

Distribution of apolipoprotein E in the plasma of insulin-dependent and noninsulin-dependent diabetics and its relation to cholesterol net transport

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Abstract Noninsulin-dependent diabetics, whose plasma contained no detectable beta-VLDL (very low density lipoprotein), had a proportion (0.23 ± 0.04) of plasma apolipoprotein E in the form of an abnormal lipoprotein not recognized by antibodies to apoB-100 from LDL (low density lipoprotein) or apoA-I from HDL (high density lipoprotein). This lipoprotein, abnormally rich in free cholesterol and apoE, had a calculated particle density within the low density lipoprotein range. It competed with LDL at the apoB,E receptor of normal fibroblasts and stimulated cholesteryl ester accumulation in mouse peritoneal macrophages. However, it did not compete with the binding of labeled rabbit beta-VLDL to macrophages. A much lower proportion of apoE (0.04 ± 0.03) was in this form in the plasma of patients with insulin-dependent diabetes who had a comparable degree of hyperglycemia. The diabetic lipoprotein was absent in normoglycemic control subjects. The net transport of cholesterol from cell membranes to the plasma of noninsulin-dependent diabetics (and to a lesser extent, insulin-dependent diabetics) was inhibited relative to control values, and the magnitude of this inhibition was well correlated with the concentration of the abnormal lipoprotein of diabetes in plasma ($r = 0.66$ and 0.75 , respectively). These findings suggest that diabetic plasma contains an abnormal and novel low density lipoprotein that mediates the abnormal cholesterol transport characteristic of human diabetes mellitus.—Fielding, C. J., G. R. Castro, C. Donner, P. E. Fielding, and G. M. Reaven. Distribution of apolipoprotein E in the plasma of insulin-dependent and noninsulin-dependent diabetics and its relation to cholesterol net transport. *J. Lipid Res.* 1986. 27: 1052–1061.

Supplementary key words low density lipoprotein • beta-VLDL • apoB,E receptor • fibroblasts • mouse peritoneal macrophages

Previous studies (1) demonstrated that the net transport of cholesterol from cells to plasma that occurs when cells are incubated with plasma from normal subjects was inhibited when cells were incubated with plasma from patients with noninsulin-dependent diabetes mellitus

(NIDDM). Plasma from patients with NIDDM also contained increased concentrations of apolipoprotein (apo) E, and plasma cholesterol transport was normalized when lipoproteins containing apoE were removed in vitro by immunoaffinity chromatography (1). Further evidence that the elevated plasma apoE concentration in diabetics was a factor in the functional defect in cholesterol transport came from the observation that both cholesterol transport and apoE levels were normalized when these diabetic patients were effectively treated with insulin (1). One goal of the present study was to extend these observations to patients with insulin-dependent diabetes mellitus (IDDM) with a similar degree of hyperglycemia to determine whether the defect observed in NIDDM was unique to this syndrome or simply a function of uncontrolled diabetes.

A second aim was to further define the lipoprotein characteristics of apoE in diabetes. Previous studies of normal individuals (2) had indicated that the whole of apoE in native fasting plasma is complexed either to apoA-I in high density lipoprotein (HDL) or to apoB (apoB-100) in very low or low density lipoproteins (VLDL and LDL). However, lipoproteins containing apoE unassociated with apoA-I (HDL_c) have been found in the HDL of experimental animals after cholesterol feeding (3). Furthermore, apoE-rich lipoproteins with abnormal electrophoretic, compositional, and functional properties

Abbreviations: VLDL, LDL, and HDL, very low, low, and high density lipoproteins, respectively; NIDDM, noninsulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; LCAT, lecithin:cholesterol acyltransferase.

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(beta-VLDL) have also been found in the plasma of patients with several genetic and acquired hyperlipidemias (4, 5). In the current study we have investigated whether apoE in lipoprotein form with the properties of either HDL_c or beta-VLDL was present in the plasma of patients with diabetes.

MATERIALS AND METHODS

Study selection

The study population consisted of ten patients with NIDDM, ten patients with IDDM, and a control group of ten normoglycemic, normolipidemic subjects. Diabetics were classified as having NIDDM or IDDM on the basis of the criteria of the National Diabetes Data Group (6). Patients with IDDM had a history of diabetic ketoacidosis and/or ketonuria, and had been taking insulin since diagnosis. Patients with NIDDM did not have a history of either diabetic ketoacidosis or ketonuria, and had never received insulin. Patients in both groups were studied while on their usual treatment program of insulin (IDDM) or sulfonylurea compounds (NIDDM). Fasting plasma glucose concentrations were similar in both groups of diabetics, and higher than in the control population (Table 1). The view that glycemic control was comparable in the two groups of diabetics is further supported by the fact that levels of glycosylated hemoglobin were also similar. Plasma triglyceride levels were also elevated above control values in both groups of diabetics, and again to a similar degree (Table 1). However, fasting plasma cholesterol concentrations were comparable in all three groups. Blood was obtained from diabetic or normoglycemic donors who had fasted overnight, collected into one-twentieth volume of 0.2 M sodium citrate, and immediately cooled in ice water. Plasma was obtained by centrifugation (200 g 30 min, 0–2°C) and used in the individual measurements described below.

Determination of apolipoproteins

The level of apoE in plasma was determined by specific radial immunoassay (2). ApoE was determined relative to a pure antigen standard prepared from normoglycemic plasma by molecular sieve and DEAE-cellulose column chromatography. Parallel dilution plots were obtained for diabetic and normoglycemic plasmas, and for apoE antigen standards. The sensitivity of detection was about 0.1 µg of apoE; samples were concentrated if necessary by a micro Pro-Dicon dialysis cell (Bio-Molecular Dynamics, Beaverton, OR).

To determine the proportion of apoE in plasma complexed in lipoproteins containing apoB, plasma (1 ml) was passed through a column (0.9 × 10 cm) containing antibody raised in rabbits to human apoB (apoB-100) from fasting normal LDL of flotation density 1.02–1.04 g · ml⁻¹,

covalently linked to agarose (7). This nonadsorbed fraction of plasma was then passed through a second similar agarose column containing immobilized antibodies to apoA-I. The level of apoE in the final eluate was then measured. The level of apoE in the eluate from anti-apoE affinity chromatography was also determined.

ApoA-I and apoE were determined in the plasma of diabetics and normoglycemic controls by radial immunoassay (8).

Agarose gel electrophoresis

Electrophoresis of plasma, or the nonadsorbed fractions of plasma obtained after affinity chromatography, was carried out on agarose strips developed in barbital buffer. None of the control or diabetic subjects in this study showed detectable beta-migrating VLDL in the $d < 1.006$ g/ml supernatant fraction after ultracentrifugal flotation (9).

Determination of cholesterol esterification in plasma

Cholesterol esterification in normal or diabetic plasma was measured as the rate of decrease of plasma free cholesterol when plasma, diluted fivefold with 0.15 M NaCl in a medium of 0.01 M Tris buffer, 1 mM disodium EDTA (pH 7.4), was incubated at 37°C. Pentuplicate initial samples of medium and of medium incubated for 60 min at 37°C were extracted with chloroform-methanol (10). Plasma free cholesterol was assayed by a modified fluorimetric method (11).

Determination of cholesterol net transport

The net flux of free cholesterol between cell membranes and plasma of diabetic or control subjects was determined using normal human fibroblasts cultured to near confluence in 10% fetal calf serum in 6-cm plastic dishes. The dishes contained 8–10 µg of cell cholesterol under these conditions. Plasma freed of fibrinogen by affinity chromatography (12) was diluted to 1.2% v/v with phosphate-buffered saline (13) and added to pentuplicate dishes of fibroblasts and to the same number of empty dishes. The contribution of cell membrane free cholesterol to the total free cholesterol utilized for esterification in the plasma medium was obtained from the difference in the free cholesterol decrease in the course of incubation between empty dishes and dishes containing cells. As previously reported (12), net transport from cells to plasma has a positive sign when the flux of cholesterol from cells to plasma exceeds the reverse; when the uptake of free cholesterol from plasma to cells exceeds the flux of cholesterol from cells to plasma, net transport has a negative sign. Such a reversal of the normal direction of cholesterol net transport has been previously reported for fibroblasts incubated with plasma of patients with NIDDM (1).

TABLE 1. Characteristics of IDDM and NIDDM patients and normoglycemic control subjects

Subjects	Hb A _{1c} % of total hemoglobin	Plasma (fasting)		
		Glucose	Triglyceride	Total Cholesterol
Control	3.8 - 5.5 ^a	83 ± 8	73 ± 27	183 ± 24
IDDM ^b	7.7 ± 0.3	212 ± 26	165 ± 39	200 ± 14
NIDDM ^b	8.3 ± 0.3	246 ± 19	172 ± 24	214 ± 19

^aValues for the control population are the normal range.^bThese patients are more fully described in the Methods section.

ApoE binding to fibroblast apoB,E receptors

The ability of apoE-containing lipoproteins in plasma to compete at the apoB,E receptor of up-regulated normal fibroblasts (14) was determined in terms of the effectiveness of unlabeled plasma in displacing centrifugally isolated LDL (d 1.02-1.04 g/ml) labeled with ¹²⁵I to a specific activity of 40-84 dpm/ng of protein (15). Fibroblasts were cultured to near confluence in 10% fetal calf serum in Dulbecco's modified Eagle's medium, then transferred to a medium containing lipoprotein-deficient serum (d > 1.21 g/ml infranatant solution of plasma) (4 mg/ml) for 48 hr at 37°C. The cells were then washed four times with phosphate-buffered saline containing albumin (4 mg/ml) and transferred to fresh medium containing ¹²⁵I-labeled LDL (2.5 µg/ml), lipoprotein-deficient serum (4 mg of protein/ml) and 0-2.5% (v/v) of either unmodified diabetic or normoglycemic plasma, or the same plasma from which lipoproteins containing apoA-I or apoB had been removed by immunoaffinity chromatography, as described above. In some dishes, nonspecific binding of LDL was determined by the inclusion of a 50-fold excess (125 µg/ml) of unlabeled LDL. All dishes were incubated for 3 hr at 0°C. The cells were then washed eight times with phosphate-buffered saline + albumin, and bound ¹²⁵I-labeled LDL was displaced with sodium heparin solution (10 mg/ml) by incubation for 30 min at 0°C. Solubilized radioactivity was then determined by gamma radiation spectrometry.

ApoE binding to mouse peritoneal macrophages

Macrophages were harvested in phosphate-buffered saline from unstimulated mice (16). Cells were collected by centrifugation (400 g, 10 min at room temperature), washed with Dulbecco's modified medium, and resuspended in the same medium containing 20% of heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 3 × 10⁶ cells/ml. Two ml of suspension was incubated in 35-mm plastic dishes for 2 hr at 37°C to allow the macrophages to adhere to the dishes. The dishes were then washed to remove nonadherent cells, and incubated 24 hr in the same medium. Each dish then received 0.6 ml of medium containing 0.2 mM[9,10-³H]oleic acid complexed as oleate with 2.4 mg/ml of human serum albumin, and 0.1 ml of normal or diabetic plasma or plasma from which

lipoproteins containing apoA-I and apoB had been removed by affinity chromatography. Dishes were incubated for 5 hr at 37°C, and the cells were removed by scraping and extracted with chloroform and methanol (10). Cholesteryl ester in the chloroform phase of the extract was isolated by thin-layer chromatography on silica gel layers on glass plates developed in hexane-diethyl ether-acetic acid 83:16:1 (v/v). Radioactivity in cholesteryl esters was determined by liquid scintillation spectrometry.

In some experiments, beta-VLDL obtained from a cholesterol-fed rabbit by centrifugation and electrophoresis (17) was labeled with ¹²⁵I and added to macrophages in the presence or absence of unlabeled plasma or plasma fractions; the cells were in a medium containing Dulbecco's modified Eagle's medium + 4 mg/ml albumin. Incubation was for 2 hr at 4°C. At the end of the incubation, the cells were washed with cold medium, dissociated with 0.2 M NaOH, and counted.

Chemical and physical characterization of apoE lipoprotein

Heparin agarose affinity chromatography was performed using columns (1 × 20 cm) of matrix (Pharmacia, Sweden) equilibrated with 0.15 M NaCl, 1 mM EDTA, pH 7.4. Plasma fractions were passed through the matrix, the nonadsorbed fraction was collected, and adsorbed lipoproteins were eluted with 3 M NaCl, 1 mM EDTA, and then dialyzed against 0.15 M NaCl-EDTA. The apoE content of adsorbed and nonadsorbed fractions was determined immunologically. Apoprotein molecular weight determination was by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Lipoprotein lipid composition was determined after extraction in chloroform and methanol (10). Triglyceride was determined with chromotropic acid (18), phospholipid as lipid phosphorus (19), and free and esterified cholesterol by fluorimetry (11).

RESULTS

Effect of diabetes on apoprotein composition and distribution

Relevant plasma apolipoprotein concentrations in the pa-

tients with diabetes and in the normoglycemic control subjects are shown in **Table 2**. Subjects with IDDM had significantly higher levels of apoA-I, the major protein of HDL, than did either control or NIDDM groups ($0.005 < P < 0.01$). On the other hand, the level of apoB was significantly higher in NIDDM than in the IDDM or control groups ($0.005 < P < 0.01$ and $P < 0.001$, respectively). As a result, while the ratio of apoA-I to apoB was similar in the IDDM and control groups, it was much lower in patients with NIDDM. A second difference in plasma apolipoprotein composition in the NIDDM and IDDM groups lay in the level of apoE. In spite of the fact that plasma triglyceride and total cholesterol concentrations were comparable in the two groups, the level of apoE was higher ($P < 0.005$) in patients with NIDDM. These findings indicate that characteristic differences in the plasma apolipoprotein pattern were present in NIDDM and IDDM, even when plasma triglyceride, cholesterol, and glucose levels were comparable.

Distribution of apoE in diabetic plasma

We have previously shown (2) that apoE in the plasma of fasting control subjects was present entirely in the form of complexes retained by antibodies to apoA-I or apoB. As a result, when plasma from subjects with normal glucose levels was subjected to sequential affinity chromatography on antibodies to these apolipoproteins, no detectable apoE was found in the final nonadsorbed eluate of such plasma. In contrast, a quite different pattern was seen in NIDDM. A significantly lower proportion of apoE ($P < 0.005$) was complexed with apoA-I than in normal plasma (**Table 3**), in spite of the generally similar levels of apoA-I seen in the two groups (**Table 2**). Specifically, about a quarter (0.23 ± 0.04) of total apoE in NIDDM plasma was present in a form not recognized by the immobilized antibodies to either apoA-I or apoB. Although plasma from seven of ten patients with IDDM also contained detectable levels of apoE not complexed to either apoA-I or apoB, only 0.04 ± 0.03 of total apoE was in this form. Furthermore, the amount of apoE not bound to either apoA-I or apoB was never as great as in any subject in the NIDDM group. No normoglycemic subject showed detectable levels of unbound apoE.

TABLE 2. Apolipoprotein composition of plasma from diabetics and normoglycemic controls

Subjects	ApoA-I	ApoB	ApoE
	mg/ml (\pm SD)		
Controls	1.30 ± 0.14	0.68 ± 0.17	0.05 ± 0.01
IDDM	1.53 ± 0.21	0.83 ± 0.29	0.04 ± 0.01
NIDDM	1.21 ± 0.21	1.21 ± 0.23	0.08 ± 0.02

The ratios of apoA-I/apoB were 1.91 ± 0.42 , 1.84 ± 0.81 , and 1.00 ± 0.16 in the control, IDDM, and NIDDM groups, respectively.

TABLE 3. Proportions of apoE complexed with apoA-I and apoB

Subjects	Complexed with ApoA-I (1)	Complexed with ApoB (2)	(1 + 2)
Controls	0.55 ± 0.05	0.45 ± 0.05	1.00
IDDM	0.57 ± 0.07	0.37 ± 0.07	0.95
NIDDM	0.33 ± 0.11	0.44 ± 0.10	0.78

Values are determined from apoE levels in unmodified plasma in all three groups, and in the same plasma after immunoaffinity chromatography on immobilized antibodies to apoA-I and then to apoB. Values are presented relative to the total apoE of the original plasma. The proportion of total plasma apoE not bound by antibodies to apoA-I and apoB was 0.04 ± 0.03 and 0.23 ± 0.04 for IDDM and NIDDM plasma, respectively, when apoE was measured directly from the final nonadsorbed eluate of affinity chromatography.

Cholesterol net transport in diabetic plasma

Net transport of cholesterol from cell membranes to plasma was significantly reduced in NIDDM ($P < 0.001$) relative to either the control or the IDDM groups (**Fig. 1**). Net transport rates in the control and IDDM groups were not significantly different.

The ability of cell membranes to transfer free cholesterol to plasma is dependent upon the continued activity of lecithin:cholesterol acyltransferase (LCAT) (8). The rates of cholesterol esterification in NIDDM, IDDM, and control plasma were 25.4 ± 9.0 , 25.9 ± 6.4 , and 23.7 ± 6.8 μg cholesterol esterified ml^{-1} plasma hr^{-1} , respectively (differences not significant). Thus inhibition of cholesterol transport from fibroblasts to plasma of patients with NIDDM is not the consequence of a reduced demand for free cholesterol by the LCAT reaction.

Role of apoE in the abnormal cholesterol metabolism of diabetic plasma

A plasma lipoprotein fraction containing apoE has been shown to be responsible for the inhibition of cholesterol net transport in NIDDM plasma (1). Since the unidirectional flux of cholesterol from cells to plasma is normal in these patients (1), apoE must act to stimulate the influx of free cholesterol from NIDDM plasma to cell membranes. The possible direct relationship between these observations was investigated by means of studies in which VLDL- and LDL-free plasma from patients with NIDDM was reconstituted with VLDL and LDL from control subjects, and vice versa.

When plasma from patients with NIDDM was fractionated on heparin-agarose, and the nonadsorbed fraction was incubated directly with cultured normal fibroblasts, cholesterol net transport was normalized to the rate characteristic of control plasma (**Fig. 2**). When the retained VLDL and LDL were recovered and reconstituted with the nonadsorbed fraction in the original proportions present in unmodified NIDDM plasma, the original, inhibited transport rate was restored (**Fig. 2**). These experiments indicate, firstly, that the factor inhibiting normal cholesterol net transport was retained on heparin-agarose along with

VLDL and LDL, and secondly, that such fractionation did not of itself modify the biological properties of the inhibitory factor.

A second series of experiments was carried out to compare cholesterol net transport rates in the nonadsorbed fraction of NIDDM plasma, as well as in the same plasma from which all lipoproteins containing apoE had been removed by immunoaffinity chromatography. As shown in Fig. 3, essentially identical transport rates were obtained when plasma was modified by removing apoE, or by removing VLDL and LDL. These data suggest that the inhibitory fraction was retained on heparin-agarose under the conditions of these experiments, and that it contained apoE.

A third series of experiments measured the effects of the reconstitution of diabetic plasma with VLDL and LDL isolated from control plasma, and of control plasma with diabetic VLDL and LDL. These results, seen in the left panel of Fig. 4, showed that the VLDL and LDL of patients with NIDDM inhibited cholesterol net transport in control plasma. The reciprocal effect was also demonstrated (Fig. 4, right panel); the addition of control VLDL and LDL relieved the inhibition of cholesterol net transport when they replaced endogenous VLDL and LDL in NIDDM plasma. These results indicated that the inhibitory factor retained on heparin-agarose with VLDL and LDL was effective in the inhibition of cholesterol net transport.

The removal of apoE from the adsorbed VLDL and LDL of NIDDM plasma also increased net transport by $0.68 \pm 0.10 \mu\text{g h}^{-1}$ ($n = 3$). Thus the inhibitory factor must be a lipoprotein containing apoE, which was not bound to antibodies to apoA-I or apoB but was retained with VLDL and LDL on heparin-agarose.

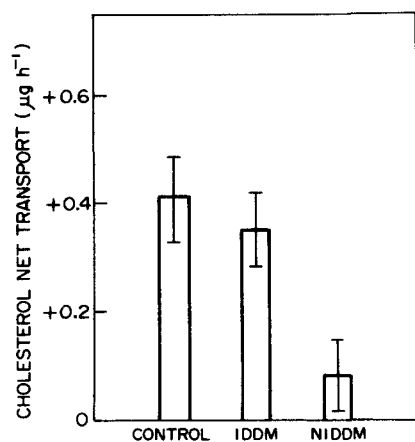


Fig. 1. Cholesterol net transport in plasma from diabetic and control subjects. Net transport was determined between defibrinated plasma (1.2% v/v in phosphate-buffered saline) and normal human fibroblasts, as described under Methods. Incubation was for 60 min at 37°C. Values are means ± 1 SD.

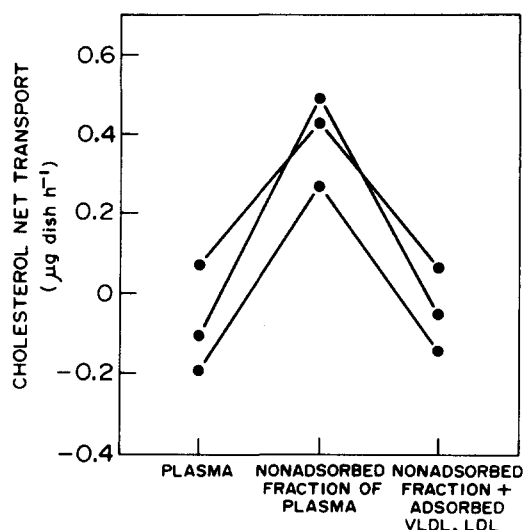


Fig. 2. The effects of heparin-agarose fractionation on cholesterol net transport in plasma from patients with NIDDM. Plasma was passed down heparin-agarose and, after washing with 0.15 M NaCl-EDTA (pH 7.4), the adsorbed VLDL and LDL were eluted with 3 M NaCl and dialyzed. Cholesterol net transport was determined as described in the legend to Fig. 1, and comparison was made of transport rates in native unfractionated plasma, in the nonadsorbed fraction of plasma from heparin-agarose chromatography, and the same nonadsorbed fraction recombined with adsorbed VLDL and LDL carried out at the same dilution (1.2% v/v relative to plasma) in each case. Incubation was for 60 min at 37°C.

Heparin-agarose chromatography of the unbound fraction of apoE

The possible identity of the fraction of apoE not retained by apoA-I or apoB, with the factor responsible for the in-

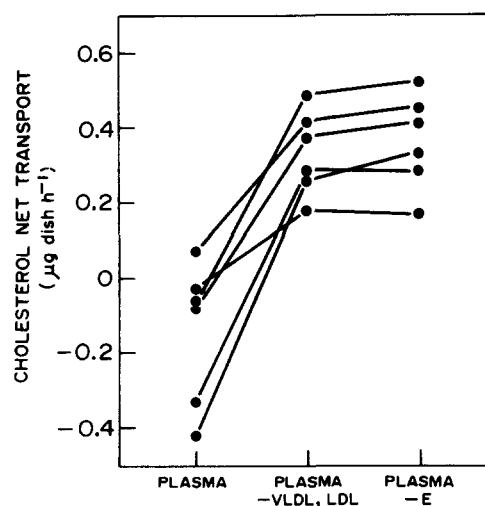


Fig. 3. Comparison of the effects of heparin-agarose chromatography and anti-apoE immunoaffinity chromatography on cholesterol net transport in plasma from patients with NIDDM. Transport rates were compared in unmodified plasma from patients with NIDDM and in the nonadsorbed fraction of the same plasma, following heparin-agarose chromatography or anti-apoE chromatography. Net transport was determined as described in the legend to Fig. 1.

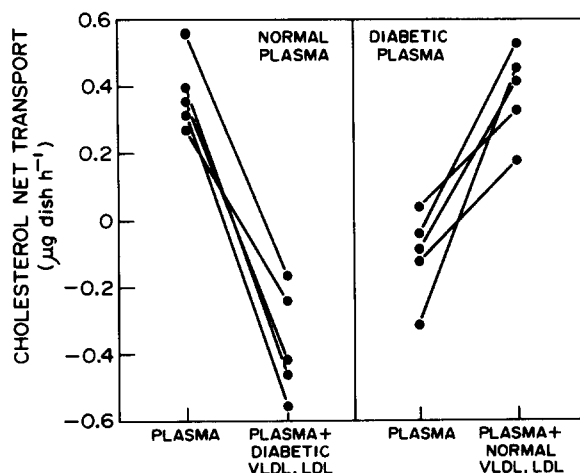


Fig. 4. Effects of VLDL and LDL from patients with NIDDM on cholesterol net transport in plasma from control subjects, and the converse. Plasma from control subjects and from patients with NIDDM was fractionated with heparin-agarose, and the nonadsorbed fraction and adsorbed VLDL and LDL were obtained as described in the legend to Table 4. Left panel: cholesterol net transport in unmodified plasma from control subjects and in the nonadsorbed fraction of such plasma reconstituted with VLDL and LDL from patients with NIDDM. Right panel: cholesterol net transport in unmodified plasma from patients with NIDDM, and in the nonadsorbed fraction of NIDDM plasma reconstituted with VLDL and LDL from control subjects. Net transport was determined as in the legend to Fig. 1.

hibition of cholesterol transport, was further investigated by determining the affinity of apoE in this fraction from NIDDM plasma for heparin-agarose, under the same conditions as used for the fractionation of VLDL and LDL (1). In five of six experiments, the apoE in the nonadsorbed fraction from immunoaffinity chromatography was quantitatively removed by heparin-agarose equilibrated in 0.15 M NaCl containing 1 mM EDTA (pH 7.4), and could be recovered with 3 M NaCl. In the remaining experiment, 0.6 of the apoE content of the fraction was retained on heparin-agarose. These studies indicate that the major part or all of apoE in the nonadsorbed fraction had the chromatographic properties on heparin-agarose of the inhibitor of cholesterol transport.

Relationship between unbound apoE and the inhibition of cholesterol net transport

The data in Fig. 5 demonstrate the presence of a close inverse relationship ($r = -0.92$) between the inhibition of cholesterol net transport and the concentration of apoE in IDDM or NIDDM plasma which was unrecognized by antibodies to either apoB or apoA-I. Furthermore, the relationship was seen in the individual patient groups with either NIDDM ($r = -0.66$) or IDDM ($r = -0.75$). It should be noted that the vertical intercept (for predicted net transport in the absence of unrecognized apoE) ($+0.38 \mu\text{g hr}^{-1}$) was similar to the mean net transport of the control group in this study ($0.41 \pm 0.08 \mu\text{g hr}^{-1}$). This rela-

tionship further suggests that the transport of free cholesterol from cell membranes to plasma would be completely inhibited when plasma apoE in this form was $21.7 \mu\text{g ml}^{-1}$, i.e., about one-quarter of total apoE present in NIDDM plasma in this study.

Chemical composition of unbound apoE

In order to define its chemical composition, the unbound fraction of apoE in NIDDM plasma was purified from other lipoproteins containing apoE by sequential affinity chromatography heparin-agarose chromatography, and flotation at $d 1.063 \text{ g/ml}$. Lipid and protein composition is shown in Table 4. Like the LDL in NIDDM plasma (which had a similar protein content), it showed a high weight ratio of free cholesterol to phospholipid (0.52 compared to 0.50) (19), but unlike diabetic LDL, it contained a high level of apoE. The other major protein of the abnormal lipoprotein of diabetes co-migrated with authentic apoB-100 of fasting normoglycemic LDL (data not shown) despite the inability of the native diabetic lipoprotein (unlike normal VLDL and LDL and the bulk of apoB in diabetic plasma) to bind to anti-apoB antibody (Table 2). The abnormal lipoprotein of diabetes also showed a much higher content of triglyceride relative to cholesteryl ester than did either control or diabetic LDL (20) (Table 4).

Reactivity of unbound apoE with fibroblast apoB,E receptors

The ability of plasma from normal subjects and patients with NIDDM to compete with ^{125}I -labeled LDL at the apoB,E receptor of fibroblasts (14) is shown in Fig. 6. Plasma from either group of subjects reduced the binding of labeled LDL in a concentration-dependent manner.

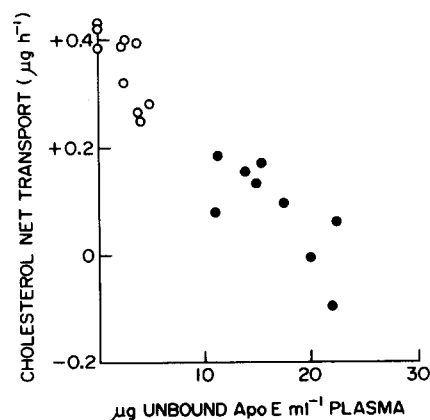


Fig. 5. The relationship between the concentration of apoE unretained by immunoaffinity columns to either apoB or apoA-I in the plasma of patients with either NIDDM or IDDM, and cholesterol net transport. The level of unbound apoE was determined following sequential chromatography on antibodies to apoB and apoA-I. Cholesterol net transport was determined as in the legend to Fig. 1. Detectable levels of unbound apoE were in no case detectable in the normoglycemic control group (data not shown).

TABLE 4. Composition of the abnormal apoE lipoprotein of NIDDM plasma

Lipoprotein	Free Cholesterol	Phospholipid	Cholesteryl Ester	Triglyceride	Protein	ApoE/Protein
	<i>weight percent</i>					
Abnormal apoE lipoprotein	12.1 ± 0.6	23.6 ± 2.0	29.5 ± 0.6	19.0 ± 0.2	15.9 ± 0.7	0.46 ± 0.06
NIDDM LDL	10.8 ± 1.5	21.5 ± 3.7	35.0 ± 3.3	7.7 ± 4.9	25.0 ± 1.7	0.01 ± 0.00

From ref. 20. Values are means ± SD (n = 5).

Half inhibition of binding (of $2.5 \mu\text{g ml}^{-1}$ of unlabeled LDL) was obtained at a lower concentration of plasma from NIDDM patients than from normal subjects. Fig. 6 also indicates that when lipoproteins reacting with antibodies to apoA-I and apoB were removed from normal plasma, the final nonadsorbed fraction caused little displacement of unlabeled LDL. Since both apoB and apoE are reactive with this receptor (21), this result indicates that the whole of apoE in such plasma (bound to either apoA-I or apoB), as well as the whole of apoB, had been removed from the plasma under these conditions. This conclusion is in agreement with earlier data (2). On the other hand, when the same experiment was carried out with plasma from patients with NIDDM, substantial displacement of labeled LDL was obtained with the nonadsorbed fraction from immunoaffinity chromatography. These data indicate that the unbound fraction of apoE present in NIDDM plasma is reactive with the apoB,E receptor of normal fibroblasts.

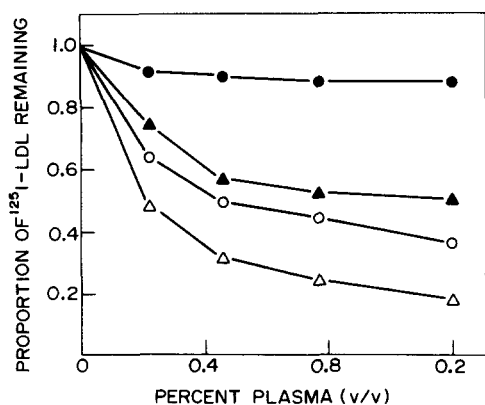


Fig. 6. Effects of immunoaffinity chromatography on the reactivity of plasma from NIDDM or control plasma with the apoB,E receptor of normal fibroblasts. Cells cultured as described under Methods were incubated with ^{125}I -labeled LDL ($2.5 \mu\text{g ml}^{-1}$ protein) and unmodified or fractionated plasma from NIDDM or control subjects. Open triangles, NIDDM plasma; open circles, NIDDM plasma from which apoA-I and apoB had been removed by affinity chromatography. Closed triangles, control plasma; closed circles, control plasma from which apoA-I and apoB had been removed by immunoaffinity chromatography. Values are expressed as the proportion of the heparin-displaceable ^{125}I -labeled LDL bound in the absence of added plasma, which remained in the presence of each reaction mixture. Nonspecific LDL binding (determined in the presence of a 50-fold excess of unlabeled LDL) was $> 2\%$ of labeled LDL binding in the absence of added plasma. Values represent the means of duplicate determinations at each experimental point.

Reactivity of unbound apoE with the macrophage beta-VLDL receptor

The ability of control plasma and plasma from patients with IDDM or NIDDM to stimulate cholesterol esterification in mouse peritoneal macrophages was compared (Fig 7). This assay estimates the rate of uptake of lipoprotein cholesterol by measuring stimulation of intracellular cholesterol esterification (22). Normal plasma showed relatively little ability to stimulate the esterification of cholesterol, and this was completely lost in normal plasma from which lipoproteins containing both apoA-I and apoB had been removed by affinity chromatography. On the other hand, NIDDM plasma promoted the synthesis of approximately ten times more cholesteryl ester under the same conditions. Furthermore, this effect was fully maintained in plasma from which apoA-I and apoB had been removed immunologically. These data indicate that the anomalous apoE of diabetic plasma is reactive with macrophages. Plasma from patients with IDDM stimulated much less cholesterol esterification than plasma from subjects with NIDDM, but the activity also persisted after apoA-I and apoB had been removed. This finding suggests that the plasma of IDDM patients contains the same abnormal lipoprotein as that of NIDDM patients, but at a significantly lower concentration.

Evidence for the role of apoE in the reaction of unbound apoE with cultured cells

When the unbound apoE fraction of NIDDM plasma was passed down an anti-apoE affinity column, the ability of such plasma to compete with the binding of ^{125}I -labeled LDL to fibroblasts was completely removed (Fig. 8A). When the same fraction was added to mouse macrophages, it was no longer active in the stimulation of cholesteryl ester synthesis in these cells (Fig. 8B). However, as shown in Fig. 8C, while diabetic plasma (even though containing no detectable beta-VLDL) did inhibit the binding of ^{125}I -labeled rabbit beta-VLDL to macrophages, this activity was not retained following anti-apoB chromatography. As a result, while the abnormal diabetic lipoprotein showed several of the properties of beta-VLDL, including its reactivity with both fibroblasts and macrophages and high content of free cholesterol and apoE, it differed from it in its flotation density and absence of reactivity with anti-apoB antibodies, or competition with rabbit beta-VLDL.

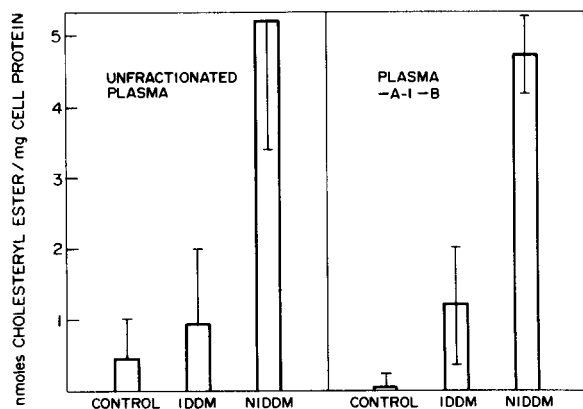


Fig. 7. Ability of diabetic or control plasma to stimulate cholesterol esterification in mouse peritoneal macrophages. Macrophages were incubated with unmodified plasma from control subjects or patients with IDDM or NIDDM, or the same plasma after sequential immunoaffinity chromatography on antibodies to apoA-I and apoB. Incubation was carried out in plasma (diluted sevenfold in Dulbecco's modified Eagle's medium) or with the nonadsorbed fraction of plasma at the same dilution, as described under Methods. Values shown represent the mass of radioactive cholesteryl ester from the cells after incubation for 5 hr at 37°C. The specific activity of tritiated oleic acid in the incubation medium was 3.7×10^6 dpm/ μ mol. Values represent means \pm 1 SD.

DISCUSSION

The net transport of free cholesterol from cell membranes to plasma of patients with NIDDM is reduced or reversed compared to that transported into plasma of normal individuals (1). A similar observation has been made in the plasma of other patients at increased risk of atherosclerotic vascular disease (13). The cause of the defect in cholesterol transport has not been further identified. In the present experiments, an average of one-fifth of total plasma apoE was in an abnormal form in patients with NIDDM, while a much lower concentration (on average about one-twentieth of total plasma apoE) was present in this form in the plasma of patients with IDDM who had a comparable hyperglycemia. Several pieces of evidence connect the presence of this lipoprotein with the abnormal transport of cholesterol in plasma from patients with diabetes.

Firstly, while all subjects with NIDDM in this study contained the abnormal fraction of apoE and showed the predicted inhibition of transport, patients with IDDM showed only low levels of this factor, and cholesterol transport rates in this group were only slightly reduced. Furthermore, the absolute level of this lipoprotein in both groups was inversely correlated with net transport rate, and the extrapolation of the relationship between these factors to zero indicated a rate of net transport comparable to that found in plasma from normoglycemic individuals when the abnormal lipoprotein was completely absent. These data suggest that a similar mechanism, different only in degree, inhibits cholesterol transport in plasma of patients with

either IDDM or NIDDM, and that the inhibitory effect mediated by the abnormal apoE lipoprotein present in diabetes is the major or only factor inhibiting normal cholesterol transport in the plasma of these patients. This concept is supported by a comparison of the different biochemical effects of this factor in NIDDM and IDDM plasma. Thus, relative to an uninhibited transport rate of $0.38 \mu\text{g hr}^{-1}$ in the control group, the NIDDM group (with a mean transport rate of $0.09 \mu\text{g hr}^{-1}$) is inhibited to a sixfold greater extent than the IDDM group (whose mean transport rate is $0.34 \mu\text{g hr}^{-1}$). This can be compared with the sixfold greater stimulation of cholesterol esterification in peritoneal macrophages by NIDDM compared to IDDM plasma. Finally, both the apoE lipoprotein and the biologically inhibitory activity bind to heparin-agarose under the same conditions and both are removed by immunoaffinity chromatography with antibodies against apoE. Taken together, these results support the concept that the abnormal fraction containing apoE from diabetics mediates the inhibition of cholesterol transport in these patients.

Although on the basis of its chemical composition the abnormal lipoprotein is of greater mean density than the beta-VLDL found in cholesterol-fed animals (3) and some human hypercholesterolemics (4, 5), it nevertheless shares with it several characteristic biochemical properties. For example, the lipoprotein in diabetic plasma displaced ^{125}I -labeled LDL from the apoB,E receptor of fibroblasts. In addition, it stimulated the esterification of cholesterol in mouse peritoneal macrophages. Finally, the lipid composition of the diabetic lipoprotein and, in particular, the presence of appreciable proportions of both cholesteryl ester and triglyceride resemble beta-VLDL. These findings suggest that diabetic plasma, although lacking classical beta-VLDL defined on a flotation basis, may still contain a comparable particle, which is unrecognized by the usual criteria because of its flotation within a higher density range. However, its lack of reactivity in the mouse macrophage system, in competition with rabbit beta-VLDL, indicates important structural differences. The sequential immunoaffinity chromatography method used here may be a convenient one for the isolation of abnormal lipoproteins from the plasma of other patient groups who have a similar abnormality of plasma cholesterol transport (13). While the nature of the ligand recognized on the abnormal diabetic lipoprotein (like that of classical beta-VLDL) is not yet understood (4, 5, 22), the diabetic lipoprotein, like beta-VLDL, has apoB-100 and apoE as its major proteins. Its lack of recognition by matrix-bound antibody to apoB-100 suggests that the conformation of the former in the native diabetic lipoprotein is highly unusual. This may be explained by other data that indicate that the expression of apoB antigenicity is highly dependent upon lipoprotein lipid composition (23, 24), which is also abnormal in this particle.

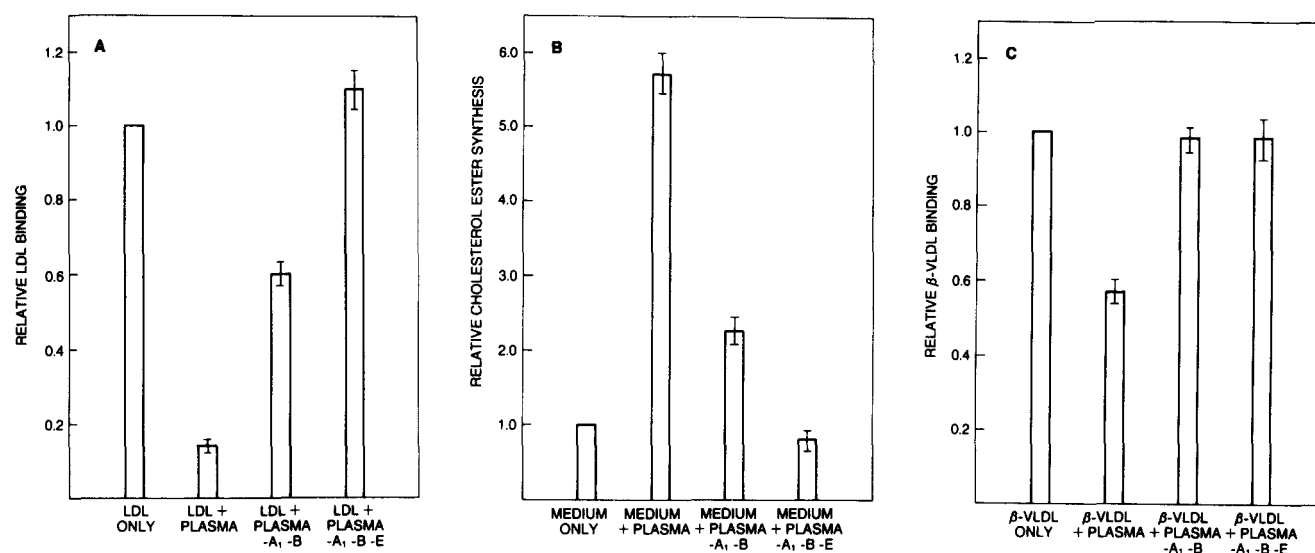


Fig. 8. Effects of removal of apoE by affinity chromatography on the reactivity of diabetic plasma with culture cells. **A.** Competition with ^{125}I -labeled LDL in fibroblasts. Cells cultured as described in the legend to Fig. 6 were incubated with ^{125}I -labeled LDL ($2\text{ }\mu\text{g/ml}$) and plasma or plasma fraction from affinity chromatography (all at a concentration equivalent to 1.2% v/v of original plasma). Original plasma, plasma -A₁-B₁ and plasma -A₁-B-E as compared. Values (relative to LDL binding in the observed added plasma) are means \pm 1 SD of quadruplicate plates. Nonspecific binding has been subtracted as described in legend to Fig. 6. **B.** Stimulation of cholesteryl ester synthesis in mouse macrophages. Plasma and its fractions were in as (A). Plasma (0.1 ml), or its equivalent from affinity chromatography, was added in a volume of 0.6 ml, and cholesteryl ester synthesis was determined as in the legend to Fig. 7. Values (relative to cholesteryl ester synthesis by unfractionated plasma) are given as means \pm 1 SD of triplicate plates. **C.** Displacement of ^{125}I -labeled rabbit beta-VLDL by diabetic plasma and its fractions from cultured macrophages. Beta-VLDL ($2\text{ }\mu\text{g/ml}$) was incubated at 4°C for 2 hr alone or together with plasma, plasma -A₁-B, or plasma -A₁-B-E, all at the equivalent of 0.5% v/v original plasma. Nonspecific binding ($< 5\%$ total binding) was determined with a 100-fold excess of unlabeled beta-VLDL and has been subtracted. Values shown (relative to ^{125}I -labeled beta-VLDL binding in the absence of added plasma or plasma fraction) are means \pm 1 SD of triplicate plates of cells.

The fact that beta-VLDL and the abnormal diabetic lipoprotein both stimulate sterol ester storage need not imply that the latter is acting through a receptor pathway in its effects on cholesterol net transport. The binding of beta-VLDL to its receptor is followed by the interiorization of the intact lipoprotein, its degradation in the lysosomes, and the esterification of most of the interiorized cholesterol within the cell. However, several pieces of evidence indicate that this is not the mechanism by means of which the diabetic lipoprotein inhibits cholesterol transport in plasma in the present studies. Firstly, normal fibroblasts lack the beta-VLDL receptor and the apoB,E receptor is expressed at comparatively low levels in fibroblasts cultured in intact serum medium, as were those in the present experiments. In addition, net transport was unchanged when fibroblasts genetically deficient in apoB,E receptors replaced normal fibroblasts in this assay (13). Secondly, uptake by fibroblasts of free cholesterol from the plasma of patients with NIDDM (1) was not accompanied by the uptake of detectable cholesteryl ester, which would have been interiorized along with free cholesterol by an endocytotic mechanism. Thirdly, selective removal of apoE from NIDDM plasma completely normalized net transport, in spite of the retention under these conditions of most of the apoB, also reactive with the apoB,E receptor. Finally, cholesterol net transport in relation to the concentration of the apoE lipoprotein of diabetic plasma showed no evidence of saturation at apoE levels as high as $20\text{ }\mu\text{g ml}^{-1}$, whereas the half-saturation

concentration for the interiorization of apoE through the apoB,E receptor is at least two orders of magnitude lower (20). In all these particulars, the reaction of the diabetic lipoprotein with fibroblasts has properties that are very different from those that have been clearly established for the apoB,E receptor present on the same cells. These data are more compatible with an alternative mechanism (25) in which the cholesterol of the diabetic lipoprotein transfers down its concentration gradient to the cell.

In summary, this study suggests that the reduced cholesterol transport out of cells to NIDDM plasma previously observed (1) is the result of an exaggerated influx of cholesterol from a novel lipoprotein particle rich in cholesterol and apoE in such plasma which stimulates cholesterol uptake and esterification.

Finally, this study shows significant quantitative differences in plasma cholesterol metabolism between patients with NIDDM and IDDM, even when the degree of hyperglycemia and hyperlipidemia is comparable. The level of the abnormal lipoprotein containing apoE, and its biological effects, are much less marked in the IDDM group. Indeed, in this study there was no overlap between the two groups. This study strongly suggest that, under equivalent conditions of hyperglycemia, subjects with NIDDM have a more clearly abnormal metabolism of cholesterol in plasma, and this includes the pathway by means of which cholesterol is transported between the plasma and cell membranes. ■

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