd. 1967.

Brown, D.: Blood lipids and lipoproteins in atherogenesis. Amer. J. Med. **46**, 691–704 (1969).

Courtice, F., Schmidt-Diedrichs, A.: Lipid deposition in the injured wall of the carotid artery in the hypercholesterolaemic-hyperlipoemic rabbit. Quart. J. exp. Physiol. **47**, 228–237 (1962).

Dayton, S., Hashimoto, S.: Movement of labelled cholesterol between plasma lipoprotein and normal arterial wall across the intimal surface. Circulat. Res. **19**, 1041–1049 (1966).

Frantz, I., Moore, R.: The sterol hypothesis in atherogenesis. Amer. J. Med. **46**, 684–690 (1969).

Fredrickson, D., Levy, R., Lees, R.: Medical progress, fat transport in lipoproteins—an integrated approach to mechanisms and disorders. New Engl. J. Med. **276**, 1–46 (1967).

Friedman, M., Byers, S.: Induction of arterial lipogenesis by red cells. Amer. J. Path. **45**, 917–927 (1964).

— — St. George, S.: Site or origin of the luminal foam cells of atherosclerosis. Amer. J. clin. Path. **45**, 238–246 (1966).

Geer, J.: Fine structure of human aortic intimal thickening and fatty streaks. Lab. Invest. **14**, 1764–1783 (1965).

Ghidoni, J., O'Neal, R.: Recent advances in molecular pathology: a review. Ultrastructure of human atheroma. Exp. molec. Path. **7**, 376–399 (1967).

Gofman, J., Lindgren, F., Jones, H., Lyon, T., Elliott, H., Strisower, B.: Blood lipids and human atherosclerosis. Circulation **2**, 161–178 (1950).

Hashimoto, S., Dayton, S.: Transfer of cholesterol and cholesteryl ester into the wall of rat aorta *in vitro*. J. Atheroscler. Res. **6**, 580–590 (1966).

Hoff, H. F.: Studies on the pathogenesis of atherosclerosis with experimental model systems. III. An ultrastructural and lipid-histochemical study on the effect of injections of physiologic saline into the lumen of the doubly-ligated rabbit common carotid artery. Virchows Arch. Abt. A Path. Anat. **351**, 179–192 (1970a).

— Effect of hypoxia on phosphoesterases, oxidative and glycolytic enzymes in the rabbit common carotid artery. A histochemical study. Histochemie **23**, 244–253 (1970b).

— Gottlob, R.: Studies on the pathogenesis of atherosclerosis with experimental model systems. I. An electron microscopy study of the effect of artificial fat-emulsion injections into the lumen of doubly-ligated rabbit carotid arteries. Virchows Arch. Abt. A Path. Anat. **347**, 1–15 (1969a).

— — Studies on the pathogenesis of atherosclerosis with experimental model systems. II. An electron microscopy study on the uptake of egg lipoproteins by endothelial and smooth muscle cells of the doubly-ligated rabbit carotid artery. Virchows Arch., Abt. A Path. Anat. **348**, 77–88 (1969b).

Imai, H., Thomas, W.: Cerebral atherosclerosis in swine: Role of necrosis in progression of diet-induced lesions from proliferative to atheromatous stage. Exp. molec. Path. **8**, 330–357 (1968).

Knierem, H. J.: Electron-microscopic study of bovine arteriosclerotic lesions. Amer. J. Path. **50**, 1035–1065 (1967).

Kritchevsky, D.: Current concepts in the genesis of the atherosclerotic plaque. In: Atherosclerotic Vascular Disease (Brest, A., and Moyer, J., eds.), p. 1–7. New York: Appelton-Century-Crofts 1967.

Lindgren, F., Nichols, A., Freeman, N., Wells, R., Wing, L., Gullberg, J.: Analysis of low density lipoproteins by preparative ultracentrifugation and refractometry. J. Lipid Res. **5**, 68–74 (1964).

Marshall, J., Adams, J., O'Neal, R., DeBakey, M.: The ultrastructure of uncomplicated human atheroma in surgically resected aortas. J. Atheroscler. Res. **6**, 120–131 (1966).

Newman, H., Zilversmit, D.: Uptake and release of cholesterol by rabbit atheromatous lesions. Circulat. Res. **18**, 293–302 (1966).

Parker, F., Odland, G.: A correlative histochemical, biochemical and electron microscopic study of experimental atherosclerosis in the rabbit aorta with special reference to the myo-intimal cell. Amer. J. Path. **48**, 197–239 (1966).

Pearse, A.: Histochemistry, theoretical and applied, 3rd ed. London: Churchill 1968.

Robertson, A. L.: Transport of plasma lipoproteins and ultrastructure of human arterial intimacytes in culture. In: Lipid Metabolism in Tissue Culture Cells. The Wistar Symposium Monograph **6**, 115–128 (1967).

Simpson, C., Harms, R.: Aortic atherosclerosis of turkeys induced by feeding of cholesterol. J. Atheroscler. Res. **10**, 63–75 (1969).

Still, W.: An electron microscope study of cholesterol atherosclerosis in the rabbit. Exp. molec. Path. **2**, 491–502 (1963).

Weller, R.: Cytochemistry of lipids in atherosclerosis **94**, 171–182 (1967).

— Clark, R., Oswald, W.: Stages in the formation and metabolism of intracellular lipid droplets in atherosclerosis. An electron microscopic and biochemical study. J. Atheroscler. Res. 8, 249–263 (1968).

Dr. Henry F. Hoff
Assistant Professor of Neurology
Baylor College of Medicine
1200 Moursund Avenue
Houston, Texas 77025, U.S.A.