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Ultrastructural findings on lipoproteins in vitro *and in xanthomatous tissue*

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Synopsis. The application of OsO_4 plus $K_3[Fe(CN)_6]$ as a secondary fixative following aldehyde fixation, permitted demonstration of the presence of 30–300 nm 'membrane-bound' particles in xanthomatous tissue.

With the same fixation method, isolated low density lipoprotein particles in a fibrin matrix could be observed in the transmission electron microscope in a way permitting comparison with similarly fixed tissue. However, isolated particles of very low density lipoproteins treated in the same way as low density particles had an irregular appearance and a diameter varying between 30 and 80 nm.

Introduction

Lipoproteins may play an initiating role in atherosclerosis and xanthomatosis (Parker & Odland, 1968; Smith & Slater, 1972; Stein & Stein, 1973; Walton *et al.*, 1973; Wolff & Braun Falco, 1973; Walton *et al.*, 1976). Furthermore, the morphology of the xanthomas and their incidence in cardiovascular diseases differ between patients with increased very low density lipoproteins (VLDL) and those with increased low density lipoproteins (LDL) (Polano *et al.*, 1969; Fredrickson & Levy, 1972; Hessel *et al.*, 1976; Vermeer *et al.*, 1978).

Although the ultrastructural morphology of lipoproteins *in vitro* has been extensively investigated by several workers with negative staining techniques (Forte & Nichols, 1972; Pasquali-Ronchetti *et al.*, 1975), more information on the preservation, visualization, and differentiation of LDL and VLDL in ultrathin sections of atheromatous and xanthomatous tissues is needed.

Hoff & Gaubatz (1975) used an immunoperoxidase technique to demonstrate the

presence of particle-bound apoprotein B in human atheroma at the ultrastructural level. Moreover the visualization of lipids can be increased by the use of OsO_4 plus $K_4[Fe(CN)_6]$ as second fixative (De Bruijn & Den Breejen, 1975). Using this fixative the membranous structure of liposomes and 'membrane bound' particles with a diameter of 30–100 nm in the subendothelial aortic space in experimental atheromatosis in rabbits could be demonstrated (De Bruijn & van Mourik, 1975; Vermeer *et al.*, 1978).

In order to answer the question as to whether such particles were also present in xanthomatous tissue and whether these particles represent serum lipoproteins, isolated human VLDL and LDL particles were embedded by polymerizing fibrinogen and processed in the same way as xanthomatous tissue, to permit comparison.

Materials and methods

Isolation of lipoproteins used in the experiments and embedding of the lipoproteins in the fibrin matrix

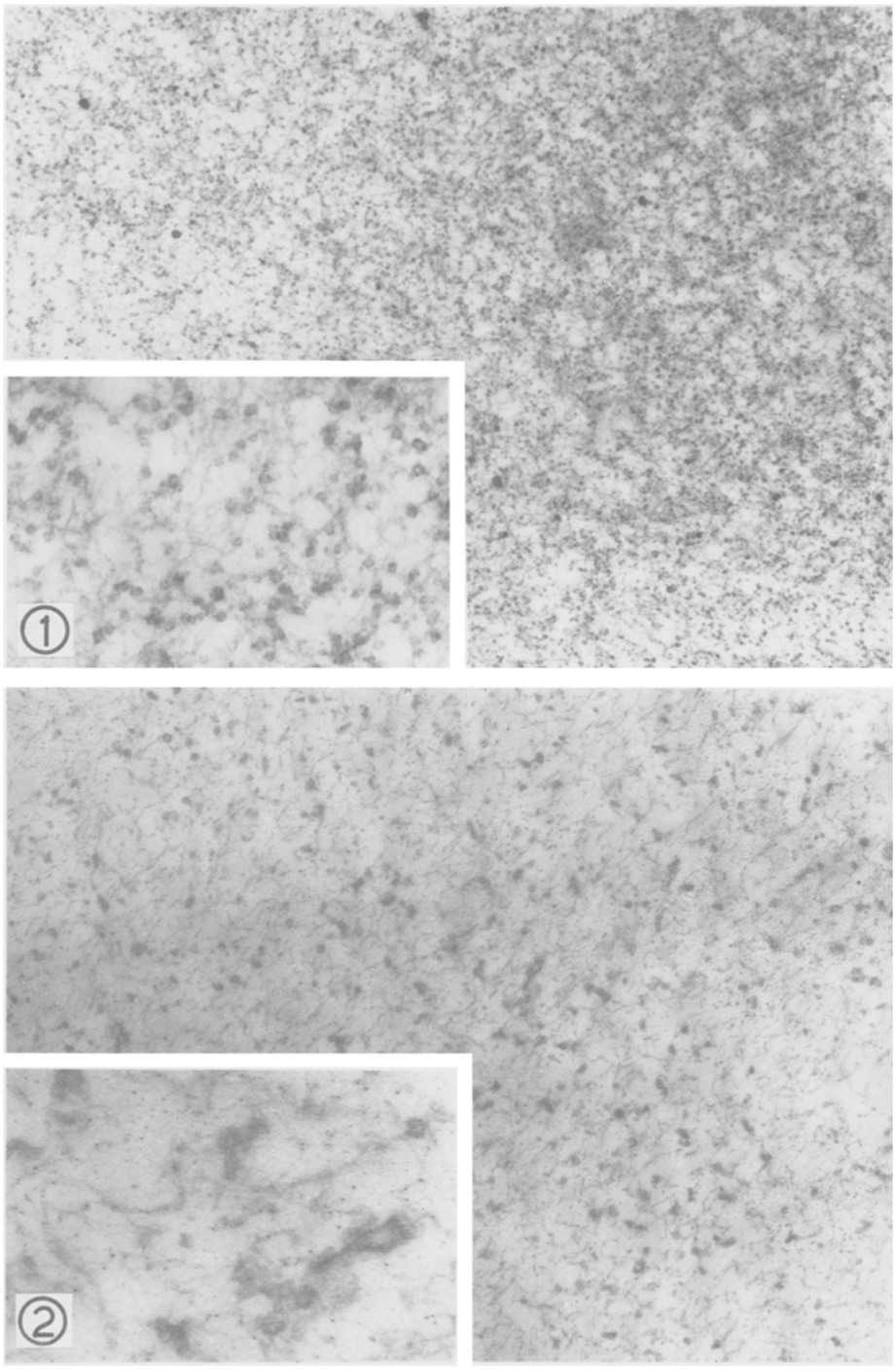
Low density lipoproteins (LDL) present in a pooled serum from patients with hyperlipoproteinaemia phenotype IIa (W.H.O., 1970) were separated by preparative ultracentrifugation according to van der Bijl & van Gent (1975). The very low density lipoproteins (VLDL) were isolated according to van Gent (1972) from the serum of a patient with hyperlipoproteinaemia phenotype IV (W.H.O., 1970).

1.4 ml VLDL in 0.9% NaCl or 1.4 ml LDL in a 9% NaCl solution at a maximal concentration of 20 mg/ml was mixed with 4.6 ml of a 1.3% bovine fibrinogen solution; this solution was solidified by the addition of 0.3 ml thrombin and the obtained fibrin matrix was prepared for electron microscopical investigations as described before (Vermeer *et al.*, 1978).

Abbreviations: PAG = 2% paraformaldehyde, 2.5% glutaraldehyde, in 0.1 M cacodylate buffer (pH 7.4) + 0.05% $CaCl_2$; (+) = Addition of 0.2% digitonin to PAG; (–) = No addition of digitonin to PAG; Os = 1% OsO_4 in cacodylate buffer (0.1 M, pH 7.4) + 0.05% $CaCl_2$; Os^{tc3} = 1% OsO_4 + $K_3[Fe(CN)_6]$ in cacodylate buffer (0.1 M, pH 7.4); Os^{tc4} = 1% OsO_4 + $K_4[Fe(CN)_6]$ in cacodylate buffer (0.1 M, pH 7.4); Eth 70 = Partial dehydration; Ac 100 = Dehydration in an acetone series without the use of propylene oxide; E = Epon.

Figure 1. The LDL particles are homogeneously distributed after isolation and embedding in a fibrin matrix.
PAG/-/Os^{tc4}/Eth 70/E. x 16 000
Inset: the LDL particles have a round vesicular structure and a diameter of 30 nm.
PAG/-/Os^{tc4}/Eth 70/E. x 48 000

Figure 2. The VLDL particles are homogeneously distributed after isolation and embedding in a fibrin matrix.
PAG/-/Os^{tc4}/Eth 70/E. x 16 000
Inset: The VLDL particles are irregularly shaped and have different sizes (30–80 nm).
PAG/-/Os^{tc4}/Eth 70/E. x 48 000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Treatment of tissue samples for electron microscopy

Xanthomatous tissue was collected surgically from patients with various types of hyperlipoproteinaemia and fixed immediately in Flickinger's fixative (1967) (without digitonin) for 23 h at 4°C. After 3 washes for 5 min each, with cacodylate buffer (0.5 M, pH 7.4), the tissue was post-fixed with 1% OsO_4 plus $K_3[Fe(CN)_6]$ in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4°C, dehydrated in an acetone series (up to 100%) without propyleneoxide, and embedded in Epon.

Results

Ultrastructural observations on LDL and VLDL in vitro

After isolation and embedding in a fibrin matrix VLDL and LDL were fixed with Flickinger's fixative and were post-fixed with the OsO_4 plus $K_3[Fe(CN)_6]$.

The LDL material was homogeneously distributed in the fibrin matrix and consisted of darkly stained round vesicular structures with a diameter of 30 nm (Fig. 1). The VLDL material was also homogeneously distributed but the particles were irregularly shaped and differed in size, varying between 30–80 nm (Fig. 2).

Ultrastructural observations on xanthomas

Various types of xanthomas from patients with hyperlipoproteinaemia were investigated.

In the perivascular tissue of the dermis and also below the dermal–epidermal junction zone of eruptive xanthomas, many small particles were present (Figs. 3 & 4). At a higher magnification these small particles were round to oval, varying in diameter from 30 to 300 nm, and surrounded by one or more electron-positive bilayers composed of polar lipids (Fig. 5).

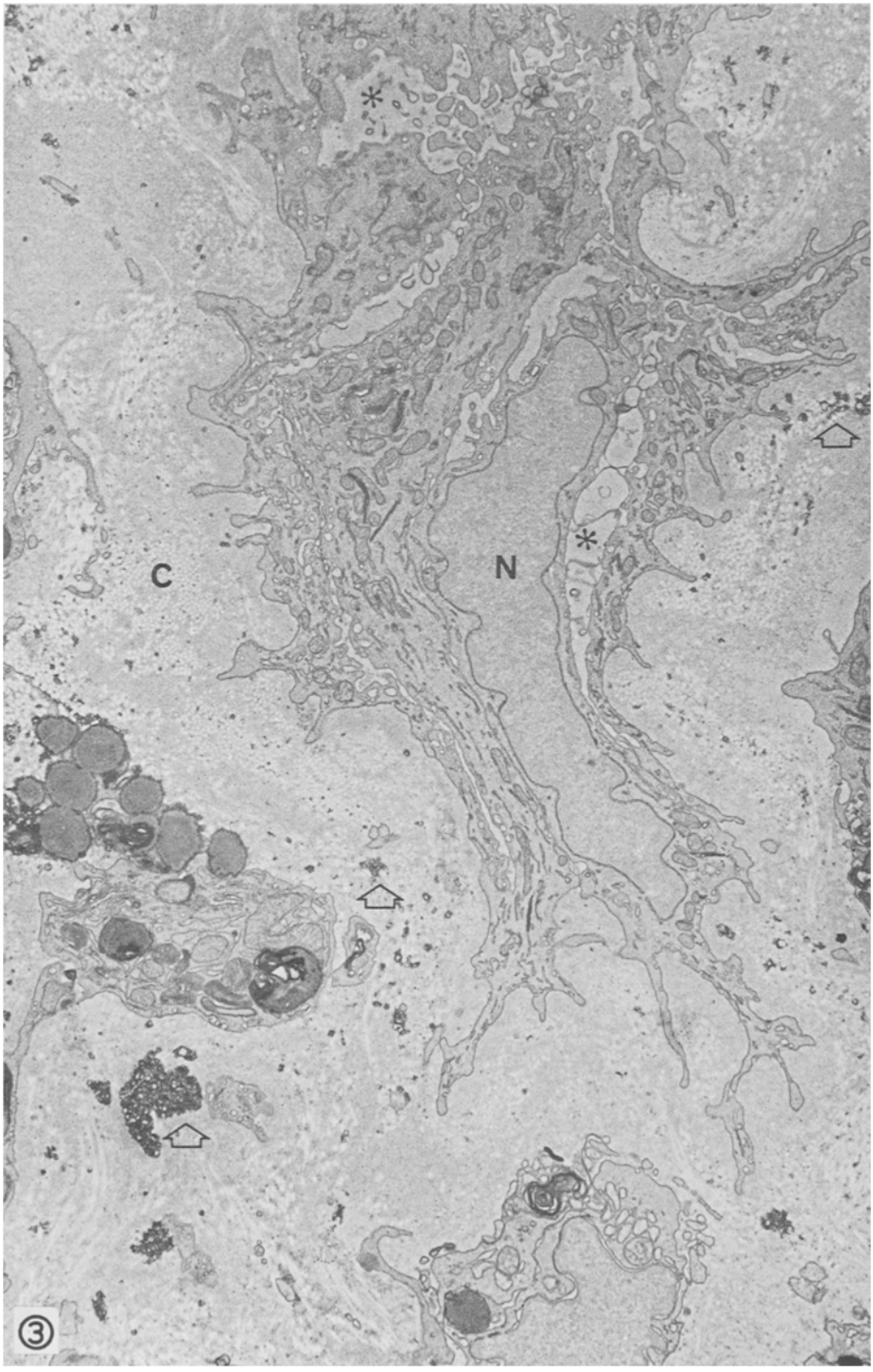
For convenience, we will call these particles membrane-bound, but we are aware that they do not necessarily represent the plasma membrane.

Figure 3. Tissue of eruptive xanthoma showing several small particles (arrows) in the perivascular tissue of the dermis. The asterisks indicate the vascular lumen. C = collagen fibers; N = nucleus.
PAG/-/Os^{tc3}/Ac 100/E. The tissue specimen was also incubated in DAB-containing medium for 1 h after the prefixation step. x 13 000

Figure 4. Eruptive xanthoma. Many small particles (arrows) are localized adjacent to the basement membrane of the epidermis. The asterisk indicates a cholesterol or cholesterolester crystal in basal cell of epidermis. N = nucleus; BM = basement membrane.
PAG/-/Os^{tc3}/Ac 100/E. x 10 000

Figure 5. Detail of particles in Fig. 4, which are surrounded by one or more electron-positive bilayers ('membrane-bound' particles).
PAG/-/Os^{tc3}/Ac 100/E. x 28 000

Figure 6. Tendinous xanthoma with several cholesterol needles (asterisk) and some small particles (arrow).
PAG/-/Os^{tc3}/Ac 100/E. x 31 000


C
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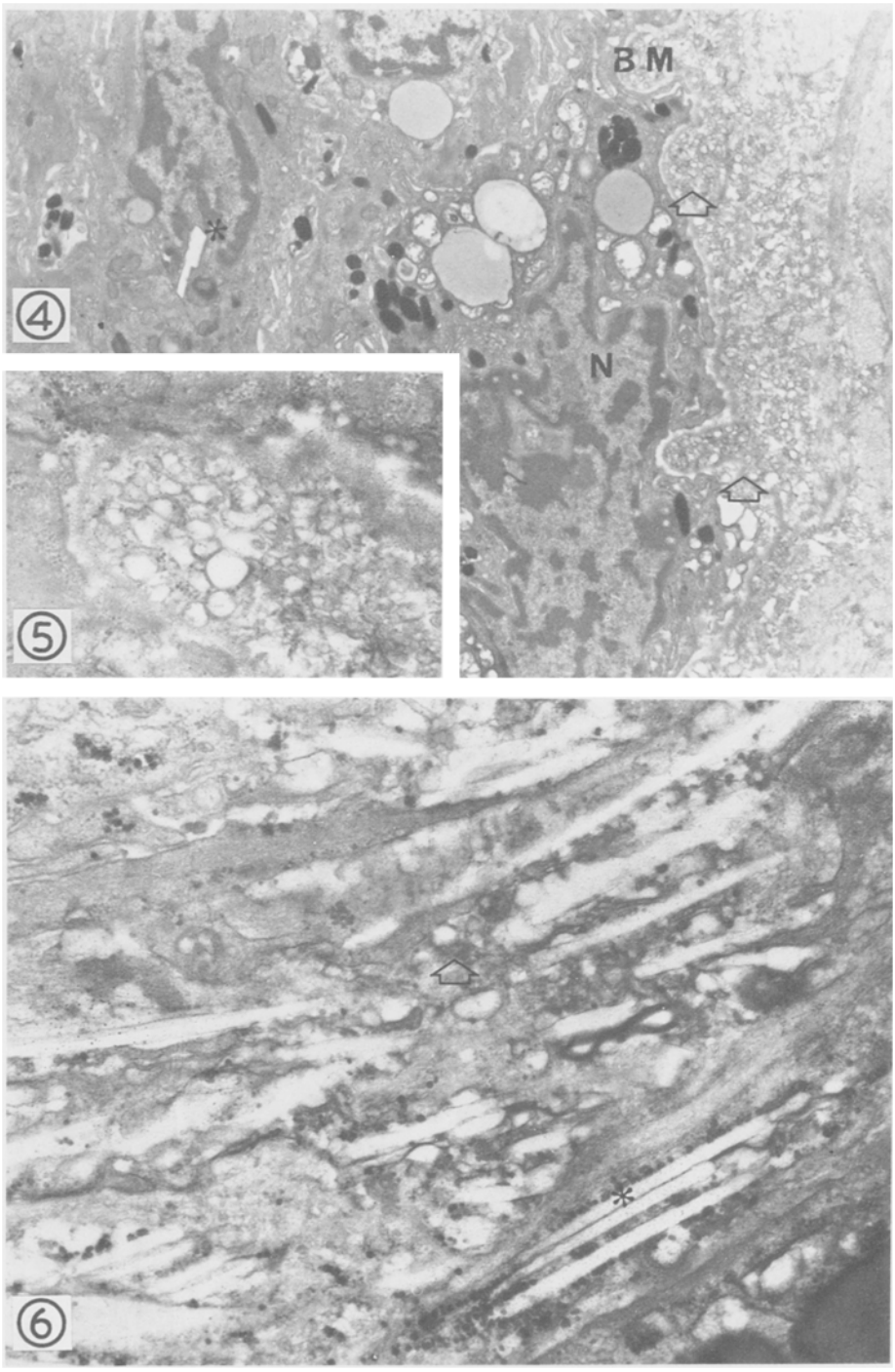
BM
N
④
⑤
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Between the collagen fibres of a tendinous xanthoma and an eruptive xanthoma, several particles of the same size and appearance were found, and many cholesterol clefts were also present in this material (Fig. 6).

Discussion

The increased visualization by the $K_4[Fe(CN)_6]$-containing fixative made it possible to detect LDL embedded in fibrin by conventional transmission electron microscope. Their size and appearance (round vesicle-like structures with a diameter of 30 nm) was in accordance with other investigations on the structure of LDL done with different techniques (Jackson *et al.*, 1976). However, in spite of the fact that we used the same fixation and preparation as for LDL, VLDL, although detectable, was irregular in shape and size. The appearance of these macromolecules was inhomogeneous and round vesicular structures were not found as in other structural studies on VLDL (Schneider *et al.*, 1973). Nevertheless, the variation in the VLDL particle size is in agreement with the results obtained for VLDL with negative staining techniques (Pasquali-Ronchetti *et al.*, 1975). It is conceivable that the fibrin matrix or the isolation procedure influences the shape of the VLDL such that no round vesicular structures remained.

Using a $K_3[Fe(CN)_6]$-containing double fixative for the ultrastructural investigation of xanthomatous tissue, we found 30–100 nm particles consisting of an electron-translucent core surrounded by a membrane-like structure. The same 30–100 nm particles were also observed in experimental atheromatosis in rabbits by de Bruijn (1969) and de Bruijn and van Mourik (1975). Particles of the same size and localization were found in xanthomatous tissue by Parker and Odland (1969) and Braun Falco (1973).

One is tempted to consider these structures as lipid-containing particles capable of passing through the vascular wall and representing lipoproteins and/or components of these, but there is no evidence to support this assumption.

Further characterization of the particles in the xanthomatous tissue is required. So far, only three criteria are available for differentiation between VLDL and LDL particles: differences in mean particle size, differences in lipid composition, and differences in apoproteins. Therefore, as we demonstrated, that digitonin does not contribute to the detection of cholesterol (Vermeer *et al.*, 1978), other methods are needed to localize and differentiate the lipoproteins in tissue on the ultrastructural level. Promising results at the light microscopic level were recently published (Emeis *et al.*, 1977).

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