Interaction of Tangier lipoproteins with cholesteryl ester-laden mouse peritoneal macrophages

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Abstract Cholesterol efflux was studied from cholesteryl ester-laden mouse peritoneal macrophages in the presence of Tangier lipoproteins derived from fasting and postprandial sera of three patients homozygous for Tangier disease (analphalipoproteinemia). The d > 1.063 g/ml fractions isolated from fasting patients and 3 hr and 18 hr after an oral fat load were all effective in cellular cholesterol removal. By contrast, the d > 1.063 g/ml fractions isolated 6 hr and 12 hr after fat ingestion did not affect net removal of cellular cholesterol. The d > 1.21 g/ml protein fractions derived from fasting as well as postprandial sera were all effective in removing cholesterol. D 1.063–1.21 g/ml fractions from fasting Tangier patients contained HDL_T. In the corresponding postprandial fractions, in addition to HDL_T, apoB-100- and apoB-48-containing lipoproteins were present. Futhermore, the 6 hr and 12 hr postprandial Tangier HDL fractions contained apoB-immunoreactive proteins of lower molecular weight. The abnormal activity of the elastase/α_1-antitrypsin proteolytic system and the abnormal fibronectin concentration we found in Tangier plasma suggests a possible relationship to the in vivo degradation of apoB. The peculiar type of membrane-bound lipid droplets in Tangier splenic macrophages points to a lipoprotein source of lipid accumulation which possibly originates from the uptake of chylomicrons or chylomicron-derived particles. It is concluded that cholesteryl ester storage in Tangier macrophages results from an imbalance of cholesterol influx and efflux. In the absence of HDL, the net increase of cholesterol caused by abnormal lipoproteins in certain postprandial states cannot be fully compensated by effective efflux and ultimately leads to macrophage cholesteryl ester accumulation.—**Schmitz, G., G. Assmann, B. Brennhausen, and H-J. Schaefer.** Interaction of Tangier lipoproteins with cholesteryl ester-laden mouse peritoneal macrophages. *J. Lipid Res.* 1987. **28:** 87–99.

Supplementary key words Tangier disease • high density lipoproteins • cholesteryl ester accumulation • apolipoprotein B • macrophages • cholesterol influx and efflux

There is increasing evidence that macrophages play an important role in the pathogenesis of the atherogenic process. These cells take up and degrade cholesterol either by phagocytosis of whole cells or fragments of membranes containing cholesterol or by receptor-mediated endocytosis of cholesterol-containing lipoproteins. After cellular degradation, the liberated cholesterol is excreted from the cells.

When macrophages take up more lipoprotein cholesterol than they can excrete, cholesterol is stored in the cytoplasm in the form of cholesteryl ester droplets, leading to the formation of foam cells. The mechanism of this process has been extensively studied in recent years with macrophages isolated from the peritoneal cavity of mice and monocytes from human blood (1–5). These studies shed new light on the mechanism of foam cell formation in vivo and its possible relationship to atherosclerosis (4). The uptake of lipoprotein-bound cholesterol by macrophages occurs through the process of receptor-mediated endocytosis (1–4). However, the mechanism of cellular cholesterol regulation of macrophages differs significantly from that in B,E-receptor cells. B,E-receptor cells, such as cultured fibroblasts, are strongly regulated in their cellular cholesterol content via the presence of the LDL receptor (B,E-receptor) and the rate of cellular cholesterol synthesis (HMG-CoA reductase activity). Therefore, these cells do not need potent cholesterol acceptors for cellular cholesterol homeostasis. By contrast, macrophages, when challenged by cholesterol influx, apparently depend on the presence of effective cholesterol removal carriers in the surrounding medium to prevent foam cell formation.

The cholesteryl esters stored in the cytoplasm of macrophages undergo a continual cycle of hydrolysis and reesterification (6). Hydrolysis is mediated by the cytoplasmic neutral cholesteryl ester hydrolase (NCEH). Reesterification is mediated by the membrane-bound enzyme

Abbreviations: HMG-CoA, hydroxymethyl-glutaryl coenzyme A; apo, apolipoprotein; VLDL, very low density lipoprotein(s); LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); HDL_T, HDL-Tangier (apoA-II particle); LPDS, lipoprotein-deficient serum; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; HPTLC, high performance thin-layer chromatography; SDS, sodium dodecyl sulfate; ACAT, acyl-CoA:cholesterol acyltransferase; NCEH, neutral cholesteryl ester hydrolase.

acyl-CoA:cholesterol acyltransferase (ACAT) that transfers a fatty acid from fatty acyl-coenzyme A to cholesterol. When the extracellular fluid contains a substance such as HDL that is capable of binding cholesterol, the free cholesterol is not reesterified or stored, but rather is excreted from the cell. When no cholesterol acceptor is available, the free cholesterol is reesterified for storage, and the cycle of hydrolysis and reesterification continues (6). Since it is known that an acceptor is necessary for cholesterol excretion from the cells, several agents have been tested for their uptake capacity (7). Human LDL, albumin, γ-globulins, and lecithin/sphingomyelin liposomes did not affect the cholesterol content of cholesteryl ester-laden macrophages, whereas human HDL, FCS, fetal calf and human LPDS, erythrocytes, casein, and thyroglobulin were able to decrease the cellular cholesterol content (7). The finding that human LPDS removes cholesterol left the question unresolved which particles in this fraction may be responsible for the uptake. It has been speculated that apoA-I-containing particles might play such a role (7).

Recent experiments suggest that HDL bind to specific cellular receptors on fibroblasts, arterial smooth muscle cells, hepatocytes, kidney cells (for a review see reference 8), and macrophages (9–11). We could demonstrate that HDL bind with their apolipoprotein A-I moiety to the receptor site of macrophages and are internalized via a nonlysosomal pathway into the cytoplasm where they take up cholesterol and are then secreted from the cells as native lipoproteins. The HDL receptors increase in number upon cellular cholesterol accumulation and inhibition of ACAT (9, 11). In monocyte-derived macrophages of Tangier patients, retroendocytosis is inoperative and HDL are erroneously directed into lysosomes leading to the extracellular absence of these lipoproteins and the storage of cholesterol in various cells (10).

Patients with Tangier disease (12, 13) store cholesteryl esters predominantly in macrophages, Schwann cells, and nonvascular smooth muscle cells (14). Hypocholesterolemia (< 80 mg/dl), hypertriglyceridemia (> 200 mg/dl), and the complete absence of normal high density lipoproteins are the major serum abnormalities (15–22). In Tangier disease, HDL deficiency is associated with the occurrence of a delayed chylomicron clearance from serum (19) and the presence of abnormal triglyceride-rich lipoproteins, presumably chylomicron remnants, in postprandial serum.

The underlying defect in Tangier disease is not fully understood, but may relate to hypercatabolism of apoA-I-containing lipoproteins (10, 15). Theoretically, macrophage cholesteryl ester storage in Tangier patients may be due to the excessive uptake of abnormal lipoproteins from serum, and impaired removal mechanisms in the absence of normal HDL.

The cholesteryl ester storage in macrophages leads to a significant increase in the secretory activity of these cells including neutral proteinases, growth factors, blood clotting proteins, and other substances (23). There is recent evidence that an elastase released from human blood polymorphonuclear cells cleaves the apoA-II moiety of HDL (24) as well as the apoB moiety of LDL (25). Moreover, it has been shown that lipoprotein lipase, which is also secreted by macrophages, induces the degradation of apoB and apoE of human VLDL (26). With respect to the excessive activation of the macrophage system in Tangier patients, the question arises whether similar mechanisms occur under in vivo conditions in these patients.

We have studied cholesterol efflux from cholesterol-laden macrophages incubated with Tangier d > 1.063 g/ml lipoproteins, with or without addition of normal HDL. Furthermore, Tangier d > 1.21 g/ml serum fractions were investigated for their cholesterol removal capacity. All lipoprotein fractions were derived both from fasting and postprandial (3, 6, 12, 18 hr) Tangier sera and were characterized by various analytical techniques. A variety of macrophage-derived secretory products were quantitated in the plasma of all three Tangier homozygotes. In addition, the intracellular pattern of lipid storage was studied in bone marrow macrophages, spleen tissue, and endocervical ectopia tissue obtained from homozygous Tangier patients.

MATERIALS AND METHODS

Patients

Tangier serum was obtained from three patients homozygous for HDL deficiency: patient I, 49 years old, male, with typical symptoms as described earlier (13–16) (triglycerides, 1.58–2.24 mmol/l; cholesterol, 1.16–1.50 mmol/l); patient II, 52 years old, female, sister of patient I (13–16) (triglycerides, 2.94–4.89 mmol/l; cholesterol, 2.02–2.67 mmol/l); patient III: a 33-year-old Pakistani, male, not related to patients I and II (12) (triglycerides, 3.42–3.88 mmol/l; cholesterol, 1.50–2.02 mmol/l). Apolipoprotein E polymorphism was studied in these three patients. They were homozygous for the ϵ-3 allele. Postprandial serum was drawn at different time intervals after a 2000 kcal breakfast consisting of 60% of calories as fat. Spleen tissue was taken from a male homozygous Tangier patient (J. S.) immediately after splenectomy (16). Bone marrow was obtained from two Tangier patients (patient I and II) by sternal suction. From patient II, a small biopsy was obtained from a yellow colored endocervical ectopia.

Materials

Cells were harvested from male NMRI-SPF mice (25–35 g). FCS was obtained from Gibco Bio-Cult Ltd. (Cat. No. 629). DMEM and PBS were purchased from Flow Laboratories (Cat. No. 10-331-25 and 18-610-24, respectively). Cholesterol (CH-S), cholesteryl linoleate (C-9003), and cholesteryl formate (C-9398 for internal standardization

as well as penicillin (PEN-NA) and streptomycin (S-6501) were purchased from Sigma Chemical Co. Tissue culture equipment was obtained from Falcon. Separation of lipids for densitometric analysis was performed on HPTLC plates (27) 10 × 20 cm from Merck, Darmstadt, West Germany (Silica gel 60, Cat. No. 5642). All solvents (LiChrosolv®-quality) were supplied by Merck.

Lipoproteins

Human VLDL (d < 1.006 g/ml), LDL (d 1.019–1.063 g/ml), HDL (d 1.063–1.21 g/ml), 1.063-infranatant (d > 1.063 g/ml), and LPDS (d > 1.21 g/ml) were isolated from sera of normolipidemic apolipoprotein E-3 homozygote volunteers or from Tangier patients I–III by sequential ultracentrifugation in a Beckman L8-70 ultracentrifuge using a 70 Ti rotor (Beckman) at 4°C. The lipoprotein fractions were dialyzed against 0.15 M NaCl/5 mM EDTA, pH 7.4, and a final dialysis against normal saline, when used in tissue culture experiments, or against 0.05 M Tris-HCl–0.15 M NaCl–0.01% EDTA, pH 7.4, when prepared for electrophoresis. Human LDL were acetylated with repeated additions of acetic anhydride as described by Ho, Brown, and Goldstein (7) and Basu et al. (28). The modified LDL showed enhanced mobility on electrophoresis in agarose gel at pH 8.6. Lipoprotein concentrations are given in terms of protein content (29, 30).

Mouse macrophage monolayers

Mouse peritoneal macrophages were obtained from unstimulated mice by peritoneal lavage in PBS containing 0.5 U of heparin/ml. The fluid from all mice for one experiment was pooled and the cells were centrifuged (400 *g*, 10 min, room temperature) (7), washed once with DMEM, and resuspended in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Aliquots of the cell suspension (0.5 ml) corresponding to the cell number of three mice were dispensed into 60 × 10 mm plastic Petri dishes with 3 ml of DMEM containing the additions mentioned above and then incubated in a humidified incubator at 37°C (5% CO_2). On the second day, each dish was extensively washed with 2 ml of DMEM without serum until there were no nonadherent cells visible under the microscope. The experiment was then started. Each dish contained approximately 200–500 μg of total cell protein.

Lipid extraction of cells

At the end of the indicated incubation time, medium was removed from the dishes and the cells were washed three times with 2 ml of DMEM and once with PBS. Cells were harvested with a rubber policeman in 500 μl of PBS and transferred to a conical glass tube. The cells of each collection were pooled (two dishes = 1 ml) and sonicated with a Branson sonifier three times for 20 sec in ice water at an intensity of 30 watts (27). After protein determination, an aliquot (0.3–0.8 ml) containing 200–500 μg of cell protein was delipidated by a modified Folch procedure (27). The organic solvents were evaporated at 40°C under vacuum and the samples were stored overnight in a desiccator.

Chromatographic separation and quantification of lipids

The evaporated samples were redissolved in 50 μl of chloroform. Then 0.2 or 0.5 μl was applied to the HPTLC plates. Separation of neutral lipids was performed in n-hexane–n-heptane–diethylether –acetic acid 63:18.5:18.5:1 (v/v). Spots were detected using a manganese chloride–sulfuric acid reagent (31). Quantification was performed by densitometry with a Camag TLC-scanner combined with a Spectra-Physics SP 4100 basic integrator equipped with a Kerr minifile 4100 D for data storage (27, 32).

Analytical isotachophoresis

The analyses were performed on a modified LKB 2127 Tachophor system. One hundred μl of serum or concentrated medium was mixed with 50 μl of a Sudan black B solution (1% in ethylene glycol) (33). After incubation at 4°C for 30 min, 0.5–1.0 μl of the stained sample was injected. The separation was started at 100 μA,4 KV and reduced after 6 min to 50 μA. After 15 min voltage had reached a value of 20 KV. Light absorption at 570 nm and conductivity were monitored with a Hewlett-Packard 9826 A calculator, and printed with a Hewlett-Packard 2671 printer-plotter.

SDS gradient polyacrylamide gel electrophoresis

Samples were twice delipidated in ethanol–ether 3:1 (v/v), once in ether, and redissolved in 0.0375 M Tris, 5% SDS, 4% β-mercaptoethanol, and 0.05% bromophenol blue, pH 8.3. A molding chamber was prepared by fixing a polyester plate with sample slits and a glass plate covered with a Gel-Bond sheet (LKB) separated by 1 mm spacers. The chamber was filled with a concave gradient by mixing a dense acrylamide solution (T = 15%, C = 4%, 20% glycerol, 0.375 M Tris, 0.1% SDS, 0.0025% NaN_3, pH 8.8) and a light buffer solution (0.375 M Tris, 0.1% SDS, 0.0025% NaN_3, pH 8.8) with a gradient mixer (34). After polymerization, 10 μl of the samples was placed into the sample slits. Electrophoresis was carried out in 25 mM Tris, 14 g/l glycerol, 0.1% SDS, 0.001% NaN_3, pH 8.3, in an LKB Multiphor electrophoresis chamber with an initial setting of 600 V,70 mA, until the marker had reached the lower end of the gel. Unless used for Western blotting, the gel was stained in 0.2% Coomassie G 250 (Serva) in 50% methanol–12% acetic acid and destained in 20% ethanol.

Western blotting

Electrophoretic transfer of apolipoproteins from SDS gradient gels to a nitrocellulose sheet and subsequent de-

tection with monospecific apolipoprotein antibodies (against apoA-I, apoA-IV, apoB, apoC-III), and a fluorescent-labeled second antibody against rabbit IgG (from goat, Sigma) was performed according to the method of Burnette (35).

Light microscopy

For light microscopy, frozen sections and smears were prepared from the tissue specimens and stained by Oil Red O and processed by conventional histological techniques.

Electron microscopy

For electron microscopy, tissue was immediately fixed for 2 hr with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, washed in the same buffer containing 7% sucrose, and post-fixed in 2% osmium tetroxide, phosphate-buffered to pH 7.2. Semifine and thin sections obtained from material embedded in Epon 812 were stained for light and electron microscopy by usual procedures. Tangier HDL fractions were extensively dialyzed against PBS. The lipoprotein particles were visualized by negative staining using phosphotungstic acid. Electron microscopy was carried out with a Hitachi H 500 electron microscope.

Other methods

Protein content of lipoprotein fractions and cells was determined by a modified Lowry method (30) using bovine serum albumin as a standard. Standards and samples were delipidated by addition of 100 μl of a 2.5% Triton X-100 (Serva) solution to each tube prior to photometric measurement. Triglycerides and total cholesterol were determined on a Technicon SMAC® Autoanlayzer by enzymatic methods. Triglycerides were corrected for free glycerol. Elastase was measured immunologically with the PMN-elastase ELISA from Merck, Darmstadt (Cat. No. 15689). α_1-Antitrypsin activity was determined with the chromogenic method from Boehringer Mannheim (Cat. No. 416 509). α_1-Antitrypsin concentration (Behring, Marburg, Cat. No. OSIE 04/05) and α_2-macroglobulin concentrations (Behring, Marburg, Cat. No. OSAM 14/15) were measured with a turbidimetric method in a Hoffmann-La Roche Cobas Bio centrifugal analyzer. Fibronectin was determined by electroimmunoassay using specific antisera from Behring, Marburg (Cat. No. OSIE 04/05). N-Acetyl-β-glucosaminidase (β-NAG) was measured colorimetrically with the substrate p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside from Koch-Light Laboratories Ltd. (Cat. No. 426 500).

RESULTS

To investigate the ability of Tangier lipoprotein fractions to take up cholesterol from cholesteryl ester-laden macrophages, tissue culture experiments were performed with mouse peritoneal macrophages. Tangier lipoprotein fractions were derived from three Tangier patients. The serum triglyceride and cholesterol levels before and after ingestion of an oral fat load are presented in **Fig. 1.** Whereas serum triglyceride levels increased to almost double fasting concentrations 6–8 hr postprandially, serum cholesterol concentrations remained almost constant.

First, the d > 1.21 g/ml density fractions derived from Tangier and control patients were analyzed for their cholesterol uptake capacity. As shown in **Fig. 2,** all fractions studied were similarly effective in net cholesterol removal. In particular, there was no difference between fasting or postprandial Tangier and control fractions.

The study of the d > 1.063 g/ml lipoproteins revealed a more complex situation. As shown in **Fig. 3,** the d > 1.063 g/ml fractions dervied from Tangier sera 0 hr, 3 hr, and 18 hr after an oral fat load were all effective in cellular cholesterol removal. The observed decrease of cellular total cholesterol concentration (Fig. 3, left) was mainly due to a decrease of cholesteryl esters (Fig. 3, middle), whereas cellular free cholesterol (Fig. 3, right) remained almost constant. By contrast, the d > 1.063 g/ml fractions isolated 6 hr and 12 hr after fat ingestion did not lower total cellular cholesterol (Fig. 3, left) or cholesteryl ester concentrations (Fig. 3, middle). However, cholesterol removal could be achieved when small amounts (10–40 μg of HDL cholesterol/ml medium) of ultracentrifugally isolated normal HDL were added to these fractions (**Fig. 4**). Control experiments (not shown) were performed with d > 1.063 g/ml fractions derived from normolipidemic E-3 homozygous controls. In contrast to our observations with Tangier d > 1.063 g/ml fractions, the control d > 1.063 g/ml fractions from fasting as well as postprandial sera (3, 6, 12,

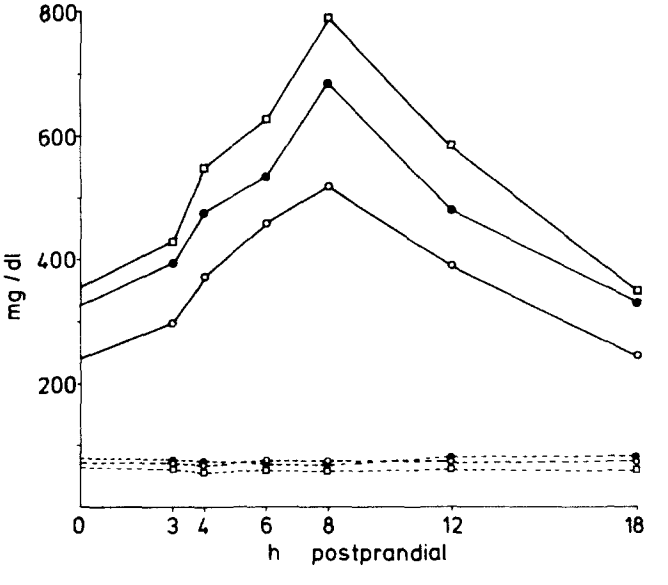

Fig. 1. Serum values for triglycerides (—) and total cholesterol (---) following an oral fat load in Tangier patients I (○), II, (□), and III (●).

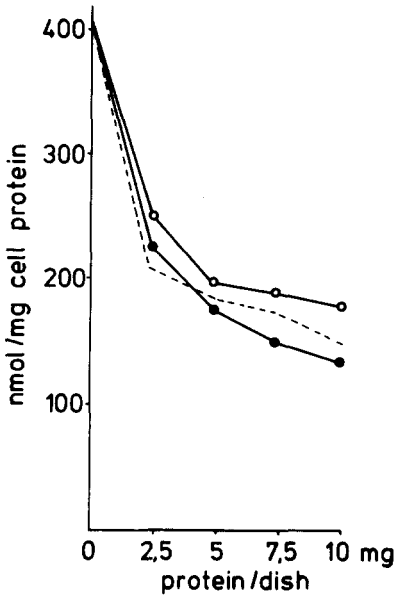

Fig. 2. Decline in the content of total cholesterol in macrophages after incubation with Tangier and control d > 1.21 g/ml serum fractions. Monolayers were prepared as described. After preincubation for 18 hr at 37°C with 3 ml of DMEM and 35 μg/ml of acetyl-LDL, cells were washed three times with DMEM. They were then incubated at 37°C for 18 hr with 3 ml of DMEM containing the indicated amount of d 1.21-infranatant from control serum (---) and from Tangier serum obtained under fasting conditions (●) and 6 hr (○) after an oral fat load. After the incubation, the cells were washed and harvested, and their lipid content was measured by HPTLC. The figure shows representative data from three sets of tissue culture experiments.

18 hr) did not differ in their cholesterol removal capacity and yielded results comparable to fasting Tangier fractions.

In view of discrepant results obtained with the various postprandial Tangier d > 1.063 g/ml fractions, lipoproteins contained in the d 1.063–1.21 g/ml density class of these patients were further characterized.

As previously described (36, 37), fasting Tangier HDL fractions contain several populations of lipoprotein particles. This heterogeneity in the morphologic pattern of Tangier HDL was confirmed by our electron microscopic studies (**Table 1**): in fasting serum, the particle composition of Tangier HDL appeared more homogenous, consisting predominantly of 50–75 Å particles while larger particles and discs were almost absent. In postprandial plasma the group of large particles and discoidal particles increased in concentration. The ratio of large versus small particles was higher in 3 hr compared to 6 hr postprandial Tangier HDL.

Fasting and postprandial Tangier sera were further analyzed by analytical capillary isotachophoresis, an analytical technique recently applied in our laboratory for the analysis of lipoprotein subfractions in whole serum (33). **Fig. 5** presents a representative isotachophoretic pattern of lipoproteins contained in fasting and postprandial Tangier sera (panels B–F in Fig. 5) as well as in control serum (panel A in Fig. 5). With this technique, control LDL was resolved into two major subfractions which were also present in fasting Tangier sera (Fig. 5, panel B). Tangier sera lack the normal HDL subfractions (Fig. 5, panel A).

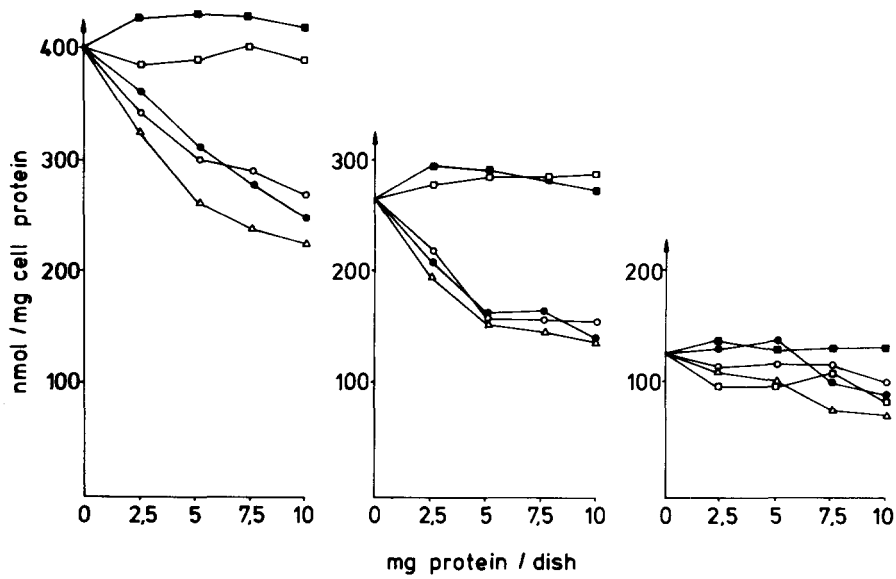

Fig. 3. Decline in the content of total cholesterol (left), esterified cholesterol (middle), and free cholesterol (right) in macrophages after incubation with Tangier d > 1.063 g/ml serum fractions. Monolayers were prepared as described. After preincubation for 18 hr at 37°C with 3 ml of DMEM and 35 μg/ml of acetyl-LDL, cells were washed three times with DMEM. The cells were then incubated with the indicated concentrations of Tangier 1.063-infranatant drawn after an 18-hr fasting period (●), and 3 hr (△), 6 hr (■), 12 hr (□), and 18 hr (○) after an oral fat load. The figure shows representative data from three sets of tissue culture experiments.

TABLE 1. Particle distribution of Tangier HDL

Time	Distribution of Particles		
	"Discoidal Particles"	50–75 Å	170–230 Å
hr		%	
0	2	94	4
3	15	52	23
6	11	73	16
12	7	78	15

Percent particle distributions calculated from negative stains of isolated Tangier HDL under fasting (0 hr) and postprandial (3 hr, 6 hr, and 12 hr) conditions. Tangier HDL was isolated by ultracentrifugation and subjected to electron microscopy. The preparations contained three major groups of particles: the diameter of the smallest particles ranged between 50 and 75 Å; a second group consisted of extremely large, often amorphous particles with a diameter between 170 and 230 Å; a third group consisted of discoidal particles with a tendency to form stacks. In addition to these major groups of particles, a few extremely large particles (> 500 Å) were also present in the Tangier HDL fraction. Normal plasma (not included in Table 1) appeared homogeneous in size (90–150 Å). The particle distribution was calculated as mean values based on the measurement of 500 particles on 10 different electron microscopic pictures from each Tangier HDL fraction.

In postprandial Tangier sera (Fig. 5, panels C–E) additional lipoprotein fractions migrating between the two major LDL subpopulations (labeled 2) and with slower mobility compared to normal HDL (labeled 1) were detected. These fractions were not present in fasting Tangier sera and 18 hr after ingestion of a fat meal. Subfractionation of Tangier lipoproteins by preparative ultracentrifugation and subsequent isotachophoretic analysis revealed that lipoproteins labeled as 1 in Fig. 5 occurred in the d < 1.006 g/ml density class, while lipoproteins labeled as 2 were contained in the d 1.063–1.21 g/ml density fraction.

The apolipoprotein composition of fasting and postprandial Tangier HDL was characterized by SDS-gradient polyacrylamide gels in combination with the Western blotting technique, utilizing monospecific antibodies against apoB, apoA-I, apoE, and apoA-IV. As demonstrated in **Fig. 6**, the 3-hr postprandial Tangier HDL fraction contained apolipoprotein B-100 as a major apolipoprotein. This apolipoprotein was almost absent from fasting HDL fractions. The concentration of apoB-100 decreased 6 hr and 12 hr after the meal and additional bands with lower molecular weight and apoB-immunoreactivity appeared. At 18 hr postprandially, the apolipoprotein composition of the Tangier HDL fraction was indistinguishable from fasting conditions in the HDL fractions derived from normal controls after fat feeding no apoB immunoreactive fragments could be observed (not shown).

A variety of macrophage-derived secretory products and related substances (23) were determined by standard laboratory procedures in freshly drawn plasma from all three Tangier patients. These included plasminogen acti-

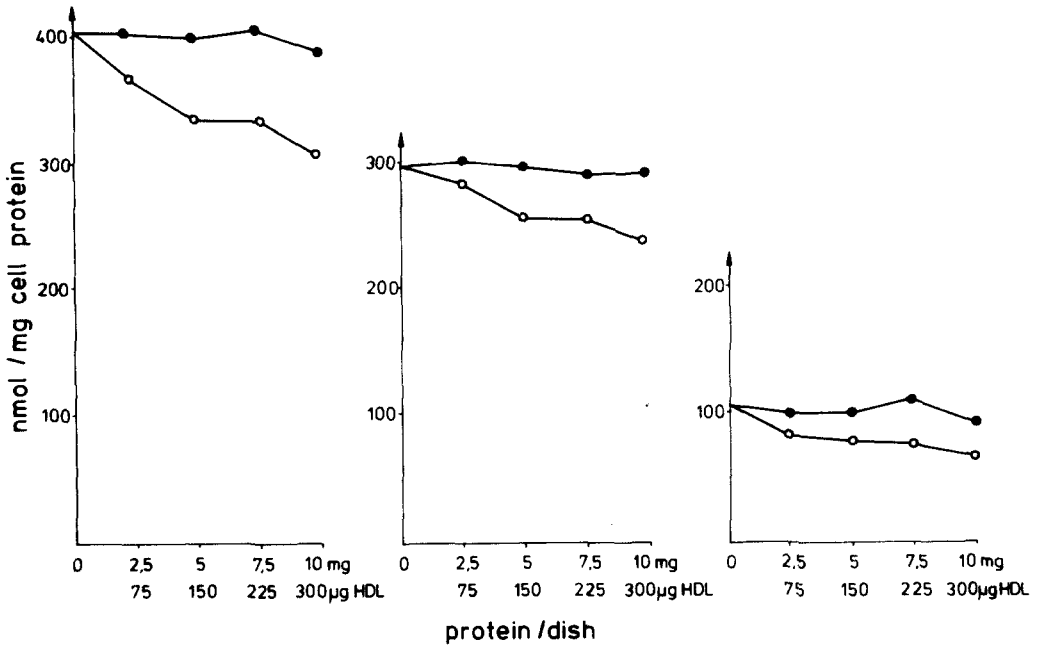

Fig. 4. Content of total cholesterol (left), esterified cholesterol (middle), and free cholesterol (right) in macrophages after incubation with Tangier d > 1.063 g/ml serum fractions in the presence and absence of normal HDL. Monolayers were prepared as described. After preincubation for 18 hr at 37°C with 3 ml DMEM and 35 µg/ml of acetyl-LDL, cells were washed three times with DMEM. After preincubation, the cells were incubated with the indicated amount of Tangier 1.063-infranatant drawn 6 hr after an oral fat load without any further addition (●) and with the addition of the indicated amount of normal HDL (○). The figure shows representative data from three sets of tissue culture experiments, each of them with serum from all three patients.

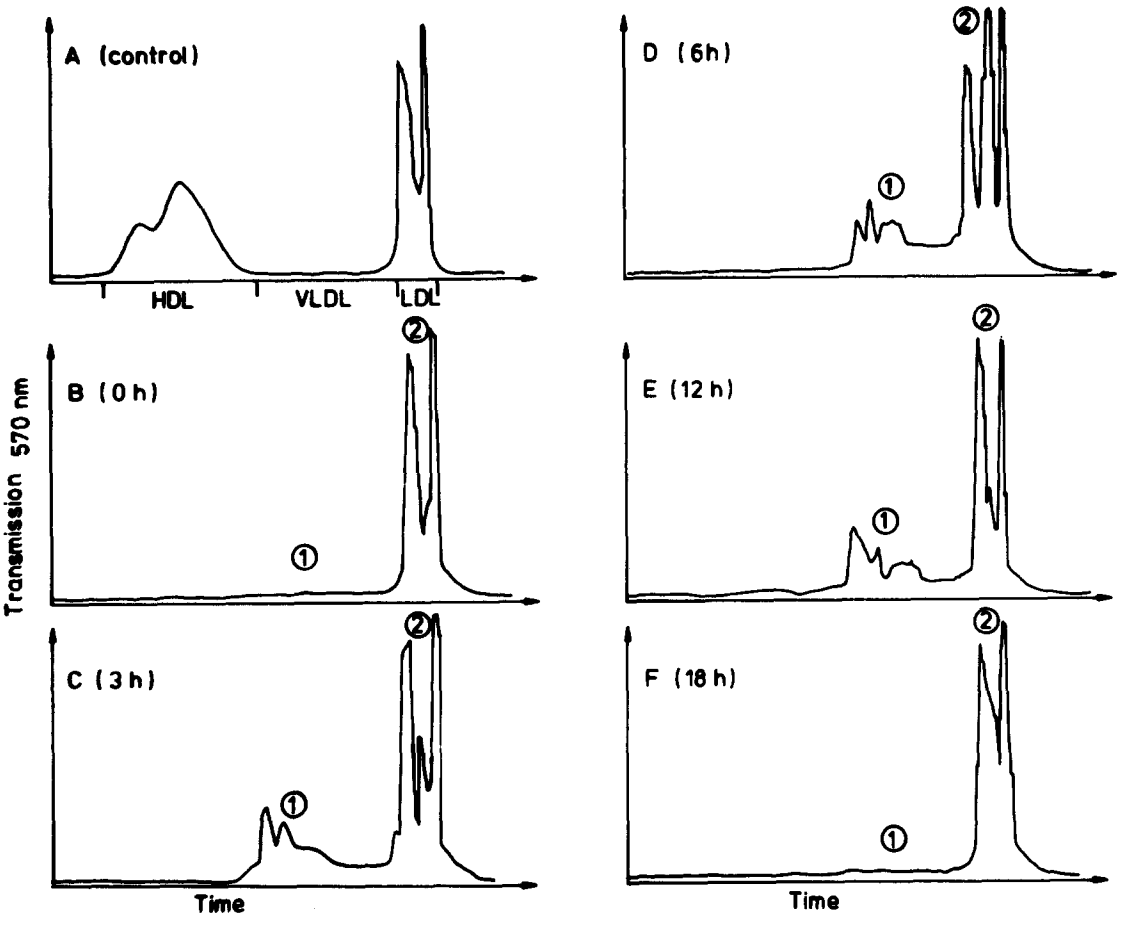

Fig. 5. Analytical capillary isotachophoresis of control serum (A) and Tangier serum: fasting (B); 3 hr postprandial (C); 6 hr postprandial (D); 12 hr postprandial (E); and 18 hr postprandial (F). Experimental details are described in Materials and Methods.

vator, elastase, lysozyme, β-NAG, angiotensin converting enzyme, clotting factors V, VII, IX, X, complement components C1q, C3c, and C4, fibronectin, α_1-antitrypsin concentration and activity, α_2-macroglobulin, and C-reactive protein. Most of these products were normal in our patients. Those compounds that revealed abnormal plasma concentrations are shown in **Fig. 7**. All three patients had a significantly elevated elastase activity concomitant with a reduction of the proteinase inhibitors α_1-antitrypsin (concentration and activity) and α_2-macroglobulin. Fibronectin was elevated in patients I and II and reduced in patient III. β-NAG was elevated in patients II and III and normal in patient I. These data indicate a possible activation of the macrophage secretory system leading to a higher activity of the neutral proteinase elastase in Tangier plasma.

To investigate the intracellular distribution and the possible origin of lipids stored in Tangier macrophages, bone marrow obtained by sternal suction, spleen tissue, and biopsy material from a yellow-colored endocervical ectopia were examined by light and electron microscopy. In all tissues studied, macrophages were found to exhibit a foamy cytoplasm filled with numerous lipid droplets. Under polarized light they showed a maltese cross birefringence. In addition there were birefringent needle-shaped crystals. These crystalline inclusions were more numerous in the cervical mucosa and in bone marrow macrophages than in the spleen. In the bone marrow, all macrophages associated to erythroblastic isles were transformed to foam cells. In the cervical mucosa, foam cells resided in an interstitial tissue infiltrated by small lymphocytes and plasmocytes due to chronic inflammation. Lipid-storing spleen macrophages were found within the pulpe cords. Sinus endothelial cells, as well as all the non-macrophage cells, appeared normal.

All foam cells, as studied by electron microscopy, contained numerous transparent lipid droplets not surrounded by a limiting membrane (**Fig. 8** and **Fig. 9**). However, the crystalline inclusions present in endocervical and bone marrow macrophages were rimmed by a dense matrix bound by a trilaminar unit membrane. These cells also contained other pleomorphic inclusions

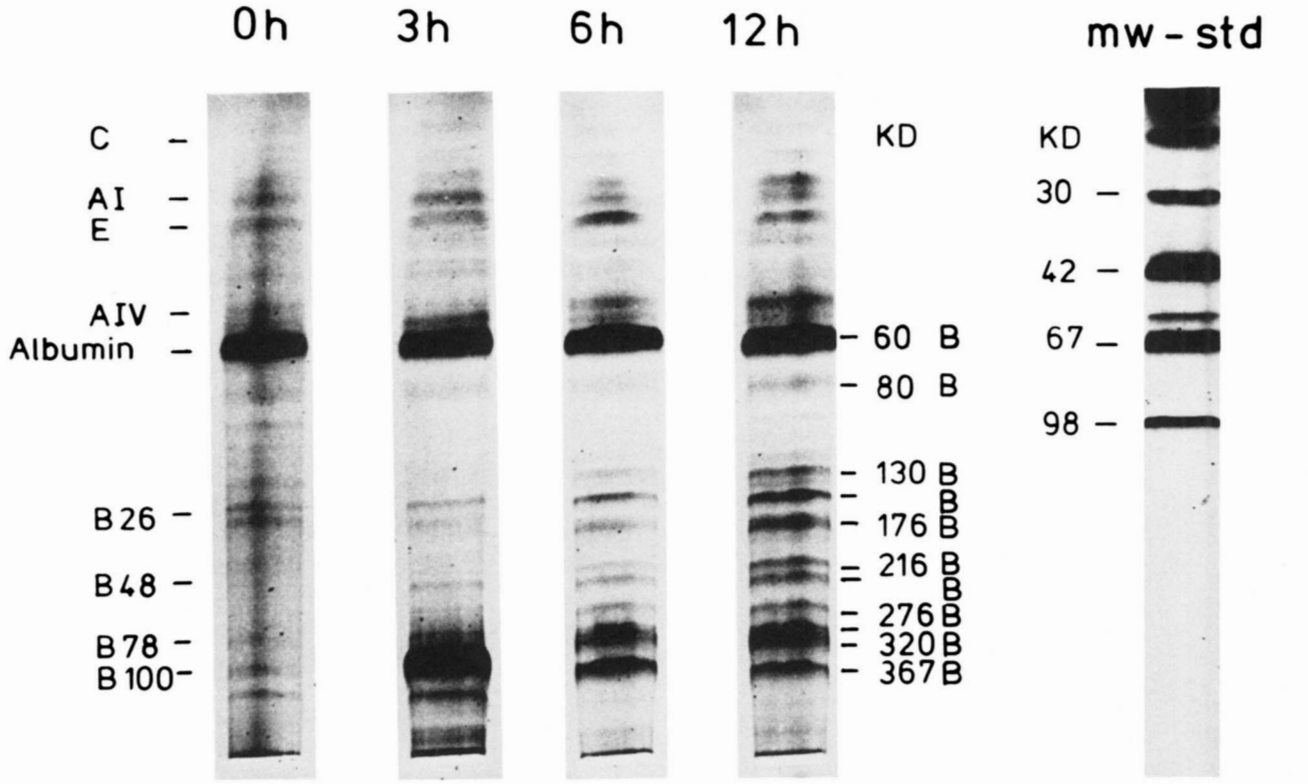

Fig. 6. SDS gradient (3%–15%) gel electrophoresis of Tangier HDL (d 1.063–1.21 g/ml) derived from fasting (0 hr) and postprandial (3 hr, 6 hr, 12 hr) Tangier sera isolated by ultracentrifugation. Apolipoproteins and albumin were identified by Western blot analysis. The protein bands that react with anti-apoB on the Western blot are indicated with B. Molecular weights were calculated by use of standard proteins. To avoid proteolytic degradation of apolipoproteins during sample preparation, a proteinase inhibitor mixture, as previously described by Walter and Blobel (38), was added to all samples.

reminiscent of phagolysosomes or phagocytotic residual bodies (Fig. 8).

A peculiar type of lipid droplet was found to occur exclusively in splenic macrophages. These droplets measured 1–2 μm in diameter, and they were surrounded by a dense matrix obviously resulting from fusion with dense lysosomal granules. The contents of these droplets appeared more opaque as compared with the more transparent lipids present within the non-membrane-bound vacuoles (**Fig. 10**). Besides these lysosome-associated lipid vacuoles, splenic macrophages also contained free lipid droplets with a transparent content.

DISCUSSION

Patients with Tangier disease lack normal HDL, probably due to hypercatabolism of these particles, and store cholesteryl esters in macrophages. Theoretically, cholesteryl ester storage might be caused by a higher ingestion of cholesterol-carrying lipoproteins into tissue macrophages or, alternatively, might be due to the lack of HDL- and/or other apoA-I-containing serum lipoproteins as acceptors for macrophage cholesterol.

To distinguish between these two mechanisms, we have studied the effect of various Tangier lipoprotein fractions on the cholesterol metabolism of tissue culture macrophages. Cells were incubated with fasting and postprandial Tangier d > 1.063 g/ml lipoproteins, in the presence or absence of normal HDL, as well as Tangier d > 1.21 g/ml serum fractions. The following observations were made.

1. Despite the almost total absence of apoA-I in Tangier serum, the d > 1.21 g/ml fractions isolated from fasting as well as postprandial (6 hr) Tangier serum were all effective in cholesterol removal and, in this regard, were indistinguishable from control d > 1.21 g/ml fractions.

2. D > 1.063 g/ml fractions isolated from Tangier serum 0, 3, and 18 hr after an oral fat load promoted cellular cholesterol efflux. By contrast, the d > 1.063 g/ml fractions isolated 6 hr and 12 hr after fat ingestion did not affect net removal of cholesterol from cells. The failure of cholesterol net removal of these fractions was related to the deficiency of HDL. Addition of normal HDL to Tangier d > 1.063 g/ml lipoproteins significantly enhanced cholesterol net removal.

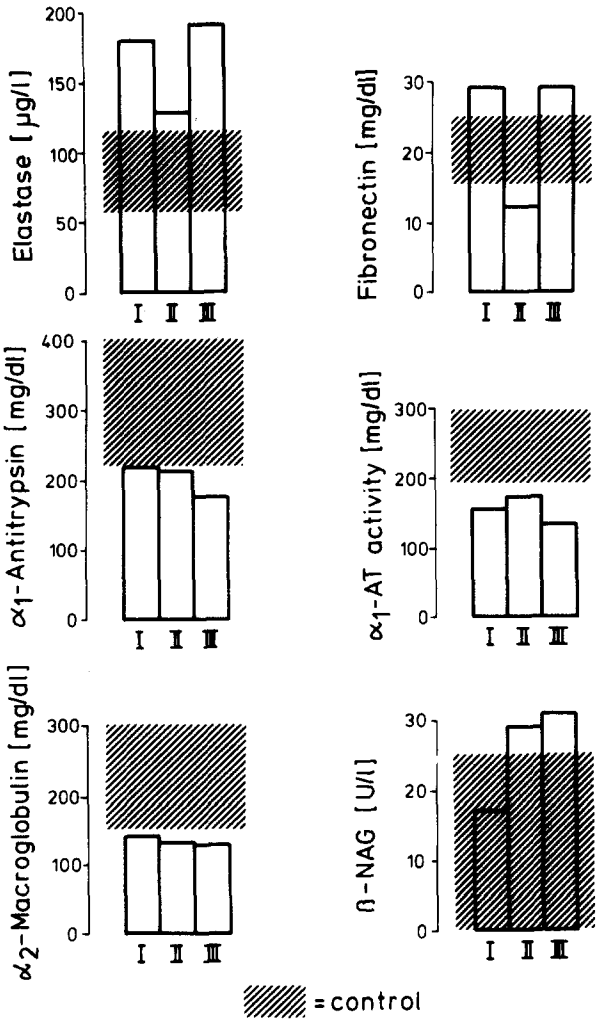

Fig. 7. Plasma concentrations of elastase (µg/l), fibronectin (mg/dl), α_1-antitrypsin concentration (mg/dl), α_1-antitrypsin activity (mg/dl), α_2-macroglobulin concentration (mg/dl) and N-acetyl-β-glucosaminidase (β-NAG) activity (U/L) from three Tangier patients (I, II, III). Normal laboratory reference values are indicated by the shaded areas.

These discrepant results (regular net removal of cholesterol in the presence of fasting, 3 hr, and 18 hr d > 1.063 g/ml fractions, and impaired net removal in the presence of 6 hr and 12 hr postprandial fractions) necessitated characterization of lipoproteins contained in the d 1.063–1.21 g/ml density class. As demonstrated by electron microscopy, the lipoprotein particle composition within the HDL density class of Tangier patients changed profoundly after an oral fat load. The particles predominantly present in the d 1.063–1.21 g/ml fasting fraction had a diameter of 50–75 Å. Small amounts of large particles (170–230 Å) and bilayer discs were also present. By contrast, the d 1.063–1.21 g/ml fractions isolated 3 hr, 6 hr, and 12 hr postprandially showed a marked increase of the large particles as well as the discoidal lipoproteins. Eighteen hours after the meal, the relative distribution of the three particle populations resembled that of fasting conditions with a predominance of the small spherical lipoproteins.

Analytical isotachophoresis in part confirmed these findings. Lipoproteins with abnormal migration properties (designated 1 and 2 in Fig. 5) were present in postprandial conditions of Tangier patients (3 hr, 6 hr, 12 hr) and could not be detected in control or in fasting Tangier sera. One of these abnormal lipoproteins (fraction 2) occurred in the d 1.063–1.21 g/ml density class upon ultracentrifugation (presumably the apolipoprotein B-containing, 170–230 Å particle) and revealed in analytical isotachophoresis a net electric mobility similar to normal LDL. Preparative isotachophoretic isolation will be required to obtain further compositional data of these abnormal postprandial lipoproteins.

The changes in the particle distribution pattern were accompanied by profound changes in the apolipoprotein composition of the fasting versus postprandial Tangier HDL fractions. A striking finding was the occurrence of large amounts of apolipoprotein B-100 in the 3 hr postprandial HDL fraction and the subsequent occurrence of apolipoprotein B proteolytic fragments. These apolipoprotein B fragments were absent from fasting or postprandial HDL fractions of normolipidemic controls though they were isolated under strictly identical conditions. It must be concluded that they were generated in vivo as a probable result of the delayed clearance of apoB-containing, triglyceride-rich lipoproteins in Tangier serum.

We have recently observed that the elastase/α_1-antitrypsin proteolytic system is activated in the plasma of our patients affected by Tangier disease (Fig. 7). With respect to the recent in vitro observations published by various authors (23–26), one can postulate that elevated plasma elastase activity is related to an increased secretion of the enzyme from macrophages upon cholesterol accumulation, leading to an in vivo apoB degradation by neutral proteinases as observed in our patients. This theory needs to be further substantiated.

Though at present unproven, one could assume that these abnormal lipoproteins and apolipoproteins in the serum of Tangier patients, generated in the course of alimentary lipemia, provoked increased cholesterol influx into macrophages. In the absence of normal HDL, the cholesterol efflux capacity present in the d > 1.21 g/ml fraction apparently cannot fully balance total cellular cholesterol concentrations.

This theory is, in part, supported by our morphologic findings. The occurrence of birefringent non-membrane-bound lipid vacuoles, as present in Tangier macrophages, represents a general phenomenon of foamy histiocytes that has been formerly studied in full detail (37, 39). These investigators demonstrated that mouse peritoneal macrophages loaded with acetylated LDL develop, in the absence

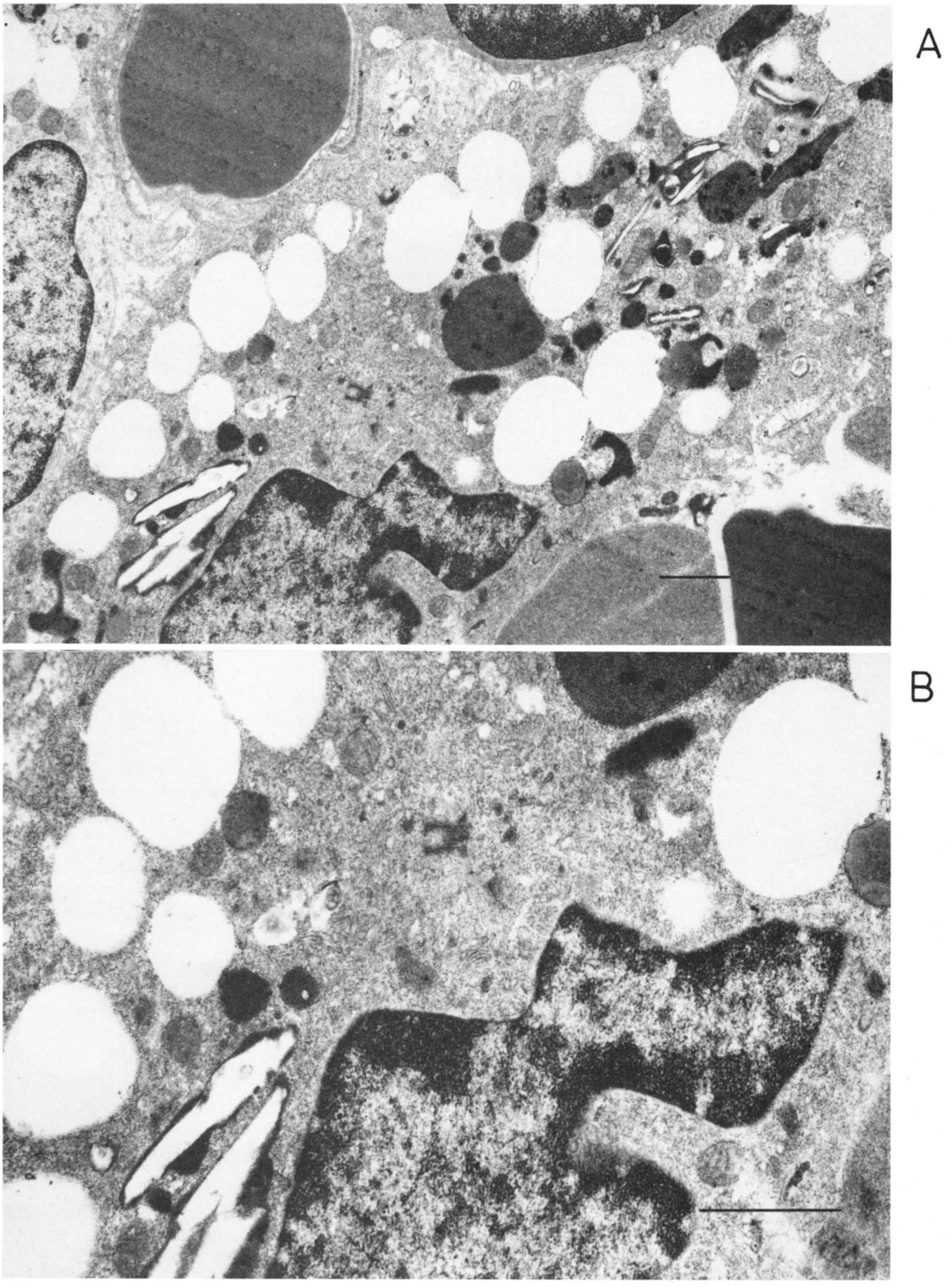

Fig. 8. Electron microscopic view of lipid-storing bone marrow macrophages in Tangier disease. A. There are transparent crystals and free lipid droplets. B. Crystals are surrounded by a dense matrix. (Bar = 1 μm)

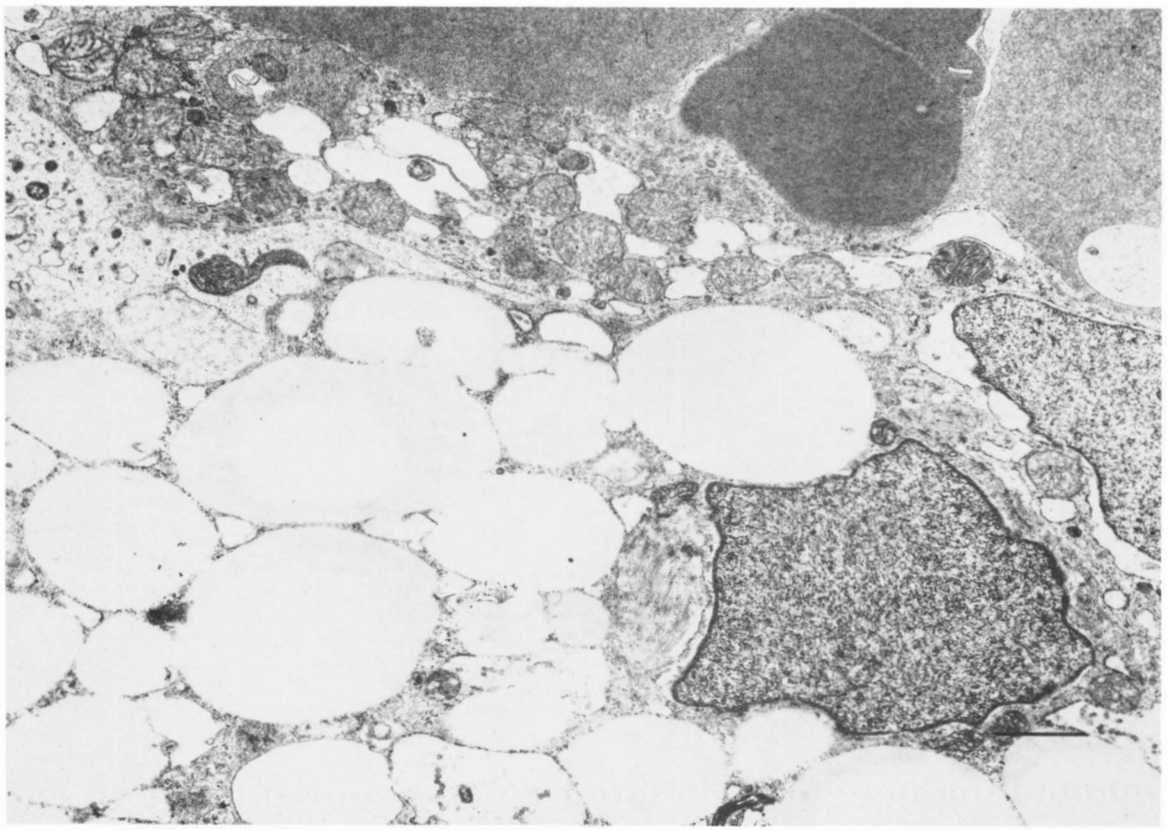

Fig. 9. Electron microscopic view of spleen macrophages containing many lipid droplets of the transparent type. (Bar = 1 μm)

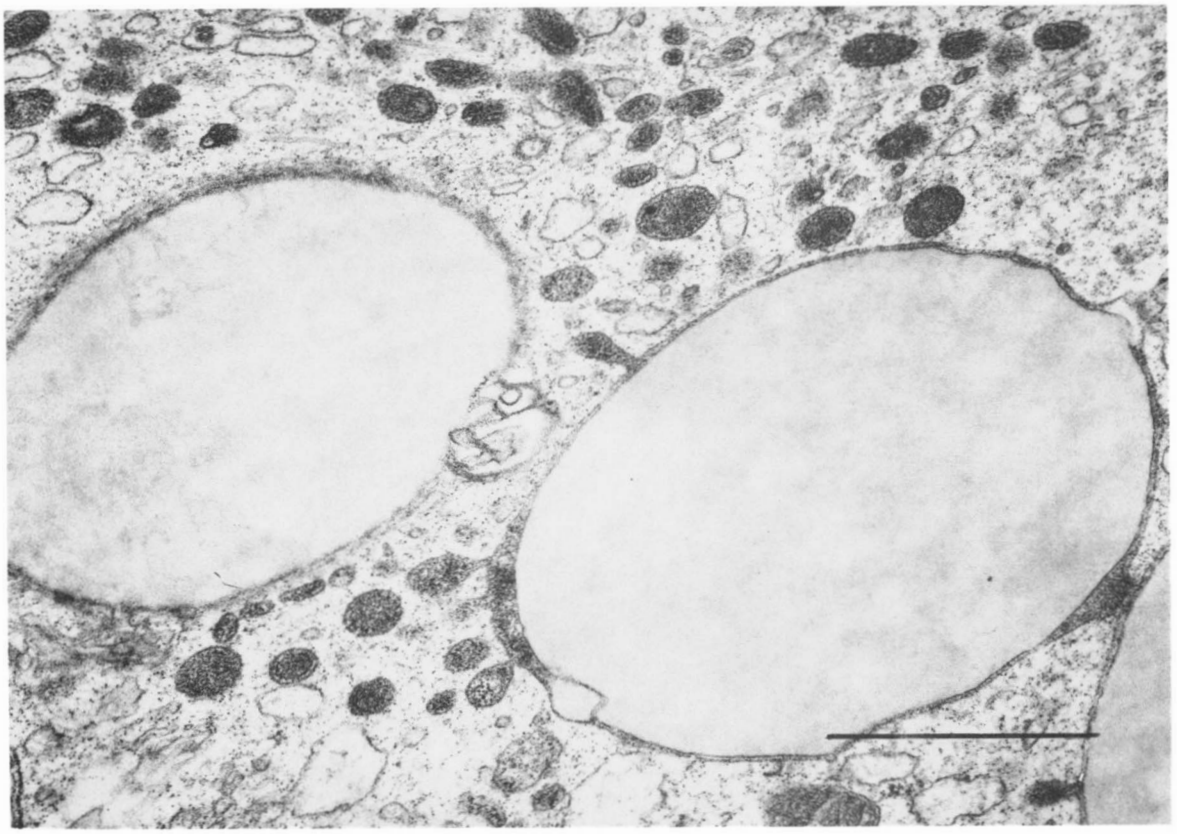

Fig. 10. Higher electron microscopic magnification of the cytoplasm of a spleen macrophage depicting opaque lipid vacuoles fusing with dense lysosomal granules. (Bar = 1 μm)

of HDL, a lipid storage pattern identical to that observed in our tissue studies. The numerous translucent, nonmembrane-bound lipid droplets apparently represent excess cholesterol reesterified by ACAT. Part of these cholesteryl esters are hydrolyzed by NCEH to form a pool of free cholesterol leaving the cell only in presence of HDL. In Tangier disease, the lack of HDL may account for the retention and accumulation of free lipid droplets in macrophages challenged by high cholesterol influx.

In Tangier disease, instead of acetyl-LDL, ingestion of degraded lipoproteins and possibly apoA-I-containing remnants originating from triglyceride-rich lipoproteins by macrophages in vivo may cause a progressive nonregulated phagocytotic influx of cholesterol that ultimately leads to the deposition of cytoplasmic cholesteryl esters (translucent lipid droplets).

The peculiar type of membrane-bound lipid droplets in the splenic macrophages points to a lipoprotein source of lipid accumulation. The content of certain splenic macrophage inclusions appears more opaque on osmocation. It seems reasonable that these lipids are not derived from an ACAT reesterification. ACAT-derived fatty acids are more or less saturated and therefore fail to reduce OsO_4. In consideration of their dimension and their presumptively higher content of unsaturated fatty acid, these lipid inclusions can be derived from the uptake of chylomicrons or chylomicron-derived particles via phagocytosis. Due to their intimate exposure to the circulating blood, splenic macrophages may be involved in the abnormal removal of chylomicrons from blood. Since chylomicrons are bound to the intravascular space, the extravascular macrophages of bone marrow and endocervical tissue have no access to circulating chylomicrons. In the latter tissues, no electron-dense lipid inclusions were detectable in macrophage-derived foam cells.

Ultimate proof of this theory depends on the isolation of the abnormal lipoproteins in postprandial Tangier serum to homogeneity and characterization of their interaction with cells. Nevertheless, the fact that only in certain postprandial situations isolated serum fractions of Tangier patients cause imbalance of cholesterol influx versus efflux highlights the role of alimentary lipemia and possibly nutrition in the pathogenesis of cholesteryl ester storage in tissue macrophages of Tangier patients.

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