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Research Article

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Relationship of an Abnormal Plasma Lipoprotein to Protection from Atherosclerosis in the Cholesterol-fed Diabetic Rabbit

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ABSTRACT Alloxan-diabetic rabbits develop a pronounced hypercholesterolemia and hypertriglyceridemia in response to cholesterol feeding. Despite higher levels of plasma cholesterol, these animals have much less atherosclerosis than cholesterol-fed nondiabetics. To determine whether this effect is due to properties of the lipoproteins, we compared chemical, physical, and metabolic characteristics of a very low density lipoprotein (VLDL) fraction ($d < 1.019$ g/ml) from the diabetic and nondiabetic cholesterol-fed rabbits. The molar ratio of triglyceride to cholesteryl ester in the particles from diabetic animals ranged from 2:1 to 6:1, and this ratio remained constant in subfractions from individual rabbits. Triglyceride from nondiabetic control animals was a minor component. Differential scanning calorimetry showed a distinct order-disorder phase transition for cholesteryl ester at ~42°C in the fractions from control animals, whereas in fractions from most of the diabetics no such transition was observed, indicating that both triglyceride and cholesteryl ester are present in the core of the same particle. The relative amount of apoprotein E in particles from diabetic animals was much less than that of cholesterol-fed controls. The ability of the lipoproteins from both groups to stimulate cholesteryl ester formation in mouse peritoneal macrophages also was tested. Lipoproteins from cholesterol-fed controls stimulated cholesteryl ester formation in a dose-dependent manner, but particles from the diabetic group had little or no effect. The results suggest that the presence of unusual VLDL particles in diabetic cholesterol-fed rabbits is responsible, at least in part, for the reduced incidence of atherosclerosis in this animal model.

INTRODUCTION

The rabbit is a convenient animal model for dietary-induced atherosclerosis, because a marked hypercholesterolemia develops several days after intake of a cholesterol-fed diet and visible aortic lesions appear within 2 mo (1). Early studies using cholesterol-fed rabbits made diabetic by alloxan administration showed that, despite the presence of hypercholesterolemia in these animals, there was a decrease in the severity of atherosclerosis when compared with nondiabetic control rabbits fed a similar diet (2, 3). This inhibitory effect of alloxan diabetes on the development of atherosclerosis was minimized by treatment with insulin, and it was shown that the inhibition was associated with the diabetic state induced by alloxan rather than with any other effect of to alloxan per se (4, 5). The effect of diabetes in the cholesterol-fed rabbit differed from that in man, since in man diabetes appears to increase both the rate of occurrence and severity of atherosclerotic disease.

The alloxanized cholesterol-fed rabbit was noted to differ from the cholesterol-fed control rabbits by a degree of hypertriglyceridemia in the diabetics much greater than that of the controls (2). This lipemia, when associated with the hypercholesterolemia, was proposed as a factor stabilizing the cholesterol in blood and thereby minimizing deposition in the wall (6). Previously, Gofman et al. (7) examined the ultracentrifugal properties of the plasma lipoproteins in cholesterol-fed rabbits and noted an association between the accumulation of larger and less dense particles (rate of flotation at $d = 1.063$ g/ml, 26°C, expressed in Svedberg

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units [S_f][1] = 12–30) and the subsequent development of atherosclerosis. When Pierce (8) compared the ultracentrifugal properties of plasma lipoproteins in alloxanized and in control cholesterol-fed rabbits, he noted that the lipemia in the diabetics was associated with the appearance of very large particles ($S_f > 100$) and that such particles were not present in the nondiabetic control rabbits. He proposed that the diabetic animals had a metabolic block in the conversion of lipoprotein of $S_f > 80$ into lipoprotein of lower S_f classes (8).

Although much is known about the hypercholesterolemia in nondiabetic cholesterol-fed rabbits, few studies have been reported on the alloxanized model within the past 30 yr. The hypercholesterolemia in nondiabetic rabbits results from the accumulation of an abnormal very low density lipoprotein, designated β-VLDL, that is characterized by a very low density, β-mobility upon electrophoresis, size heterogeneity, and relatively high proportions of cholesteryl ester and apoprotein E (9–11). Recent studies on the interaction of lipoproteins with cultured macrophages show that rabbit β-VLDL undergoes a receptor-mediated uptake by these cells, resulting in cholesteryl ester synthesis and accumulation (12). The data suggest that β-VLDL are atherogenic particles responsible, at least in part, for the accumulation of cholesteryl ester characteristic of atherosclerotic lesions.

In the present study we compared the $d < 1.019$ g/ml fractions between alloxanized cholesterol-fed rabbits and nondiabetic cholesterol-fed control rabbits to determine whether the reduced incidence of atherosclerosis in the diabetic might be related to changes in the physicochemical properties of the lipoproteins present in this fraction.

METHODS

Animal treatment. Male New Zealand white rabbits (2–2.5 kg) were purchased from Pine Acres Farm (Brattleboro, VT). Animals were housed individually and fed Purina Rabbit Chow (Ralston Purina Co., St. Louis, MO) ad lib. for 2–3 wk before any treatment. To produce diabetes, the animals were administered alloxan monohydrate (150 mg/kg body wt) by infusion of a 0.3 M aqueous solution through the lateral ear vein. 2 wk after alloxan administration, rabbits with plasma glucose levels >300 mg/100 ml were fed a diet containing 0.5% cholesterol and 8% corn oil and were kept on this diet for 8–12 wk. Control animals were not given alloxan, but were otherwise treated like the diabetics with respect to diet, housing, and feeding schedule. Fasting blood samples were taken weekly or bimonthly from the lateral ear vein and collected in 0.1% EDTA.

At the end of the treatment period, animals were killed by guillotine after an overnight fast, and the aorta removed from the arch to the renal artery. The extent of lesion formation was assessed visually, the adventitia was removed, and the intima-media was homogenized in 10 mM Tris, pH 7.4. Cholesterol content was measured on a lipid extract (13) prepared from an aliquot of the homogenate.

Isolation and characterization of lipoproteins. Lipoproteins were obtained from plasma drawn into EDTA after an overnight fast. Plasma was adjusted to a density of 1.019 g/ml with NaCl and centrifuged at 45,000 rpm for 18 h at 4°C in a 50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The $d < 1.019$ layer was obtained with a tube slicer. To separate subclasses of VLDL, 1–2 ml of plasma adjusted to a density of 1.063 g/ml was layered beneath 30 ml of a solution adjusted to 1.019 g/ml in a thick-walled polyallomer tube. Centrifugation conditions were chosen with reference to the nomogram of Dole and Hamlin (14) to accomodate a SW-28 rotor and to collect three lipoprotein fractions in sequence: $S_f > 400$, $S_f = 180–400$, and $S_f = 20–180$.

Plasma lipoproteins were separated on a Corning agarose electrophoresis system (Corning Medical, Corning Glass Works, Medfield, MA), with agarose gels cast onto a thin clear plastic film and supplied in dehydrated form. The gels were equilibrated with 0.05 M sodium barbital buffer, pH 8.6, containing 0.05% sodium azide. 2-μl samples were placed into the sample wells and electrophoresis was performed for 35 min; they were then dried in an oven at 55°C for 20 min and stained with 0.05% Sudan Black B (Fisher Scientific Co., Pittsburgh, PA) in absolute methanol and 0.1 N NaOH (1:5 vol/vol).

Lipid analyses were performed on extracts of the lipoprotein fractions according to the method of Folch et al. (13). Cholesterol was determined by the method of Rudel and Morris (15), triglycerides were measured by the procedure of Fletcher (16), and phospholipids were estimated as described by Bartlett (17), with a factor of 25 to convert phosphorus to phospholipid. Protein was assayed by the method of Lowry et al. (18) and, when samples were turbid owing to excess lipid, extraction with chloroform was performed before determining optical density. Plasma glucose was measured enzymatically (19).

The fatty acid composition of the cholesteryl ester and triglyceride fractions were determined after separation of the major lipid classes on silica gel G thin-layer plates with hexane/diethyl ether/glacial acetic acid (70:30:1, vol/vol/vol) as the developing solvent. Fractions were eluted from the gel by three extractions with chloroform/methanol (2:1 vol/vol). Transmethylation was performed by refluxing with boron trifluoride at 80°C for 30 min (20). The methyl esters were extracted into hexane and analyzed by gas-liquid chromatography with 5% diethylene glycol succinate polyester on Chromosorb WHP (Supelco Inc., Bellefonte, PA) run at a temperature of 160°C on a Hewlett-Packard Co. (Palo Alto, CA) model 700 equipped with a hydrogen flame ionization detector. Retention times were standardized with a known mixture of methyl esters (Supelco Inc.) and the relative amounts of methyl esters were determined with an auxilliary integrator.

For polyacrylamide gel electrophoresis (PAGE), lipoprotein fractions were delipidated with 40 vol of ethanol:diethyl ether (3:2 vol/vol) at −15°C overnight, then centrifuged at 2,000 rpm for 30 min at 4°C. The resulting pellet was washed several times with 40 ml ether at −15°C. Electrophoresis was performed with 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (21). Gels were stained for 3

[1] *Abbreviations used in this paper:* apo, apoprotein; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; S_f, rate of flotation at $d = 1.063$ g/ml, 26°C, expressed in Svedberg units.

h with 1.5% Coomassie Blue in 50% methanol, 10% acetic acid, then overnight with 0.05% Coomassie Blue in 20% propan-2-ol, 1% acetic acid.

Calorimetric studies were performed on a Perkin-Elmer Corp., Instrument Div. (Norwalk, CT) DSC2 differential scanning calorimeter as previously described (22). Briefly, samples were hermetically sealed in Perkin-Elmer stainless steel 75-μl sample pans and run with 0.9% NaCl in the reference pan at a sensitivity of 0.2 mcal/s with heating and cooling rates of 5°C/min. Enthalpy measurements were derived from the peak areas by planimetry and related to the area of the crystal-liquid melting transition of an indium standard.

Preparation of macrophages. Mouse peritoneal macrophages were obtained from adult male outbred mice (CDl, Charles River Breeding Laboratories, Inc., Wilmington, MA) by flushing the peritoneal cavity with 6 ml of Hanks' balanced salt solution. The cells were harvested essentially by the method described by Mahley et al. (12). 20 mice were used for each experiment, yielding ~3 × 10^6 cells/mouse. The pooled cells were washed once in Dulbecco's modified Eagle's medium (DMEM) containing penicillin and streptomycin, and resuspended in DMEM with 20% fetal calf serum at a cell concentration of 1.2–1.6 × 10^6 cells/ml. 2-ml aliquots were added to 35-mm petri dishes, incubated at 37°C for 2 h, and the cells washed twice with DMEM to remove nonadherent cells. At this stage, >95% of the cells were positive for nonspecific esterase activity, as determined by the method of Koski et al. (23). The cells were incubated overnight with 2 ml DMEM containing 20% fetal calf serum, washed twice with DMEM, and then incubated for 5 h with 2 ml DMEM containing 0.7 mM [1-^{14}C]oleate (sp act 2,100–2,600 cpm/nmol), complexed to bovine serum albumin at a fatty acid/albumin molar ratio of 5:1. When indicated, the designated amount of lipoprotein was added directly to the petri dish at the beginning of a 5-h incubation. After incubation, the macrophages were washed three times with saline and scraped into centrifuge tubes. A lipid extract of the cell pellet was prepared (13), the lipid classes separated by thin-layer chromatography, and the amount of cholesteryl [1-^{14}C]oleate formed during the incubation was determined (24).

RESULTS

Animals were administered alloxan intravenously and routinely monitored for 2 wk before being fed the cholesterol-containing diet. Approximately 75% of the alloxan-treated animals survived this 2-wk period. In these animals, fasting plasma glucose increased from 110 to 420 mg/100 ml, plasma triglyceride rose from 40 to 420 mg/100 ml, and plasma cholesterol increased slightly from 40 to 95 mg/100 ml (averages for 12 animals). After cholesterol feeding began, plasma lipids rose sharply; after 1 wk on the diet, plasma triglycerides and cholesterol averaged 4,500 and 1,020 mg/100 ml, respectively. During the subsequent 2 mo on the 0.5%-cholesterol diet, plasma triglyceride levels in the diabetics were extremely variable between animals, ranging from 500 to 17,000 mg/100 ml. Glucose levels in several animals reverted to the normal range during the dietary treatment, and these animals were removed from the study. The nondiabetic animals fed cholesterol had normal glucose levels throughout the study (120–150 mg/100 ml), triglyceride values remained relatively low (30–210 mg/100 ml), and plasma cholesterol rose progressively to values between 600 and 1,400 mg/100 ml.

After being maintained on the diet containing 0.5% cholesterol for 8–11 wk, animals were killed and the severity of atherosclerosis was assessed by measuring the total cholesterol content of the aorta from the arch to the renal bifurcation. As shown in Table I, although the average plasma cholesterol values in the nondiabetic control animals were less than half that of the diabetics at death (1,030 vs. 2,120 mg/100 ml), the aortic cholesterol content was significantly higher in

TABLE I
Effect of Cholesterol Feeding for 9–12 wk on Aortic Cholesterol Content in Control and Diabetic Rabbits

	0.5%-Cholesterol diet		1.5%-Cholesterol diet
	Nondiabetic (n = 8)	Diabetic (n = 6)	Nondiabetic (n = 5)
Plasma cholesterol, (mg/100 ml)	1,030±105	2,120±350	1,980±290
Plasma triglyceride, (mg/100 ml)	109±23	3,050±610	26±5
Plasma glucose, (mg/100 ml)	131±6	420±45	122±5
Aortic cholesterol, (mg/g/wet wt)	6.81±1.26	2.31±0.65	20.0±4.6

Data represent mean±SE for the designated number of animals in each group. Measurements were performed on samples obtained from fasted animals 1 d before they were killed.

the nondiabetics (2.3 vs. 6.8 mg/g wet wt, $P < 0.02$). The effect of diabetes on the development of atherosclerosis was even more obvious if compared with a nondiabetic group fed a 1.5%-cholesterol diet; the plasma cholesterol values at death in this group were comparable to that of the diabetics (1,980 mg/dl vs. 2,120 mg/dl). The nondiabetic animals had aortic cholesterol contents almost eightfold greater than the diabetics, showing a clear dissociation between plasma cholesterol and aortic lesion development.

We compared the properties of the $d < 1.019$ g/ml fraction from rabbit plasma, which contain the β-VLDL particles characteristic of cholesterol-fed animals. Because the plasma lipid levels varied considerably during the period of cholesterol feeding of the diabetic animals, we chose to focus on the plasma obtained between 3 and 7 wk after initiating the diet, since plasma triglycerides of the diabetic group were at the highest levels during this period. Fig. 1 shows representative electrophoretograms of the plasma lipoproteins in the cholesterol-fed animals. The major lipoprotein in the nondiabetic control was a band migrating between human low density lipoproteins (LDL) and VLDL. In the diabetics, a less distinct band was seen in this region and material was also seen at the origin, even though the plasma was obtained from fasted animals.

Previous studies on cholesterol-fed rabbits showed that much of the plasma cholesterol was associated with lipoproteins having a density of <1.019 g/ml. As shown in Table II, >80% of the total plasma lipids in diabetic rabbits fed cholesterol for 4–7 wk was contained in the $d < 1.019$ g/ml fraction, and ~50% of total plasma lipids from the control group was in this fraction. Analysis of the lipid composition of this fraction indicated that cholesteryl ester was the predominant core lipid in the control group, with triglyceride forming <5% of total lipid. In contrast, triglyceride was the predominant lipid in the particles isolated from the diabetic animals, and the molar ratio of triglyceride to cholesteryl ester was ~2:1. In both groups, approximately equimolar amounts of the surface lipids, cholesterol, and phospholipid were present. Although more protein floated at $d < 1.019$ g/ml in the diabetic group, the weight ratio of protein to total lipid in the diabetic group actually was less than that of the control group.

The fatty acid composition of cholesteryl ester and triglyceride contained in the $d < 1.019$ g/ml fractions is shown in Table III. Consistent with other studies (11), we found that oleic acid was the most abundant fatty acid esterified to cholesterol in the cholesterol-fed control group and the ratio of oleate to linoleate was >2:1, in marked contrast to the corn oil used in the diet (oleate/linoleate ratio, 0.4). A relative enrichment of oleate also was seen in the triglyceride fraction

TABLE II
Lipid Composition of $d < 1.019$ g/ml Lipoproteins from Control and Diabetic Cholesterol-fed Rabbits

	Control (n = 11)	Diabetic (n = 6)
Total plasma lipid (mg/ml)	26.2±4.7	151±49
Total lipid in $d < 1.019$ g/ml fraction (mg/ml)	12.7±2.0	129±40
Protein content in $d < 1.019$ g/ml fraction (mg/ml)	1.1±0.2	5.1±0.9
Lipid composition of $d < 1.019$ g/ml fraction (wt %)		
Triglyceride	4.7±1.4	58.3±5.8
Cholesteryl ester	68.8±2.5	26.7±4.8
Cholesterol	9.4±5.1	4.7±0.6
Phospholipid	16.2±1.8	10.3±1.2

Data represent mean±SE for the designated number of animals in each group. Plasma was obtained from animals maintained on a diet containing 0.5% cholesterol for 4–7 wk.

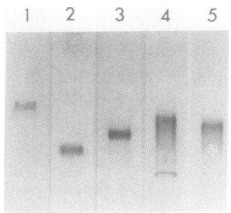

FIGURE 1 Agarose gel electrophoresis of lipoproteins from cholesterol-fed control and diabetic $d < 1.019$ g/ml fractions. Staining was performed with Sudan Black B. Lane *1*, human VLDL; *2*, human LDL; *3*, control rabbit; *4* and *5*, diabetic rabbit (two separate samples).

TABLE III
Distribution of Fatty Acids in Neutral Lipids from Control and Diabetic Cholesterol-fed Rabbits

	Triglyceride		Cholesterol ester	
Fatty acid	Control	Diabetic	Control	Diabetic
		%		
14:0	5.5±3	0.5±0.1	1.4±0.3	0.6±0.1
16:0	34.0±4	23.2±1	19.6±1	18.3±2
18:0	10.4±1	6.2±1	7.2±1	6.9±0.3
18:1	29.1±3	28.3±2	47.6±1	34.0±1
18:2	21.7±2	42.6±1	22.5±1	39.0±2

Data represent mean±SE for three animals from each group. Larger, more unsaturated fatty acids formed <3% of the total in all samples tested and were not considered in the calculations.

of the control group. In the diabetic animals, linoleate was the most abundant fatty acid both in cholesteryl ester and triglyceride fractions, and the oleate/linoleate ratios averaged 0.9 and 0.7, respectively. The saturated fatty acids were ~50% of the total in the triglyceride fraction of the nondiabetic group, but were <40% of the total fatty acids in triglycerides from diabetics and cholesteryl ester from both groups.

To address heterogeneity within the major lipoprotein classes, plasma was subfractionated by ultracentrifugation and the lipid composition of the lipoproteins obtained by flotation was determined (Table IV). In the diabetic animals, >60% of total plasma lipid was associated with particles of $S_f > 400$ and >90% of total lipids had $S_f > 20$. Although progressively less neutral lipid was associated with the smaller, more dense particles isolated between $S_f = 180–400$ and $S_f = 20–180$, the ratio of triglyceride to cholesteryl ester ranged from 5:1 to 4:1 and appeared to vary inversely with particle size. The greater amount of triglyceride than cholesteryl ester within the different fractions suggested that both core lipids were associated with the same heterogeneous particle population. The molar ratio of cholesterol to phospholipid was an inverse function of particle size and was much lower in the $S_f < 20$ fraction. For comparison, plasma from nondiabetic animals fed the 0.5%-cholesterol diet also was fractionated (Table IV). In the samples analyzed, which had an average plasma cholesterol of 660 mg/100 ml, only 6% of total plasma lipid was in the $S_f > 400$ fraction and almost 50% had an $S_f < 20$. Cholesteryl ester was the predominant core lipid in all fractions and, as with the diabetic particles, the molar ratio of unesterified cholesterol to phospholipid was close to 1.

If the lipid compositions of the lipoprotein fractions shown in Table IV are plotted on triangular coordinates (Fig. 2), important information may be obtained about the core and surface compositions of the lipoproteins (25, 26). If all the data points for an individual set of subfractions fall on a single tie line, these fractions are in equilibrium with respect to core and surface composition and only the size of the particle is different (25). The core and surface compositions are given by the intersections of the line through the composition points with the phase boundaries of the model system (25). Thus, fractions 1, 2, and 3 fall on tie lines whose core and surface composition are given by the intersections with the phase boundaries. Note that, whereas cholesterol-fed control and the cholesterol-fed diabetic animals had similar surface compositions, the core compositions of the diabetic-fed control had only about half as much free cholesterol. This is probably related to the high cholesteryl ester content of the core in cholesterol-fed controls, since an increased cholesteryl ester content of the core causes an increased partitioning of free cholesterol into the core (25).

Comparisons of the $d < 1.019$ g/ml fractions from nondiabetic and diabetic animals also were made with differential scanning calorimetry. Fig. 3 *A* shows a rep-

TABLE IV
Lipid Composition of Lipoprotein Subfractions from Control and Diabetic Cholesterol-fed Rabbits

	Fractions (S_f)			
	1 (>400)	2 (180–400)	3 (20–180)	4 (<20)
Diabetic				
Total lipid (mg/ml plasma)	106±22	29.4±6.5	9.5±2.3	9.5±2.0
Lipid composition (wt %)				
Triglyceride	71.9±4.7	65.7±4.4	59.6±2.4	45.0±2.8
Cholesteryl ester	13.9±5.0	14.6±4.5	15.3±2.7	17.1±1.9
Cholesterol	4.8±0.3	6.1±0.2	7.3±0.4	6.9±0.3
Phospholipid	9.1±0.8	13.6±1.5	17.7±2.1	30.8±2.4
Nondiabetic				
Total lipid (mg/ml plasma)	2.2±0.6	4.0±0.7	8.3±1.1	15.6±2.1
Lipid composition (wt %)				
Triglyceride	0.5±0.2	7.2±2.0	3.1±0.5	0
Cholesteryl ester	88.5±3.2	71.2±1.8	70.7±1.2	48.2±3.1
Cholesterol	5.3±2.5	7.9±0.7	8.6±1.2	6.4±0.7
Phospholipid	8.2±1.6	13.4±0.5	17.6±1.6	45.5±2.4

Data represent mean±SE for three separate experiments, with a different animal as the source of plasma for each experiment.

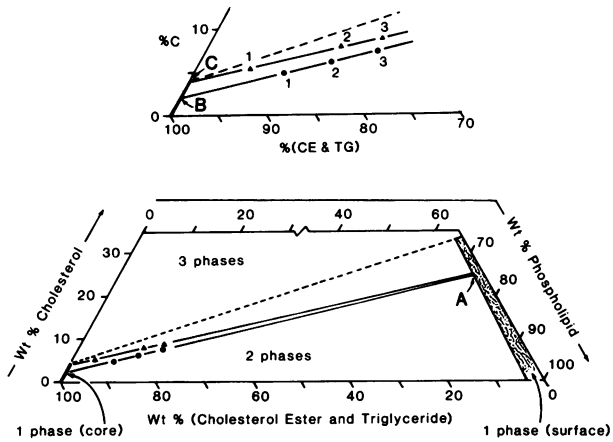

FIGURE 2 The composition of lipoprotein subfractions of S_f > 20 from cholesterol-fed control animals and cholesterol-fed diabetic animals plotted on the lipoprotein lipid phase diagram. The lower part of the lipoprotein lipid phase diagram (25), phospholipid-cholesterol-(triglyceride plus cholesteryl ester) is shown at the bottom of the figure. The top of the figure is an expansion of the lower left-hand corner. The lipoprotein compositions of fractions (Table IV) from diabetic cholesterol fed animals (●) and cholesterol-fed controls (▲) are shown (for the method of plotting, see reference 26). The dotted line is the boundary between the two-phase zone (core and surface) and a three-phase zone, which contains not only core and surface but can form cholesterol crystals (25, 26). Fractions 1, 2, and 3 in both groups fall on lines, indicating that the fractions within a given group are in equilibrium and differ only in size. The largest lipoproteins are on the left-hand side and the smallest on the right. The intersection of these lines with the phase boundaries on the left and right sides of the diagrams indicate the core and surface compositions of these lipoprotein particles, respectively. Thus, the surface compositions of both groups are similar (~73% phospholipid, 25% cholesterol), 2% (cholesterol ester plus triglyceride) (*A*), whereas the core compositions are different. The diabetic cholesterol-fed animals that have a high triglyceride concentration (Table IV) have only ~2% cholesterol in the core (*B*), while the cholesterol-fed controls that contain a very high content of cholesterol ester in the core contain ~4% of free cholesteryl (*C*). The finding that increased cholesteryl ester content in the core increases the distribution of free cholesterol into the core was predicted from model studies on such emulsion systems (25, 26).

resentative curve obtained with nondiabetic plasma as the lipoprotein source. A single reversible transition was observed in scans from 4 to 55°C. The endotherm started at ~22°C, peaked between 38 and 45°C (42±1°C, mean±SE, for eight separate samples), and returned to base line at ~47°C. The enthalpy of the transition averaged 0.79 cal/g cholesterol ester, and, in general, the calorimetry data were consistent with a previous report on β-VLDL from cholesterol-fed rabbits (27), suggesting a particle with a core of predominantly monounsaturated cholesteryl ester. The calorimetric data from the $d < 1.019$ g/ml fraction of diabetic animals showed two general patterns, both in marked contrast to the nondiabetic fractions. In some samples, a small transition was seen between 28 and 33°C, with an average enthalpy for the transition of 0.24 cal/g cholesteryl ester in four such examples. A representative tracing is shown in Fig. 3 *B*. In other samples, no detectable transition for cholesteryl ester or triacylglycerol was observed between 0 and 60°C, despite the presence of >12 mg core lipid in the calorimetry pan (Fig. 3 *C*). The absence of any transition is consistent with a particle population containing both triglyceride and cholesteryl ester in the core of the same particles (22, 27). If samples from diabetic animals showing no transition were mixed with samples from a control animal showing a transition, the transition for the control sample still was observed at the appropriate transition temperature (~42°C).

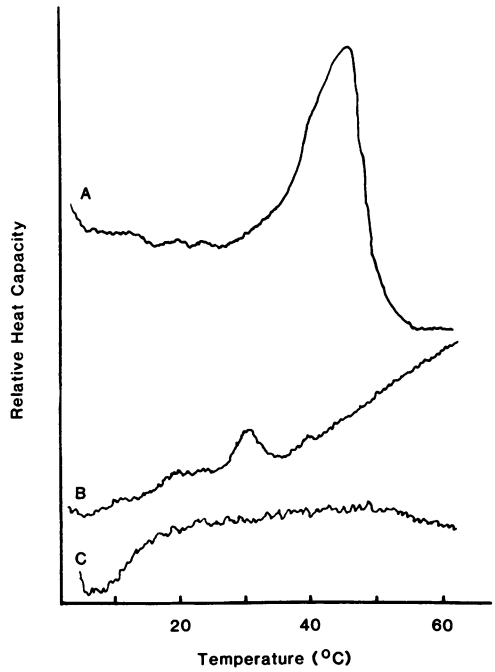

FIGURE 3 Differential scanning calorimetry curves of d > 1.019 g/ml fractions from cholesterol-fed control and diabetic rabbit plasma. Heating scans were run at 5°C/min at a sensitivity of 0.2 mcal/s. (*A*) Cholesterol-fed control, (*B* and *C*) diabetic (two separate samples).

A qualitative assessment of the apoprotein (apo) composition of the $d < 1.019$ g/ml fractions was made using SDS-PAGE. Fig. 4 shows representative gel patterns from control and diabetes fractions. The major apoprotein from control animals was apo E, and lesser amounts of apo C was also detected, consistent with previous studies (9). In contrast, the ratio of apo E to apo C in the diabetes fraction was much less than the control group, reflecting a relative lack of apo E.

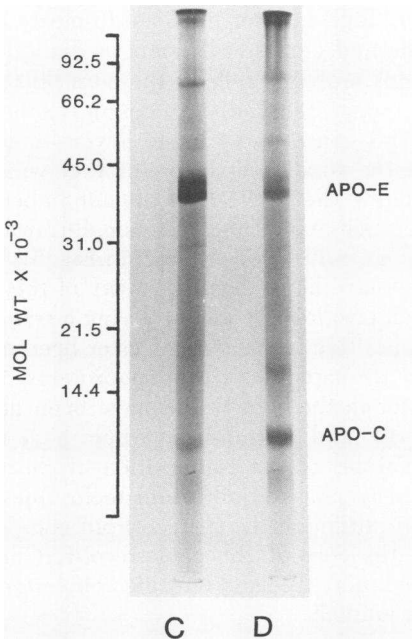

FIGURE 4 SDS-PAGE of apoproteins from cholesterol-fed control (*C*) and diabetic (*D*) $d < 1.019$ g/ml fractions. Molecular weight standards in order of decreasing size were phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

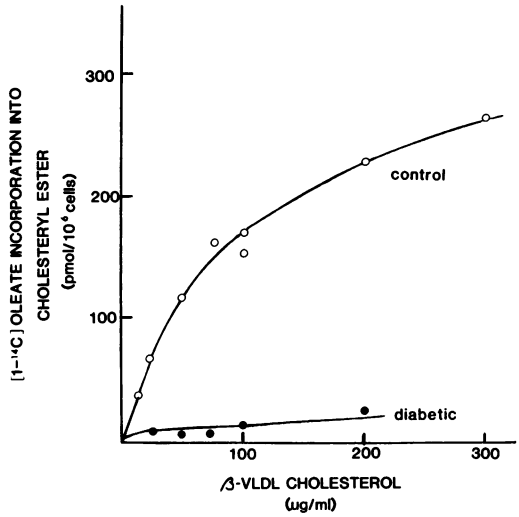

FIGURE 5 Effect of cholesterol-fed control or diabetic $d < 1.019$ g/ml fractions on the incorporation of [1-^{14}C]oleate into cholesteryl ester by mouse peritoneal macrophages. Each point represents the average of duplicate determinations. Samples from two rabbits from each group were used and the amount of lipoprotein added is expressed as total cholesterol in the fraction.

The data presented above documented differences in the chemical and physical properties of the $d < 1.019$ g/ml fractions from diabetic and nondiabetic animals. Metabolic comparisons also were made by examining the ability of the lipoprotein fractions to stimulate the incorporation of added [1-^{14}C]oleate into cholesterol ester by mouse peritoneal macrophages. The lipoprotein fraction from nondiabetic animals stimulated cholesterol esterification in the macrophages in a dose-dependent manner proportional to the amount of total cholesterol contained in the added lipoprotein (Fig. 5). This stimulatory effect was optimal at ~100 μg cholesterol/ml incubation medium and appeared to correspond to saturation of receptors for β-VLDL on the macrophages (12). When equivalent amounts of cholesterol from diabetic plasma lipoproteins were added, stimulation of cholesterol esterification was barely measurable and no evidence for a saturable process was detected.

Table V summarizes data from a series of experiments where $d < 1.019$ g/ml fractions from four separate animals in each group were added to macrophages and the incorporation of labeled oleate into cholesteryl ester and triglyceride was determined. In separate experiments, it was shown that incorporation of labeled oleate into both cholesteryl ester and triglyceride was linear for at least 5 h in the presence of lipoprotein. When lipoprotein was not added to the macrophages, incorporation of labeled oleate into cholesteryl ester was negligible, whereas significant amounts of radioactivity were associated with the triglyceride fraction. Addition of β-VLDL from either diabetic or control animals had only a slight effect on incorporation into triglycerides, despite the presence of between 190 and 420 μg triglyceride in the added β-VLDL from diabetic animals. Incorporation of labeled oleate into cholesteryl ester was increased only slightly by the diabetic β-VLDL, whereas all fractions from cholesterol-fed controls produced dramatic increases in cholesteryl ester formation.

TABLE V
Effect of $d < 1.019$ g/ml Lipoproteins on [1-^{14}C]Oleate Esterification by Mouse Peritoneal Macrophages

Addition	[1-^{14}C]Oleate incorporation	
	Cholesterol ester	Triglyceride
	nmol · 10^6 cells	
No addition	6±2	910±60
Control	180±12	720±50
Diabetic	18±4	1250±63

Data represent mean±SE for four separate experiments, each using lipoproteins obtained from different animals. In each experiment 80–100 μg cholesterol contained in the lipoprotein fraction was added and incubation was for 5 h.

If mixing experiments were performed, the presence of diabetic samples did not affect the response of the macrophages to the β-VLDL from control animals (data not shown).

DISCUSSION

The diabetic cholesterol-fed rabbit developed a marked elevation of plasma cholesterol and plasma triglycerides, yet had a low incidence of aortic atherosclerosis, consistent with earlier observations (2, 3). Since a major difference between nondiabetic and diabetic cholesterol-fed animals was the pronounced hypertriglyceridemia of the diabetic animals, it seemed possible that an alteration in the composition of the circulating lipoproteins could be a factor in the development of atherosclerosis. The major new findings of this study were the unique chemical and physical properties of the VLDL fraction from diabetic animal model and the lack of any obvious interaction of this lipoprotein fraction with mouse peritoneal macrophages.

An objective of the present study was to determine whether the lipoproteins from the diabetic cholesterol-fed rabbit contained different cholesteryl ester-rich and triglyceride-rich particles, or whether both neutral lipids coexisted in the core of the same particle. Chemical analysis of different subfractions obtained by sequential ultracentrifugation of the diabetic plasma showed only minor changes in the ratio of triglyceride to cholesteryl ester, apparently ruling out the possibility that large triglyceride rich particles resembling chylomicrons were present along with smaller, cholesteryl ester-rich β-VLDL particles.

More direct evidence that both neutral lipids were in the core of the same particle was obtained by calorimetry, where the order-disorder transition characteristic for cholesteryl ester in cholesteryl ester-rich particles (22) was usually absent in the lipoproteins from diabetic animals. The most plausible explanation for this effect is that triglyceride and cholesteryl ester are both contained in the same particle, and the presence of triglyceride prevents the ordering of cholesteryl ester necessary to form a liquid crystalline phase at the temperatures studied (22). Triglycerides have been shown to produce such effects in model systems and in certain lipoprotein particles (22, 27–30). The fatty acid composition of cholesteryl esters also determines thermal behavior and the diabetic fraction did have more cholesteryl linoleate than the nondiabetic fraction, which should lead to a lower transition temperature. However, the cholesteryl ester composition of the diabetic fraction was similar to that of normal human LDL, which is known to show a characteristic transition between 20 and 40°C (22, 27), and such transitions were absent in the diabetic fractions. The relatively high ratio of linoleate to oleate in both triglyceride and cholesteryl esters of diabetic rabbit lipoproteins probably reflects the corn oil contained in the diet, suggesting a dietary origin for the large particles. This contrasts with the lower linoleate/oleate ratio of the smaller lipoproteins that we and others (11) noted in the β-VLDL of the nondiabetic animals. An occasional animal showed a small transition of low enthalpy at ~30°C (see trace *B* in Fig. 3). These samples may have had a small quantity of the cholesteryl ester-rich fraction capable of giving a transition. Had the composition of cholesteryl ester been equally distributed throughout all the lipoproteins, the cholesteryl ester in the core would have been diluted with triglyceride and no transition would have been noted (28). Analyses of the composition of the lipoprotein fractions by phase equilibrium techniques (25) indicate that, although the surface lipid composition was similar, the core of the cholesterol-fed nondiabetics contained more free and esterified cholesterol than the diabetic animals.

The β-VLDL fraction from nondiabetic animals stimulated the incorporation of [1-^{14}C]oleate into cholesteryl ester by macrophages in a saturable, dose-dependent manner. This effect was similar to previous reports using β-VLDL from dogs or rabbits (12), and it was established by others that a macrophage receptor for β-VLDL exists, and that binding, internalization, and lysosomal degradation of core cholesteryl esters precede intracellular cholesteryl ester formation (31). The lipoprotein fraction from diabetic animals was ineffective in stimulating cholesteryl ester synthesis, suggesting that these particles did not undergo a receptor-mediated internalization and subsequent lysosomal degradation. Since our studies focused only on oleate incorporation into cholesteryl ester, we cannot define at which step of the pathway the diabetic fraction differed from the β-VLDL.

It is conceivable that lipoprotein lipase, an enzyme recently shown to be associated with macrophages (32), could have degraded the triglyceride contained in the diabetic fraction, resulting in formation of unlabeled fatty acid, thereby diluting the [1-^{14}C]oleate in the medium and causing an apparent reduction of labeled oleate into cholesteryl ester. This sequence of events is unlikely, since the concentration of [1-^{14}C]oleate in the medium (0.7 mM) was usually severalfold greater than the triglyceride contained in the added lipoprotein and the incorporation of labeled oleate into macrophage triglyceride was not reduced as a result of adding lipoproteins (Table V).

The relatively low amount of apo E in the lipoprotein fraction from diabetic animals may explain why these particles did not influence cholesteryl ester formation in macrophages. Apo E, a major apoprotein in

β-VLDL from nondiabetic cholesterol-fed models, has been shown to be recognized by lipoprotein receptors in fibroblasts (33) and liver (34). Qualitatively, the ratio of apo E to apo C in the particles from diabetic plasma was much less than that in the cholesterol-fed controls, and this ratio could determine binding to a macrophage receptor in a manner analogous to binding of emulsions or remnant particles to hepatocyte lipoprotein receptors (35, 36).

In man, a positive correlation between hypertriglyceridemia and atherosclerosis is well established. Recent studies from several laboratories have shown that patients with hypertriglyceridemia have unusual triglyceride-rich lipoproteins. Holdsworth et al. (37) isolated an abnormal lipoprotein from patients with chronic renal failure or severe hypertriglyceridemia (type V) that contained an increased content of sialylated apo C III and was a less efficient substrate for lipoprotein lipase. Chung et al. (38) used vertical spin centrifugation to show that VLDL from type IV patients were defective in their interaction with bovine milk lipoprotein lipase. Gianturco et al. have shown that VLDL from hypertriglyceridemia patients differed from normal VLDL when added to cultured fibroblasts (39) or macrophages (40). Hypertriglyceridemic VLDL of S_f = 100–400 was shown to interact with fibroblasts by a receptor-mediated process, and apo E was implicated in the process (41).

Redgrave and Snibson (42) demonstrated an impaired clearance of chylomicron triglyceride in diabetic rats and suggested that incompletely degraded chylomicron remnants accumulate in plasma and contribute to the development of hypertriglyceridemia. A possible explanation for the formation of the particles in the diabetic cholesterol-fed rabbit is the occurrence of a functional defect in lipolysis that prevents these particles from being cleared from plasma. This deficiency could be due to reduced lipoprotein lipase activity, an altered apoprotein distribution, or impaired lipoprotein receptor function. If the surface characteristics of the retained dietary particles do not exhibit the appropriate ligands for recognition by macrophages, this could retard the development of foam cell lesions in the diabetic animals.

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REFERENCES

1. Anitschkow, N. 1933. Experimental arteriosclerosis in animals. *In* Arteriosclerosis: A Survey of the Problem. E. V. Cowdry, editor. Macmillan, Inc., New York. 271–322.
2. Duff, G. L., and G. C. McMillan. 1949. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. I. The inhibition of experimental cholesterol atherosclerosis in alloxan diabetes. II. The effect of alloxan diabetes on the retrogression of experimental cholesterol atherosclerosis. *J. Exp. Med.* 89:611–630.
3. McGill, H. C., Jr., and R. L. Holman. 1949. The influence of alloxan diabetes on cholesterol atheromatosis in the rabbit. *Proc. Soc. Exp. Biol. Med.* 72:72–75.
4. Duff, G. L., D. J. H. Brechin, and W. E. Finkelstein. 1954. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. IV. The effect of insulin therapy on the inhibition of atherosclerosis in the alloxan-diabetic rabbit. *J. Exp. Med.* 100:371–380.
5. Cook, D. L., L. M. Mills, and D. M. Green. 1954. The mechanism of alloxan protection in experimental atherosclerosis. *J. Exp. Med.* 99:119–124.
6. Duff, G. L., and T. P. B. Payne. 1950. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. III. The mechanism of the inhibition of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits. *J. Exp. Med.* 92:299–317.
7. Gofman, J. W., F. Lindgren, H. Elliott, W. Mantz, J. Hewitt, B. Strisower, and V. Herring. 1950. The role of lipids and lipoproteins in atherosclerosis. *Science (Wash. DC).* 111:166–187.
8. Pierce, F. T., Jr. 1952. The relationship of serum lipoproteins to atherosclerosis in the cholesterol-fed alloxanized rabbit. *Circulation.* 5:401–407.
9. Shore, V. G., B. Shore, and R. G. Hart. 1974. Changes in apolipoprotein and properties of rabbit very low density lipoproteins on induction of cholesteremia. *Biochemistry.* 13:1579–1584.
10. Camejo, G., V. Bosch, and A. Lopez. 1974. The very low density lipoproteins of cholesterol-fed rabbits. A study of their structure and in vivo changes in plasma. *Atherosclerosis.* 19:139–152.
11. Rodriguez, J. L., G. C. Ghiselli, D. Torreggiani, and C. R. Sirtoli. 1976. Very low density lipoproteins in normal and cholesterol-fed rabbits: lipid and protein composition and metabolisms. Part 1. Chemical compositions of very low density lipoproteins in rabbits. *Atherosclerosis.* 23:73–83.
12. Mahley, R. W., T. L. Innerarity, M. S. Brown, Y. K. Ho, and J. L. Goldstein. 1980. Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. *J. Lipid Res.* 21:970–980.
13. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497–509.
14. Dole, V. P., and J. T. Hamlin III. 1962. Particulate fat in lymph and blood. *Physiol. Rev.* 42:674–701.
15. Rudel, L. L., and M. O. Morris. 1973. Determination of cholesterol using *o*-phthalaldehyde. *J. Lipid Res.* 14:364–366.
16. Fletcher, M. J. 1968. A calorimetric method for estimating serum triglycerides. *Clin. Chim. Acta.* 22:393–397.
17. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* 234:466–468.
18. Lowry, O. H., N. J. Rosenbrough, R. L. Farr, and R. J. Randle. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
19. Raabo, E., and T. C. Terpildsen. 1960. On the enzymatic determination of blood glucose. *Scand. J. Clin. Lab. Invest.* 12:402–407.
20. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids

with boron fluoride-methanol. *J. Lipid Res.* 5:600–608.
21. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulphate polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
22. Deckelbaum, R. J., G. G. Shipley, and D. M. Small. 1977. Structure and interactions of lipids in human plasma low density lipoproteins. *J. Biol. Chem.* 252:744–754.
23. Koski, I. R., D. G. Poplack, and R. M. Blaese. 1976. A non-specific esterase stain for the identification of monocytes and macrophages. *In* In Vitro Methods in Cells Mediated and Tumor Immunity. B. R. Blood and J. R. David, editor. Academic Press, Inc., New York. 359–362.
24. Lichtenstein, A. H., and P. Brecher. 1980. Properties of acyl CoA:cholesterol acyltransferase in rat liver microsomes. Topological localization and effect of detergents, albumin and polar steroids. *J. Biol. Chem.* 255:9098–9104.
25. Miller, K. W., and D. M. Small. 1983. Triolein-cholesteryl oleate-cholesterol-lecithin emulsions: Structural models of triglyceride-rich lipoproteins. *Biochemistry.* 22:443–451.
26. Miller, K. W., and D. M. Small. 1982. The phase behavior of triolein, cholesterol, and lecithin emulsions. *J. Colloid Interface Sci.* 89:466–478.
27. Waugh, D. A., and D. M. Small. 1982. Rapid method for determining cholesteryl ester transitions of apo β-containing lipoproteins. *J. Lipid Res.* 23:201–204.
28. Deckelbaum, R. J., A. R. Tall, and D. M. Small. 1977. The interaction of cholesterol ester and triglyceride in human plasma very low density lipoproteins. *J. Lipid Res.* 18:164–168.
29. Kroon, P. A. 1981. The order-disorder transition of the core cholesteryl esters of human plasma low density lipoprotein. *J. Biol. Chem.* 256:5332–5339.
30. Kroon, P. A., and J. Seidenberg. 1982. Organization of the core lipids of lipoprotein from normal and cholesterol-fed rabbits. A proton nuclear magnetic resonance study. *Biochemistry.* 21:6483–6488.
31. Goldstein, J. L., Y. K. Ho, M. S. Brown, T. L. Innerarity, and R. W. Mahley. 1980. Cholesteryl ester accumulation in macrophages resulting from receptor mediated uptake and degradation of hypercholesterolemic canine β-very low density lipoproteins. *J. Biol. Chem.* 255:1839–1848.
32. Mahoney, E. M., J. C. Khoo, and D. Steinberg. 1982. Lipoprotein lipase secretion by human monocytes and rabbit alveolar macrophages in culture. *Proc. Natl. Acad. Sci. USA.* 79:1639–1642.
33. Innerarity, T. L., and R. W. Mahley. 1978. Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. *Biochemistry.* 17:1440–1447.
34. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes metabolically distinct apo-E and apo-B, E receptors. *J. Biol. Chem.* 256:5646–5655.
35. Windler, E., Y. Chao, and R. J. Havel. 1980. Regulation of the hepatic uptake of triglyceride-rich lipoproteins in the rat. Opposing effects of homologous apolipoprotein E and individual C apoproteins. *J. Biol. Chem.* 255:8803–8807.
36. Shelburne, F., J. Hanks, W. Meyers, and S. Quarfordt. 1980. Effect of apoproteins on hepatic uptake of triglyceride emulsions in the rat. *J. Clin. Invest.* 65:652–658.
37. Holdsworth, G., J. Stocks, P. Dodson, and D. J. Galton. 1982. An abnormal triglyceride-rich lipoprotein containing excess sialylated apoliprotein C-III. *J. Clin. Invest.* 69:932–939.
38. Chung, B. H., J. T. Cone, and J. P. Segrest. 1982. Defective in vitro lipolyses of type IV hyperlipidemia human plasma by purified milk lipoprotein lipase. Studies by single vertical spin centrifugation. *J. Biol. Chem.* 257:7472–7480.
39. Gianturco, S. H., F. B. Brown, A. M. Gotto, Jr., and W. A. Bradley. 1982. Receptor-mediated uptake of hypertriglyceridemic very low density lipoprotein by normal human fibroblasts. *J. Lipid Res.* 23:984–993.
40. Gianturco, S. H., W. A. Bradley, A. M. Gotto, Jr., J. D. Morrisett, and D. L. Peavy. 1982. Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis and accumulation in mouse peritoneal macrophages. *J. Clin. Invest.* 70:168–178.
41. Gianturco, S. H., A. M. Gotto, S.-L. C. Hwang, J. B. Karlin, A. H. Y. Lin, S. C. Prasad, and W. A. Bradley. 1983. Apolipoprotein E mediates uptake of S_f 100–400 hypertriglyceridemic very low density lipoproteins by the low density lipoprotein receptor pathway in normal human fibroblasts. *J. Biol. Chem.* 258:4526–4533.
42. Redgrave, T. G., and D. A. Snibson. 1977. Clearance of chylomicron triacylglycerol and cholesteryl ester from the plasma of streptozotoxin-induced diabetic and hypercholesterolemic hypothyroid rats. *Metab. Clin. Exp.* 26:493–503.