BIOCHEMICAL AND ULTRASTRUCTURAL INVESTIGATIONS ON HYPERLIPOPROTEINAEMIA AND XANTHOMATOSIS

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A.A.H. KASSENAAR, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 24 OKTOBER 1979 TE KLOKKE 16.15 UUR

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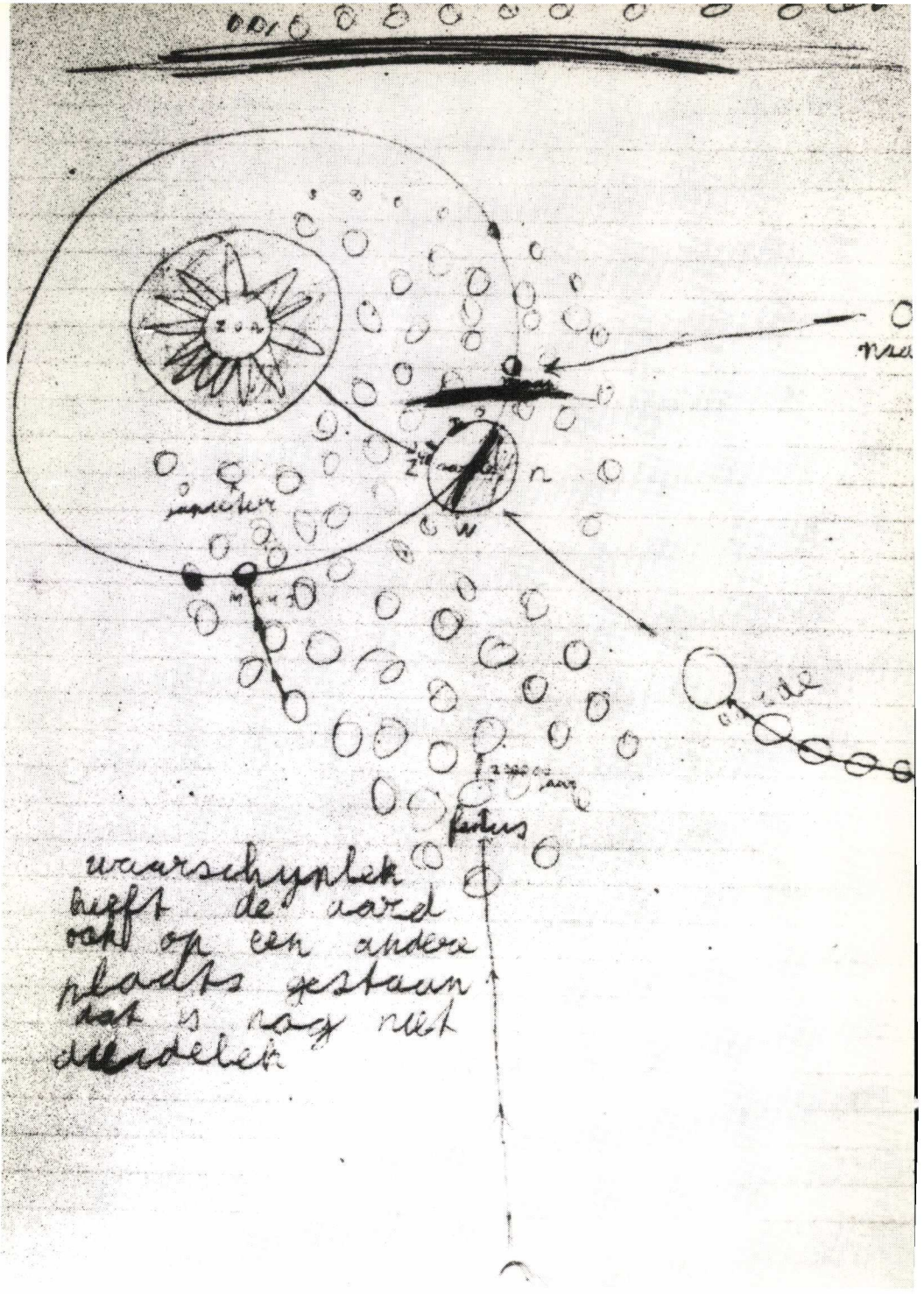
The studies presented in this thesis were performed in the Departments of Electronmicroscopy and Dermatology of the Leiden University Hospital, and in the Gaubius Institute T.N.O., Leiden.
The manuscript was prepared by Clara Horsting-Been, and Gerda Roest, and mrs. I. Seeger-Wolf read the manuscript.

To Anneli
Maarten
Margreetje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CONTENTS

Definitions and abbreviations

Hyperlipidaemia	=	elevated total lipid serum levels
Hyperlipaemia	=	elevated triglyceride serum levels
Hyperlipoproteinaemia	=	elevated lipoprotein serum levels
Hypercholesterolaemia	=	elevated cholesterol serum levels
Fasting serum	=	serum taken at 9 a.m. after overnight fasting and after 48 hours, without the use of C_2H_5OH
Phenotype HLP	=	type of hyperlipoproteinaemia found in fasting serum
HLP	=	hyperlipoproteinaemia
Apo B	=	apoprotein B
Apo AI	=	apoprotein A1
VLDL	=	very low density lipoprotein ($0.94 < d < 1.006$)
LDL	=	low density lipoprotein ($1.006 < d < 1.063$)
HDL	=	high density lipoprotein ($1.063 < d < 1.21$)
IDL	=	intermediate density lipoprotein
ASCVD	=	atherosclerotic vascular disease
WHO	=	World Health Organization

When God made time, He made plenty of it.

(African proverb)

An August midnight

A shaded lamp and a waving blind,
And the beat of a clock from a distant floor:
On this scene enter-winged, horned, and spined-
A longlegs, a moth, and a dumbledore;
While 'mid my page there idly stands
A sleepy fly, that rubs its hands .....

Thus meet we five, in this still place,
At this point of time, at this point in space,
-My guests besmear my new-penned line,
Or bang at the lamp and fall supine.
'God's humblest, they!' I muse. Yet why?
They know Earth-secrets that know not I.

Th. Hardy, 1899.

Chapter I

GENERAL INTRODUCTION

Review of the literature

§ 1 Clinical investigations

The correlation between cardiovascular lesions and xanthomatosis (yellow skin tumors) was described as early as 1872 by Fagge. Xanthomas proved to be histologically characterized by the presence of many foam cells in the dermis (Touton, 1885). Since the publications of Pincus and Pick in 1908 it has been known that most patients with xanthomatosis have hypercholesterolaemia. The extensive studies done by Anitschkow (1913a,b) in rabbits with alimentarily-induced hyperchohesterolaemia showed that many deposits of cholesterol and other lipids resembling atherosclerosis and xanthomatosis occurred in the vascular wall and skin. Speculations on common pathogenic factors that might play a role in atherosclerosis and xanthomatosis were put forward in 1924 by Anitschkow.

Since then, there have been many investigations on xanthomatosis and atherosclerosis in which an attempt was made to find the postulated common pathogenic factors. The xanthomas offer the advantage that the lesions can be easily observed and examined throughout the patient's life.

In 1921, Siemens found that xanthomatous skin lesions could also be present in persons with normal lipid levels. These xanthomas, later called normocholesterolaemic xanthomas, could be distinguished on the basis of clinical criteria from the xanthomas found in hyperlipidaemic patients (Thanhauser and Magendantz, 1938; Montgomery and Osterberg, 1938; Polano, 1940), and it was postulated that the pathogenesis was different for these two types of xanthoma. Only

the xanthomas occurring in patients with elevated lipid levels (hyperlipidaemia) will be discussed in this thesis.

Schaaf and Werner (1930,1931) and Polano (1934,1936, 1940) reported that not only the cholesterol but also the serum phospholipid levels were elevated in xanthomatosis. These findings were regarded as an indication that the emulsification of cholesterol by phospholipid might play an important role in the pathogenesis of lipid depositions.

For more than a century, xanthomatosis was considered an inherited disease (Addison and Gull, 1851), but eventually the familial clustering of patients showing the combination of xanthomas, premature coronary artery disease, and hypercholesterolaemia was recognized (Thanhauser and Magendantz, 1938; Muller, 1939). The condition characterized by elevated cholesterol levels and xanthomas was named hypercholesterolaemic familial xanthomatosis by Thanhauser and Magendantz (1938). The skin lesions found in these patients were called xanthoma tuberosum multiplex (Polano, 1934,1940; Montgomery and Osterberg, 1938).

It was already known that in patients with severe diabetes a special type of xanthomas could be found, and in addition to glucosuria these patients presented lactescence of the serum due to hypertriglyceridaemia. Patients with elevated serum levels of triglycerides and an elevation of serum cholesterol with and without secondary eruptive xanthoma were described by Thanhauser and Magendantz in 1938, and diagnosed by Thanhauser (1958) as idiopathic hyperlipaemia in adults. This clinical syndrome was also occasionally accompanied by glucosuria.

This group differed from patients with xanthomatosis, hyperlipaemia, and severe untreated diabetes and ketosis, who were considered to suffer from symptomatic hyperlipaemia with

secondary eruptive xanthoma. Thanhauser also recognized three other types of symptomatic hyperlipaemia with skin xanthomas: 1) hypercholesterolaemic xanthoma secondary to liver disease, as already described in 1851 by Addison and Gull; 2) eruptive xanthoma secondary to symptomatic hyperlipaemia in chronic pancreatitis (Marcus, 1937), and, 3) eruptive xanthoma secondary to symptomatic hyperlipaemia in lipid nephrosis (Friedman et al., 1949; Crocker, 1951).

The xanthomatosis in symptomatic hyperlipaemia will not be discussed in this thesis.

Only after Gofmann et al. found in 1954 that lipoproteins in which lipids are transported in the blood could be separated and quantitated by analytical ultracentrifugation did further classification of the syndromes including hyperlipidaemia and xanthomas become possible. The lipoproteins can be isolated by ultracentrifugation and electrophoresis, and their names refer to these techniques.

Various authors have described the lipoproteins and their composition (Havel et al., 1955; Carlson and Ericsson, 1975). We shall use here the description given by Fredrickson et al. (1978) (see Tables I and II).

Using the paper-electrophoretic findings as discriminatory criteria, Fredrickson and Lees proposed in 1965 a system for phenotyping of patients suffering from elevated serum levels of lipoproteins (hyperlipoproteinaemia = HLP). In their 1967 paper Fredrickson et al. described five different types of hyperlipoproteinaemia on the basis of paper-electrophoresis and preparative ultracentrifugation. This classification system was a slightly modified version of the one published in 1965, and its essence was accepted in 1970 by the World Health Organization (WHO). Only one type of hyperlipoproteinaemia (HLP type IIb) has been added (in 1970).

Table I Major lipoprotein families

Family name	Ultracentrifugal definition*	Electrophoretic definition†	Range of particle size Å§
Chylomicrons	$d < 0.94$ $S_f^\circ > 400$	Remain at origin in most systems‡	750-12,000
Very low density lipoproteins (VLDL)	$0.94 < d < 1.006$ S_f° 20-400	Pre-β-lipoproteins	300-700
Low density lipoproteins (LDL)	$1.006 < d < 1.063$ S_f° 0-20	β-Lipoproteins	180-300
High density lipoproteins (HDL)	$1.06 < d < 1.21$ $F^\circ_{1.20}$ 0-9	α-Lipoproteins	50-120

*Expressed as densities (d) in grams per milliliter; S_f° is the corrected flotation rate at d = 1.063, expressed in Svedbergs $[10^{-13}$ cm/(sec.dyne.g)]; $F^\circ_{1.20}$ is the corrected flotation rate at d = 1.20.

†On paper, agarose gel, polyacrylamide, and cellulose acetate.

‡On free or starch-block electrophoresis, chylomicrons have alpha mobility.

§As determined by electron microscopy.

Table II Chemical and apolipoprotein composition of the lipoprotein families, percent of dry weight

	Chylomicrons	VLDL	LDL	HDL
Lipoprotein constituents				
Unesterified cholesterol	1-3	4-8	6-8	3-5
Phospholipid	3-6	15-20	18-24	26-32
Protein	1-2	6-10	18-22	45-55
Esterified cholesterol	2-4	16-22	45-50	15-20
Triglyceride	80-95	45-65	4-8	2-7
Apoprotein constituents*				
A-I	Major	Minor	Minor	Major
A-II	Major	Minor	Minor	Major
B	Major	Major	Major	Minor, if present
C-I	Major	Major	Minor	Minor
C-II	Major	Major	Minor	Minor
C-III	Major	Major	Minor	Minor
D	Unknown	Minor, if present	Minor	Minor
E	Unknown	Major	Minor	Minor, if present

*The nomenclature used is described in the text. "Major" refers to proteins making up 7 percent or more of the total protein.

From the Metabolic basis of inherited disease, fourth edition by Fredrickson et al., 1978, p 545.

These six types of hyperlipoproteinaemia defined by the WHO are shown in Table III.

Table III The major abnormal lipoprotein patterns* and their type numbers

Type	Chylomicrons	LDL (β-lp)	VLDL (pre-β-lp)	Floating β-lipo-proteins**
I	+			
IIa		+		
IIb		+	+	
III				+
IV			+	
V	+		+	

*Indicates which lipoprotein "family" (families) occurs in concentration above "normal" in the different abnormal patterns.
**Also known as "broad β-lipoproteins".

From WHO Bulletin 1970, p. 892.

With the exception of hyperlipoproteinaemia III, this classification is based on the amount of lipoprotein lipids in fasting serum under standardized conditions. However, no mention is made of the method required for the quantification of the lipoprotein classes. This caused much confusion in the use of the classification system.

As far as the etiology of the hyperlipoproteinaemia is concerned, the WHO report makes a distinction between primary and secondary hyperlipoproteinaemias. The primary hyperlipoproteinaemias are considered to be due to genetically determined defects in lipid or lipoprotein metabolism or to be caused by environmental factors via an unknown mechanism. In

this system the secondary hyperlipoproteinaemias are associated with other known diseases (pancreatitis, biliary cirrhosis, hypothyreoidy, and also diabetes). Recently, two different monogenic types of hyperlipoproteinaemia IIa and IIb have been described (Glueck et al., 1973; Goldstein et al., 1973; Fredrickson et al., 1978).

It must be kept in mind, however, that the hereditary forms of hyperlipoproteinaemia constitute only a minority of the population presenting the phenotypes hyperlipoproteinaemia IIa and IIb (Motulsky, 1976). Furthermore, because the metabolic defects responsible for the different types of hyperlipoproteinaemia are unknown, use must be made of a descriptive system for their classification.

§ 2 Experimental investigations

Animal model

To study the development of xanthomatous lesions, many investigators have used the xanthomas occurring in rabbits with alimentarily-induced hypercholesterolaemia. These experimental induced xanthomas are predominantly localized on the footsoles and in artificially irritated areas of the skin (Wang, 1957). The investigations of Walton et al. (1973) showed that xanthomas in hypercholesterolaemic rabbits could also be provoked at other sites by increasing the vascular permeability by subcutaneous injection of histamine and bradykinin. Analyses of lipid levels in the serum of rabbits fed with cholesterol showed that the levels of cholesterol, phospholipids, and triglycerides are elevated (Schaaf, 1938; Polano, 1942, and Snellen, 1954). The lipids are transported in lipoproteins whose levels proved to be elevated as well. Contrary to observations in man, a clear distinction between VLDL and LDL could not be made in rabbits (Walton et al., 1973).

Morphological studies

The xanthomatous lesions could conceivably develop by the flow of lipoproteins from the blood into the perivascular tissue with subsequent transformation of perithelial cells and macrophages into foam cells in which the lipoproteins are degraded. This hypothesis is based on the results of the following experiments.

Histologic and histochemical studies done by Weidman (1927), Plewes (1934), Schaaf (1938), and Polano and Snellen (1954) demonstrated an accumulation of lipid-containing cells around the small vessels of the dermis in advanced experimental xanthomas in rabbits. These lesions resemble the histologic findings in human xanthomas.

Extensive ultrastructural studies on experimental xanthomas performed by Parker and Odland (1968) showed that the development of foam cells in the dermis is preceded by a widening of the space between pericytes and endothelial cells and that subsequently lipid droplets can be observed in the perivascular space. The pericytes ultimately changed into foam cells. Foam cells with a different origin are also found in the perivascular space. The results of the ultrastructural studies on human xanthomas (Imaeda, 1960; Parker and Odland, 1969,1973) were in accordance with these findings in experimental xanthomas.

To visualize the lipoproteins in xanthomatous tissue specifically, Walton et al. (1973) used an immunofluorescence technique that reacted monospecifically with apoprotein B (protein moiety of LDL and VLDL). Results obtained with the immunofluorescence technique on the light-microscopical level led Walton et al. (1973) to conclude that in experimental xanthomas apoprotein B occurs in the extracellular compartment in close proximity to the foam cells. Older experimental

xanthomas did not show immunofluorencence (Walton et al., 1973). In human xanthomas only very weakly positive immunofluorescence with anti-apo B was found in some xanthomas (foam cells) by Ishikawa and Sato (1974). Positive immunofluorescence with anti-apo B in the extracellular compartment was seen in human xanthomas by Hoff (personal communication).

To establish the origin of the foam cells, detailed studies on the morphological and enzyme-cytochemical characteristics of these cells have been performed by Parker and Odland (1968) and Wolff and Braun-Falco (1970). The finding of a large number of lysosomes, the presence of microvilli on the surface of the cell, and the absence of a surrounding basal lamina, was regarded as ultrastructural evidence that most foam cells in the perivascular space are transformed macrophages. Cytochemical studies on the early foam cells already containing lipids or cholesterol crystals showed that the cytoplasmic enzyme activity pattern (acid phosphatases and Na-As-D-acetate esterase) was also characteristic for macrophages (Braun-Falco, 1970).

Radioactive labeling and biochemical analysis

Using ^{125}I LDL for in vivo studies, Scott and Winterbourn (1967) showed that an accumulation of radioactivity occurred in actively growing xanthomatous lesions of patients with hyperlipoproteinaemia IIa. The long-term kinetics of serum and xanthoma cholesterol radioactivity in patients with hyperlipoproteinaemia IIa were investigated by Samuel et al. (1972) and Bhattacharya et al. (1976). Isotopic equilibrium between serum and xanthomatous tissue was reached after 70 days and the final half-time decay curves of the xanthomas were significantly longer (200 days) than the slowest half-time decay curves of serum (90 days) (Samuel et al.,

1972). However, using cholesterol radioactivity, Bhattacharya et al. (1976) found an isotopic equilibrium between xanthomas and serum after 50 days and a half-time decay curve of only 90 days for xanthomas. Moreover, in the tuberous and tendinous xanthomas of one patient with hyperlipoproteinaemia III the isotopic equilibrium was much more rapid (< 24 days) and the half-time decay curve was longer (< 70 days).

It was found by lipid analyses of different types of xanthomas that the lipid contents varied considerably (5-10%) (Fletcher and Gloster, 1974; Baes et al., 1968). There was no consistent difference between tendinous and tuberous xanthomas, but xanthomatous lesions younger than 6 months contained relatively more triglyceride (> 10%) and relatively less free cholesterol (< 14%) and less cholesterolesters (< 40%) than xanthomas older than 6 months (Baes et al., 1968). Maintenance of a high fat diet for 10 days in a patient suffering from hyperlipoproteinaemia V led to an increase of up to 40% in the triglyceride content of an eruptive xanthoma (Parker and Short, 1970). Moreover, Parker et al. (1970) also showed that when the eruptive xanthomas resolved during treatment, a small but distinct decrease in the proportion of triglyceride and a definite increase in the proportion of esterified cholesterol occurred. Analyses of the cholesterolester fatty acid pattern in human xanthomas have all shown that the linoleic acid content is decreased and the oleic content increased compared with the cholesterolester fatty acid pattern in plasma (Fletcher and Gloster, 1964; Baes et al., 1968; Parker et al., 1970; Smith and Slater, 1973).

Thus, the radioactive labeling studies and the biochemical analyses also favour the hypothesis that xanthomatous lesions develop under the influence of a flow of lipo-

proteins and that almost all cholesterol is exchangeable with the cholesterol in plasma. In the tissue a cholesterol transesterification takes place, and the removal or breakdown of triglyceride is much more rapid than the clearing of cholesterol and cholesterolesters.

§ 3 Relationship between xanthomatous lesions and atherosclerotic vascular disease

A common pathomechanism shared by atherosclerosis and xanthoma formation was already suggested by Anitschkow (1924). Several aspects will be mentioned here, but without any attempt to be comprehensive.

The ultrastructural findings of Parker (1960), Geer et al. (1961), Geer (1965), and Parker and Odland (1966) suggested that the foam cells which play a primary role in the development of atherosclerosis are derived from smooth muscle cells and macrophages.

The cytochemical investigations of Peters and de Duve (1974), Goldfisher et al. (1975), and Adams and Bayliss (1976), also showed that lysosomal activity, which is characteristic for macrophages, is present in atherosclerotic lesions.

As already mentioned (page 8), the foam cells in xanthomatous lesions are mainly derived from pericytes and macrophages. Presumably, the same cell types are involved in both atherosclerotic and xanthomatous lesions.

Immunochemical investigations using antiserum against apoprotein B showed that apoprotein B was present in the extracellular region of atherosclerotic lesions (Walton and Williamson, 1968; Hoff and Gaubatz, 1975; Walton et al. 1976). Ultrastructural, cholesterol-labeling, biochemical, and quantitive immunochemical studies have all demonstrated

that the flow of lipoproteins into the arterial wall is mainly responsible for the cholesterol accumulation in atherosclerotic lesions (Duncan and Buck, 1959; Adams, 1967; De Bruyn, 1969; Smith and Slater, 1972; Stein and Stein, 1973; Ross and Glomset, 1976; Hoff et al., 1977).

However, in contrast with the findings of Bhattacharya et al. (1976) for xanthomatous tissue, the cholesterol-labeling studies of Jaganatthan et al. (1974) showed no isotopic equilibrium between atherosclerotic lesions and serum cholesterol. The cholesterolester fatty acid pattern was similar in serum and in atherosclerotic lesions (Parker et al., 1966; Parker and Odland, 1966; Smith and Slater, 1972a,b). In contrast with these findings, the cholesterolester fatty acid pattern in the fat-filled cells in the caps of atherosclerotic plaques and in fatty streaks is identical to this pattern in xanthomatous tissue and at variance with it in serum (Smith and Slater, 1973).

On all these grounds it seems reasonable to regard the xanthomatous lesions as a suitable model for the investigation of the accumulation and removal of intracellular lipids, a process which also plays a role in the pathogenesis of atherosclerosis.

The following topics were chosen for investigations:

Clinical

Ia. A natural subdivision within the group of patients with hyperlipoproteinaemia and xanthomatous lesions.

Ib. Recognition of characteristic distribution patterns of the xanthomatous lesions in patients with elevated levels of different classes of lipoprotein.

Experimental

II. Determination of the apoprotein and lipid content of interstitial fluid.

IIIa. Investigations to find out whether digitonin-containing fixative is suitable for specific ultrastructural demonstration of cholesterol.

IIIb. Visualization of the various lipoprotein classes by transmission electron microscopy.

IV. Specific visualization of LDL (apoprotein B) binding to the plasma membrane of cultured (normal and pathologic) cells on the light-microscopical and ultrastructural levels.

§ 4 Clinical investigations

Although laboratory techniques made it possible to distinguish between the six different types of hyperlipoproteinaemia defined by WHO, the question of whether the six different phenotypes of hyperlipoproteinaemia have characteristic clinical features remained to be answered.

The extensive study of Fredrickson et al. in 1967 showed a difference in the appearance of xanthomatous lesions in patients suffering from different types of hyperlipoproteinaemia. In this study the lipoprotein levels were quantified

by the determination of cholesterol in the different lipoprotein classes separated by preparative ultracentrifugation. The presence of the so-called floating beta lipoprotein, which was regarded as a characteristic criterion for hyperlipoproteinaemia III, was determined by paper-electrophoresis of VLDL. Polano et al. (1969) gave a different classification system for hyperlipoproteinaemias with concomittant xanthomatosis, the weights of total lipids in the lipoprotein classes isolated by preparative ultracentrifugation being used to quantify the lipoproteins. To define the different types of hyperlipoproteinaemia more precisely, further investigations were needed. In a study performed in 74 patients with hyperlipoproteinaemia (see Chapter II, section 1) we attempted to find a natural subdivision between patients with hyperlipoproteinaemia and xanthomas. In addition, the clinical features of xanthomatous patients with elevated LDL serum levels were compared with those of xanthomatous patients with elevated VLDL serum levels. Further study was needed to explore the possibility of further differentiation of patients with elevated VLDL levels. The results of this done in patients with elevated VLDL levels and making use of the cholesterol VLDL/triglyceride VLDL ratio as additional discriminatory criterion (Hazzard et al., 1972) are described in section 2 of Chapter II.

Unlike in earlier work of our group and in accordance with Fredrickson et al. (1972), we included patients with an abnormal glucose tolerance in this study. A patient with very extensive xanthomatosis who showed improvement after the withdrawal of insulin is described in Chapter II, section 3.

§ 5 Experimental investigations

I. *Determination of lipids and apoproteins in interstitial fluid*

Few data are available concerning the lipid or apoprotein content of interstitial fluid in man (Reichl et al., 1973). To obtain an impression of the interactions between dermal cells and the lipoproteins present in interstitial fluid, more information about the lipid and apoprotein content in this fluid is needed. As a model for investigation of the composition of interstitial fluid, we used the suction blister fluid obtained by mild suction on the skin (Kiistala, 1968). The results are described in section 1 of Chapter III.

II. *Visualization of lipoproteins*

Several authors have advocated the use of a digitonin-containing fixative for cholesterol retention and as a specific marker for the ultrastructural demonstration of cholesterol (Okros, 1968; Frühling et al., 1969; Scallen and Dietert, 1969). The digitonin-containing fixative has also been used for ultrastructural studies on atherosclerosis and xanthomatosis (Triilo, 1971; Albert and Rucker, 1975; Parker and Odland, 1973; Braun-Falco, 1973). However, Levy et al. (1967), Frühling et al. (1970), and Frederik and Klepper (1975) showed that digitonin can also damage this tissue. We investigated the effect of digitonin on the retention of free cholesterol and cholesterolesters when used in the preparation of tissue for ultrastructural studies and, also the ultrastructural appearance of xanthomatous tissue incubated with and without a digitonin-containing fixative. The findings are reported in Chapter IV, section 1. Because it was found that digitonin-containing fixatives were not suitable as a specific marker for cholesterol, other methods were

needed to visualize the lipoproteins. To obtain an impression of the appearance of lipoproteins as seen by transmission electron microscopy, we treated isolated lipoproteins with a post-fixation procedure described by de Bruyn (1969). Xanthomatous tissue treated with the same fixation procedure was also investigated. The findings are described in Chapter IV, section 2. Although similar electron-dense particles were observed both in vitro and in vivo, a more specific visualization procedure for the lipoproteins was still required.

To fill this need, we developed an indirect immunoperoxidase technique for apoprotein B (the protein moiety of LDL and VLDL). This technique enabled us to visualize specifically the apoprotein B on the light-microscopical and ultrastructural levels.

III. *Interaction of cultured cells with LDL*

Since the publications of Goldstein and Brown (1973, 1974), the interaction of cultured cells with lipoproteins has been studied extensively by Stein and Stein (1974), Fogelman et al. (1975), and Mahley et al. (1977). The use of biochemical methods showed that the LDL is bound to the plasma membrane of the cultured cell by a high affinity process. This high affinity process is called specific binding of LDL (for a review, see Goldstein and Brown, 1977).

The purpose of our investigations was to visualize the specific binding to the plasma membrane of cultured fibroblasts. For this purpose, use was made of an indirect immunoperoxidase technique. The results of these studies are described in sections 1 and 2 of Chapter V.

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PRIMARY HYPERLIPOPROTEINEMIA IN XANTHOMATOSIS

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Summary

Blood lipid values, clinical data and effects of therapy are reported on 74 patients with hyperlipidemia and xanthomatosis. A natural subdivision into two groups was observed on the basis of low density lipoprotein lipid values: one corresponding to Frederickson's type II, characterized by elevated low density lipoproteins, tendinous xanthomata, absence of eruptive xanthomata and a high incidence of cardiovascular diseases and the other resembling Frederickson's type III, with elevated very low density lipoproteins, eruptive xanthomata, xanthomata striata palmaria, elevated cholesterol/triglyceride ratios in the very low density lipoproteins and irregular appearance of floating beta lipoproteins. The latter group consisted of 32 patients in whom cardiovascular symptoms were relatively rare, despite mean cholesterol levels of 500 mg/dl.

Introduction

Quantitative evaluation of lipoprotein patterns according to the Frederickson system [1] or the modified Frederickson system [2] has been of great value for the classification of primary hyperlipoproteinemias. Limitations, already recognized by its initiator, arise from uncertain lipid cutoff values, from shifts in phenotype in a given person and, most fundamentally, because the system differentiates between symptoms, not diseases. Particularly for type III, diagnostic criteria are still a subject of debate [3] and several new types of hyperlipoproteinemia have recently been recognized [4].

Our contributions in this field since 1967 have centered on the relationship between primary hyperlipoproteinemia and xanthomatosis [5,6,7]. Discrimina-

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tory analysis of blood lipid values in a series of 41 hyperlipidemic patients with xanthomata revealed a natural division into two groups. This differentiation was most clear-cut when the percentage of low density lipoproteins (LDL) as a fraction of total blood lipids was used as a parameter. A subdivision of either the high percentage LDL group (into types IIa and IIb) or the low percentage LDL group (into types III and IV) was less obvious [8].

In the present paper, we extend and confirm our observations on a larger group of 74 patients, all presenting with xanthomata and primary hyperlipoproteinemia. We also include effects of various types of therapy on serum lipid and lipoprotein levels and on clinical symptoms.

Patients

Our study includes all patients with xanthomata and primary hyperlipidemia (total lipids more than 900 mg/dl) referred to the dermatological department or the cardiological department of Leiden University in the period 1966 to 1974. Their mean age at the time of first analysis was 41 years, range 21—70 years. Exclusion of secondary hyperlipoproteinemias, classification of the various types of xanthomata and diagnosis of cardiovascular diseases were carried out as described earlier [7].

Analytical methods

Blood sampling and lipid and lipoprotein analyses were carried out as before [7]. No advantage was obtained by separating very low density lipoproteins (VLDL) into two fractions [7]; in our present study, these are combined.

The presence of floating beta lipoproteins (beta VLDL) was ascertained as follows: VLDL were isolated by centrifugation of 2 ml serum overlayered with 3 ml of 0.9% saline in a 5 ml tube [9]. A portion of the floating VLDL layer and a portion of the infranatant HDL + LDL fraction were applied side by side to paper strips and submitted to concurrent electrophoresis according to Lees and Hatch [10]. If the VLDL band did not move beyond the LDL band, floating beta lipoproteins were considered to be present.

The ratio of cholesterol to triglyceride (TG) in VLDL fractions was determined by densitometry of silica gel thin-layer chromatograms after visualization by spraying with sulphuric acid followed by heating at 200°C [11].

Results

Classification

Full data for LDL and VLDL lipid concentrations under standardized conditions are given in Figs. 1a and 1b. The distribution of cardiovascular diseases is shown in Fig. 1a and of the various types of xanthomata in Fig. 1b. The two diagrams are superimposable. The distribution of LDL lipid concentrations shown as a bar diagram in Fig. 2 is bimodal with a minimum at 600 mg/dl. This minimum coincides with the 95% upper limit of LDL lipid concentrations in a previously assembled reference group [7] of 118 apparently healthy male Dutch citizens of a mean age of 56 years (range 46—66 years). This 600 mg/dl

limit, drawn as a vertical line in Figs. 1a and 1b, separates the total patient material into two groups.

Group 1 has LDL lipid levels below 600 mg/dl, elevated VLDL levels, a high incidence of xanthomata striata palmaria (27 out of 32) and xanthomata eruptiva (24 out of 32) near absence of xanthomata tendinea (2 out of 32) and a moderate incidence of cardiovascular diseases (7 out of 32).

Group 2 has LDL levels above 600 mg/dl, a high frequency of xanthomata tendinea (37 out of 42), hardly any xanthomata eruptiva (1 out of 42) or

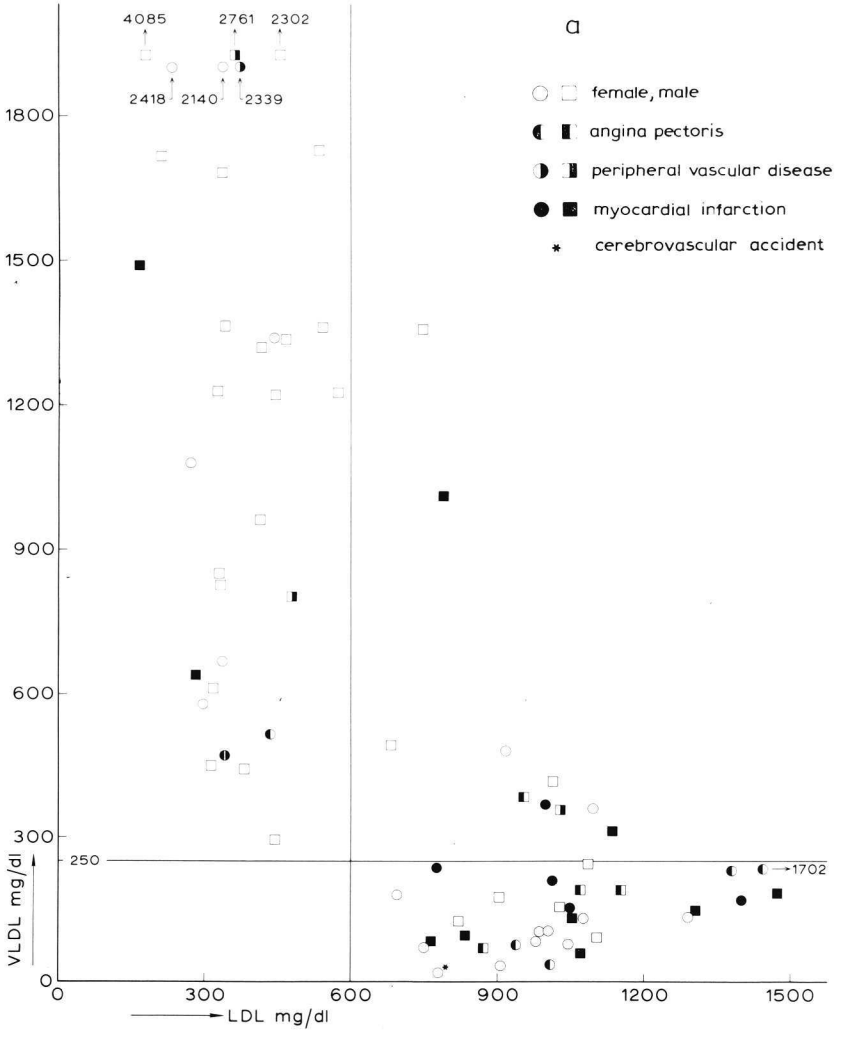

Fig. 1. a. Lipoprotein lipid levels in 74 patients with xanthomatosis and hyperlipidemia. Distribition of cardiovascular symptoms.

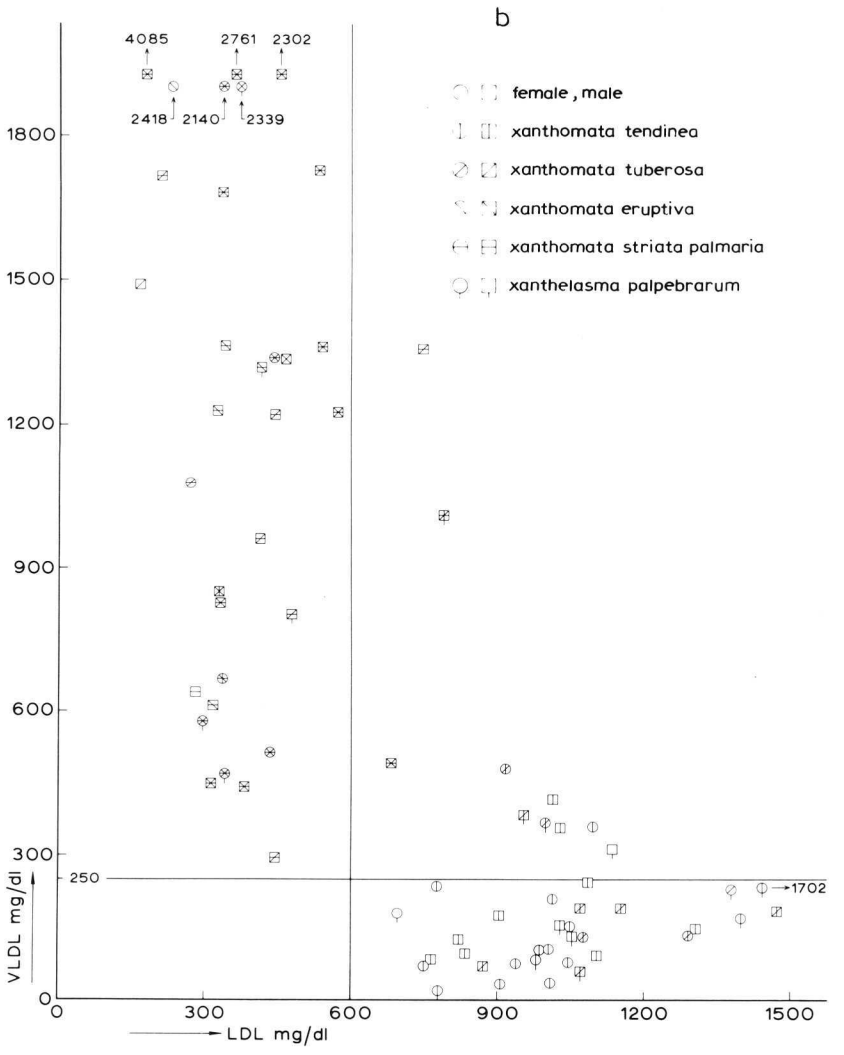

Fig. 1. b. Lipoprotein lipid levels in 74 patients with xanthomatosis and hyperlipidemia. Distribution of xanthomata.

xanthomata striata palmaria (3 out of 42) and a high incidence of cardiovascular diseases (23 out of 42) (Table I).

The mean age of patients with cardiovascular diseases in group 1 was 52 years and, in group 2, 46 years. Group 1 corresponds to Frederickson's types III and/or IV and group 2 to Frederickson's type II. Frederickson's types I and V were not observed in our material.

[illegible] the percentage of LDL lipid as a fraction of total serum lipids is used as a

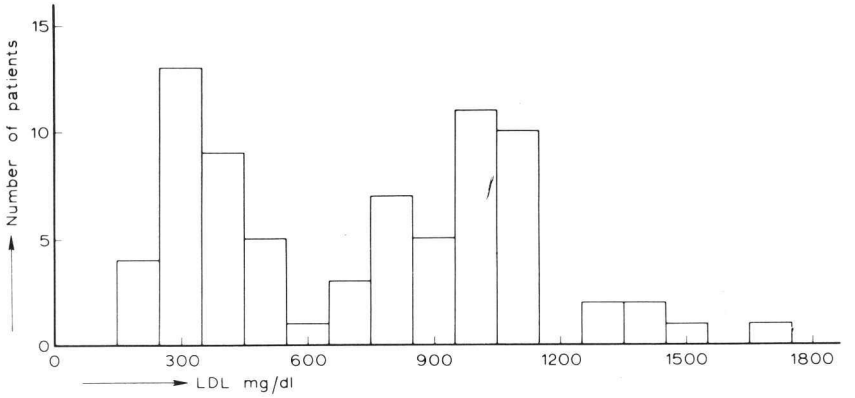

Fig. 2. Frequency distribution of LDL lipid levels in 74 patients with xanthomatosis and hyperlipidemia.

criterion for pattern recognition, almost the same division into two groups is obtained. Thirty-three patients have an LDL percentage below 40; with the exception of the two patients with LDL > 600 mg/dl and VLDL > 1000 mg/dl, these all belong to group 1 above. Only two patients have an LDL percentage between 40 and 58 (i.e. the patients with 440 mg/dl LDL, 290 mg/dl VLDL and 680 mg/dl LDL, 490 mg/dl VLDL). Thirty-nine patients have an LDL percentage above 58; all of these belong to group 2 above.

A further subdivision of group 1 into types III and IV hyperlipoproteinemia was not possible. In half of these patients, beta VLDL appeared and disappeared unpredictably and they were not observed in eight patients.

Thus, using the presence of beta VLDL as a criterion for type III [1], half of the patients in group 1 would be unclassifiable because of inconsistency of this symptom; eight patients would be classified unequivocally as type IV hyperlipoproteinemic in that case.

The VLDL cholesterol/triglyceride ratio of all patients in group 1 was always

TABLE I

DISTRIBUTION OF XANTHOMATA AND CARDIOVASCULAR DISEASES IN 74 HYPERLIPIDEMIC PATIENTS

	Group 1 LDL < 600 mg/dl (n = 32)	Group 2 LDL > 600 mg/dl	
		Subgroup 2A VLDL < 250 mg/dl (n = 32)	Subgroup 2B VLDL > 250 mg/dl (n = 10)
Xanthelasma palpebrarum	5	9	4
Xanthomata tendinea	2	30	7
Xanthomata tuberosa	25	8	6
Xanthomata eruptiva	24	0	1
Xanthomata striata palmaria	27	0	3
Cardiovascular diseases	7	18	5

TABLE II

ALLEGED CHARACTERISTICS FOR TYPE III

Regularly attending patients with xanthomatosis, normal LDL (group 1).

	n; Chol./TG ratio in VLDL under standardized conditions					Total
	Not determined	<0.5	0.5-0.75	0.75-1.0	⩾1.0	
Floating beta observed under standard conditions	1	0	0	2	5	8
Floating beta not observed under standard conditions but appearing during therapy	2	0	2	4	1	9
Floating beta never observed	1	0	4	2	1	8
Total	4	0	6	8	7	25
Xanthoma striata palmaris	4	0	5	6	6	21

more than 0.42 whenever it was measured. According to this criterion, proposed by Hazzard et al. [14] for hypertriglyceridemic patients, they all have to be classified as type III. If the presence of xanthomata striata palmaria would be a valid criterion for type III, the prevalence of this type of xanthomata in group 1 would support this classification.

Table II shows the distribution of various alleged characteristics for type III in those patients who regularly attended the clinic. Beta VLDL were most conspicuously absent in the lower range of VLDL cholesterol/triglyceride ratios. In three of the four patients without xanthomata striata palmaria beta VLDL were repeatedly found.

In group 2, beta VLDL under standardized conditions were observed only once.

The frequency distribution of VLDL concentrations in group 2 shows a shallow minimum between 250 and 300 mg/dl. The 95% upper limit of VLDL lipid

TABLE III

SUMMARY OF LIPID ANALYSES (mg/dl) AND CLINICAL DATA OF XANTHOMA PATIENTS

Figures are presented as means ± 1 S.D. or ranges, in parentheses medians. PL, phospholipid; FFA, free fatty acids.

	Group 1, LDL < 600 mg/dl		Group 2, LDL > 600 mg/dl			
	VLDL > 250 mg/dl		Subgroup 2A, VLDL < 250 mg/dl		Subgroup 2B, VLDL > 250 mg/dl	
	Male	Female	Male	Female	Male	Female
Number	23	9	14	18	7	3
Age (year)	40 ± 8 (41)	51 ± 13 (54)	40 ± 10 (41)	47 ± 13 (47)	32—58 (44)	38—54 (54)
Total lipid	1908 ± 833 (1832)	1849 ± 810 (1585)	1335 ± 224 (1315)	1349 ± 310 (1271)	1451—2256 (1606)	1467—1632 (1552)
Total Chol.	524 ± 202 (496)	489 ± 183 (398)	504 ± 89 (515)	525 ± 116 (503)	470— 565 (541)	527— 543 (535)
Total TG	649 ± 429 (609)	630 ± 428 (555)	142 ± 46 (142)	129 ± 57 (124)	234— 770 (280)	254— 288 (282)
Total PL	455 ± 145 (437)	459 ± 155 (432)	411 ± 70 (393)	409 ± 94 (392)	450— 587 (472)	381— 505 (474)
FFA	29 ± 17 (25)	37 ± 16 (36)	23 ± 11 (21)	20 ± 7 (21)	11— 83 (17)	16— 18 (17)
VLDL	1334 ± 842 (1228)	1283 ± 814 (1078)	139 ± 54 (139)	125 ± 72 (118)	313—1354 (417)	359— 481 (367)
LDL	374 ± 109 (362)	339 ± 69 (338)	1037 ± 195 (1062)	1043 ± 258 (1007)	682—1136 (953)	916—1094 (998)
HDL	134 ± 32 (134)	152 ± 44 (143)	114 ± 33 (109)	143 ± 34 (142)	75— 221 (139)	55— 147 (107)
Total incidence of clinical cardiovascular diseases (%)	17	33	64	50	57	33
Number (male + female)	32		32		10	
Mean age (male + female)	43		44		45	

TABLE IV

RESULTS OF THERAPY OF XANTHOMA PATIENTS

The lipid levels are presented in mg/dl as medians, in parentheses ranges.

	Group 1, LDL < 600 mg/dl		Group 2, LDL > 600 mg/dl			
	VLDL > 250 mg/dl		Subgroup 2A, VLDL < 250 mg/dl		Subgroup 2B, VLDL > 250 mg/dl	
	Male	Female	Male	Female	Male	Female
Patients initially presenting	23	9	14	18	7	3
Patients regularly attending	18	7	11	14	4	1
Patients regularly attending, obese [a]	10	5	2	6	2	—
Obese patients responding [b] to dietary prescription [c]	8	3	—	1	2	
Lipid levels						
Initially						
Total lipid	1933(1082—3029)	2816(1938—2914)		1667	1606 and 2256	
Total cholesterol	533(303— 913)	604(523— 743)		653	541 and 540	
Total triglyceride	575(310—1159)	1020(526—1462)		122	306 and 770	
After normalization of body wt.						
Total lipid	914(708—1533)	897(709—1602)		1368	1392 and 1180	
Total cholesterol	254(219— 442)	259(186— 481)		529	515 and 392	
Total triglyceride	227(140— 340)	217(158— 497)		105	195 and 182	
Non obese patients submitted to dietary prescription [c]	7	1	3	3		
Lipid levels						
Initially						
Total lipid	1320(917—2043)	1284	1313(1255—1626)	1077(986—1709)		
Total cholesterol	409(232— 590)	398	521(451— 616)	474(385— 649)		
Total triglyceride	330(187— 649)	321	173(131— 175)	99(54— 205)		
Under dietary treatment						
Total lipid	747(643— 850)	790	1206(910—1445)	1026(851—1395)		
Total cholesterol	227(199— 255)	198	507(346— 549)	410(332— 558)		
Total triglyceride	141(64— 230)	213	85(82— 202)	85(72— 125)		

Non obese [d] patients submitted to diet and drug prescription	7	1	7	9	2(M)	2(M)	1F
Lipid levels							
Initially							
Total lipid	850(688—1533)	997	1445(1000—1841)	1256(851—1568)	1392 and 1556	1180 and 1537	1552
Total cholesterol	255(219— 442)	309	517(369— 692)	498(332— 612)	515 and 545	392 and 532	553
Total triglyceride	230(103— 340)	249	190(76— 212)	105(47— 156)	195 and 280	182 and 269	254
	← Clofibrate →		← Cholestyramine →			Clofibrate	Perycit
Under drug treatment							
Total lipid	650(498— 816)	783	1141(688—1482)	974(818—1510)	1200 and 1292	921 and 1597	891
Total cholesterol	202(149— 341)	219	373(227— 489)	390(294— 589)	408 and 401	276 and 498	374
Total triglyceride	92(52— 206)	196	167(96— 311)	99(58— 155)	228 and 394	182 and 277	62
Observed remission of xanthomata	17 (94%)	6 (86%)	1 (9%)	1 (7%)		2 (50%)	—

[a] Obesity defined as body weight in kg > (body length in cm − 100) × $\frac{110}{100}$.
[b] By normalization of body weight.
[c] Low cholesterol, low saturated fat for group 2; low carbohydrates for group 1; caloric restriction for obese patients.
[d] Either on entry or after dietary treatment.

in our above-mentioned reference group was 250 mg/dl. This value, drawn as a horizontal line in Figs. 1a and 1b, subdivides group 2 into patients with "normal" VLDL (Frederickson's type IIa) and patients with elevated VLDL (Frederickson's type IIb). The only difference between these two subgroups is the presence of xanthomata striata palmaria in the three patients with the highest VLDL levels. In other respects, these patients were not much different from group 2 as a whole; their lipid levels were as refractory to treatment as those of the other patients in this group.

A summary of mean lipid levels in the various groups is presented in Table III. Cholesterol levels are strongly elevated, mean values being around 500 mg/dl in all of these groups.

Therapy

Results of therapy are summarized in Table IV. Seventy-four percent of the patients initially presenting for therapy attended the clinic regularly thereafter. Patients were treated along commonly recommended lines: first with diet alone and, if no normalization of blood lipids was achieved, with drugs. Dietary prescriptions were caloric restriction for obese patients, carbohydrate restriction for the high VLDL group and cholesterol and saturated fat restriction for the high LDL group. The drug prescribed for the high VLDL group was clofibrate and, for the high LDL group, cholestyramine. In incidental cases, clofibrate or pericyte was given to patients with elevated LDL.

Adherence to dietary prescription by obese patients was 56% as measured by normalization of body weight. In patients with elevated LDL, adherence was very low: only one patient of eight attained a normal body weight.

Effects of therapy on blood lipids were measured at intervals of 1—6 months. Levels achieved by therapy as reported in Table IV represent the lowest stable values.

In all patients, total lipid and total cholesterol levels were lowered as a result of therapy. In group 1 (type III/IV), this resulted in almost complete normalization of serum lipid levels. In group 2 (type II), lowering of cholesterol levels was very modest; a decrease of only 10—20% was commonly observed, resulting in final cholesterol levels averaging about 400 mg/dl. Triglyceride levels in group 2 were affected differently by cholestyramine in the two sexes. In males, triglyceride levels increased in about 7 of 9 persons (signed rank test, $P < 0.01$); in females, triglyceride levels did not change on the average. High density lipoproteins (HDL) levels in group 1 (type III/IV) increased under diet (signed rank test, $P < 0.02$) and under drug therapy (signed rank test, $P < 0.01$). There was no significant change in HDL levels in group 2 (type II) as a result of either diet or drug administration.

Discussion

In evaluating blood lipoprotein levels, we use the total lipid content of the various lipoprotein fractions. Estimating the VLDL fraction by its TG content or the LDL and HDL fractions by their cholesterol content, as is common practice, underestimates VLDL if it contains excessive cholesterol and under-

estimates LDL as well as HDL when these fractions contain abnormally high quantities of triglycerides [4].

Our classification into two groups is based only on the bimodality of the frequency distribution of LDL lipid levels (absolute as well as proportional) and is therefore independent of arbitrarily chosen cut-off values. This bimodality seems to be the result of choosing the presence of xanthomata as a selection criterion. By this criterion, only extreme cases of hypercholesterolemia are included, as is apparent from the average cholesterol level of about 500 mg/dl in both the high VLDL as well as the high LDL group.

The minimum in the frequency distribution of LDL lipid levels coincided with the upper 95% limit of our middle-aged reference group [7].

A natural subdivision of the high LDL group on the basis of VLDL levels in this group and corresponding to Frederickson's types IIa and IIb could be observed but seemed of little diagnostic value.

Diagnostic criteria for type III have been subject to modification since 1965. In Frederickson's later systems, the presence of floating beta was said to be "the one certain test" for type III [13]. Hazzard et al. [14] advocated the use of elevated cholesterol/triglyceride ratios of the VLDL as "a more quantitative approach to its diagnosis" but only for hypertriglyceridemic patients.

In the WHO modification of the Frederickson system, both criteria must be fulfilled [2]. From the time that beta VLDL became suspected as a normal intermediate stage in VLDL catabolism, its use as a marker for type III hyperlipoproteinemia has come under increasing criticism. In a recent report from the Seattle group, the ability to detect beta VLDL is said to be more a function of the sensitivity of the electrophoretic technique than an indication of its absolute presence or absence [17]. If we base our classification on Hazzard's criterion [14], our entire group 1 would consist of type III patients and type IV would be absent. On the other hand, type IV hyperlipoproteinemia is not uncommon; its absence from the present sample could be explained by the choice of xanthomatosis as a selection criterion for the patients. The appearance of xanthomata in type IV is, according to Frederickson, restricted to cases of extreme hypertriglyceridemia [1].

The most remarkable features in the material presented are the high frequency of type III-like patients and the low incidence of cardiovascular symptoms in these patients.

Type III is usually described as a rare disease. In our sample, it was present almost as often as the common disorder type II hyperlipoproteinemia. This is unlikely to be due to the choice of xanthomatosis as a selection criterion, as the frequency of xanthomata in both diseases is said to be roughly equal [1]. If the unsuitability of beta VLDL as a marker for type III is accepted, our large number of type III-like patients could perhaps reflect a much greater prevalence of this type than is usually assumed. The predominance of males in group 1 (23 out of 32), their lower age at detection (10 years earlier than in females) and the high frequency of xanthomata striata palmaria are in accordance with other observations on type III [1]. The low incidence of cardiovascular symptoms seems at variance with reports from other investigators [1,18,19]. It cannot be explained by differences in age or male/female distribution (Table III). There is certainly no bias to low cholesterol levels in our sample: average serum

cholesterol in type III has been reported as being between 282 and 477 mg/dl; in our group 1 it was 515 mg/dl. Peripheral vascular disease is usually found in at least half of all patients with type III and some form of vascular disease in 80% of male type III patients under age 50. In our group 1, we found 5 cases out of 32 with peripheral vascular disease and 3 out of 21 males below 50 with some form of cardiovascular disease. Unless we assume that type III-like patients who develop xanthomata are less suspectible to cardiovascular complications than are comparable patients without xanthomata, we cannot offer an explanation for this finding. A programme for further metabolic studies on these patients is in progress.

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Xanthomatosis and other clinical findings in patients with elevated levels of very low density lipoproteins

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SUMMARY

Forty-six patients with xanthomatosis and elevated very low density lipoproteins (VLDL) levels (in different types of hyperlipoproteinaemia) were classified on the basis of the WHO criteria and the cholesterol/triglyceride ratio in VLDL. A large majority (31/46) of the patients referred to the Department of Dermatology could be classified as hyperlipoproteinaemia type III, only 8/46 as type IIB and 7/46 as type IV/V.

This distinction seems to be relevant as the xanthomatous lesions differed distinctly between these three types of hyperlipoproteinaemia. Xanthochromia striata palmaris was present in 29/31 cases of hyperlipoproteinaemia type III and was not found in type IV/V patients, who had distinctive papuloeruptive xanthomas. During a follow-up in 35/46 patients all xanthomas disappeared within 2 years except the xanthelasma palpebrarum and tendinous xanthomas. All type IV/V patients (7/7) but only one type III patient (1/31) had abnormal glucose tolerance. Only 2/18 type III patients less than 45 years showed claudication and none of the young type III patients had angina pectoris. In contrast, all four type IIB patients less than 45 years had clinical signs of atherosclerosis. However, angina pectoris and/or claudication were present in 5/13 type III patients over 45 years old. The mean serum cholesterol level was equally elevated in both groups but the cholesterol was mainly present in VLDL in type III and in low density lipoproteins (LDL) in type IIB. In 9/31 type III patients the LDL level was also elevated but was easily normalized by a diet low in carbohydrate, whereas the elevated LDL level in type IIB was therapy-resistant. The recognition of xanthomatous lesions, specifically xanthochromia striata palmaris, as an early sign of type III hyperlipoproteinaemia, can lead to the early diagnosis and successful treatment of these patients, and thus possibly prevent the development of premature atherosclerosis.

According to WHO (1970), the serum levels of chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL) are diagnostic criteria for hyperlipoproteinaemia (HLP) of

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types I, IIA, IIB, IV, and V. However, irrespective of the serum levels of VLDL and LDL, the presence of a cholesterol-rich VLDL with abnormal electrophoretic mobility (floating beta) is considered to be the decisive criterion for type III. Because these floating beta lipoproteins are not always detectable in the same patient and furthermore the VLDL are also elevated in most type III patients, it is often impossible to differentiate on the basis of the WHO criteria between this type of hyperlipoproteinaemia and other types in which the VLDL are also elevated.

To define this type III-specific cholesterol-rich VLDL more precisely, Hazzard, Porte & Bierman (1972) proposed the use of the ratio of cholesterol to triglyceride in the VLDL, and considered a cholesterol/triglyceride ratio in the VLDL (C/T-VLDL) higher than 0·42 in hypertriglyceridaemic patients to be specific for type III. A similar criterion was used by Fredrickson, Morganroth & Levy (1975), who considered a ratio of cholesterol in VLDL over triglycerides in serum $>0{\cdot}3$ to be specific for hyperlipoproteinaemia type III.

In addition to the biochemical findings, the xanthomatous lesions in patients with HLP can be very helpful for diagnostic purposes. This paper reports the evaluation and biochemical observations in a series of forty-six patients with elevated serum VLDL levels, xanthomatosis, and hyperlipidaemia (total lipid more than 900 mg/dl) studied between 1966 and 1977. Thirty-five of these patients were followed up for over a year.

PATIENTS AND METHODS

The forty-six patients (mean age 44 years) were referred to the Department of Dermatology of the Leiden University Medical Centre because of xanthomatosis. The age of onset was defined as the age at which hyperlipidaemia, xanthoma or definite vascular disease were first observed.

The patients were classified according to the WHO criteria (Beaumont *et al.*, 1970), taking into account the C/T-VLDL ratio as specified below, which resulted in the following groups:

HLP type III. Thirty-one patients: VLDL elevated, C/T-VLDL $>0{\cdot}5$ (see under Analytical methods). Nine patients showed a temporarily elevated LDL level which was easily normalized by a low calorie diet with carbohydrate restriction.

HLP types IV/V. Seven patients: VLDL elevated, C/T-VLDL $<0{\cdot}5$; LDL normal. HLP type IV = no detectable chylomicrons; HLP type V = detectable chylomicrons.

HLP type IIB. Eight patients: VLDL elevated, LDL constantly elevated, independent of therapy, no chylomicrons.

For follow up studies, 23/31 HLP type III, 5/7 HLP type IV/V and 7/8 HLP type IIB patients were available. The classification of the various types of xanthomas and the diagnosis of atherosclerotic cardiovascular disease (ASCVD) was carried out as described elsewhere (Polano *et al.*, 1969).

Patients were considered obese when they weighed more than 120% of ideal body weight as defined by the Metropolitan Life Insurance Co. Table (1960).

When glucosuria was combined with a capillary blood glucose level of more than 10 mmol/dl, the patient was regarded as having an abnormal glucose tolerance.

We took the upper limit of normal LDL level as total LDL lipid >550 mg/dl. On the basis of a mean cholesterol content of 40% in LDL, this value is comparable to LDL cholesterol 220 mg/dl, which has been proposed as a useful cut-off value by Carlson (1976). The upper limit of normal VLDL level was taken at total VLDL lipid >260 mg/dl. All patients had a fasting triglyceride serum level above 160 mg/dl, which is considered by our group (Hessel *et al.*, 1976) to be the upper limit of the normal level.

Analytical methods

The blood was taken after fasting for 12 h, and after a period of 2 days without consuming C_2H_5OH. The serum samples were stored at 4°C and determined within 2 weeks.

Serum lipoproteins were separated by a combination of ultracentrifugation and precipitation (van Gent, 1972). The lipids were extracted from the lipoproteins according to Folch, Lees & Sloane-Stanley (1957) and were determined by weighing.

Since the small quantity of cholesterol and triglycerides in VLDL is difficult to measure directly by routine techniques, cholesterol/triglyceride ratios in VLDL were determined by densitometry after thin-layer chromatography and charring with sulphuric acid (van Gent, 1968). This method gives a high degree of accuracy. To check the validity of this method, we compared the C/T ratio measured in serum by thin-layer chromatography with the same ratio determined by the colorimetric methods of Abell *et al.* (1952) and Giegel, Ham & Clema (1975) (acetyl acetone reaction) for cholesterol and triglycerides, respectively. For eighteen volunteers, the relationship between colorimetry and densitometry determinations in serum is given by the formula C/T (densitometry) = 0·991 C/T (colorimetry) + 0·033; (r = 0·969, n = 18). The results of colorimetric determination in our laboratory were within the WHO recommended limits (WHO reference laboratory, Prague).

To compare the C/T-VLDL ratio according to Hazzard *et al.* (1972) determined by densitometry with the cholesterol VLDL/triglyceride serum ratio determined by colorimetry according to Fredrickson *et al.* (1975), both methods were applied to the serum of seventeen patients with elevated VLDL levels. The relationship between the two methods is given by the formula C/T VLDL = 1·23 CVLDL/TG serum + 0·15 (r = 0·70, n = 17). We took a C/T VLDL ratio > 0·5 as diagnostic of type III hyperlipoproteinaemia. This ratio correlates with C VLDL/TG serum > 0·28 (Fredrickson *et al.* 1975). The distribution of the C/T VLDL among the xanthomatous patients with elevated VLDL levels is shown in Fig. 1.

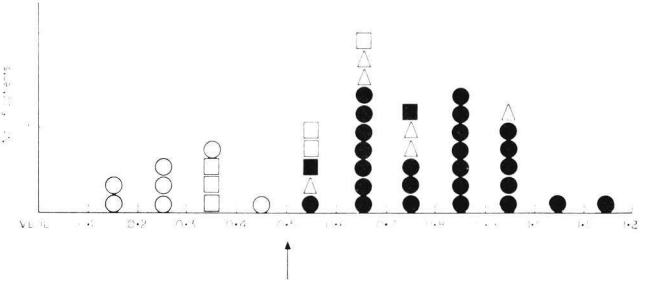

FIGURE 1. Distribution of the cholesterol/triglyceride ratio in VLDL (C/T-VLDL) in forty-six patients with elevated VLDL levels and xanthomatosis. ○ = HLP type IV; □ = HLP type IIB; ■ = HLP type IIB (Fl. $\beta+$); △ = HLP type III (Fl. $\beta-$); ● = HLP type III (Fl. $\beta+$).

RESULTS

Xanthomatous lesions in different types of hyperlipoproteinaemia (HLP types IIB, III, and IV/V)

The distribution of xanthomas and other clinical findings in the various groups of patients with elevated VLDL levels are shown in Table 1.

Xanthochromia striata palmaris (yellow creases in the palms (Fig. 2)) was found in 29/31 type III patients. In only 2/8 patients classified as type IIB (because of the presence of a constantly

TABLE 1. Relevant clinical findings in patients with elevated serum VLDL levels

	Normal LDL C/T-VLDL > 0·50 (phenotype III)	Normal LDL C/T-VLDL < 0·50 (phenotype IV/V)	Elevated LDL C/T-VLDL (not diagnostic) (phenotype IIB)
Mean age and range (years)	44 (25–65)	43 (29–51)	48 (32–60)
Males/females	22/9	6/1	5/3
Floating beta	25/31	0/7	2/8
Abnormal glucose tolerance	1/31	7/7	2/8
Obesity	15/31	7/7	4/8
Xanthochromia striata palmaris	29/31	0/7	2/8
Xanthoma tubero-eruptivum	26/31	0/7	2/8
Xanthoma papulo-eruptivum	20/31	7/7	0/8
Xanthoma tendineum	8/31	0/7	8/8
Xanthelasma palpebrarum	2/31	0/7	4/8
ASCVD	7/31	1/7	7/8
Cholesterol mg/dl	540* (232–913)	466* (326–1,393)	541* (380–569)
Triglyceride mg/dl	503* (187–1,536)	2,186* (315–3,831)	280* (190–722)

* Median values with ranges in parentheses.

elevated LDL level which did not respond to therapy) was xanthochromia striata palmaris present. However, because in these two patients floating beta LP were sometimes present, they were previously classified as type III (Marien, Hulsmans & van Gent, 1974).

The clinical appearance of the eruptive xanthomas in types III and IV/V differed distinctly. In type III patients the papulo-eruptive xanthomas were usually found on the buttocks, elbows and knees and sometimes on the palms of the hands. On the elbows and knees these eruptive xanthomas had a tendency to coalesce and to form lesions which we describe as tubero-eruptive xanthomas (Fig. 3).

In type IV/V patients the lesions were more widely distributed on the trunk, upper thighs, and feet, and were sometimes localized in groups of small papules (Fig. 4). Tendinous xanthomas were found in all type IIB patients (8/8) but only in a minority of the type III patients (8/31).

Among the hyperlipoproteinaemia type III and type IV/V patients there is a strong predominance of males whereas our small group of type IIB patients shows no predominance of either sex.

All type IV/V patients were obese and had an abnormal glucose tolerance, whereas among the type III patients only one had an abnormal glucose tolerance and not more than 50% were obese.

Prevalence and distribution of atherosclerotic and cardiovascular diseases (ASCVD) in various types of hyperlipoproteinaemia (HLP types IIB, III, and IV/V)

The prevalence of ASCVD in the age-group below 50 years is very high in patients with persistently elevated LDL levels (hyperlipoproteinaemia of types IIA and IIB) (Hessel *et al.*, 1976). In contrast, we found a lower prevalence of ASCVD in hyperlipoproteinaemia of types III and IV/V with normal LDL levels.

It is of particular interest that the mean cholesterol level was equally elevated in the different groups of patients. This cholesterol was, however, mainly present in VLDL in type III and type IV/V hyperlipoproteinaemia and in LDL in type IIB (see Table 1).

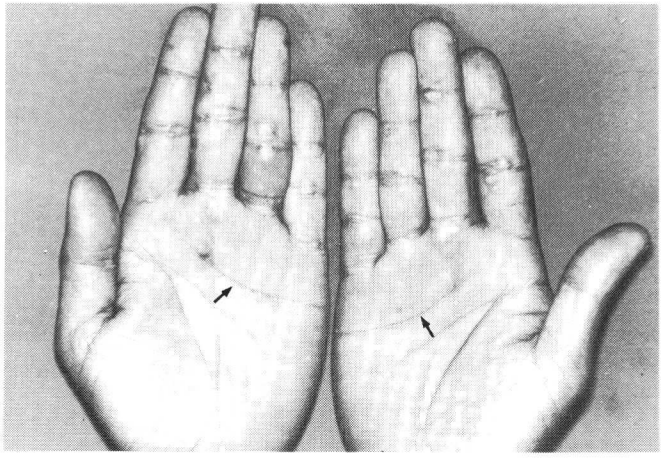

FIGURE 2. Yellow creases in the palms of the hands = xanthochromia striata palmaris (arrows). (HLP type III patient).

The prevalence and symptoms of the ASCVD distributed over the various groups with hyperlipoproteinaemia are illustrated in Table 2.

Intermittent claudication was present in 4/31 type III patients and in 2/8 type IIB patients; and symptoms of coronary sclerosis were present in 4/31 type III and in 6/8 type IIB patients.

Among the thirty-one type III patients, eighteen were less than 45 years old when first seen in the out-patient clinic and fifteen of these were male. In the thirteen older patients, however, the male to female ratio was 7:6.

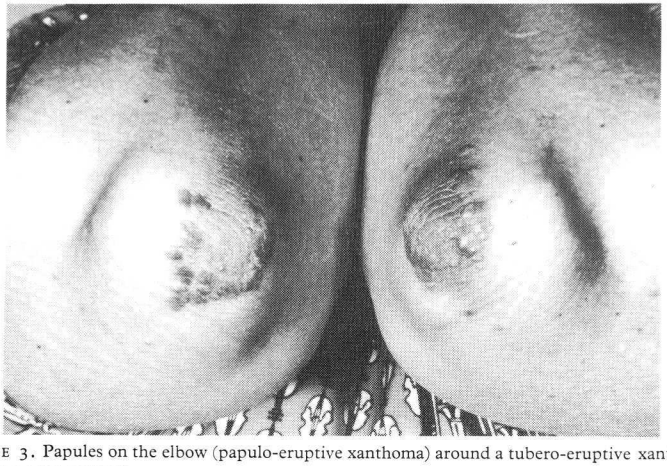

FIGURE 3. Papules on the elbow (papulo-eruptive xanthoma) around a tubero-eruptive xanthoma. (HLP type III patient).

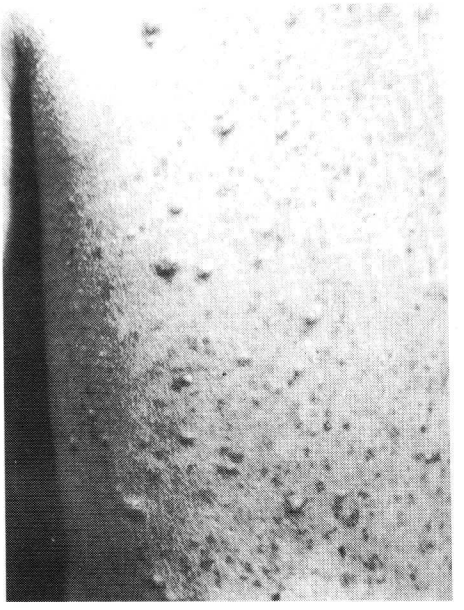

FIGURE 4. Multiple small papules on upper thigh (papulo-eruptive xanthomas) (HLP type IV/V patient).

In 9/31 type III patients the LDL lipid level was also initially elevated (total LDL lipid more than 550 mg/dl), but was easily normalized by a low calorie diet with carbohydrate restriction. This group of nine patients had no clinical features that distinguished them from the other type III patients.

Follow-up results for xanthomatous lesions, for the VLDL-cholesterol/triglyceride ratio and for floating beta-LP

Thirty-five hypertriglyceridaemic patients were available for follow-up studies. These included 23/31 HLP type III, 5/7 HLP type IV/V and 7/8 type IIB patients.

TABLE 2. Prevalence of ASCVD among patients with elevated VLDL levels and xanthomatosis

	Type IIB n = 8			Type III n = 31			Type IV/V n = 7		
Age group (years)	<35	35–45	>45	<35	35–45	>45	<35	35–45	>45
No. of patients	1	3	4	8	10	13	1	5	1
Male/female ratio	1/10	2/1	2/2	7/1	8/2	7/6	1/0	4/1	1/0
Intermittent claudication	1	0	1	0	2	2	0	1	0
Angina pectoris	0	3	2	0	0	4	0	0	0
Mycocardial infarction	0	2	2	0	0	3	0	0	0
Total ASCVD	1/1	3/3	3/4	0/8	2/10	5/13	0/1	1/5	0/1

Apart from the xanthelasma palpebrarum and tendinous xanthomas, the other xanthomatous lesions in all type III and type IV/V patients and also the xanthochromia striata palmaris present in the two type IIB patients invariably disappeared within 2 years, even when normal total lipid levels were not attained. In type III and type IV/V patients no new xanthomas appeared, but in 3/7 type IIB patients the tendinous xanthomas increased in size during the follow-up period. The tendinous xanthomas in 8/23 type II patients persisted even when total lipid and VLDL levels were normalized.

The C/T-VLDL value and the presence of floating beta on paper electrophoresis were evaluated several times during the follow-up period. The twenty-three type III patients invariably had a C/T-VLDL value higher than 0·5 during the follow-up period, irrespective of the kind of therapy applied. However, floating beta LP could not be demonstrated regularly on paper electrophoresis in the same group of patients during this follow-up period.

In 17/23 type III patients the total lipid levels became normal and in 11/17 also the VLDL levels attained normal levels during the follow-up.

The C/T-VLDL values in the four type IV/V patients available for follow-up were constantly below 0·5 except for one determination in a patient whose successive values were 0·33, 0·42 and 0·63. In this patient the high C/T-VLDL value was not correlated with a decrease in the amount of VLDL. None of the type IV/V patients showed floating beta LP at any time.

It is noteworthy that all eight type IIB patients showed marked variation of the C/T-VLDL values (between 0·3 and 0·9), both at the initial determination (Fig. 1) and during the follow-up period.

DISCUSSION

Our patients were selected on the basis of the presence of xanthomas. This might explain the high prevalence of HLP type III (31/46) in our patients with elevated VLDL levels, as xanthomatous lesions are present in a higher proportion of HLP type III in contrast to HLP types IV/V and IIB (Morganroth, Levy & Frederickson, 1975).

The clinical appearance and distribution of the xanthomas differed distinctly between the four hyperlipoproteinaemia phenotypes examined by us (HLP types IIB, III, and IV/V). In 29/31 type III patients characteristic xanthochromia striata palmaris was found and in most of the type III patients distinctive tubero-eruptive xanthomas were present on the elbows. In contrast, type IIB patients showed tendinous xanthomas and no eruptive xanthomatous lesions. In type IV/V patients both the clinical appearance and the distribution of the eruptive xanthomas are in agreement with the findings of Borrie & Slack (1974) and different from those in type III (Figs 3 and 4). The limitation of xanthochromia striata palmaris to type III patients contradicts earlier publications of our group, e.g. Polano *et al.* (1969), but is in accordance with the observations of Borrie (1969), Fredrickson *et al.* (1975) and Braun-Falco (1976).

Since we now use the VLDL cholesterol/triglyceride ratio as criterion to discriminate between type III and type IV hyperlipoproteinaemia, many patients, classified earlier by us as type IV on the basis of elevated VLDL as the decisive criterion, are now classified as type III. Formerly, patients with diabetes were excluded from our studies on primary hyperlipoproteinaemia and xanthomatosis. However, following the suggestion made by Fredrickson & Levy (1972), who included adult onset diabetic patients in their type IV, we now include patients with glucosuria and blood-sugar level above 10 mmol/dl. All patients with xanthomas classified by us as type IV have an abnormal glucose tolerance.

Closer analysis of the clinical findings in the relatively large group of type III patients showed that the xanthomatous lesions appeared at a relatively young age (before 45 years) in many male type III patients, which is in accordance with the findings of Morganroth *et al.* (1975). The absence

of females in this group might be explained by the influence of oestrogens on the VLDL catabolism, as described by Chait *et al.* (1977) and Kushwawa *et al.* (1977b).

The higher incidence of premature coronary disease and peripheral vascular disease in type III patients reported by others was not found in our type III patients younger than 45 years; only 2/18 had claudication. On the other hand all four type IIB patients with xanthomatous lesions younger than 45 years showed ASCVD. This difference is certainly not due to a bias arising from differences in the cholesterol levels in our patient groups (see Table 1), but might be explained by the fact that elevated LDL cholesterol levels have a different effect to elevated VLDL cholesterol.

Most type III patients respond well to therapy. Only one out of ten type III patients, followed for more than 5 years, developed ASCVD (a cerebro-vascular accident). This relatively good prognosis of type III patients under dietary control underlines the need for early diagnosis of type III. As already mentioned, the presence of xanthochromia striata palmaris can be very helpful in this respect.

It is noteworthy that nine type III patients had elevated LDL levels and that these levels responded rapidly to a low calorie diet with carbohydrate restriction. These elevated LDL levels in type III patients could be caused by increased levels of the LDL subfraction with values between 12 and 20, which is characteristic for this group (Slack & Mills, 1974). However, it is also possible that these patients represent a subgroup of the type III patients. Vessby *et al.* (1977) also described several type III patients with elevated LDL cholesterol levels.

During the follow-up period of more than a year in twenty-three type III patients the C/T-VLDL value determined in hypertriglyceridaemic serum remained higher than 0·5, irrespective of the kind of treatment with drugs (clofibrate) and/or diet. Paper electrophoresis showed that floating beta VLDL occurred irregularly during this period. On this basis, the C/T-VLDL was considered preferable to the demonstration of floating beta lipoprotein for the classification of patients with elevated VLDL levels and xanthomatosis, which is in accordance with other recent reports (e.g. Patsch, Jackson & Gotto, 1977).

It is remarkable that the demonstration of cholesterol-rich VLDL proved to be a useful discriminatory criterion, because this VLDL is formed as a normal intermediate stage in VLDL catabolism (Bilheimer, Eisenberg & Levy, 1972; Quarfordt, Levy & Fredrickson, 1973; Eisenberg *et al.*, 1973). Apparently, this VLDL disappears so rapidly in normal individuals that it is not demonstrable (Hazzard & Bierman, 1975).

However, during the follow-up period the C/T-VLDL in patients with elevated VLDL and elevated LDL levels (type IIB), showed inconsistently high or low C/T-VLDL levels. If the cholesterol/triglyceride ratio in VLDL were taken as the only diagnostic criterion for these patients, they would be classified as type III, but according to the WHO criteria these same patients should be classified as type IIB. Six of these eight patients had no xanthochromia striata palmaris and, as already mentioned, the classification of the other two patients is equivocal. We assigned more diagnostic value to a persistently elevated LDL level than to the C/T-VLDL in these cases.

It is noteworthy that the C/T-VLDL ratio we used and the cholesterol VLDL/total TG ratio used by Fredrickson, Morganroth & Levy (1975) have limited value, because both methods give false-positive and false-negative values even when only the presence of an elevated triglyceride level is taken into account (Mishkel, Nazir & Crowther, 1975; Albers, Warwick & Hazzard, 1977). The determination of the apoprotein E_{III}/E_{II} ratio might prove to be a more specific criterion for type III (Havel & Kane, 1973; Uterman, Hees & Steinmetz, 1977; Kushwawa *et al.*, 1977a).

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Chapter II §3

Dermatologica *151:* 43–50 (1975)

A Case of Xanthomatosis and Hyperlipoproteinemia Type V Probably Induced by Overdosage of Insulin

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Key Words. Xanthomatosis · Hyperlipoproteinemia · Diabetes · Insulin

Abstract. A woman, aged 46 years, with a hyperlipoproteinemia phenotype V, an unusual type of xanthomatosis, as well as lipemia retinalis, diabetes mellitus, and nephropathia, is discussed.

The withdrawal of 64 IU insulin and the institution of a hypocaloric diabetes diet resulted in the disappearance of the skin lesions and a regression of the eye lesion. During this treatment the total lipid-level became almost normal (6,965 mg% before treatment and 947 mg% after 10 weeks). The chylomicrons disappeared but the VLDL content remained relatively and absolutely elevated, as in type IV hyperlipoproteinemia.

Case History

The patient, a woman aged 46 years, had suffered from an unusual type of xantho matosis since 1970. She had been treated for diabetes mellitus, diagnosed during her seventh pregnancy, with 64 IU insulin novo lente and a salt-free diabetes diet since 1966. Toxicosis had occurred during several of the pregnancies. The birth weight of her children had never been more than 3.5 kg.

In 1968, the patient had had attacks of severe abdominal pain which continued after cholecystectomy. In 1970, she had shown a nephrotic syndrome with an albuminuria of $4^{0}/_{00}$, and during the last half of 1973 the vision of the left eye had diminished.

On admission, in addition to insulin, the patient was taking aldomet, lasix, and digoxin, because of hypertension and dyspnea d'effort. There was doubt as to whether she was following the diet prescribed by her physician.

[1] The lipid analyses were performed by the Gaubius Institute of the University of Leiden.

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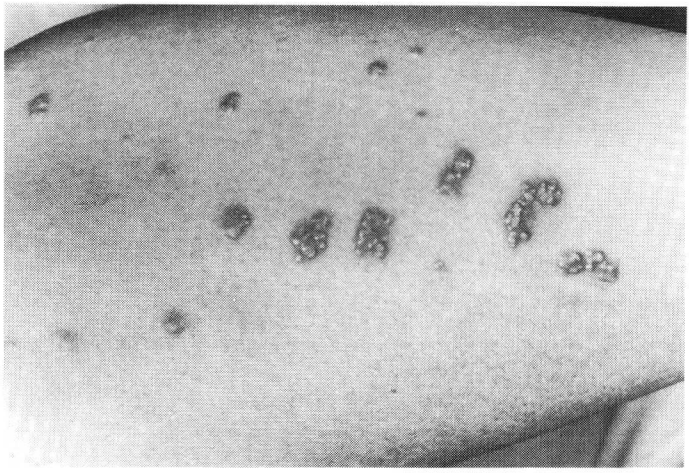

Fig. 1. Groups of yellow papules.

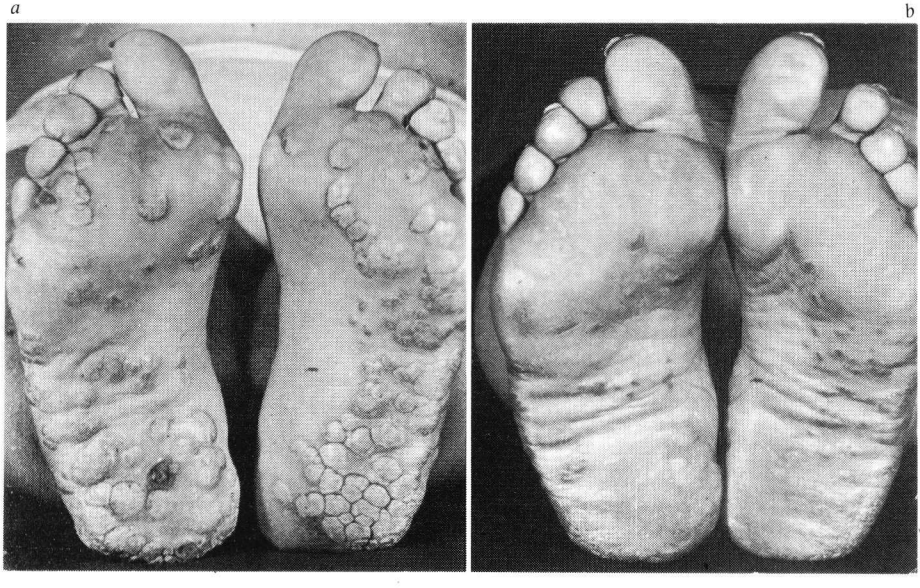

Fig. 2. a Xanthomata on foot soles on admission. *b* The same lesions 3 months later.

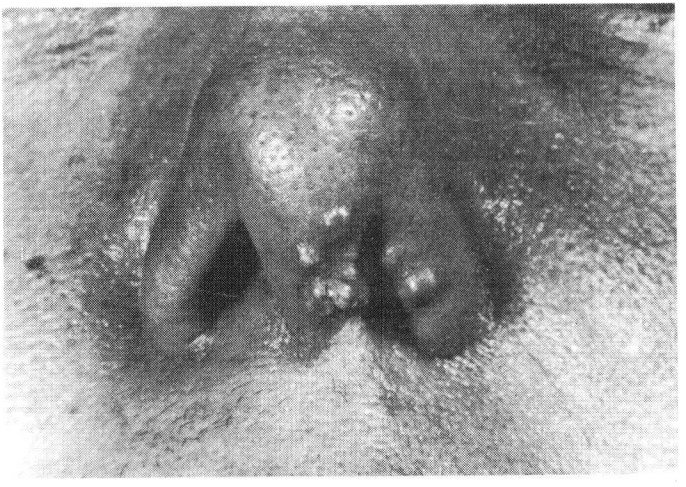

Fig. 3. Yellow papules under the apex nasi.

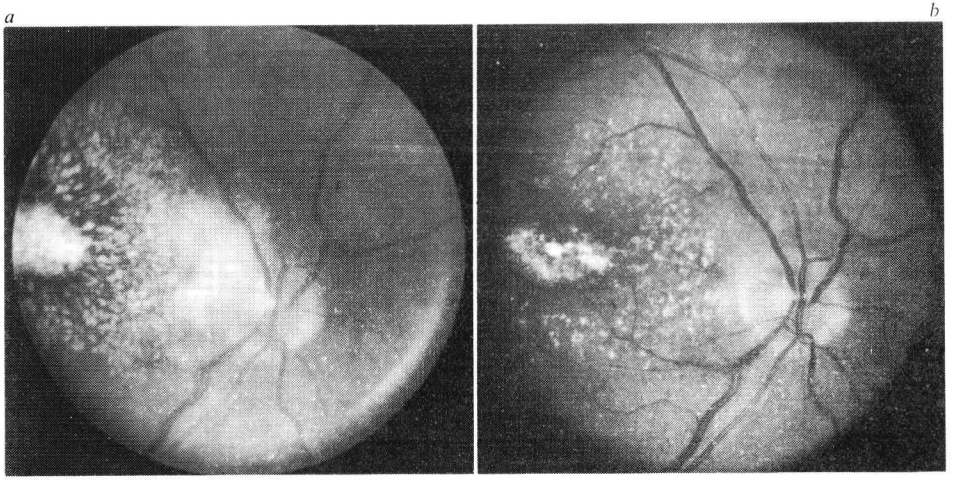

Fig. 4. a Lipemia retinalis (left eye) on admission. *b* The same lesion 3 months later.

Clinical Investigation

The skin eruption consisted of yellow papules with a red halo, coalescing in groups of three or more (fig. 1). These groups were disseminated over the elbows, dorsum of the hand, fingers, and forearms. The footsoles showed flat yellow tubera occupying almost the entire pressure area (fig. 2a). An unusual phenomenon was the presence of yellow papules under the apex nasi (fig. 3).

The examination of the left eye (Prof. J.A. OOSTERHUIS, MD) revealed an exudate between the papilla and the macula in the fundus, compatible with lipemia retinalis (fig. 4a). The right fundus showed diabetic retinopathia. The patient was markedly obese, weighing 76 kg (height 146 cm). The blood pressure was 175/120, but measured in the brachial artery proved to be normal 155/80 (cuff hypertension). Apart from a slight systolic souffle grade I at the ictus cordis, the physical examination showed no other abnormalities.

Family History

The patient's mother and one sister had died at the age of 40 and 39 years, respectively, due to a myocardial infarction (the sister's blood lipid and lipoprotein levels were known to have been normal). Of the other siblings, two sisters and one brother were obese; one of these had diabetes mellitus and hyperlipoproteinemia phenotype IV, one had hyperlipoproteinemia phenotype IIb, and one had normal lipid levels. The other brother had a normal weight and normal lipid levels. There were no relatives with xanthomatosis.

Although the hyperlipoproteinemia is classified according to the WHO classification of 1970, this should not be taken to imply that we agree completely with this classification [12].

Laboratory Investigations

On admission, the lipid and lipoprotein levels in the blood serum were grossly elevated. The serum showed a creamy layer overlying a turbid infranatant layer. At this time, the pattern of the hyperlipoproteinemia was phenotype V according to the WHO classification (table I).

The blood sugar was 116 mg% at 9.00 a.m.; the reduction in the urine was negative. The kidney function was anomalous; blood: urea 113 mg%, creatinine 3.1 mg%. The protein content of the urine was 1.5 g/24 h. There were no casts in the urine sediment. Histologically, the kidney showed some ischemic glomeruli and periglomerular fibrosis. There were no signs of diabetic glomerulosclerosis or amyloidosis. Interstitial nephritis and fibrosis was considered the most likely diagnosis. The biopsy specimen was investigated by Dr. G. BRUTEL DE LA RIVIÈRE and Dr. P.C.J. VAN BREDA VRIESMAN.

The function of the liver, thyroid gland, and adrenal glands was normal. There were no signs of paraproteinemia; the calcium and diastase levels were normal and the uric acid was slightly elevated (7.1 mg%).

Table I. Fasting lipoprotein and lipid values in serum before and after admission[1]

	On admission		10 weeks later	
	mg%	% of total lipids	mg%	% of total lipids
Lipoprotein lipids				
Chylomicrons	1,324	19	9	1
VLDL	5,363	77	473	50
LDL	139	2	313	33
HDL	139	2	152	16
Lipid levels				
Phospholipids	1,045	15	265	28
Free sterol	557	8	76	8
Sterolesters	1,393	20	322	34
Triglycerides	3,831	55	265	28
Free fatty acids	139	2	19	2
Total lipids	6,965		947	
Total sterol	1,393	20	265	28

[1] The lipoproteins were determined according to VAN GENT *et al.* [6].

Table II. Lipid values of xanthomatous lesion[1]

	Before treatment	In regression
Dry weight xanthoma, mg	2.34	1.50
Lipids in xanthoma, %	22.4	15.8
Phospholipids, %	17	31
Free sterol, %	9	11
Sterolesters, %	18	18
Triglycerides, %	43	20
Free fatty acids, %	13	20
Total sterol, %	20	22

[1] The lipids were determined according to BAES *et al.* [1].

Histological investigation of a xanthoma showed many cells with foamy cytoplasm in the upper dermis. The foam cells were separated by fibrous tissue and stained positively with oil red 0. A second biopsy specimen of a xanthoma taken 6 weeks after admission, showed a decrease in the number of foam cells.

The chemical analysis of the first biopsy specimen showed a relatively high level of triglycerides; the second biopsy sample showed a decrease of this level (table II). The results of both analyses are in accordance with the findings of BAES *et al.* [1] for eruptive xanthomata.

Course

The patient was put on a salt-free diabetes diet amounting to 1,030 cal (65 g protein, 38 g fat, 103 g carbohydrate). During the 6-week period of hospitalization, her weight dropped from 76 to 71 kg. The insulin (64 IU) was cautiously withdrawn over a period of 3 weeks, during which the blood sugar values remained the same and the reduction in the urine remained negative. This withdrawal of insulin was done in collaboration with Mrs. Dr. J. TERPSTRA, head of the Diabetes Department.

The administration of aldomet, digoxin, and lasix was also discontinued, because there was no real hypertension, and the poor kidney function made this therapy undesirable.

Under this management the patient's condition improved remarkably, the xanthomatous lesions disappeared within 10 weeks, the lipemia retinalis showed signs of regression, and the visus improved (fig. 2b, 4b).

The lipid levels dropped and became almost normal. A remarkable finding is the increase of LDL from 139 to 313 mg% while the VLDL decreased from 5,363 to 473 mg% (table I). The lipoprotein pattern changed from phenotype V to IV (normal LDL, elevated VLDL). The chylomicrons disappeared completely and the VLDL diminished very considerably. Three months after admission, the patient had no complaints. The diet, which had finally become the only form of treatment, was continued.

Discussion

The serum lipoprotein pattern in this patient is compatible with a hyperlipoproteinemia phenotype V. However, it is not possible to state whether she has a primary familiar hyperlipoproteinemia. The heredity factors, the nephropathia, the diet, and the unnecessary use of insulin, in combination

with the diabetes mellitus, may all have contributed to the clinical picture. Diabetes mellitus, elevated uric acid, attacks of abdominal pains, lipemia retinalis, and xanthomatosis are all found in hyperlipoproteinemia phenotype V [2, 5, 8]. The localization of the xanthomata at the apex nasi and on the soles of the feet as well as the absence of the eruptive xanthomata on the nates, are very unusual [8].

A hypocaloric diet is the treatment of choice for hyperlipoproteinemia phenotype V [3, 5]. This treatment, combined with the withdrawal of insulin, was also very successful in our patient. As to the question of whether an overdosage of insulin can influence the triglyceride level, we can state that insulin affects both the production and catabolism of triglycerides. It is a well-known fact that in insulinopenic diabetes patients the triglyceride level is lowered by the administration of insulin [7]. The disappearance of xanthomatous lesions and lipemia retinalis is also a well-known feature under this treatment [11, 5]. NIKKILÄ [7] and other authors [4, 9, 10] postulate that a state of hyperinsulinism may also cause hypertriglyceridemia. The lowering of the triglyceride level and the disappearance of the xanthomatous lesions and the lipemia retinalis after the withdrawal of insulin in our patient support this last hypothesis.

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THE DETERMINATION OF LIPIDS AND PROTEINS IN SUCTION BLISTER FLUID*

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SUMMARY

The concentration of five different proteins in suction blister fluid and serum was determined by immuno-techniques. These proteins, varying in size and molecular weight (6,600--2,300,000) were insulin, albumin, high density lipoprotein determined as apoprotein A I, α_2-macroglobulin and low density lipoprotein measured as apoprotein B.

The difference in the blister fluid/serum concentration ratio of the proteins was dependent on the molecular weight and followed mainly the law of diffusion. Moreover, the amounts of insulin, albumin and apoproteins A I and B in suction blister fluid were the same as those reported in peripheral lymph.

The results indicate that the sieve function of the capillary basement membrane remains intact during the formation of the suction blisters. Suction blister fluid might therefore be regarded as representative of interstitial fluid.

The concentrations of four different lipids (cholesterol, cholesterolesters, triglycerides and phospholipids) were also determined and their blister fluid/serum concentration ratio proved to have a fairly constant value of 0.25.

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INTRODUCTION

Interactions between cells (e.g. smooth muscle cells, macrophages) and lipoproteins seem to play an important role in the development of atherosclerosis and xanthomatosis (1,4). It is important to keep in mind that only endothelial cells are in direct contact with the blood; the smooth muscle cells in the vascular wall and cells in the dermis are surrounded by interstitial fluid.

It has been suggested that blister fluid obtained by mild (-200 mm Hg) suction represent interstitial fluid. So far, only a study by Kiistala (5) has demonstrated an inverse relationship between molecular weight and blister-to-serum concentration ratios of proteins, thus indicating that the sieve function of the vascular wall remained intact. We have determined the blister/serum concentration ratios of five proteins ranging in molecular weight from 6,600 (insulin) to 2,300,000 (low density lipoprotein), to find out whether the afore mentioned suggestion is also valid for these proteins.

We assumed that the concentrations of apoprotein B and apoprotein A I were representative for the concentrations of low density lipoprotein (LDL) and high density lipoprotein (HDL). Separately, we also determined the concentration in the blister fluid of various lipid substances which are present in the lipoproteins.

MATERIALS AND METHODS

Suction blister fluid (0.5-0.8 ml) was collected between 9.00 a.m. and 11.00 a.m. from 6 fasting volunteers by mild suction (-200 mm Hg) with the aid of the suction blister device described by Kiistala (6). The blister fluid (0.5-0.8 ml)

obtained from 7 blisters per person was checked for the presence of cells in order to exclude damage to the vascular wall in individuals with a high susceptibility for this type of suction. Only when no cells were found the fluid was used for analyses. Blood was collected from the fasting subjects at 11.00 a.m. by venepuncture of the cubital vein and allowed to clot.

The $\frac{\text{concentration blister fluid}}{\text{concentration serum}}$ = CB/CS of α_2-macro-

globulin and apoprotein A I (the major protein component of HDL) was determined by radial immunodiffusion according to Mancini (7) using monospecific antisera prepared in our laboratory (8,9). These antisera were tested for monospecificity by means of immunoelectrophoresis and Ouchterlony double diffusion. The CB/CS of apoprotein B (the major component of LDL) and albumin were determined on commercially available immunodiffusion plates (Partigen-Behringwerke A.G., Marburg am Lahn, W. Germany). Samples of blister fluid and serum were applied to the same Mancini plates. The two immunoprecipitate areas were measured and used to calculate the CB/CS ratio. The plates were read by a measuring projector for immuno-analysis (Behring Institute A.G., Marburg am Lahn, W. Germany). Insulin was determined by a radioimmunoassay according to Berson and Yalow (10).

Lipid composition was determined by densitometry after thin layer chromatography and charring with sulphuric acid, as previously described (11). Total lipids were determined after extraction (12) and by weighing (13).

RESULTS

The blister fluid/serum concentration ratio (CB/CS) of the proteins are shown in Figure 1 and Table I and are plotted as a function of the logarithm of their molecular weight (triangles). It is evident that the CB/CS for low density lipoproteins (LDL) with a molecular weight (MW) of circa 2,300,000 measured as apo B, as well as the CB/CS for α_2-macroglobulin (MW 800,000) is considerably lower than that of high density lipoprotein (HDL) (MW circa 300,000) and albumin (MW 69,000). Furthermore, the CB/CS ratio for insulin (MW 6,600) is 0.90 $\pm$ 0.01.

Lipid analysis showed that all major classes of lipids present in plasma (triglycerides, cholesterol, cholesterol-esters and phospholipids) are also present in blister fluid. The concentration ratio of these lipids in blister fluid was 0.20-0.27 (see Table I).

Table 1
The blister fluid/serum concentration ratio (CB/CS) of 5 proteins and 4 lipids

Proteins	CB/CS	Average SD $\pm$ 1 (n = 6)
Insulin	0.90	$\pm$ 0.01
Albumin	0.29	$\pm$ 0.04
Apo A I	0.24	$\pm$ 0.02
α_2-macroglobulin	0.14	$\pm$ 0.01
Apo B	0.16	$\pm$ 0.02
Lipids	**CB/CS**	**Average SD $\pm$ 1 (n = 6)**
Sterol	0.27	$\pm$ 0.04
Sterolesters	0.27	$\pm$ 0.04
Phospholipids	0.20	$\pm$ 0.01
Triglycerides	0.21	$\pm$ 0.05
Total lipids	0.25	$\pm$ 0.04

The movement of a macromolecule is expressed in terms of its diffusion coefficent (D), a parameter which is inversely related to its molecular weight. We assumed that a diffusion process would be responsible for the differences in concentrations of the various proteins measured in blister fluid. Therefore, the known (14) diffusion coefficients (D) of nine globular proteins are plotted against the logarithm of their molecular weight (Fig. 1; closed circles). The nine proteins were chosen in such a way that a large range of molecular weights was covered. Moverover, the diffusion coefficients of the five proteins of which the CB/CS was measured were included.

DISCUSSION

As shown in Figure 1, the blister fluid/serum concentration ratio of proteins is dependent on the logarithm of their molecular weight, and this ratio drops sharply from 0.90 to 0.29 for substances with a molecular weight lying between 6,600 (insulin) and 60,000 (albumin). These findings are in accordance with the rapid decrease of lymph/serum concentration ratios for dextran molecules with molecular weights between 6,000 and 30,000 observed by Grotte (15).

Moreover, the further slow decrease of the blister fluid/serum concentration ratio of molecules exceeding molecular weights of 60,000 was also found by Garlick and Renkin (16) in investigations on the lymph/serum concentration ratios for dextran molecules with molecular weights ranging between 60,000 and 500,000.

From these observations it can be concluded that the capillary pore system with a diameter of 45 nm and the

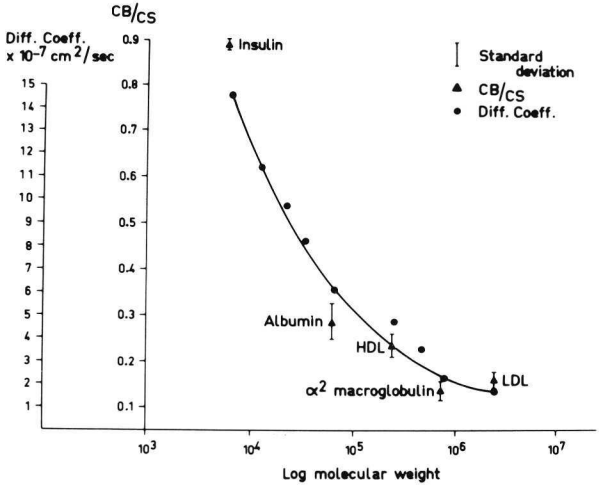

Figure 1

The blister fluid/serum concentration ratio (CB/CS) of five different proteins ▲-▲, and the diffusion coefficients (Diff. Coeff.) (D = 10^{-7}cm^2/sec), at 38°C of nine globular proteins ●-●, plotted as a function of the logarithm of their molecular weight.
The nine globular proteins ascending in molecular weight were respectively: 1) insulin (6,600); 2) cytochrome C (13,300); 3) trypsinogen (23,560); 4) carboxypeptidase (34,280); 5) albumin (68,460); 6) HDL (250,000); 7) apoferritine (466,900); 8) α_2-macroglobulin (775,500); 9) LDL (2,300,000). See ref. (14).

system responsible for transport of large molecules remain intact under the condition of our experiment (17). Furthermore, the line drawn through the diffusion coefficients of globular proteins (Fig. 1) could be superimposed on the measured data shown in Fig. 1. The concentration of high molecular weight substances in blister fluid therefore seems to be determined mainly by passive diffusion, which is in

agreement with the findings of Garlick and Renkin (16) for peripheral lymph.

In addition, it was found by Herfst and van Rees (18) that the flow of low molecular weight substances (6,000) into the blister fluid also obeyed the law of diffusion.

If we consider peripheral lymph to represent the interstitial fluid, we must keep in mind the fact that the protein content in peripheral lymph is variable and is dependent on the lymph flow (19). The blister fluid/serum concentration ratio we found for albumin, i.e., 0.3, is in accordance with the findings of Kiistala (5). This ratio was also found for peripheral lymph at a lymph flow which is maximal during exercise (19).

Comparison of the concentraton ratio of 0.16 (± 0.02) for Apo B and of 0.24 (± 0.02) for Apo A I with those given by Reichl et al. (20) for peripheral lymph, shows that their values are somewhat lower than ours (Apo B = 0.10; Apo A I = 0.20) but the difference is very small. Moreover, Reichl et al. (21) showed that on the basis of biological activity, the concentration of LDL is approximately 10% of the LDL concentration in serum.

The concentration ratio (CB/CS) of various lipids in blister fluid were in agreement with the values obtained for peripheral lymph of rabbits (22) and sheep (23). Reichl's (24) values for total cholesterol and triglycerides in human peripheral lymph are 5-10% of those in plasma. Whether this difference must be attributed to variations in the composition of peripheral lymph under various conditions or to differences in the method used for lipid determination remains to be answered. By determining four different classes of lipid, we have shown that there is no selectivity for the lipid classes in the blister fluid.

The lipid and apoprotein levels found in blister fluid suggest that the composition of the lipoproteins in interstitial fluid differs from that of the lipoproteins in blood. This assumption is supported by the difference in the electrophoretic mobility of apoprotein B-containing lipoproteins in peripheral lymph found by Reichl et al. (25).

The pathogenesis of atherosclerosis and xanthomatosis is studied extensively using cultured cells exposed to different lipoproteins (26). However, the incubation of the cells with the various lipoproteins should resemble as closely as possible the in vivo situation. The measurement of the different lipoprotein concentrations in suction blister fluid is of importance to determine the correct incubation conditions for cultured cells, which are in vivo surrounded by interstitial fluid.

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Chapter IV §1

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The effect of digitonin-containing fixatives on the retention of free cholesterol and cholesterol esters

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Synopsis. The influence of several fixation and dehydration procedures on the retention of free cholesterol and cholesterol esters was studied in filter paper preparations. The retention of free cholesterol by the filter paper proved to be decreased by the addition of digitonin to the aldehyde fixative (aqueous phase) and was only slightly enhanced by partial dehydration (alcoholic phase, up to 70% ethanol). Furthermore, digitonin or the presumably formed cholesterol–digitonide complex bound hardly any osmium oxides in glass-fibre paper.

Up to 26% of the cholesterol esters was mobilized during the aqueous phase when digitonin was added to the aldehyde fixative. When the glass-fibre papers containing the digitonin cholesterol–ester–osmate complexes were stored in distilled water after fixation, the fluid became turbid. Particulate material isolated from this turbid solution showed ultrastructurally a close resemblance to the 'whorls' observed by several authors in tissue fixed by a digitonin-containing aldehyde fixative.

Digitonin also changed the ultrastructural appearance of liposomes, containing lecithin: cholesterol: phosphatidic acid; in a molar ratio 7:2:1. Our observations lead to the conclusion that the use of digitonin-containing fixatives should be abandoned, because they give results which cannot be interpreted. By the use of $K_4[Fe(CN)_6]$ containing OsO_4 in the post-fixation step we were able to demonstrate an increase in the visualization of membranous structures (liposomes).

Introduction

For electron microscopical investigations on cholesterol-containing tissue specimens, Ökrös (1968) introduced an adaption of the Windaus digitonin reaction (1910), which was originally performed in ethanol. Since cholesterol retention in tissue specimens

was repeatedly reported to be increased by the addition of digitonin to the glutaraldehyde fixative (Scallen & Dietert 1969; Frühling *et al.*, 1969), several authors (Napolitano *et al.*, 1969; Williamson, 1969; Napolitano & Scallen, 1970; Darrah *et al.*, 1971; Szabo *et al.*, 1971; Triilo, 1971; Braun Falco, 1973; Parker & Odland, 1973; Albert & Rucker, 1975) have used a digitonin-containing fixative for the retention and ultrastructural localization of free sterol in tissue.

The interpretation of the observed spicules and whorls as cholesterol was based on the assumption that cholesterol-digitonide complexes were visualized in such structures. However, a destructive action of digitonin on the outer membrane of mitochondria was reported by Levy *et al.* (1967).

As we planned to use digitonin for the localization of cholesterol in very low density or low density lipoprotein particles (VLDL, LDL) and in xanthomatous tissue, model experiments were designed to test the validity of the digitonin fixation technique. The results of this investigation are reported in this paper, whereas ultrastructural investigations on VLDL, LDL and xanthomatous tissue are reported in the following paper.

Material and methods

IN VITRO EXPERIMENTS PERFORMED TO EVALUATE THE CURRENTLY USED METHODS

Determination of free cholesterol and cholesterol oleate

1 mg free cholesterol or 1 mg cholesterol oleate dissolved in 0.1 ml chloroform was applied to 1 cm^2 pieces of cellulose-fibre filter paper (Machery Nagel and Co., Düren, W. Germany; paper no. 866) and allowed to evaporate completely. These pieces and also control pieces of untreated paper were exposed to the fixation and dehydration procedure to be investigated. After incubation in Flickinger's fixatives [see procedure (a)] with and without digitonin, the papers were washed 3 times for 10 min each in a 0.5 M cacodylate buffer (pH 7.4) containing 0.05% $CaCl_2$. Some of the papers were then subjected to the dehydration procedure. The lipids retained in the paper were extracted according to Folch *et al.*, (1957).

The lipid solution was dried under nitrogen gas and the amount of cholesterol retained in the residue was determined according to Huang *et al.*, (1961).

Determination of osmium on glass-fibre paper

Cellulose-fibre filter paper is not suitable for the quantitative measurement of OsO_4, because large proportions of this substance bind to the paper and these amounts are influenced by lipids applied to the paper.

Glass-fibre filter paper was found to bind only very small amounts of OsO_4. Therefore, cholesterol and cholesterol-esters were applied as described for cellulose-fibre filter paper. As a control for the retention of OsO_4 by digitonin, 1 mg digitonin in ethanol was applied.

After evaporation of the solvents, these pieces of filter paper and untreated controls were subjected to the fixation procedures to be investigated. After the post-fixation with 1% OsO_4 the papers were stored in distilled water at room temperature. In some

experiments with cholesterol oleate, double fixation was performed with 1% OsO_4 containing 0.05 M $K_4[Fe(CN)_6]$. These pieces of glass-fibre paper were stored in 0.05 M $K_4[Fe(CN)_6]$ in distilled H_2O at room temperature.

The osmium remaining on the glass-fibre paper was determined according to Burkl and Schliechl (1968). The extinction values of 100 and 350 γ OsO_4 were used as reference.

Comparison of investigated fixative and dehydration procedures

The procedures used were as follows:

(a) 2% paraformaldehyde, 2.5% glutaraldehyde, 0.05% $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4) (Flickinger's fixative, 1967). Fixation duration: 21 h at room temperature. Called PAG/–.

(b) Flickinger's fixative with 0.2% digitonin added, as described by Scallen and Dietert (1969). Fixation duration: 21 h at room temperature. Called PAG/+.

(c) Fixation procedure as PAG/– followed by partial dehydration in an ethanol series up to 70%, as described by Idelman (1964), at room temperature. Called PAG/–/Eth 70.

(d) Fixation procedure as PAG/+ followed by partial dehydration. Called PAG/+/Eth 70.

(e) Double fixation procedure. PAG/+ followed by 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) containing 0.05% $CaCl_2$ for 90 min at 4°C followed by partial dehydration. Called PAG/+/Os/Eth 70.

(f) Double fixation procedure PAG/+/Os followed by complete dehydration in an ethanol series up to 100% without propylene oxide. Called PAG/+/Os/Eth 100.

Preparation of the fibrin matrix and embedding of the liposomes in this matrix

First step: 4.6 ml of a 1.3% bovine fibrinogen solution plasminogen-free and buffered (veronal buffer, pH 7.75) was mixed in a flat Petri dish with 1.4 ml of the liposomal solution containing 72 mg/ml lipid.

The veronal buffer was composed of 0.05 M Na-veronal, 0.093 M NaCl, 1.66 mM $CaCl_2$, and 0.69 mM $MgCl_2$

Second step: 0.3 ml thrombin (20 NIH/ml in 0.87% NaCl and 0.25% gelatin) was rapidly mixed with the fibrinogen solution and allowed to clot at room temperature for 30 min. The control fibrin matrix consisted of a solution of 6 ml 1% fibrinogen and 0.3 ml thrombin. The bovine fibrinogen solution contained negligible amounts of sterols (5 γ/ml cholesterol and 12 γ/ml cholesterol-esters). The liposomes were kindly provided by Dr E. Wisse and Dr G. Gregoriadis. The lipid composition was lecithin: cholesterol: phosphatidic acid, in a molar ratio of 7:2:1.

Processing of the fibrin matrix for electron microscopy

4 mm ϕ discs were punched out of the fibrin matrix (about 1 mm thick) and fixed in Flickinger's fixative (1967) with and without 0.2% digitonin for 5 h at 4°C. After 3 washes for 5 min each with cacodylate buffer (0.5 M; pH 7.4), the specimen was post-fixed with 1% OsO_4 + $K_4[Fe(CN)_6]$ in 0.1 M cacodylate buffer (pH 7.4) for 12 h at 4°C (de Bruijn, 1973).

The specimens were partially dehydrated in an ethanol series (up to 70%) at room

temperature, according to Idelman (1964), and embedded in Epon. Ultrathin sections stained with uranyl acetate and lead hydroxide were examined in a Siemens Elmiskop II operating at 80 kV with a 50 μm objective aperture.

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 (with 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 propylene oxide, and embedded in Epon.

Results

Determination of the loss of free cholesterol during fixation and dehydration procedures in vitro

As described above, pieces of filter paper impregnated with cholesterol were incubated in the various fixatives and washing solutions to mimic the situation of the tissue cholesterol in the various steps of the procedure.

The results of these experiments are summarized in Table 1. All of the free cholesterol impregnated in the untreated filter paper was recovered. The loss of cholesterol from the filter paper exposed to the aqueous solutions amounted to 1%, but addition of digitonin to the fixative increased this loss to 9%. Although the loss of cholesterol during the alcoholic phase up to 70% ethanol was reduced from 42% to 25% by the addition of digitonin to the fixative, this effect was partially reversed by the digitonin-induced mobilization effect on cholesterol during the aqueous phase. Double fixation with OsO_4 had no influence on the retention of cholesterol by the filter paper. Extension of the alcoholic dehydration to 100% ethanol increased the cholesterol loss considerably, only 8% free cholesterol being retained despite the use of a digitonin-containing aldehyde fixative.

Determination of the loss of cholesterol oleate during fixation and dehydration procedures in vitro

In order to investigate the influence of digitonin on cholesterol esters, comparable experiments were done with cholesterol oleate. The results are shown in Table 2.

All of the cholesterol oleate impregnated in untreated filter paper was recovered. Exposure to the aqueous solution gave a loss of 9%. The digitonin-containing Flickinger's fixative had a mobilizing effect on cholesterol oleate, causing a loss of 26%. The additional loss of 15–18% cholesterol oleate during subsequent exposure to the alcoholic solutions (up to 70% ethanol) was roughly similar with and without digitonin, but was reduced from 18% to 7% by use of the OsO_4 double fixation procedure. Despite double fixation, use of the alcoholic phase up to 100% ethanol caused an additional loss of cholesterol oleate amounting to 25%.

In the preceding experiments the pieces of filter paper were treated like tissue and washed 3 times for 10 min in buffer after incubation in Flickinger's fixative. In an additional experiment in which the washing was omitted, the mobilizing effect of

Table 1. Distribution of the loss of free cholesterol during fixation and dehydration procedures*

Procedure	*Fixation and dehydration procedure*†	*Total retention on filter paper* (%)	*Loss during fixation + washing (aqueous phase)* (%)	*Additional loss during dehydration (alcoholic phase)* (%)
C	Controls	100	–	–
(a)	PAG/–	99	1	–
(c)	PAG/–/Eth 70	57	1	42
(b)	PAG/+	91	9	–
(d)	PAG/+/Eth 70	66	9	25
(e)	PAG/+/Os/Eth 70	66	9	25
(f)	PAG/+/Os/Eth 100	8	9	83

*All measurements were performed in quadruplicate (variation less than 1%).
†Plus sign indicates the presence of digitonin.

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.
Eth 100 = Dehydration in an ethanol series up to 100%.

digitonin on cholesterol oleate was checked by the determination of cholesterol in the Flickinger's fixative itself after incubation of the cholesterol oleate-containing filter paper.

No cholesterol was detectable in the fixative without digitonin, whereas the fixative with digitonin contained 4.6% of the cholesterol originally applied as cholesterol oleate to the paper, the corresponding filter paper yielding 95% of the cholesterol and the total recovery being 99.6%. Evidently, the cholesterol oleate loss during the aqueous phase occurs partially during the fixation procedure and partially during washing of the filter paper.

Determination of osmium on glass-fibre paper

To find out whether the free cholesterol-digitonide complex could bind the additional amount of OsO_4 needed for better visualization, the osmium oxide content of the material remaining on the impregnated glass-fibre filter paper was determined.

Table 2. Distribution of the loss of cholesterol oleate during fixation and dehydration procedures‡

Procedure	*Fixation and dehydration* §	*Total retention on filter paper* (%)	*Loss during fixation + washing (aqueous phase)* (%)	*Additional loss during dehydration (alcoholic phase)* (%)
	Controls	100	–	–
(a)	PAG/–	91	9	–
(c)	PAG/–/Eth 70	76	9	15
(b)	PAG/+	74	26	–
(d)	PAG/+/Eth 70	56	26	18
(e)	PAG/+/Os/Eth 70	67	26	7
(f)	PAG/+/Os/Eth 100	49	26	25

‡All measurements were performed in quadruplicate (variation less than 1%).
§The plus sign indicates the presence of digitonin.

Table 3. Retention of osmium by glass-fibre paper*

	21 h in Flickinger's fixative		
Controls	*Without digitonin + 90 min 1% OsO_4*	*With digitonin + 90 min 1% OsO_4*	*Without digitonin + 90 min $K_4[Fe(CN)_6]$ containing 1% OsO_4*
Glass-fibre paper	7–22 γ	30–32 γ	n.p.†
Glass-fibre paper + 1 mg digitonin	20–32 γ	n.p.	n.p.
Glass-fibre paper + 1 mg free cholesterol	30–32 γ	25–25 γ	n.p.
Glass-fibre paper + 1 mg cholesterol oleate‡	242–303 γ	153–243 γ	280–322 γ

*The papers were stored for 12 h in distilled water, except where $K_4[Fe(CN)_6]$ containing OsO_4 was used, when storage was in 0.05 M $K_4[Fe(CN)_6]$ in distilled water.
†Not performed.
‡The reproducibility of this experiment was apparently affected by the reported mobilizing effect of aqueous solutions on the cholesterol esters in these pieces of papers.

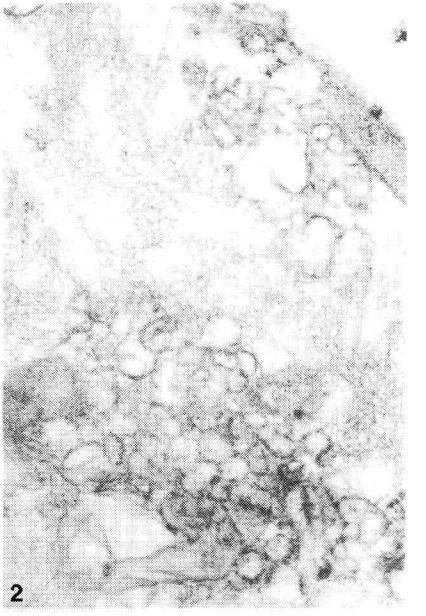

Figure 1. Cholesterol oleate–digitonin–osmate complex in fibrin matrix. Note similarity to whorl-like structures as shown in Fig. 2. PAG/+/Os/Eth 70/E. x 96 000
Figure 2. Whorl-like structures between collagen fibres in a tendinous xanthoma treated with a digitonin-containing aldehyde fixative. PAG/+/Os^{tc3}/Ac 100/E. x 56 000

The osmium retention in the controls (Table 3) and the digitonin- and cholesterol-impregnated glass-fibre paper was invariably very low. It was obvious that the retention of osmium was not increased by the cholesterol–digitonide complexes assumed to be formed during the fixation procedure using digitonin in the aqueous phase. On the other hand, osmium was clearly retained by the cholesterol oleate-impregnated glass-fibre paper (Table 3, column 1), and addition of digitonin to the preceding Flickinger's fixative slightly lowered the amount of osmium retained (Table 3, column 2).

In addition, the distilled water used for 12 h after the post-fixation with 1% OsO_4, became turbid in the experiment using cholesterol oleate on the glass-fibre paper and the digitonin-containing Flickinger's fixative. It is evident that the cholesterol oleate–digitonin–osmate complex is more soluble in the aqueous phase than the cholesterol oleate–osmate complex. The turbid material was isolated by centrifugation for 30 min at 4800*g* and embedded in a fibrin matrix for electron microscopy. Darkly osmium-stained crystals and particles with an electron-translucent central core and an electron-dense outer rim were found in the matrix. Several particles with a size of 200–400 nm observed in cross-sections had a circular appearance and resembled the whorl-like structure found in digitonin-treated tissues (Figs. 1 & 2).

In addition, with the use of the $K_4[Fe(CN)_6]$ containing OsO_4 fixative there was a tendency for more osmium to be retained (Table 3, column 3).

ULTRASTRUCTURAL EFFECTS OF DIGITONIN ON LIPOSOMES

Because the experiments presented so far are open to the criticism that the free cholesterol and the cholesterol oleate were impregnated in paper and were not bound in a 'structure', cholesterol-containing liposomes were embedded in a fibrin matrix and small pieces of this material were treated in the same way as the filter paper.

When the double fixation procedure (1% OsO_4 after Flickinger's fixative) was performed, the liposomes could hardly be seen electron microscopically in spite of uranyl acetate and lead citrate staining, but when the primary Flickinger's fixative was followed by OsO_4 plus $K_4[Fe(CN)_6]$ according to de Bruijn and den Breejen (1975), the liposomes were clearly observed. Most of these liposomes were round, and all were limited by 4 to 6 osmiophilic lines surrounding an electron-translucent core. When digitonin was added to the primary fixative the liposomes had a heterogenic morphology and were invariably limited by only two osmiophilic lines (Wisse *et al.*, 1978) (see Figs. 3 & 4).

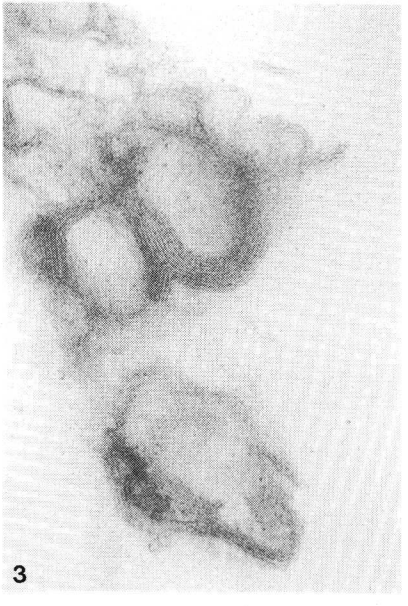

Figure 3. Liposomes embedded in fibrin matrix and post-fixed with OsO_4 plus $K_4[Fe(CN)_6]$. The liposomes show the multilamellar structure. PAG/−/Os^{tc4}/Eth 70/E. x 17 000

Figure 4. Liposomes embedded in fibrin matrix and limited by only two osmiophilic lines. Note the signs of destructive action of the digitonin-containing aldehyde fixative. PAG/+/Os^{tc4}/Eth 70/E. x 17 000

It is therefore clear that the cholesterol-phospholipid membrane is altered by the digitonin solution and that lipid material is mobilized.

Discussion

If digitonin could serve to localize and visualize free cholesterol, the following three prerequisites should be fulfilled.

(1) Digitonin should greatly reduce the loss of free cholesterol during both the aqueous and the alcoholic dehydration phases of the tissue-processing procedure.

(2) Digitonin–cholesterol complexes formed in the aqueous aldehyde fixation phase should react with the second fixative, OsO_4, to visualize the preserved complexes.

(3) Digitonin should not mobilize or dislocate cholesterol and/or other lipids during any step in tissue processing in any way leading to uninterpretable artifacts.

Our finding that digitonin had no retaining effect on free cholesterol during the aqueous phase *in vitro* makes it hard to believe that under similar conditions digitonin would specifically bind free cholesterol *in vivo*. The cholesterol loss in the alcoholic phase of the procedure was not prevented by the presence of digitonin in the aqueous phase in our *in vitro* experiments. In the same experiments the loss of cholesterol and cholesterol esters was reduced by partial dehydration (70% ethanol) instead of complete dehydration (100% ethanol). These results are in agreement with the findings made *in vivo* for cholesterol and cholesterol esters by Idelman (1964) and Frühling *et al.* (1969).

Although the presence of digitonin, in the aqueous phase, was not expected to influence cholesterol ester retention, our model experiments demonstrated a mobilization of cholesterol esters by digitonin during this step of the procedure. In accordance with the literature, the use of the OsO_4 double fixation, in our model experiments, decreased the loss of cholesterol esters during the alcoholic phase.

With respect to free cholesterol, our osmium determinations showed that free cholesterol, the presumably formed cholesterol–digitonide, and digitonin itself, were all unable to bind appreciable amounts of osmium under normal fixation conditions. The same experiments also demonstrated that when an unsaturated fatty acid is esterified to cholesterol, e.g. cholesterol oleate, osmium was retained and could be detected.

In addition to the mobilizing effect of digitonin on cholesterol ester, it was found that digitonin mobilized the osmium-cholesterol ester complexes in such a way that the aqueous solutions became cloudy, and the reaction product could be isolated and embedded in Epon.

Frühling *et al.* (1970) had already demonstrated *in vitro* that the use of digitonin as a marker for the ultrastructural localization of free cholesterol is debatable. They argued that among the three morphological structures observed (lamellae, spicules, and 'whorls'), only the lamellae are related to the presence of cholesterol, the other two structures possibly being related to interaction between digitonin and other lipids.

Our results indicate that these 'whorls' (Fig. 1) represent lipids dislocated by the action of digitonin. This effect was also demonstrated by the changes induced by digitonin in liposomal membranes. These whorl-like structures can cause confusion

because they resemble serum lipoprotein particles (VLDL or LDL) embedded in fibrin (Vermeer *et al.*, 1978).

Our results obtained in model experiments on cholesterol retention are not in accordance with the findings of others concerning cholesterol retention in tissue (Scallen & Dietert, 1969; Frühling *et al.*, 1969; Darrah *et al.*, 1971; Parker & Odland, 1973). These authors reported 80–90% retention of free cholesterol in tissue when digitonin was used in the aqueous phase, even after complete dehydration. In these experiments the retention of free cholesterol in tissue without the use of digitonin was 3–8%. Using labelled digitonin in the aqueous phase Napolitano *et al.*, (1972) found that digitonin is also retained in the tissue, which suggests the formation of a complex of cholesterol and digitonin in water. In contrast with these results, Casley-Smith & Day (1966) found no effect on cholesterol retention in tissue when digitonin was added to the alcoholic instead of the aqueous phase.

According to the literature, the solubility of cholesterol and its digitonides in water and in ethanol is as follows:

Cholesterol in water: 4 μg/l (Gilbert *et al.*, 1975);
Cholesterol–digitonide in water: 600 μg/l (Windaus, 1910);
Cholesterol in 96% ethanol: 260 g/l (Windaus, 1910);
Cholesterol–digitonide in 96% ethanol: 0.2 g/l (Windaus, 1910), 0.11 g/l (Scallen & Dietert, 1969);
Cholesterol–digitonide in 100% ethanol: 0.9 g/l (Windaus, 1910).

The solubility of cholesterol in water is clearly increased by the presence of digitonin, whereas the solubility of cholesterol in ethanol is decreased by the presence of digitonin.

In addition, it must be taken into account that after the aqueous phase, 1 mm^3 tissue specimens are exposed to relatively large (10 ml) amounts of ethanol. In this solvent all of the presumably formed cholesterol–digitonide complexes could be washed out, in spite of their very low solubility (0.2 g/l). Contrary to the reports on cholesterol retention in tissue, the results of our model experiments are completely in accordance with the above solubility data.

The retention of cholesterol in tissue induced by the addition of digitonin to the aqueous phase cannot be attributed to the Windaus reaction but must be caused by some other, still unknown, reaction. Moreover such retention reactions might have dislocated the tissue cholesterol.

The findings of Frühling *et al.* (1970) and our experiments (Fig. 1) have shown that whorls and spicules can also be formed *in vitro* by the action of a digitonin-containing fixative on lecithin or cholesterol esters in the absence of free cholesterol.

The loss of free cholesterol during the dehydration procedure is so great that the losses during infiltration in Epoxy monomers have only a very small influence on the free cholesterol retention in our model experiments and was, therefore, not determined.

It must be concluded that digitonin-containing fixatives are not appropriate for the localization of cholesterol in tissue on the ultrastructural level, and the presence of

so-called spicules and whorl-like structures in digitonin treated tissue specimens should be regarded as uninterpretable digitonin-induced artifacts.

No adequate method for the preservation of free cholesterol during the fixation and dehydration needed for ultrastructural observations by the conventional transmission electron-microscope is available. The use of the $K_4[Fe(CN)_6]$ containing OsO_4 in the post-fixation step was introduced as this fixative is known to increase the visualization for investigations by conventional transmission electron microscopy (De Bruijn, 1973). This increase in visualization is caused by the formation of $Os^{VI}Fe^{II}$ complexes as proposed by De Bruijn & Den Breejen (1975), whereas a slight increased retention of osmium was demonstrated in our experiments. However the $K_4[Fe(CN)_6]$ containing OsO_4 is by no means a fixative which reacts selectively with free cholesterol. The ultrastructural investigations by the conventional transmission electron microscopy on lipoproteins *in vitro* and on xanthomatous lesions using the previously mentioned fixation procedure are reported in the following paper.

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Chapter IV § 2

Histochemical Journal, **10**, (1978), 299–307

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, 1970; 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:)

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

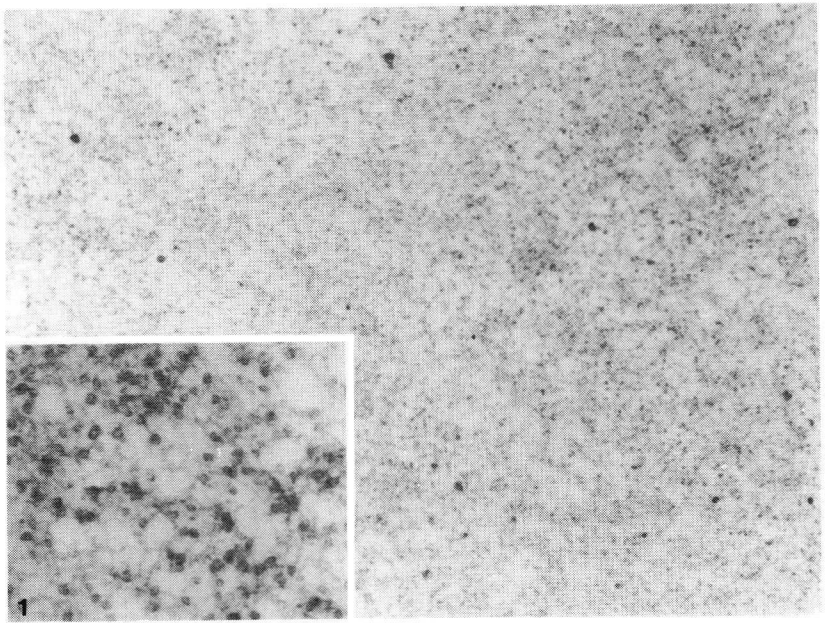
Low density lipoproteins (LDL) present in a pooled serum from patients with hyperlipoproteinaemia phenotype IIa (W.H.O., 1970) were separated by preparative ultracentrifugation according to van der Bijl & van Gent (1975). The very low density lipoproteins (VLDL) were isolated according to van Gent (1972)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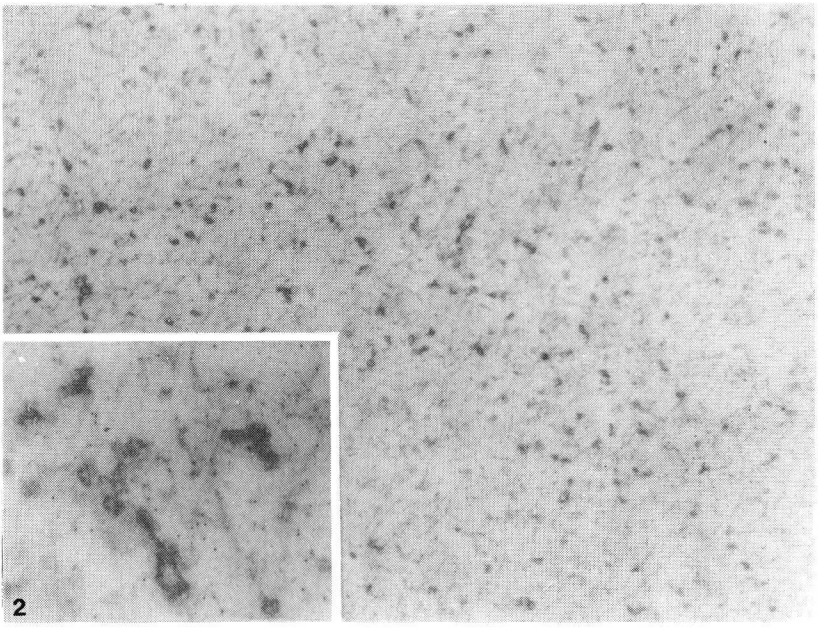

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Treatment of tissue samples for electron microscopy

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 $K_4[Fe(CN)_6]$.

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).

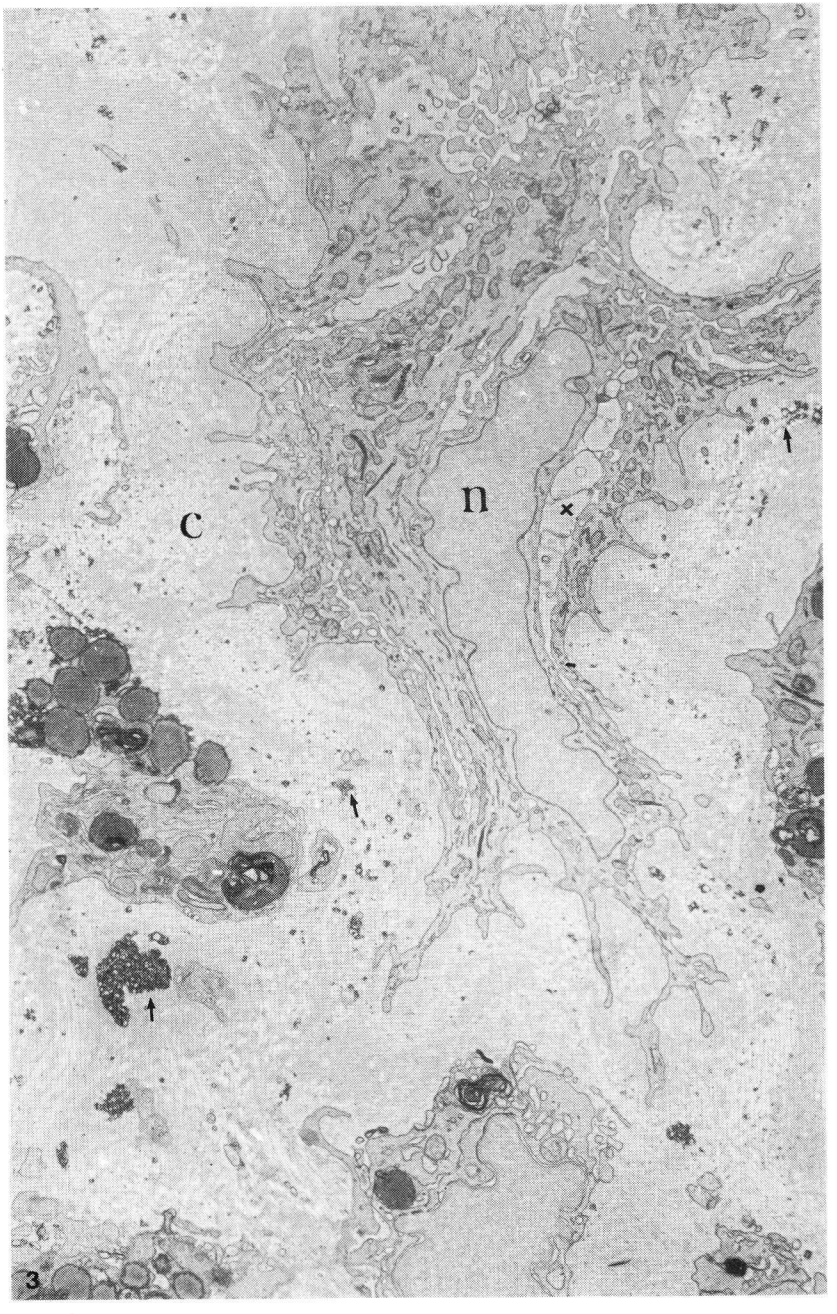
For convenience, we will call these particles membrane-bound, but we are aware that they do not necessarily represent the plasma membrane.

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 8000

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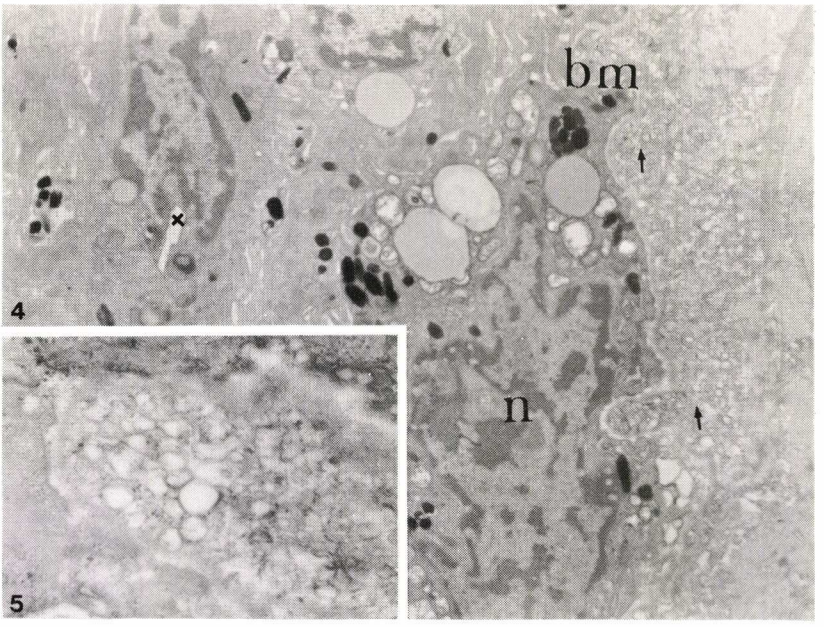
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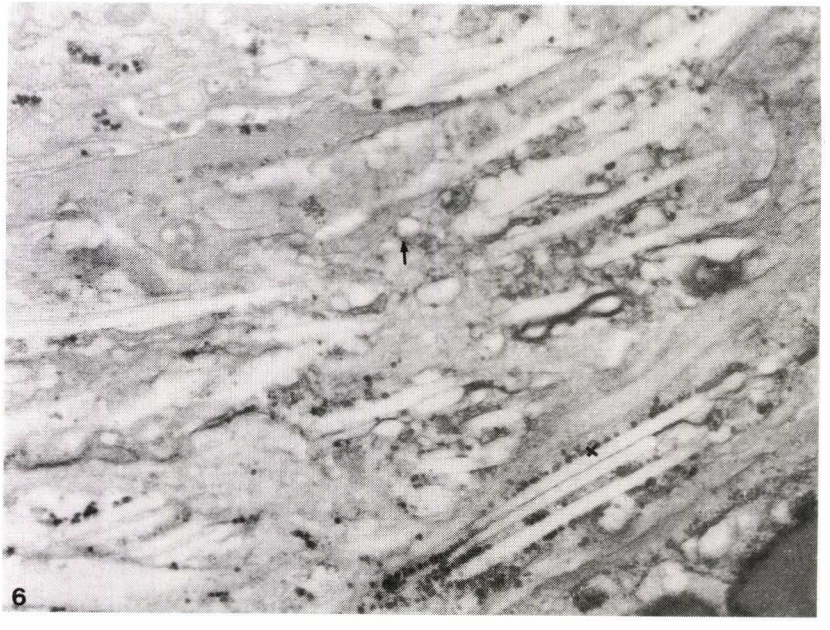
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Between the collagen fibres of a tendinous xanthoma and an eruptive xanthoma, several particles of the same size and appearance were found, and many cholesterol clefts were also present in this material (Fig. 6).

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 30–300 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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Chapter V §1

Histochemistry 56, 197–201 (1978)

Histochemistry

Immunoenzymehistochemical Demonstration of the Binding of Low Density Lipoproteins to Cultured Human Fibroblasts

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Summary. Using an indirect cytochemical immunoperoxidase technique, we were able to demonstrate the binding of low density lipoprotein to cultured human fibroblasts. With this technique, fibroblasts from a patient suffering from homozygous hyperlipoproteinaemia type IIa did not show this binding.

The method described here allows study of the localization of unmodified low density lipoproteins binding to cultured fibroblasts.

Introduction

The existence of specific receptors for human low density lipoproteins (LDL) on the plasma membrane of normal human fibroblasts in culture has been shown by biochemical studies (reviewed by Brown and Goldstein, 1976a). Brown et al. (1976b) also demonstrated the binding of LDL to fibroblasts by using an indirect immunofluorescence technique. Ultrastructural studies with modified (ferritin-conjugated or cationized LDL) have shown the binding of this LDL to coated regions of plasma membrane and the internalization of ferritin-labeled LDL in endocytotic vesicles (Anderson et al., 1976; Basu et al., 1977). We investigated the binding of unmodified LDL to cultured normal and homozygous hyperlipoproteinaemia type IIa human fibroblasts at the light-microscopical level with an indirect immunoperoxidase technique.

Materials and Methods

(a) *Cells.* A permanent cell strain of normal human foreskin fibroblasts was maintained in the form of confluent monolayers in 90 mm plastic petri dishes provided with 10 ml medium [Hams's F 10 growth medium containing 15% v/v newborn calf serum (NBCS)]. Fibroblasts from a skin biopt of a patient homozygous for hyperlipoproteinaemia type IIa were cultured and maintained in the same way.

0301-5564/78/0056/0197/$01.00

(b) *Lipoprotein and Delipidated Newborn Calf Serum.* Human low density lipoproteins (LDL) (d 1.03–1.05 g/ml) were isolated according to Hatch and Lees (1968) from freshly obtained EDTA plasma from a healthy volunteer, in a Sorvall OTD 2 ultracentrifuge with Ti 865 rotor (Ivan Sorvall, Inc., Newtown, Conn., U.S.A.). Two washes were carried out successively at 50,000 rpm in the same ultracentrifuge and rotor. Newborn calf serum was delipidated by ultracentrifugation at density 1.25 g/ml for 36 h at 50,000 rpm. The $d > 1.25$ g/ml fraction was exhaustively dialysed against isotonic saline and then sterilized by millipore filtration (0.2 μ).

(c) *Preparation of Human Apoprotein B.* Crude apolipoprotein B was obtained as the insoluble fraction of an apo VLDL isolation (Chung and Scanu, 1974). This material was solubilized in 0.002 M decylsulphate in 0.05 M Tris-HCl (pH 8.2) and run on Sephadex G 200. The peak eluting at the void volume was pooled, dialysed against 0.005 M NH_4HCO_3 (during dialysis apolipoprotein B precipitated), lyophilized, taken up in 0.002 M decylsulphate in 0.005 M Tris-HCl (pH 8.2), and again chromatographed on Sephadex G 200. The peak eluting at the void volume of the column was pooled and dialysed as described above. Polyacrylamide electrophoresis in SDS revealed no low molecular weight impurities (apo C, arginine rich peptide). The amino acid composition was determined according to Spackman et al. (1958) and in agreement with published data (Lee, 1976).

(d) *Antiserum Preparation.* Immunization of rabbits with apoprotein B was carried out as described by Nieuwenhuizen et al. (1977). The antiserum reacted only with LDL and VLDL on immunoelectrophoresis and Ouchterlony double diffusion. The antiserum did not react with low molecular weight apo-lipoproteins from VLDL or with apo A–I, apo HDL, or albumin.

(e) *Experimental Procedure.* On day 1, about 1.10^5 trypsinized cells (5th–10th passage) were seeded on 4 glass coverslips (9 × 22 mm) in a 90 mm petri dish and grown as confluent monolayers in 10 ml medium at 37° C. On day 3, the medium was replaced with 10 ml growth medium containing 15% v/v delipidated NBCS. On day 5 the cells were incubated for 30 min at 4° C, the delipidated medium was replaced by precooled delipidated medium containing freshly isolated human LDL (protein concentration 0.1 mg/ml), and the cells were incubated for 2 h at 4° C. As a control, cells were treated in the same way except that on day 5 they were incubated for 2 h at 4° C in delipidated medium *without LDL*.

After the incubation procedure the cells were extensively washed with a buffer containing albumin, according to Goldstein et al. (1976).

(f) *Immunoenzyme Procedure.* Glass coverslips with washed fibroblasts were mounted on glass slides and fixed in 1% paraformaldehyde in PBS (pH = 7.4) for 10 min at 4° C. All subsequent steps were carried out at room temperature. A conventional indirect immunoperoxidase technique was used, i.e., with rabbit-anti-human apoprotein B diluted 1:20 in the first step and goat-anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:50 in the second step (Taylor and Burns, 1974; Nieuwenhuyzen Kruseman et al., 1975). Cell-bound peroxidase was visualized with 3.3′-diaminobenzidine 4 HCl (Merck, Darmstadt, W. Germany) and H_2O_2 (Graham and Karnovsky, 1966). No counter stain was used. As a staining control, cells were incubated either in the first step with normal rabbit serum or in the second step with unconjugated antibodies, followed by incubation with conjugated antibodies (Sternberger, 1974). The goat-anti-rabbit IgG-peroxidase conjugate was obtained from Nordic (Tilburg, The Netherlands). The coverslips were examined by conventional light microscopy and phase-contrast microscopy.

Results

With an indirect immunoperoxidase technique, normal fibroblasts incubated for 48 h in a delipidated medium at 37° C and subsequently exposed to LDL-containing delipidated medium for 2 h at 4° C showed an intense brown staining (Fig. 1). The reaction product was distributed diffusely over the whole cell surface and no predilection sites were observed.

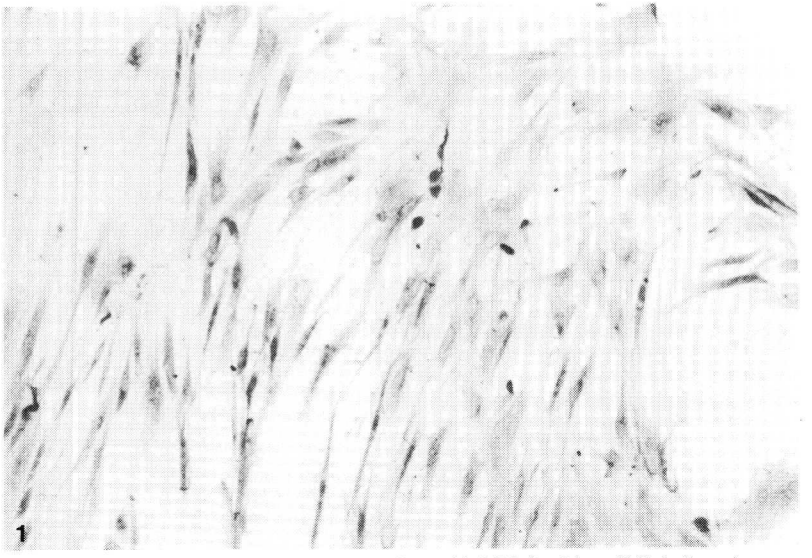

Fig. 1. Fibroblasts incubated in delipidated medium with LDL for 2 h at 4° C. Indirect immunoperoxidase staining. ×140

Fig. 2. Fibroblasts incubated for 2 h at 4° C in delipidated medium without LDL. Indirect immunoperoxidase staining. ×140

In contrast with these results, the fibroblasts of the homozygous hyperlipoproteinaemia IIa patient did not show any reaction product. The normal fibroblasts and the homozygous cells which had been exposed for 2 h at 4° C to delipidated medium *without* LDL were not stained by the immunoperoxidase technique and resembled the homozygous cells incubated in the LDL-containing delipidated medium (Fig. 2).

The staining controls were negative in all cases.

Discussion

The LDL-specific receptors of various types of cultured cells have been extensively studied since Brown and Goldstein (1976a) demonstrated that these receptors are present on normal fibroblasts but absent on cultured fibroblasts of familial type IIa hyperlipoproteinaemia homozygotes (Ho et al., 1976; Stein et al., 1976).

When the temperature is kept at 4° C during the incubation of fibroblasts with LDL, the internalization of LDL is prevented (Brown and Goldstein, 1976a) and the cell-bound LDL remains on the cell surface. The aspecifically bound LDL is removed by washing with albumin-containing buffer (Goldstein et al., 1976). In accordance with the results obtained by Brown et al. (1976b), with an indirect immunofluorescence technique, our studies done with an indirect immunoperoxidase technique showed the binding of unmodified LDL to normal fibroblasts at the light-microscopical level. This technique is easy to apply at the light-microscopical level and can also be used for ultrastructural investigations. Furthermore, with this technique the binding and internalization of unmodified LDL can be studied. On light and electron microscopical level studies are in progress.

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Chapter V §2

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BINDING OF UNMODIFIED LOW-DENSITY LIPOPROTEINS TO HUMAN FIBROBLASTS

AN INVESTIGATION BY IMMUNOELECTRON MICROSCOPY

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Summary

The binding of unmodified low density lipoproteins to the plasma membrane of fibroblasts was studied at the ultrastructural level. The bound low density lipoprotein was visualized by an indirect immunoperoxidase technique, with the use of an antiserum against apoprotein B. Immunoreactive regions representing bound apoprotein B were found on the plasma membrane, in indented regions with a diameter of 0.15—0.30 μm and a fuzzy coat on the cytoplasmic side. Fibroblasts from a patient homozygous for hyperlipoproteinaemia type IIa showed no immunoreactive material in the indented regions. The specific ^{125}I-labelled low density lipoprotein binding to these homozygous fibroblasts was 7% compared to control fibroblasts.

Using biochemical techniques, Brown et al. [1] showed that cultured normal human fibroblasts possess high-affinity binding sites for low-density lipoproteins (LDL). These binding sites were also demonstrated with the same techniques on cultured smooth-muscle cells [2], lymphocytes [3], and endothelial cells [4], but not on macrophages [5]. Fibroblasts and lymphocytes from patients homozygous for hyperlipoproteinaemia type IIa [6], have defective or no high-affinity LDL binding sites (Refs. 1, 3; for review, see Ref.

Abbreviation: LDL, human low density lipoprotein (density range 1.019—1.063 g/ml) prepared by ultracentrifugation [10].

7). Ultrastructural studies with ferritin-conjugated LDL showed the binding of these conjugates to specific regions of the fibroblast plasma membrane [8]. However, the conjugation procedure may influence the biological activity of LDL to such a degree that ferritin-conjugated LDL (in contrast to native LDL) will have an inhibitory effect on the 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in fibroblasts from patients homozygous for hyperlipoproteinaemia IIa [9]. Therefore, we developed an indirect immunoenzymecytochemical method for ultrastructural studies of the binding of unmodified LDL to fibroblasts.

Monolayers of normal human skin fibroblasts were grown to confluency at 37°C on pieces of Melinex plastic in 90-mm plastic petri dishes in 10 ml medium (Hams's F 10 growth medium containing 15% v/v new born calf serum), in an atmosphere of 95% air/5% CO_2. Human low-density lipoproteins (d = 1.03—1.05 g/ml) were isolated from fasting normal human EDTA plasma by preparative ultracentrifugation in a Sorvall OTD 2 ultracentrifuge with Ti 865 rotor (Ivan Sorvall, Inc., Newton, CT, U.S.A.) [10]. The new born calf serum was delipidated by ultracentrifugation at a density of 1.25 g/ml for 36 h at 50 000 rev./min, after which the bottom fraction was dialyzed against isotonic saline and sterilized by millipore filtration.

Antiserum against apolipoprotein B was obtained as described by Vermeer et al. [11]. For the biochemical studies, human LDL were isolated from human serum in an SW 40 Rotor according to Redgrave et al. [12]. ^{125}I-labelled LDL was prepared at pH 10 according to a modification [13] of the ^{125}I chloride labelling method of McFarlane [14]. The iodine/protein ratio was 0.64 atom/mol for human LDL, and 92—94% of the radioactivity was protein bound [15]. Confluent fibroblasts were incubated for 48 h at 37°C in 10 ml medium containing 15% (v/v) delipidated new born calf serum prior to the experimental procedure. For the immunohistochemical procedure, the cells were cooled for 30 min at 4°C and subsequently incubated for 2 h at 4°C in delipidated precooled medium containing freshly isolated human LDL (protein concentration: 0.1 mg/ml medium) [11].

After incubation, the cells were washed 4 times with 5 ml 0.2% (v/v) bovine serum albumin in 0.15 M NaCl and then twice with 5 ml 0.15 M NaCl without albumin, in order to remove aspecifically bound LDL [16]. Next, the cells were fixed in 1% paraformaldehyde in phosphate-buffered saline at 4°C for 10 min, and stained by the following indirect immunoenzyme procedure at room temperature:

(a) incubation (20 min) in normal goat serum (diluted 1:8);
(b) incubation (20 min) in rabbit-anti-human apoprotein B (diluted 1:20);
(c) refixation (10 min) in 0.5% glutaraldehyde in phosphate-buffered saline. Glutaraldehyde fixation can only be used after the immunological reaction with apoprotein B has taken place, because glutaraldehyde destroys the immunoreactivity of apoprotein B [17];
(d) incubation (20 min) in goat-anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:50), (Nordic, Tilburg, The Netherlands);
(e) visualization of cell-bound peroxidase with 3,3′-diaminobenzidine·4 HCl (Merck, Darmstadt, F.R.G.) and H_2O_2 [18];

(f) postfixation (30 min) in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) containing 0.05% $CaCl_2$, at 4°C.

Between each of these steps, the cells were washed twice with phosphate-buffered saline, except after glutaraldehyde fixation, when the cells were washed with 0.1 M Tris-HCl buffer (pH 7.4) to remove free aldehyde groups [19]. After postfixation, the cells were partially dehydrated in an ethanol series to 70%, in order to reduce the lipid loss [20], and embedded in situ according to Mariano and Spector [21].

Because an antiserum against apoprotein B (the main apoprotein of LDL) was used in the first step of the immuno procedure, immunoreactive regions of the plasma membrane can be regarded as showing LDL binding.

As can be seen from Fig. 1, immunoreactive material is present on indented regions of the plasma membrane. In lead-stained sections (Figs. 2,3) these indented regions have a fuzzy coat on their cytoplasmic sides, and closely resemble the coated regions described by Anderson et al. [8]. The width of the indented regions is fairly constant, ranging between 0.15 and 0.30 μm. Immunoreactive material was only found very occasionally on the plasma membrane outside these indented regions.

The immunoreactive indented regions were unevenly distributed over the cell surface and were sometimes found in 'clusters' at peripheral cellular margins. Moreover, some cells had many peroxidase-positive regions, whereas neighbouring cells did not show such regions. This uneven distribution is in agreement with the autoradiographic findings of Anderson et al. [8].

In the controls (cells incubated with normal rabbit serum instead of rabbit-anti-apoprotein B or cells exposed to peroxidase (50 μg/ml) instead of LDL) no peroxidase-positive indented regions were found.

In additional biological control experiments, use was made of cells from a patient homozygous for hyperlipoproteinaemia IIa who had extensive tuberous xanthomas and serum LDL cholesterol level of 700 mg/dl. Biochemically, these fibroblasts showed an almost complete absence of high-affinity LDL binding (see below). Ultrastructural immunoenzymehistochemical studies performed with these cells showed indented regions which invariably lacked immunoreactive material (Figs. 4,5).

To quantitate the binding of LDL to the control and homozygous cells, biochemical studies were done with iodine-labelled LDL. According to the method described by Stein et al. [22], the cells were incubated for 2 h at 37°C with ^{125}I-labelled LDL (14 μg protein/ml). The total specific LDL binding was determined by incubation with ^{125}I-labelled LDL in both the absence and presence of 500 μg unlabelled LDL. At 14 μg protein ^{125}I-labelled LDL/ml, the specific binding of the homozygous cells was 7% of the control values. The degradation of ^{125}I-labelled LDL, measured as trichloric acetic acid soluble radioactivity, was, under the conditions applied, virtually zero.

The results of our ultrastructural studies are in accordance with the findings of Anderson et al. [8], who used ferritin-conjugated LDL. In contrast to Anderson's method (under our conditions) the fibroblasts are exposed to unmodified LDL.

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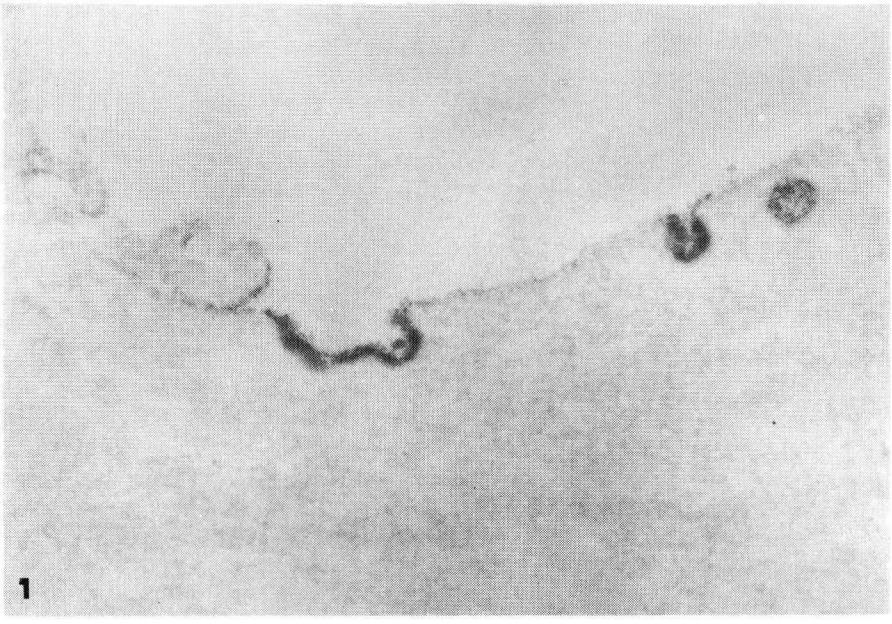
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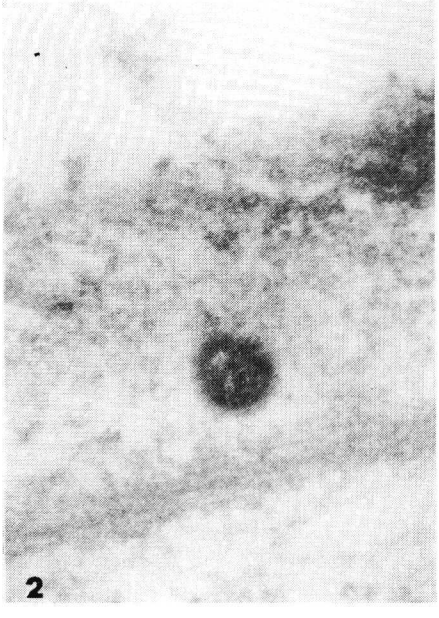
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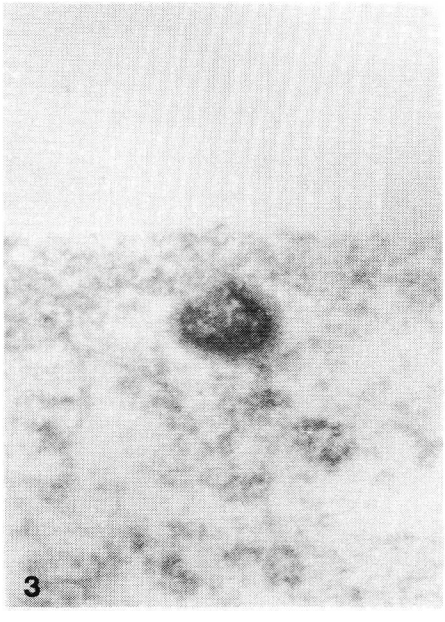
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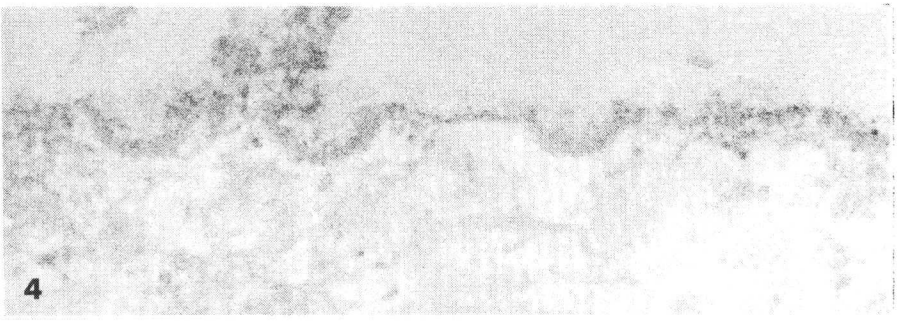

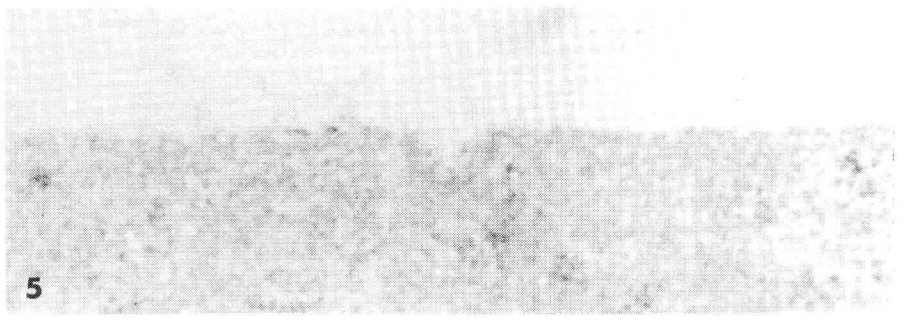

Foreskin fibroblasts, grown to confluency, were cultured in delipidated medium for 48 h, and then exposed for 2 h at 4°C to LDL (0.1 mg protein/ml). After washes with albumin-containing buffer, the cells were fixed for 10 min at 4°C in 1% *p*-formaldehyde, incubated with normal goat serum followed by rabbit-anti-human apoprotein B, refixed for 10 min in 0.5% glutaraldehyde, and re-incubated with goat-anti-rabbit IgG conjugated with horse-radish peroxidase. After cytochemical visualization of the cell-bound peroxidase [18], the cells were postfixed for 30 min in 1% OsO_4, partially dehydrated in ethanol, and embedded and sectioned according to Mariano and Spector [21]. Fig. 1. An unstained ultrathin section of a normal human foreskin fibroblast showing several immunoreactive indented regions with a diameter of 0.15—0.30 μm. × 25 000. Figs. 2 and 3. A lead citrate-stained section of a normal human foreskin fibroblast showing a fuzzy coat on the cytoplasmic side of the plasma membrane in the immunoreactive region. × 60 000. Figs. 4 and 5. An indented region of the plasma membrane of a fibroblast from a patient homozygous for hyperlipoproteinaemia IIa. The indented region shows a fuzzy coat but no immunoreactive material. Lead citrate-stained section. × 60 000.

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Chapter VI

DISCUSSION

§ 1 Clinical investigations

Biochemical analysis of the serum of patients with xanthomatosis and hyperlipoproteinaemia showed a bimodal distribution of the LDL total lipid serum levels. The minimum between the two peaks lays at a concentration of 550-600 mg total lipid LDL/dl serum. A natural subdivision of patients with xanthomatous lesions was found, i.e., a group with normal and a group with elevated LDL levels. The LDL concentration of 550-600 mg total lipid/dl serum appears to represent the cut-off value of the normal LDL total lipid levels in these patients. This cut-off value obtained in such a way is preferable to upper-limit normal levels, which are defined as 95th percentile of reference groups. The cut-off value found by natural subdivision in our investigations corresponds with the upper-limit normal levels of LDL cholesterol (220-240 mg/dl serum) as described by Fredrickson et al. (1978).

However, a natural subdivision of VLDL total lipid levels was not found in the same group of patients with xanthomatosis. Therefore, the cut-off value of 250 mg/dl VLDL total lipid was based upon the 95% upper-limit of VLDL lipid in a reference group, as determined earlier (Polano et al., 1969). According to the upper-limit normal levels of VLDL cholesterol (33 mg/dl) and VLDL triglyceride (160 mg/dl) given by Carlson and Ericsson (1975), the upper-limit normal VLDL total lipid level would be 240 mg/dl serum which is in good accordance with our value. The cut-off values for VLDL and LDL influence the classification of different types of hyperlipoproteinaemia among our patients with xanthomatosis. This is especially relevant for the differentiation between

hyperlipoproteinaemia IIa and IIb (Carlson, 1976).

Using the above-defined cut-off values for LDL and VLDL total lipid levels, we were able to recognize characteristic patterns in the distribution of hyperlipoproteinaemia. For their designation we use the terms of the WHO classification of 1970, although we are aware of the limitations of this classification. The WHO accepts the presence of floating beta lipoprotein as diagnostic for hyperlipoproteinaemia III. However, because these floating beta lipoproteins are not always detectable in the same patient and, furthermore, the VLDL are also elevated in most HLP type III patients, it is often impossible to differentiate on the basis of the WHO classification system between this type of hyperlipoproteinaemia and other types in which the VLDL are also elevated (Hazzard et al., 1972; Fredrickson et al., 1975). For this reason, we classified hyperlipoproteinaemia III according to the cholesterol VLDL/triglyceride VLDL ratio as advocated by Hazzard et al. (1972).

A cholesterol VLDL/triglyceride VLDL ratio higher than 0.5 in patients with elevated VLDL levels was regarded as diagnostic for hyperlipoproteinaemia III. If this ratio was lower than 0.5, the patients were classified as hyperlipoproteinaemia IV/V. However, contrary to the findings in hyperlipoproteinaemia III and hyperlipoproteinaemia IV/V, we found that the cholesterol VLDL/triglyceride VLDL ratio had no diagnostic value in 8 patients with elevated VLDL and elevated therapy resistant LDL levels. According to the WHO criteria, these patients would be classified as IIb.

The relatively high cholesterol content in VLDL found in patients with hyperlipoproteinaemia III is in all likelihood caused by the intermediate density lipoprotein present in the isolated "VLDL" serum fraction of these patients (Hazzard and

Bierman, 1975).

This intermediate density lipoprotein (IDL) is characteristic for hyperlipoproteinaemia III, according to Quarfordt et al. (1973). The IDL is found not only in the VLDL fraction of the serum but also in the LDL fraction (Slack and Mills, 1974). The elevated LDL levels we found in 9/31 patients diagnosed as hyperlipoproteinaemia III are probably caused by the IDL also present in the LDL fraction. These 9 patients did not differ clinically from the other hyperlipoproteinaemia III patients (see Chapter II, § 2), and these "elevated LDL" levels could easily be normalized by a low caloric diet. These findings suggest that our 9 patients do not represent a distinctive subgroup of the hyperlipoproteinaemia III.

Recent investigations of Demacker et al. (1978) have demonstrated that, using agarose gel electrophoresis, two variants in type III hyperlipoproteinaemia could be distinguished. Moreover, these two groups differed considerably as to the presence of xanthomas. Studies of the apoproteins in the lipoproteins isolated at $d < 1.006$ in hyperlipoproteinaemia III have shown that these lipoproteins are enriched in apolipoprotein E (Havel and Kane, 1973). Using analytical isoelectric focusing it was demonstrated by Utermann et al. (1975,1977) that in patients with hyperlipoproteinaemia III as well as in their relatives a defect of apolipoprotein E III could be found. As yet we have not been able to screen our patients for this specific criterion.

The clinical findings in the 78 patients with xanthomatosis and hyperlipoproteinaemia classified according to the above-described criteria are summarized in Table I, from which it is clear that the clinical pictures of patients suffering from hyperlipoproteinaemia IIa and IIb resemble

Table I

Clinical features of 78 patients with hyperlipoproteinaemia and xanthomatosis

	HLP IIa *LDL elevated **VLDL normal	HLP IIb LDL elevated VLDL elevated	HLP III LDL normal or temporarily elevated VLDL elevated *** IDL present	HLP IV/V LDL normal VLDL elevated
Chol.VLDL/Tg.VLDL	n.p. n = 32	n.d. n = 8	> 0.5 n = 31	< 0.5 n = 7
Mean age (year)	44	48	44	43
Males/females	14/18	5/3	22/9	6/1
ASCVD	18	7	7	1
Cholesterol mg/dl	510	541	540	466
Xanthochromia striata palmaris	0	2	29	0
Xanthoma tubero eruptivum	8	2	26	0
Xanthoma papulo eruptivum	0	0	20	7
Xanthoma tendineum	30	8	8	0
Xanthelasma palpebrarum	9	4	2	0

*LDL, elevated = total lipid LDL > 550 mg/dl serum

**VLDL, elevated = total lipid VLDL > 260 mg/dl serum

***IDL, = Intermediate Density Lipoprotein

n.p. = not performed

n.d. = not diagnostic

each other. Both groups of patients are characterized by elevated LDL levels, tendinous xanthomas, and a high incidence of atherosclerotic vascular disease (ASCVD).

Recently, Fredrickson et al. (1978) no longer distinguished the hereditary forms of hyperlipoproteinaemia IIa and IIb in the hereditary disease for which he uses the term familial hypercholesterolaemia coined by Thanhauser in 1938. It is clear from Table I that the patients with elevated VLDL levels have a much lower incidence of ASCVD compared with the patients characterized by elevated LDL levels. However, the cholesterol value is almost the same in both groups. For this reason we think that the term familial hypercholesterolaemia is not very appropriate, and would prefer the term familial hyper-low density lipoproteinaemia.

The clinical appearance of the xanthomatous lesions also differs distinctly between the patients with elevated LDL (HLP IIa and HLP IIb), IDL (HLP III), or VLDL (HLP IV/V) levels. Moreover, the presence of xanthochromia striata palmaris is highly characteristic for hyperlipoproteinaemia III. The pattern of distribution of the eruptive xanthomas is distinctive in hyperlipoproteinaemia IV/V (see page 38). Consequently, these clinical findings already allow us to discriminate between patients with elevated IDL levels (HLP III) and patients with elevated VLDL levels (HLP IV/V). According to Morganroth et al. (1975), the incidence of xanthomatous lesions in hyperlipoproteinaemia III is almost 70%. Moreover, the appearance of these characteristic xanthomas will often be the first sign of this hereditary disease. The high incidence of xanthomatosis in HLP III patients might also explain the large number of these patients in our series compared with the low proportion of HLP IV/V patients with xanthomas in our material. In the

latter group, the patients all show glucosuria and very strongly elevated triglyceride levels. Very often, chylomicronaemia is found at the first visit. A low caloric diet with carbohydrate restriction can be used to lower the very high VLDL levels, and the chylomicrons disappear at the same time. Consequently, the elevated triglyceride and cholesterol levels are also lowered by this diet. This good response to therapy is also found in hyperlipoproteinaemia III, but is in sharp contrast with the resistance to therapy found in patients with hereditary forms of elevated LDL levels.

As an example of the improvement of extremely elevated levels obtained with a low caloric diet, one patient with HLP V and xanthomatosis is described in Chapter II, § 3. In this patient the insulin could be withdrawn, and it is conceivable that the iatrogenic high insulin serum levels had also contributed to the elevated VLDL levels in the serum.

When using the system of phenotyping the different types of hyperlipoproteinaemias we must always keep in mind the fact that with exception of HLP I and HLP III, the phenotypes are not genetically specific and may denote monogenic, polygenic, or acquired disorders (Jensen and Blankenhorn, 1972; Motulsky, 1976).

In conclusion we can state that three groups of patients with different clinical findings could be distinguished and that these patients were characterized by elevated VLDL, IDL, or LDL serum levels. The group of patients with elevated LDL levels show, despite almost equal cholesterol values, a markedly higher incidence of ASCVD than the other two groups. Our clinical findings suggest that VLDL, IDL, and LDL have different effects on dermal and vascular tissue.

This led us to investigate the lipid (lipoprotein) content of the interstitial fluid from which the cells

present in xanthomatous lesions internalize lipids and/or lipoproteins.

§ 2 Insterstitial fluid

As described in Chapter III, the suction blister fluid/-serum concentration (CB/CS) ratio for apoprotein B (MW 2,300,000) was 0.14 and the corresponding value for apoprotein AI (MW 300,000) was 0.24. This divergence was dependent on the molecular weight. The amounts of apoprotein found by us in suction blister fluid are similar to those reported for peripheral lymph by Reichl et al. (1975).

On the basis of these findings it was concluded that suction blister fluid reflects the composition of interstitial fluid. Moreover, Kiistala's (1969) finding that the albumin/globulin ratio in suction blister fluid resembles this ratio in pheripheral lymph also supports this conclusion. The higher concentration ratio for Apo AI representing HDL compared with Apo B respresenting LDL and VLDL is most probably due to the fact that the smaller HDL is more easily transported through the vascular wall than the larger LDL (Grotte, 1956). The cells surrounded by interstitial fluid are therefore exposed to an Apo AI/Apo B ratio which is different from that in serum. The relatively greater amount of apo AI in interstitial fluid probably favours the release of cholesterol from these cells (Werb and Cohn, 1972; Stein et al., 1976).

Studies on cultured cells incubated with LDL have shown that this LDL can be internalized by a high-affinity process that is saturated at low LDL levels (Goldstein and Brown, 1977). The low apoprotein B concentration we found in suction blister fluid therefore suggests that cells surrounded by interstitial fluid will take up most of the apo B (LDL) by

means of the high-affinity process. In addition to a high--affinity uptake, the LDL may also be internalized by a low--affinity process. The rate of this process is proportional to the LDL concentration. Presumably, the low-affinity uptake process only plays an important role at pathological LDL concentrations.

The Apo AI and Apo B we estimated were regarded as representing HDL and LDL in the interstitial fluid. On the basis of the fact that the lipid composition of these two lipoproteins differs distinctly, it is reasonable to assume that this difference would also be reflected in a selectivity of the blister fluid/serum concentration ratio of various lipid classes. However, we did not find a similar difference for the various lipid classes, where concentration blister fluid/serum ratio was always about 0.25. These findings could be explained by assuming that the lipid composition of the lipoproteins in interstitial fluid differs from its composition in serum.

When we consider the Apo AI and Apo B content in suction blister fluid or peripheral lymph, another consideration must be taken into account. Apoprotein AI and/or apoprotein B can react with other substances in the interstitial fluid, in all probability acid mucopolysaccharides (Gero et al., 1960; Amenta and Waters, 1960). As a result of this interaction, the apoprotein-containing particle can be retained in the interstitial space and is not found in the suction blister fluid or peripheral lymph. Recent investigations have shown that apoprotein B-containing particles are trapped in atherosclerotic lesions and arterial walls (Hoff et al., 1978; Avila et al., 1978).

§ 3 Morphological studies

On the basis of the experiments described in Chapter IV, § 1, we concluded that digitonin is not suitable for the specific ultrastructural visualization of cholesterol in tissue. Therefore, alternative methods were needed to visualize the lipoproteins in vitro by conventional transmission electron microscopy. For this purpose, a post-fixation procedure (de Bruyn, 1969) which increases the contrast needed for ultrastructural studies seemed suitable. To make it possible to prepare ultrathin sections of the material, the lipoproteins were enrobed in a fibrin matrix and embedded in Epon. In this way the LDL and VLDL isolated from serum by preparative ultracentrifugation could be studied with a conventional transmission electron microscope. As shown in Chapter IV, § 2, the LDL particles had a relatively regular shape and consisted of darkly stained round structures with a diameter of 30 nm. The VLDL particles homogeneously distributed in the fibrin matrix had an irregular shape and varied in size between 30 and 80 nm. The findings concerning the LDL particles are in accordance with the observations of other authors who used physicochemical and negative staining techniques (Forte and Nichols, 1972; Pasquali-Ronchetti et al., 1975; Jackson et al., 1976).

The application of the same post-fixation procedure (de Bruyn, 1969) to xanthomatous tissue showed that several round particles with a diameter of 30 nm were present in the extracellular space. However, because the staining of the lipoproteins was obtained in a non-specific way (de Bruyn and den Breejen, 1975) it was impossible to say whether, despite their morphological similarity to the in vitro material, the 30-nm particles really represented the LDL.

In addition to these smaller particles, many membrane-

-bound structures with a translucent core and a larger diameter were found in the xanthomatous tissue as well. After the same fixation procedure, both the 30-nm particles and the larger membrane-bound structures were also observed in experimental atherosclerotic lesions of rabbits as described by de Bruyn (1969) and de Bruyn and van Mourik (1975).

No method has yet been proposed to enable us to visualize the lipoproteins in a specific way. Since it was thought that demonstration of the apoproteins might serve as a specific marker for the different lipoproteins, we developed an immuno-enzymehistochemical method, which allowed us to visualize the apoprotein B reŕesenting LDL or VLDL on the ultrastructural levels. However, preliminary light-microscopical studies on xanthomatous lesions, in which immunoperoxidase was used, showed only uninterpretable deposits of this tissue. Moreover, we found that the antigenic binding site of apoprotein B was easily destroyed by the glutaraldehyde used in preparing tissue for electron microscopy. This was also described by Hoff and Gaubatz (1975) and Alexander and Hamilton (1976). Because pre-fixation with glutaraldehyde is indispensable to obtain satisfactory preservation of xanthomatous tissue for ultrastructural studies, we started to develop an immunoperoxidase technique, first using an in vitro model. For this purpose, cultured fibroblasts were incubated with LDL, as described in the following section.

§ 4 LDL binding sites

Biochemical studies have shown that cultured fibroblasts possess a high-affinity binding site for LDL on the plasma membrane which is saturated at low levels of LDL (for review, see Goldstein and Brown, 1977). The internalized LDL is subsequently degraded in lysosomes and cholesterol is formed.

The LDL-cholesterol thus formed induces a suppression of the cellular cholesterol-synthesizing enzyme 3-hydroxy-3-methyl--glutaryl coenzyme. A reductase (HMG CoA red). Furthermore, the activity of the cholesterol-esterifying enzyme fatty acyl CoA: cholesteryl acyl transferase (ACAT) is increased by the LDL-cholesterol. The synthesis of high-affinity LDL binding sites is suppressed after the internalization and degradation of LDL. In this way the normal cell is able to utilize the LDL-cholesterol and to prevent excessive cholesterol accumulation.

The above-described feedback mechanism of high-affinity binding of LDL proved to be absent or defective in fibroblasts and lymphocytes from patients with homozygous familial hypercholesterolaemia (Goldstein and Brown, 1974; Ho et al., 1976).

Ultrastructural studies on LDL binding have been performed by Anderson et al. (1976) and Orci et al. (1978) in cultured fibroblasts incubated with ferritin-conjugated LDL. These authors found that the ferritin-labeled LDL was bound to the plasma membrane in an indented region with a diameter of 0.2 μm and with a fuzzy coat on the cytoplasmic side. However, the procedure they used has the drawback that the cultured cells are incubated with ferritin-conjugated LDL which is a modified LDL. According to the findings of Fung et al. (1978), the biological activity of ferritin-conjugated LDL is different from that of non-conjugated LDL. The use of an immunotechnique made it possible to incubate the cultured cells with unmodified LDL. The LDL binding sites with a diameter of 0.15-0.30 μm found with our method proved to be similar to those observed by Anderson et al. (1977a). With our methods the binding sites were also characterized by a fuzzy coat on the cytoplasmic side.

In our studies on cultured fibroblasts of a patient with homozygous familial hypercholesterolaemia we found several coated indented regions. However, after incubation with LDL no apoprotein B complexes were present in these regions or on the plasma membrane outside this region.

§ 5 Perspectives

The nature and chemical composition of the indented coated regions (coated pits) on the plasma membrane of cells is still a matter of speculation. Roth and Porter (1964) and Roth et al. (1976) have shown that oocytes possess a specific binding site for maternal proteins, a site which resembles the above-described coated regions. Moreover, in a recent paper by Maxfield et al. (1978) specific α_2-macroglobulin patches situated over indented coated regions in the plasma membrane of 3T3-4 cells are described. These findings suggest that an indented coated region represent a specific binding site on the plasma membrane for different macromolecules in solution.

The specific binding to these sites is biochemically characterized by a high-affinity process that is saturated at low concentrations of the substrate. The subsequent selective and concentrating uptake of these solutes is called adsorptive endocytosis or adsorptive pinocytosis by Silverstein et al. (1977). This uptake process might take place via invagination of these indented coated regions, which then acquire the morphological characteristics of coated vesicles (Roth and Porter, 1964; Fawcett, 1965; for review, see Goldstein et al., 1979).

The ultrastructural studies of Anderson et al. (1977a) showed that receptor-bound LDL conjugated with ferritin was internalized by fuzzy-coated vesicles which ultimately fused

with lysosomes. This internalization was completed in approximately 10 minutes at 37°C. In recent investigations it was found that all LDL specifically bound to the plasma membrane was internalized within 10 to 20 minutes (Vermeer et al., 1979).

However, it is important to keep in mind that coated vesicles may also be involved in functions other than endocytosis. As shown by Friend and Farquhar (1967), coated vesicles can be found as Golgi-associated vesicles. Rodewald (1973) observed in studies on neonatal cells that maternal immunoglobulin enters the intestinal cells in smooth-surface invaginations and is discharged from coated vesicles into the intercellular space.

It remains a question whether the coated pits represent a discrete structural entity as a site for endocytosis or represent secondary changes of the plasma membrane following attachment of LDL or other macromolecules to its receptor, or both. In this respect the following findings are relevant.

The ultrastructural observations of Anderson et al. (1976, 1977a) showed that the LDL was bound to coated pits on the plasma membrane when the fibroblasts were incubated for a short time with LDL at a temperature of 4°C or even when the cells were pre-fixed with formaldehyde. During both experimental procedures, lateral movement of LDL on the plasma membrane into coated pits is retarded (Frye and Edidin, 1970; Abbas et al., 1975; Singer, 1979). These findings support the hypothesis that a coated pit represents a discrete structural entity.

However, immunofluorescence studies on other substances (insulin, epidermal growth factor, and α_2-macroglobulin) which are also characterized by a specific binding and selective uptake, showed that these substances bind diffusely

at 4°C to receptors on the cell surface (Maxfield et al., 1978). When the cells are warmed to 23 or 37°C, the bound substances rapidly form immobile patches on the cell surface and are internalized.

In this context the observation of Brown and Goldstein (1976) of one very exceptional patient homozygous for familial hypercholesterolaemia is important. This patient has a "normal specific binding of I^{125} labeled LDL", but no specific internalization took place. In cultured fibroblasts of this patient it was found that LDL was bound to the plasma membrane in a random fashion and was not present in the indented coated regions (Anderson et al., 1977b). The authors postulated that clustering of LDL into the coated regions occurs due to recognition at the cytoplasmic side of the LDL membrane receptor.

This hypothesis is consistent with the fact that in normal fibroblasts 30% of the LDL can be found on the plasma membrane outside the invaginated regions. These specifically bound LDL found outside the coated regions are virtually absent in homozygote cells (Anderson, 1977a; Orci et al., 1978). It therefore remains possible that this specifically bound LDL is migrating into pre-existing coated pits or induces the formation of a new coated pit.

The latter hypothesis is in accordance with the report by Roth et al. (1976) that oocytes show a dramatic increase of indented coated regions during the period of active protein sequestration. These findings are supported by the irregular distribution of coated pits and LDL-containing coated pits on the plasma membrane, as we found in our studies (see Chapter V, § 2).

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SUMMARY

In this thesis clinical and biochemical investigations performed in patients with xanthomatosis and hyperlipoproteinaemia are reported. It was found that at a serum concentration of 550-600 mg/dl LDL* total lipid there is a natural subdivision by which patients with xanthomas and elevated LDL serum levels can be distinguished from patients with xanthomas and "normal" LDL serum levels. On the basis of earlier investigations, the cut-off value for normal VLDL** total lipid serum levels was taken as 250 mg/dl. In 46 patients with elevated VLDL levels the cholesterol VLDL/-triglyceride VLDL ratio was used as an additional discriminating criterion.

A cholesterol/triglyceride ratio of more than 0.5 in the VLDL was considered to be diagnostic for patients with elevated IDL*** levels. This abnormality was found in 31 out of 46 patients. In 9 out of these 31 patients the LDL level was initially elevated but was easily normalized by a low caloric diet. Unlike these 9 patients, in 8 out of 46 patients the elevated LDL levels proved to be therapy-resistant. In the latter group the ratio of cholesterol VLDL to triglyceride VLDL was not of further diagnostic help. In addition, 7 out of 46 patients had elevated VLDL levels, normal LDL levels, and a cholesterol VLDL to triglyceride VLDL ratio lower than 0.5. Each of these groups of xanthoma patients with hyperlipoproteinaemia proved to have its own characteristic pattern of xanthomatosis and frequency of ASCVD.

Comparison of our findings with the 1970 WHO classification gave the following groups:

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*LDL = Low Density Lipoprotein $1.006 < d < 1.063$.
**VLDL = Very Low Density Lipoprotein $0.94 < d < 1.006$.
***IDL = Intermediate Density Lipoprotein.

A. Patients with elevated LDL levels (therapy-resistant), sometimes combined with elevated VLDL levels, comparable with WHO type HLP IIa (n=32) or HLP IIb (n=8), respectively.

B. Patients with elevated VLDL levels and with detectable IDL levels (ratio cholesterol VLDL/triglyceride VLDL > 0.5; n=31) comparable with HLP III in the WHO classification. In 9 out of 31 patients the LDL levels were also elevated, but normalized easily on a diet.

C. Patients with elevated VLDL levels, normal LDL levels, and a cholesterol VLDL/triglyceride VLDL ratio < 0.5, comparable with HLP IV/V (WHO; n=7).

In all three groups the elevation of the cholesterol levels was caused by different classes of lipoprotein. Despite equally elevated cholesterol serum levels and the same mean age of the patients, the clinical findings differed distinctly between these three groups. In patients with therapy-resistant elevated LDL levels (HLP IIa, IIb) the incidence of atherosclerotic vascular disease (ASCVD) was very high. These patients showed only tendinous and tuberous xanthomas.

In the group with elevated VLDL levels the groups HLP III and HLP IV/V could be distinguished not only biochemically but also by a different xanthoma pattern. In both, the incidence of ASCVD was much lower than in HLP IIa or IIb. In HLP III the presence of xanthochromia striata palmaris was characteristic, and in HLP IV/V the papular eruptive xanthomas were distributed in a distinctive way. Moveover, all HLP IV/V patients with xanthomatosis showed an abnormal glucose tolerance. These clinical findings suggest a different effect of VLDL, IDL, and LDL on dermal and vascular tissue.

To obtain more information about the distribution of the different lipoproteins in the interstitial fluid to which dermal cells are exposed, the concentration of apoproteins and various lipid classes were determined in suction blister fluid. The results showed that the blister fluid/serum concentration ratio of the different proteins was dependent on their molecular weight (representing different molecular sizes). The blister fluid/serum concentration ratio of apoprotein AI (protein moiety of HDL*) was 0.24 and that of apoprotein B (protein moiety of LDL and VLDL) was 0.14. In contrast with the selectivity of the concentration ratios for these proteins, the suction blister fluid/serum concentration ratio for the different lipid classes had a fairly constant value of 0.25. No explanation could be found for this phenomenon.

An attempt was also made to visualize the different lipoproteins in xanthomatous tissue and in vitro by using a transmission electron microscope.

Because it has been suggested in the literature that digitonin-containing aldehyde fixatives retain and visualize cholesterol in tissue, this possibility was investigated. In our in vitro studies we found that the loss of cholesterol by the addition of digitonin to the aldehyde fixative during dehydration was only decreased very slightly. In the in vitro model even 26% of the cholesterolesters was mobilized when digitonin was added. The mobilized cholesterolester complexes were isolated and showed ultrastructurally a close resemblance to the "whorls" observed by several authors in tissue fixed with a digitonin-containing aldehyde fixative.

Because digitonin produced uninterpretable artefacts and

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*HDL = High Density Lipoprotein $1.063 < d < 1.21$.

mobilization of lipids, other methods to visualize the lipoproteins were sought. The application of OsO_4 plus $K_3Fe(CN)_6$ as a secondary fixative after aldehyde fixation, permitted the demonstration of the presence of 30-300 nm membrane-bound particles in xanthomatous tissue. These particles resembled those seen in the atherosclerotic lesions of hypercholesterolaemic rabbits. When the same fixation procedure was used on isolated LDL in a fibrin matrix, darkly stained structures with a diameter of 30 nm were observed. When isolated VLDL particles were treated in the same way they showed an irregular appearance and a diameter varying between 30 and 80 nm. Because the staining obtained in this way was insufficiently specific, an indirect immunoperoxidase technique was developed in order to visualize the apoprotein B.

In cultured fibroblasts incubated with LDL the apoprotein B could be demonstrated at both the light-microscopical and ultrastructural levels. The immunoperoxidase--positive binding sites were irregularly distributed on the plasma membrane. These sites might represent different metabolic activity of cultured cells exposed to lipoproteins. More detailed studies showed that the LDL specifically bound to the plasma membrane proved to be localized mainly in indented regions with a diameter of 0.15-0.30 μm and a fuzzy coat on the cytoplasmic side. Using ^{125}I, we found that cultured fibroblasts of a patient homozygous for familial hypercholesterolaemia did not show specific binding of LDL. The ultrastructural studies on these fibroblasts showed the same morphological structures (indented coated regions) on the plasma membrane, but no apoprotein B complexes were found in their indented coated regions.

The findings described here suggest that the indented coated regions are structures which not exclusively take up LDL. The study of cell cultures exposed to specific lipoprotein classes or to specifically modified lipoproteins might result in the recognition of factors playing a role in the accumulation or removal of cholesterolesters in and from cells and tissues.

SAMENVATTING

In dit proefschrift wordt een beschrijving gegeven van klinische en biochemische onderzoekingen, die bij patienten met xanthomen en hyperlipoproteinaemieën zijn verricht. Een concentratie van 550-600 mg lipiden in de LDL* per deciliter serum bleek een natuurlijke onderverdeling te maken tussen patienten met xanthomen en verhoogde LDL serumspiegels en patienten met xanthomen en normale LDL serumspiegels. In navolging van eerdere onderzoekingen werd een lipidengehalte van 250 mg/dl serum in de VLDL** als bovenste grens van de normale waarde beschouwd.

Op deze wijze konden in een populatie van xanthoompatienten met hyperlipoproteinaemieën 46 patienten met verhoogde VLDL serumspiegels onderscheiden worden. Met het oogmerk een nadere onderverdeling in deze patientengroep te maken werd als tweede criterium de ratio cholesterol/triglyceride in de VLDL gebruikt. Een verhoogde VLDL serumspiegel en een cholesterol/triglyceride VLDL ratio hoger dan 0,5 werd als diagnostisch criterium voor verhoogde IDL*** serumspiegels beschouwd. Deze afwijking werd bij 31 van de 46 patienten gevonden in het serum. Bij 9/31 patienten werd aanvankelijk bovendien een verhoogde LDL serumspiegel gevonden. Deze verhoogde LDL serumspiegels konden bij deze patienten vrij gemakkelijk door een vermageringsdieet tot normale waarden worden teruggebracht.

In tegenstelling tot deze patienten met tijdelijk verhoogde LDL spiegels hadden 8 van de 46 patienten met verhoogde VLDL serumspiegels ook verhoogde LDL spiegels die thera-

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*LDL = Low Density Lipoprotein $1{,}006 < d < 1{,}063$.
**VLDL = Very Low Density Lipoprotein $0{,}94 < d < 1{,}006$.
***IDL = Intermediate Density Lipoprotein.

peutisch niet beïnvloedbaar waren. Bij deze 8 patienten bleek de ratio cholesterol/triglyceride in de VLDL geen verdere diagnostische betekenis te hebben. Tenslotte bleken de resterende 7/46 patienten met verhoogde VLDL spiegels een normale LDL serumspiegel en een ratio cholesterol/triglyceride in de VLDL te hebben die lager was dan 0,5.

Indien wij onze gegevens samenvatten en deze vergelijken met de WHO classificatie van 1970, worden de volgende patientengroepen gevonden:

A. Patienten met verhoogde LDL spiegels (n=40) (therapeutisch niet beïnvloedbaar) soms gecombineerd met verhoogde VLDL spiegels. Deze patienten komen overeen met de WHO classificatie als hyperlipoproteinaemie IIa (n=32) en hyperlipoproteinaemie IIb (n=8).
B. Patienten met verhoogde VLDL serumspiegels en met aantoonbaar IDL in het serum (n=31) (ratio cholesterol/triglyceride in de VLDL groter dan 0,5). Deze patienten komen overeen met de WHO classificatie hyperliproteinaemie III. Bij 9/31 patienten waren de LDL serumspiegels aanvankelijk ook verhoogd.
C. Patienten met verhoogde VLDL serumspiegels, normale LDL serumspiegels en een ratio cholesterol/triglyceride in de VLDL kleiner dan 0,5 (n=7). Deze patienten komen overeen met de WHO classificatie hyperlipoproteinaemie IV/V.

Het is belangrijk om vast te stellen dat bij al deze drie groepen de cholesterol-serumspiegels verhoogd waren. De verhoogde cholesterolwaarden zijn echter door verschillende lipoproteineklassen veroorzaakt respectievelijk LDL, IDL en VLDL.

De klinische bevindingen verschilden ook aanzienlijk tussen de genoemde drie patientengroepen, ondanks het feit dat de cholesterol-serumwaarden gelijkelijk verhoogd waren.

De gemiddelde leeftijd was in de drie patientengroepen nagenoeg dezelfde. Er werd een zeer hoge frequentie van klinisch waarneembare atherosclerotische vaatafwijkingen gevonden bij xanthoompatienten met therapeutisch niet beïnvloedbare verhoogde LDL serumspiegels (hyperlipoproteinaemie IIa en IIb). Bij deze xanthoompatienten werden alleen tendineuze en tubero-eruptieve xanthomen gevonden.

In de patientengroepen met verhoogde VLDL serumspiegels konden de hyperlipoproteinaemieën III en IV/V zowel biochemisch als door middel van de xanthomen onderscheiden worden. Bij deze hyperlipoproteinaemieën was de frequentie van klinisch waarneembare atherosclerotische vaatafwijkingen veel lager dan bij de hyperlipoproteinaemieën IIa of IIb. De aanwezigheid van xanthochromia striata palmaris werd bijna uitsluitend gevonden bij patienten met hyperlipoproteinaemie III, en de papulo-eruptieve xanthomen waren op een voor deze groep kenmerkende manier verdeeld bij patienten met hyperlipoproteinaemie IV/V. Daarenboven werd bij alle patienten met xanthomen en een hyperlipoproteinaemie IV/V een abnormale glucosetolerantie gevonden. De beschreven klinische bevindingen suggereren dat respectievelijk VLDL, IDL en LDL een verschillend effect hebben op het weefsel van de dermis en van de vaatwand.

Om meer informatie te krijgen over de verdeling van de verschillende lipoproteinen in interstitiële vloeistof waaraan de dermale cellen zijn blootgesteld, werden de concentraties van apoproteinen en verschillende lipidenklassen in zuigblaarvocht bepaald. Het bleek dat de blaarvocht/serumconcentratie ratio van de verschillende proteinen bepaald werd door hun moleculair gewicht. Het moleculair gewicht is representatief voor de grootte van het molecuul. De blaarvocht/-serumconcentratie ratio van apoproteinen AI (= proteinedeel

van HDL*) bedroeg 0,24 en van het grotere apoproteine B (= proteinedeel van LDL en VLDL) was deze ratio 0,14. De blaarvocht/serumconcentratie ratio voor de verschillende lipidenklassen was vrij constant en bedroeg 0,25. Voor deze bevindingen kon geen verklaring gevonden worden.

Door gebruik te maken van een transmissie electronenmicroscoop werd getracht de verschillende lipoproteinen in xanthoomweefsel en in vitro zichtbaar en herkenbaar te maken.

In de literatuur wordt aangegeven dat digitonine-bevattende aldehyde fixatievloeistoffen in staat zouden zijn cholesterol in weefsel vast te houden en dientengevolge electronenmicroscopisch herkenbaar te maken. Gezien deze literatuurgegevens werd het effect van digitonine-bevattende fixatieven nagegaan. In vitro konden wij aantonen dat het verlies van cholesterol dat optreedt tijdens expositie aan alcohol gedurende de dehydratieprocedure slechts minimaal werd beïnvloed door de toevoeging van digitonine aan het aldehyde fixatief. Daarentegen bleek bij het in vitro model dat 26% van de cholesterolesters gemobiliseerd werd door de toevoeging van digitonine. De aldus gemobiliseerde cholesterolestercomplexen werden geïsoleerd, en bij electronenmicroscopisch onderzoek bleken deze complexen te bestaan uit spiraalvormige structuren. Deze structuren werden ook door verscheidene auteurs waargenomen in weefsel dat met een digitonine-bevattend aldehyde fixatief was gefixeerd. De toevoeging van digitonine aan een aldehyde fixatief veroorzaakte dus zowel artefacten die niet interpreteerbaar waren, als een mobilisatie van verschillende lipiden. Dit noodzaakte ons om andere methoden te gebruiken om lipoproteinen te visualiseren. De toepassing van OsO_4 met $K_3Fe(CN)_6$ als dubbel fixatief volgend

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*HDL = High Density Lipoprotein $1{,}063 < d < 1{,}21$.

op de aldehyde fixatie stelde ons in staat de aanwezigheid van 30-300 nm door een membraan omgeven deeltjes in xanthoomweefsel aan te tonen. Deze deeltjes geleken op de deeltjes zoals deze gevonden worden in atherosclerotische laesies van hypercholesterolaemische konijnen. Indien LDL geïsoleerd uit serum ingebed werd in een fibrinematrijs en met OsO_4 $K_4Fe(CN)_6$ werd gefixeerd, konden met de electronenmicroscoop donkergekleurde deeltjes met een diameter van 30 nm worden gevonden. VLDL geïsoleerd uit serum werd op dezelfde wijze als LDL behandeld en bestudeerd. De VLDL manifesteerden zich als onregelmatige deeltjes met een diameter die varieerde tussen 30 en 80 nm. Hoewel de lipoproteinen wel zichtbaar waren gemaakt, waren deze lipoproteinen niet op specifieke wijze gekleurd zodat deze niet met zekerheid van andere deeltjes onderscheiden konden worden. Voor specifieke herkenbaarheid van deze lipoproteinen werd een indirecte twee-staps immunoperoxidasetechniek ontwikkeld. Deze techniek stelde ons in staat het apoproteine B specifiek zichtbaar te maken.

Bij gekweekte fibroblasten die met LDL geïncubeerd werden was het mogelijk het apoproteine B zowel op lichtmicroscopisch als electronenmicroscopisch niveau aan te tonen. De specifieke binding van LDL aan de plasma membraan bleek vooral plaats te vinden in ingedeukte regio's. Deze ingedeukte regio's hadden een diameter van 0,15-0,30 µm en waren gekenmerkt door een "fuzzy coat" die zich aan de cytoplasmakant bevond. De immunoperoxidase-positieve bindingsplaatsen waren niet regelmatig verdeeld over de plasma membraan en zouden op verschillen in de metabole activiteit van de gekweekte cellen kunnen wijzen. Van gekweekte fibroblasten van een patient, homozygoot voor familiaire hypercholesterolaemie, werd eerst met behulp van gejodeerd LDL de specifieke binding bepaald. Deze fibroblasten bleken niet in staat LDL specifiek te binden.

Bij electronenmicroscopisch onderzoek hadden deze cellen wel ingedeukte regio's met dezelfde grootte en vorm als hierboven beschreven, doch geen apoproteine B-complexen konden in deze regio's worden aangetoond. Deze bevindingen wijzen erop dat de ingedeukte regio's met fuzzy coat geen structuren zijn die alleen LDL kunnen opnemen.

De bestudering van celcultures die aan specifieke lipoproteineklassen of aan specifiek veranderde lipoproteinen zijn blootgesteld zou kunnen leiden tot de herkenning van factoren die een rol spelen bij de stapeling of verwijdering van cholesterolesters in en uit cellen en weefsels.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 10 juni 1942 te Rheden. In 1961 deed hij eindexamen gymnasium B aan het Amsterdams Lyceum te Amsterdam. In datzelfde jaar ging hij medicijnen studeren aan de Rijksuniversiteit te Groningen. In 1967 werd het doctoraal examen geneeskunde afgelegd, in maart 1970 het arts examen. Van mei 1970 tot maart 1971 was hij werkzaam als algemeen assistent (Heelkunde, Gynaecologie en Verloskunde) op het Prot.Chr. Ziekenhuis "De Lichtenberg" te Amersfoort. Als voorbereiding op werkzaamheden in de tropen in het kader van de ontwikkelingssamenwerking (DTH) werd een cursus voor Tropische Geneeskunde gevolgd op het Koninklijk Instituut voor de Tropen (Amsterdam). Van 1971 tot augustus 1973 was hij eerst werkzaam als districtsarts in Zomba en later in Lilongwe, Malawi. Vanaf 1 september 1973 tot 1 september 1977 volgde hij de opleiding tot huidarts aan de Dermatologische kliniek (Hoofd: destijds Prof. Dr. M.K. Polano, Hoofd sinds 1978 Prof.Dr. D. Suurmond) van het Academisch Ziekenhuis te Leiden. Sinds 1 september 1977 is hij in dezelfde kliniek werkzaam als wetenschappelijk hoofdmedewerker, dermatoloog.

Naast allen die expliciet in dit proefschrift genoemd zijn wil ik alle medewerkers van het Gaubius Instituut TNO, het Laboratorium voor Electronenmicroscopie en de afdeling Dermatologie bedanken voor hun hulp en de plezierige samenwerking tijdens de bewerking van dit proefschrift.