

# Roles of Apolipoproteins B and E in the Cellular Binding of Very Low Density Lipoproteins

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## Abstract

Apoproteins B and E both interact with cellular low density lipoprotein (LDL) apolipoprotein B and E (apo B,E)-receptors, and very low density lipoproteins (VLDL) contain both apo B and apo E. Our aim was to study the relative importance of apo B and apo E in the binding of VLDL subfractions to cells. Two monoclonal anti-LDL-apo B antibodies (464B1B3 and 464B1B6, 2a and 2b, respectively) and two anti-apo E antibodies (1506 A1.4 and 1907 F6.4) were used to inhibit lipoprotein-cell interactions.

In confirmation of previous findings, the binding and degradation of  $^{125}\text{I}$ -LDL by human fibroblasts were inhibited  $\sim 90\%$  by antibodies 2a or 2b or the antigen-binding fragments of 2a, whereas the cellular processing of  $^{125}\text{I}$ -VLDL<sub>3</sub> ( $S_r$  20–60),  $^{125}\text{I}$ -VLDL<sub>2</sub> ( $S_r$  60–120), and  $^{125}\text{I}$ -VLDL<sub>1</sub> ( $S_r$  >120) were inhibited by only  $\sim 50\%$ ,  $\sim 25\%$ , and  $<10\%$ , respectively. The VLDL<sub>1-3</sub> and LDL-dependent intracellular esterification of cholesterol with [ $^3\text{H}$ ]oleate were inhibited to a similar extent. Other monoclonal anti-human apo B antibodies inhibited lipoprotein-cell interactions much less effectively and nonimmune IgG isolated from mouse serum did not inhibit at all. 20-fold excesses of LDL produced about the same patterns of inhibition of degradation of  $^{125}\text{I}$ -VLDL<sub>1-3</sub> and LDL by cells as did antibodies 2a and 2b, whereas homologous unlabeled VLDL<sub>1-3</sub> in like amounts inhibited the matched  $^{125}\text{I}$ -VLDL subfraction more effectively. Two anti-apo E monoclonal antibodies and a polyclonal anti-apo E antibody inhibited cell-mediated degradation of and lipoprotein-dependent cholesterol esterification by VLDL<sub>1</sub> but not VLDL<sub>3</sub> or LDL. The results suggest that receptor recognition sites on apo E in preference to sites on apo B mediate the cellular binding of hypertriglyceridemic VLDL<sub>1</sub>. However, the proportion of particles bound via apo B seems to increase as VLDL decreases in size toward LDL, and virtually all of LDL binding is mediated by apo B.

## Introduction

Very low density lipoproteins (VLDL)<sup>1</sup> isolated from plasmas of patients with hypertriglyceridemia seem to be taken up by normal cultured human fibroblasts much more readily than

VLDL isolated from normal plasma (1, 2). Density gradient ultracentrifugation can be used to subfractionate VLDL (3, 4), and it seems that differences between the cell reactivities of normal and hypertriglyceridemic (HTG) VLDL are most readily apparent when the largest particles,  $S_r$  > 100, or VLDL<sub>1</sub> fractions are compared (5). The cellular uptake of the VLDL<sub>1</sub> fraction, which contains both apo B and apo E (6–8), is mediated by the same apo B,E receptor on fibroblasts (9–12) that recognizes low density lipoprotein (LDL) (13), which contains only apo B (13–15). Therefore, it was of great interest when Gianturco et al. (16) recently reported that the recognition of HTG-VLDL<sub>1</sub> by fibroblasts occurred via apo E rather than apo B. Gianturco et al. (16) reached this conclusion by showing that thrombin digestion of VLDL<sub>1</sub> abolished its cellular recognition. Concomitantly, VLDL<sub>1</sub>-apo E was cleaved into two major 22,000- and 12,000-mol wt peptides. Similar treatment of LDL had no effect on its recognition by cells. Furthermore, addition of intact apo E to thrombin-treated VLDL<sub>1</sub> restored its capacity to interact with cells, and addition of apo E to VLDL<sub>1</sub> isolated from normolipidemic subjects increased its recognition by cells.

We also have been interested in the interaction of apo B-containing lipoproteins with cultured cells (17–19), and have developed mouse monoclonal anti-human LDL antibodies as probes of lipoprotein-apo B structure and as inhibitors of lipoprotein-cell interactions. Two monoclonal IgG antibodies, 2a and 2b, have been reported with the following characteristics: a) both antibodies 2a and 2b and the antigen-binding fragment (Fab) of antibody 2a effectively block the apo B,E receptor-mediated binding and degradation of  $^{125}\text{I}$ -LDL in cultured normal human fibroblasts, while other anti-apo B antibodies do not inhibit cellular binding (18); b) chemical modification of the lysyl and arginyl residues of LDL destroys the recognition of LDL by antibodies 2a and 2b, and also by fibroblasts (18); c) limited proteolysis of LDL destroys the immunoreactivities of the LDL cores vis-a-vis several monoclonal antibodies, but the abilities of the LDL cores to bind to antibodies 2a and 2b and to fibroblast LDL receptors are preserved (19); and d) LDL of other species of animals bind to antibodies 2a and 2b, but not to other antibodies (20). From these findings, we conclude that antibodies 2a and 2b define epitopes on apo B that are closely related to the cell recognition domains on apo B. Apo E either in its delipidated form or reconstituted in dimyristoylphosphatidylcholine vesicles does not react with antibodies 2a and 2b (G. Schonfeld, unpublished observations). Anti-human apo E monoclonal antibodies have also been developed in this laboratory. Two of these antibodies, 1506 A1.4 and 1907 F6.4, were used in the present study in an attempt to block apo E-mediated cell binding.

In the experiments to be reported, the apo B and apo E antibodies were used to inhibit the cell recognition of VLDL subfractions and LDL, to assess the roles of apo B and apo E

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1. Abbreviations used in this paper: apo B, apolipoprotein B; apo E, apolipoprotein E; Fab, antigen-binding fragment; HTG, hypertriglyceridemic; TMU, tetramethylurea.

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in the binding of these lipoproteins to fibroblasts. We conclude that apo B plays a very small role compared with apo E in the cellular binding of the largest VLDL fractions, VLDL<sub>1</sub>, but that the binding of progressively increasing proportions of the smaller and more dense VLDL fractions is mediated by apo B and not apo E, and that nearly all of LDL is recognized via apo B.

## Methods

**Lipoprotein donors.** Lipoproteins of six subjects with hypertriglyceridemia and six normal controls were studied. Clinical characteristics are given in Table I. Lipoprotein lipid values are representative of those obtained for each subject at diagnosis. Patients were not taking medications and none had visible chylomicronemia.

**Isolation of lipoproteins.** Blood collected in 0.1% EDTA was obtained from subjects after 12–14 h of fasting. After separation of cells by centrifugation, 10  $\mu$ M phenylmethylsulfonyl fluoride, 50 mg/l chloramphenicol and 50 mg/l gentamycin were added to the plasma (16, 21). VLDL was immediately isolated by ultracentrifugation at  $d = 1.006$ . LDL was isolated between the densities of 1.019 and 1.050 (22). Two ultracentrifugations were carried out at each density. VLDL density subfractions were isolated from VLDL that had been centrifuged only once, by zonal ultracentrifugation using a linear density gradient of 1.00–1.15 g/ml (17, 23). The fastest floating fraction ( $S_{120-400}$ ) was designated fraction VLDL<sub>1</sub>; the  $S_{60-120}$  fraction, VLDL<sub>2</sub>; and  $S_{20-60}$  fraction, VLDL<sub>3</sub>. On occasion, VLDL fractions were obtained by ultracentrifugation in a salt gradient formed in an SW40 swinging bucket rotor (3). After dialysis against 0.15 M sodium chloride and 1 mM EDTA (pH 8.2), the lipoproteins were concentrated by dry analysis (Aquacide IIA; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA), dialyzed against EDTA-saline, filtered through 0.45- $\mu$ m filters (Acrodisc; Gelman Sciences, Inc., Ann Arbor, MI), and stored in EDTA-saline containing the above mentioned concentrations of antibacterial agents at 4°C.

The purities of the isolated VLDL and LDL preparations were assessed by 3–10% or 3–20% sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis of the individual samples (24). VLDL apo B/E ratios were calculated from these gels in some experiments. In LDL, three bands corresponding to apo B-100, B-74, and B-26 (8) were found by Coomassie Blue staining, but none of the LDL samples

contained detectable amounts of B-48, serum albumin, or non-apo B apolipoproteins (21). The HTG-VLDL preparations used in the studies reported here contained apo B-100, trace amounts of apo B-48 and B-26 (Fig. 1), apo E, and apolipoprotein C. The apo B contents of the VLDL subfractions and LDL were determined by tetramethylurea (TMU) precipitation (8). Apo E contents were quantitated by radioimmunoassay (RIA) after pretreatment of the VLDL with 6 M urea (25). Total protein contents were measured by modifications of the method of Lowry et al. (26, 27).

**Preparation of purified antibodies and Fab fragments.** Affinity purified human apo B monoclonal antibodies and Fab fragments were prepared as previously described (18). The purity of the Fab fragments was checked by sodium dodecyl sulfate gel electrophoresis in the presence of 1% 2-mercaptoethanol (100°C for 2 min), which yielded two bands of 26,500 and 25,000 apparent molecular weight. The anti-apo B antibodies used in the present study are 464B<sub>1</sub>B<sub>3</sub> (2a) and 464B<sub>1</sub>B<sub>6</sub> (2b), which inhibit binding of LDL to receptors and also define closely related but not identical epitopes 2a and 2b. Antibodies 457C<sub>4</sub>D<sub>1</sub> (1a), and 467D3D5 (6), which do not inhibit LDL binding and define two other spatially independent epitopes called 1a and 6, were also used as controls.

Monoclonal antibodies were raised against human apo E. The details of their preparation are described elsewhere (Krul, E. S., M. J. Tikkanen, and G. Schonfeld, manuscript in preparation). The anti-apo E monoclonal IgG fraction of ascitic fluid was purified by chromatography on either DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) (28) or Protein-A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) (29). The apo E antibodies used in the present study are 1506 A1.4, 1907 F6.4, and 1363 C3A10, and are all of the IgG<sub>1</sub> subclass. Six IgM antibodies were also used (827, 1365, and 1366 series).

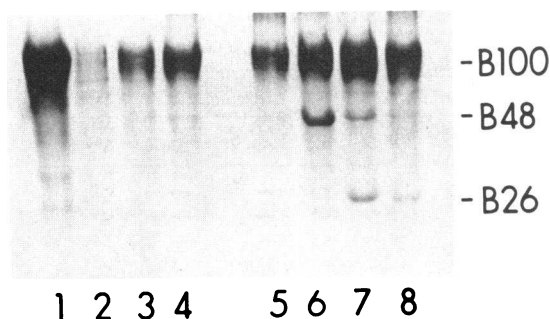
Polyclonal anti-human apo E antiserum (R224-3) was produced in rabbits (25) and the IgG was purified by ion exchange chromatography on cellulose (DE52; Whatman Chemical Separation, Inc., Clifton, NJ). Nonimmune immunoglobulins were isolated from nonimmunized mouse serum by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation (20–40% of saturation), followed by chromatography on Protein-A Sepharose (29).

**<sup>125</sup>I-labeling of lipoproteins and antibodies.** VLDL fractions and LDL used in the cell assays were <sup>125</sup>I-labeled to a specific activity of ~100 cpm/ng using the iodine monochloride method (30, 31). >90% of the <sup>125</sup>I-lipoproteins were precipitable by 10% TCA. The lipid solvent (chloroform/methanol, 2:1 vol/vol) extractable counts for <sup>125</sup>I-VLDL were 9–17%, and for <sup>125</sup>I-LDL, were 7–9%.

Table I. Lipoprotein Donors

Subject	Age	Sex (M/F)	Height	Wt	Lipoprotein lipids				
					TG	TC	VLDL-C	LDL-C	HDL-C
	yr		cm	kg	mg/dl				
1	54	M	188	128	1,207	348	288	75	28
2	41	F	166	66	1,413	280	190	32	16
3	54	F	164	49	3,648	729	672	49	24
4	62	F	166	65	694	319	199	83	37
5	29	M	173	69	754	220	119	91	25
6	44	M	182	82	475	239	85	123	32
7	44	F	178	61	143	239	29	125	85
8	28	F	165	52	90	176	18	95	63
9	26	F	170	61	76	181	15	111	55
10	35	M	170	69	75	219	19	158	42
11	34	M	173	66	105	168	21	109	38
12	38	M	168	71	232	190	46	91	53

TG, triglycerides; TC, total cholesterol; VLDL-C, LDL-C, and HDL-C, cholesterol in respective lipoproteins.



**Figure 1.** Apo B of VLDL preparations analyzed by 3–10% polyacrylamide gradient gel electrophoresis and stained by Coomassie Blue. Lanes from left to right are: 1, LDL; 2, VLDL<sub>1</sub>; 3, VLDL<sub>2</sub>; and 4, VLDL<sub>3</sub> of HTG subject 5; 5, VLDL ( $d < 1.006$ ) of control subject 10; 6 and 7, VLDLs of two subjects with type III ( $E_2/E_2$ ) hyperlipoproteinemia (shown for comparison); 8, VLDL ( $d < 1.006$ ) of HTG subject 4.

**Human fibroblast cultures.** Monolayer cultures of normal human skin fibroblasts were grown and maintained as previously described (32, 33).  $\sim 7.5\text{--}10.0 \times 10^4$  cells were seeded into  $35 \times 15\text{-mm}$  dishes (Costar, Cambridge, MA) with Eagle's Modified Essential Medium (15% newborn calf serum). After 5 d, the cells were washed with saline and the medium was replaced with medium containing 10% lipoprotein-deficient human serum for 48 h.

**Binding and degradation studies.** Fibroblasts were grown in dishes as described above and experiments begun after growth for 48 h in medium containing lipoprotein-deficient serum. Experiments with antibodies were conducted in two ways. In some experiments, the medium was removed from each cell dish and replaced with medium containing the indicated amounts of purified antibodies, followed 5 min later by  $^{125}\text{I}$ -labeled lipoproteins (VLDL<sub>1</sub>, VLDL<sub>2</sub>, VLDL<sub>3</sub>, or LDL) (18). Alternatively, lipoproteins were incubated with the indicated concentrations of purified antibodies for 30 min at  $37^\circ\text{C}$  before being added to the cells. Incubations of lipoproteins with cells were carried out at  $37^\circ\text{C}$  for 4 h in duplicate or triplicate dishes. The proteolytic degradation of  $^{125}\text{I}$ -lipoproteins by the fibroblasts was determined by measuring the TCA-soluble material in the spent medium after removal of free iodide with chloroform (32, 33). At the end of the incubations, the cells were washed as described (33), dissolved in 0.1 M sodium hydroxide, and aliquots were taken for determination of cell protein and cell-associated radioactivity (binding). Nonspecific degradation in the absence of cells was determined in no-cell control dishes that had been precoated with the incubation medium. Binding and degradation were also determined in the presence of 20-fold excesses of nonlabeled homologous lipoproteins. 20-fold of nonlabeled LDL also were added together with the different  $^{125}\text{I}$ -labeled lipoprotein fractions in some experiments. Results are expressed as nanograms of  $^{125}\text{I}$ -labeled lipoprotein bound or degraded per milligram of cell protein. In one experiment, various nonlabeled lipoproteins were assessed for their abilities to compete with  $^{125}\text{I}$ -labeled LDL for binding to the fibroblasts at  $4^\circ\text{C}$ . Experiments were essentially carried out as described above with the medium containing 25 mM Hepes as buffering agent. Increasing concentrations of nonlabeled lipoproteins were added to the cells followed 5 min later by  $^{125}\text{I}$ -labeled lipoproteins.

**Incorporation of [ $^3\text{H}$ ]oleate into cellular cholesteryl oleate.** Indicated concentrations of lipoproteins were incubated with cultured fibroblasts for 5 h before the addition of [ $^3\text{H}$ ]oleic acid (0.14 mM, 20–40 cpm/pmol) bound to defatted bovine serum albumin (BSA). After an additional 18 h, the cells were washed three times with saline, scraped off the dishes, and aliquots were taken for protein determination. The concentration of intracellular cholesteryl [ $^3\text{H}$ ]oleate was determined by thin layer chromatography as described (34, 35).

**Chromatography of VLDL on monoclonal antibody affinity column.**

Monoclonal antibodies were coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the directions of the manufacturer. Specifically, IgG from 2.5 ml of ascites fluid (antibody 2b) was precipitated with 50%  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was dissolved in protein coupling buffer (0.1 M  $\text{NaHCO}_3$ , 0.5 M NaCl, pH 8.3) and incubated with  $\sim 3.5$  ml of CNBr-activated Sepharose gel for 2 h at room temperature with gentle shaking. Remaining active groups on the gel were blocked with 1 M ethanolamine, pH 8.0, for 2 h at room temperature. The gel was washed with 0.1 M sodium acetate buffer, pH 4.0, and coupling buffer alternatively to remove excess absorbed protein. The gel was finally washed with 0.01 M phosphate-buffered saline (PBS), pH 7.2, buffer and stored in the same buffer containing antibiotics at  $4^\circ\text{C}$ .

Radioiodinated lipoproteins ( $2.5 \times 10^6$  cpm, 20–50  $\mu\text{g}$  total protein) were loaded onto the column in 3% BSA-PBS, pH 7.2, and left at  $4^\circ\text{C}$  overnight. The column was then eluted and washed with several gel bed volumes of PBS. 2-ml fractions were collected. Specifically bound proteins were eluted with 6 M thiocyanate, 10 mM Tris, pH 7.2, followed by regeneration of the column with extensive washings with PBS. Aliquots of column fractions were counted for  $^{125}\text{I}$ -radioactivity.

## Results

**Stimulation of cholesterol esterification by various VLDL preparations.** Initially, the abilities of various VLDL ( $d < 1.006$ ) preparations isolated from normolipidemic and hyperlipidemic subjects to esterify cholesterol with [ $^3\text{H}$ ]oleate were examined. At equivalent VLDL protein concentrations (25  $\mu\text{g}/\text{ml}$ ) in the medium, HTG-VLDL stimulated esterification more effectively than normal VLDL. The values were  $2.03 \pm 0.41$  and  $0.50 \pm 0.12$  nmol cholesteryl ester formed per milligram cell protein per 18 h for HTG-VLDL (subjects 1–4) and normal VLDL (subjects 7–12), respectively ( $P < 0.001$ ). HTG-VLDL also contained more cholesterol per mass of protein (cholesterol/protein ratios were  $1.63 \pm 0.25$  vs.  $0.88 \pm 0.34$  for HTG and normal VLDL, respectively ( $P < 0.01$ ). A positive correlation ( $r = 0.81$ ) was obtained between nanomoles of cholesterol esterified and the lipoprotein cholesterol/protein ratio for the four HTG and five normolipidemic VLDLs added at equivalent protein doses. In one experiment, the apo B/apo E ratios were determined by scanning densitometry after electrophoresis of the HTG and normal VLDL protein on 3–20% polyacrylamide gels. The HTG-VLDL contained proportionately more apo E than normal VLDL, and apo B/apo E ratios were  $2.4 \pm 1.5$  vs.  $5.0 \pm 1.2$ , respectively,  $P < 0.01$ . Interestingly, the cholesterol esterification rate was inversely correlated with the VLDL apo B/apo E ratio ( $r = -0.77$ ,  $n = 8$ ). These results are compatible with those reported by Gianturco et al. (1) and suggest that HTG-VLDL in general are more potent in stimulating cholesterol esterification than are normal VLDL, and that apo E may be important in mediating the interaction of lipoproteins with cells. However, these experiments did not explain whether differences between HTG-VLDL and normal VLDL may be due to differences in the size and/or density distributions of VLDL subpopulations. Therefore, VLDL subfractions were examined.

**Inhibition by anti-apo B antibodies and Fab fragments of the interaction of VLDL subfractions and LDL with cultured human fibroblasts.** To ascertain the antibody dosage required to obtain maximum inhibition, increasing amounts of antibody 2b were added to dishes containing 25  $\mu\text{g}/\text{ml}$  of each VLDL subfraction and of LDL (Fig. 2). Maximum inhibition was achieved at 10  $\mu\text{g}/\text{ml}$  of antibody for all VLDL and LDL

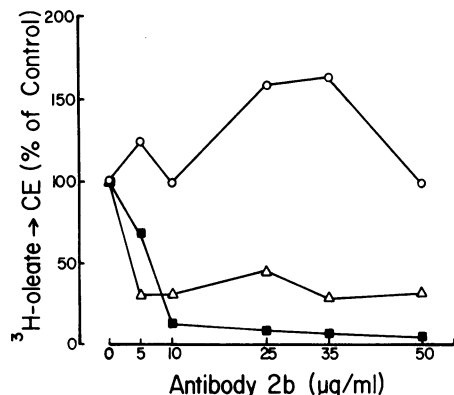


Figure 2. Inhibition by monoclonal anti-apo B antibody 2b of lipoprotein-dependent esterification of cholesterol in cultured human fibroblasts. Lipoproteins were isolated from the plasma of HTG subject 5 and VLDL subfractions were prepared by zonal ultracentrifugation. Increasing doses of antibody 2b were incubated with 25 µg/ml of lipoprotein for 30 min at 37°C before being added to the cells. The basal rate of cholesterol esterification (CE) for the fibroblasts was  $0.27 \pm 0.05$  nmol CE/mg cell protein/18 h. Net uninhibited esterification rates for VLDL<sub>1</sub>, VLDL<sub>3</sub>, and LDL were 1.32, 1.56, and 10.8 nmol CE/mg cell protein/18 h, respectively. Mean coefficient of variation was 12% for each point in duplicate dishes. —○—, VLDL<sub>1</sub>; —△—, VLDL<sub>3</sub>; —■—, LDL.

fractions. Antibody 2b inhibited LDL-stimulated cholesterol esterification by ~90% (18). VLDL<sub>1</sub>-stimulated esterification was not inhibited, while stimulation by VLDL<sub>3</sub> was inhibited by 70%. In agreement with the above, when in other experiments 10–25 µg/ml of lipoprotein proteins were incubated with 25 and 50 µg/ml of antibodies 2a, 2b, or Fab fragments of 2a (to ensure saturation of antibody binding sites on the lipoprotein particles), the binding and degradation by normal human fibroblasts of <sup>125</sup>I-VLDL<sub>1</sub>, <sup>125</sup>I-VLDL<sub>2</sub>, <sup>125</sup>I-VLDL<sub>3</sub>, and <sup>125</sup>I-LDL were inhibited least for <sup>125</sup>I-VLDL<sub>1</sub> and most for <sup>125</sup>I-LDL (Table II and Fig. 3). The same antibodies and Fab fragments also inhibited cholesterol esterification dependent on VLDL<sub>3</sub> by 35–73%, on VLDL<sub>2</sub> by <5–32%, and on VLDL<sub>1</sub> by <5–12% (Table II). The action of whole VLDL was inhibited by 33%. Preincubations of antibody with lipoproteins for 30 min yielded the same results as when no premixing or 5 min of premixing were employed. These results suggest that the lipoproteins were saturated with antibodies or Fab under the experimental conditions employed, i.e., that any lack of antibody-induced inhibition of lipoprotein-cell interactions was not due to lack of opportunity for maximal antibody-lipoprotein interaction. Antibodies 1a and 6, which were previously shown (18) to be much less effective in inhibiting the cellular processing of <sup>125</sup>I-LDL, were much less effective inhibitors here, too, with all lipoprotein fractions. The Ig fraction of nonimmune mouse serum did not inhibit at all.

Incubations of fibroblasts in the presence of normolipidemic lipoproteins and antibodies were also carried out (Fig. 4 A–C). Stimulation of cholesterol esterification by these VLDL subfractions was minimal (over the basal rates), suggesting that differences in size and/or density distributions between HTG-VLDL and normal VLDL did not account for the differences in their interactions with cells. However, VLDL<sub>3</sub> tended to stimulate better than VLDL<sub>1</sub> or VLDL<sub>2</sub>. This may be due to the presence of IDL in VLDL<sub>3</sub> as shown by Gianturco et al.

Table II. Inhibition by Antibodies of Lipoprotein Dependent Cholesterol Esterification

Added lipoprotein	Experiment 1			Experiment 2		Experiment 3
	Aby 2b*	Fab 2a	Irr†	Aby 2b	Aby 6	Aby 2a
VLDL <sub>1</sub>	<5	12	<5	—	—	<5
VLDL <sub>2</sub>	<5	<5	<5	—	—	<5
VLDL <sub>3</sub>	73	54	<5	—	—	62
Whole VLDL	—	—	—	33	3	—
LDL	86	92	<5	82	26	95

Basal esterification (<sup>3</sup>H]oleate → CE) in the absence of lipoproteins was  $0.45 \pm 0.05$  (Experiment 1,  $n = 5$ );  $1.00 \pm 0.18$  (Experiment 2,  $n = 4$ ); and  $0.27 \pm 0.05$  (Experiment 3,  $n = 12$ ) nmol/mg protein/18 h (means ± SD). Control rates in the presence of 25 µg/ml VLDL<sub>1</sub>, VLDL<sub>2</sub>, VLDL<sub>3</sub>, or LDL (subject 5), respectively, were 1.24, 1.08, 1.39, and 10.3 (Experiment 1), and 1.31, 1.05, 1.55, and 10.8 (Experiment 3). Rates in the presence of 25 µg/ml whole VLDL or LDL were 2.1 and 5.5 for Experiment 2. Basal rates have been subtracted from control rates to obtain uninhibited rates. Results are given as mean percent reduction of uninhibited rates produced by 25 and 50 µg of antibody 2b or 8.3 µg and 16.7 µg/ml Fab of antibody 2a. The two doses each gave similar degrees of inhibition. In Experiment 1, antibodies 2b, Fab 2a, or Irr were added to cells 5 min before addition of lipoproteins. In Experiments 2 and 3, antibodies 2b, 6, and 2a were preincubated with lipoproteins for 30 min at 37°C before the mixture was added to the cell dishes. Each point was performed in duplicate dishes. Coefficients of variation were <12%.

\* Aby, antibody.

† Irr, a nonimmune mouse IgG fraction purified on Staphylococcal Protein-A Sepharose CL4B, added at equivalent concentrations.

(1). Inhibiting effects of antibodies on the lipoprotein-dependent cholesterol esterification were difficult to discern in view of the low rates of stimulated esterification. However, where stimulation of esterification was appreciable (Fig. 4 B), antibody 2b demonstrated inhibition similar to that observed for HTG-VLDL<sub>3</sub>.

*Inhibition by homologous VLDL subfractions and by LDL of the cellular interactions of <sup>125</sup>I-VLDL subfractions and of <sup>125</sup>I-LDL.* Each VLDL subfraction was able to inhibit the cellular degradation of its labeled homologue (Table III). Inhibition in the presence of 20-fold excesses of nonlabeled VLDL subfraction ranged from 40 to 64% (Table III). The relatively poorer ability of unlabeled VLDL<sub>1</sub> to inhibit <sup>125</sup>I-

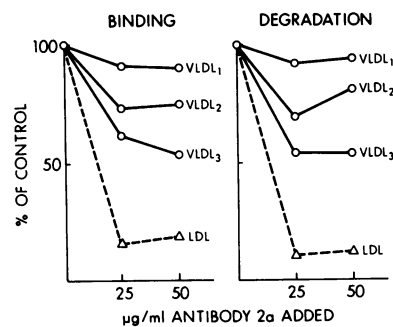


Figure 3. Inhibition of binding and degradation of <sup>125</sup>I-labeled HTG-VLDL<sub>1</sub>, VLDL<sub>2</sub>, and VLDL<sub>3</sub> (each at 10 µg/ml medium) and of <sup>125</sup>I-LDL (5 µg/ml) by antibody 2a in cultures of human fibroblasts. Lipoproteins were isolated from the plasma of subject 6 and VLDL subfractions were prepared by zonal ultracentrifugation. Respective 100% values (nanograms per milligram of cell protein) for binding were 121, 122, 215, and 187, and for degradation were 525, 224, 488, and 631 for VLDL<sub>1-3</sub> and LDL, respectively. Mean coefficient of variation was <8% for duplicate dishes.

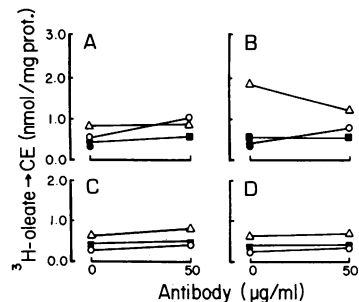


Figure 4. Effect of addition of monoclonal anti-apo B and anti-apo E antibodies on normolipidemic lipoprotein-dependent esterification of cholesterol in cultured human fibroblasts. VLDL subfractions were isolated from the plasma of normolipidemic subjects 8 (A), 10 (B), and 7 (C and D) by density gradient ultracentrifugation in an SW40 rotor as described in Methods. Monoclonal antibodies (50  $\mu\text{g/ml}$ ) were incubated with 25  $\mu\text{g/ml}$  of lipoprotein for 30 min at 37°C before being added to the cells. Apo B antibody 2b was used in the experiments depicted in A–C, while apo E antibody 1363C3A10 was used in D. The basal rate of cholesterol esterification for the fibroblasts ( $0.31 \pm 0.05$ ,  $n = 5$ ) (nmol CE/mg cell protein/18 h) is indicated by the solid circles. Mean coefficient of variation for points determined in duplicate dishes was 18%. —○—, VLDL<sub>1</sub>; —■—, VLDL<sub>2</sub>; —△—, VLDL<sub>3</sub>; —●—, basal.

tracentrifugation in an SW40 rotor as described in Methods. Monoclonal antibodies (50  $\mu\text{g/ml}$ ) were incubated with 25  $\mu\text{g/ml}$  of lipoprotein for 30 min at 37°C before being added to the cells. Apo B antibody 2b was used in the experiments depicted in A–C, while apo E antibody 1363C3A10 was used in D. The basal rate of cholesterol esterification for the fibroblasts ( $0.31 \pm 0.05$ ,  $n = 5$ ) (nmol CE/mg cell protein/18 h) is indicated by the solid circles. Mean coefficient of variation for points determined in duplicate dishes was 18%. —○—, VLDL<sub>1</sub>; —■—, VLDL<sub>2</sub>; —△—, VLDL<sub>3</sub>; —●—, basal.

VLDL<sub>1</sub> degradation is probably due to an exchange of radio-labeled apoproteins between the tracer and cold VLDL<sub>1</sub> (16). As the percentage of TMU-soluble apoproteins decreases, i.e., VLDL<sub>2</sub> → VLDL<sub>3</sub> (22), the potential for exchange would diminish and could account for the increasing inhibiting effect of unlabeled VLDL<sub>2</sub> and VLDL<sub>3</sub> on tracer degradation.

In contrast with unlabeled VLDL used as inhibitors, 20-fold excess of unlabeled LDL inhibited  $^{125}\text{I}$ -VLDL by <5%,  $^{125}\text{I}$ -VLDL<sub>2</sub> by only 9–27%, and  $^{125}\text{I}$ -VLDL<sub>3</sub> by only 24–47%, while LDL inhibited the degradation of  $^{125}\text{I}$ -LDL by ~90%. Thus, the patterns of inhibition produced by antibodies 2a, 2b, and Fab fragments were similar to the patterns produced by excess unlabeled LDL.

To document that the VLDL cell interactions were occurring at the cellular LDL (apo B,E)-receptor,  $^{125}\text{I}$ -LDLs were incubated at 4°C in the presence of competing nonlabeled LDL and VLDL (Table IV). VLDL and LDL were equally

Table IV. Competition for Binding of  $^{125}\text{I}$ -LDL to Human Fibroblasts

Lipoprotein competitor	Cell associated	
$\mu\text{g/ml}$	ng/mg cell protein	% of control
0	129	100
FH-LDL		
5	95	74
10	71	55
25	66	51
100	40	31
200	24	19
Subject 4-VLDL		
5	103	80
25	64	49
100	41	31

$^{125}\text{I}$ -LDL (FH) at 5  $\mu\text{g/ml}$  was added to all dishes along with the indicated concentrations of competitor lipoproteins. Incubations were for 2 h at 4°C and cell associated counts were determined. FH is a subject with homozygous familial hypercholesterolemia; 4 is an HTG subject (see Table I). VLDL ( $d < 1.006$ ) from subject 4 was obtained by ultracentrifugation and washed by a second ultracentrifugation at  $d 1.006$ . Results are means of triplicate dishes with coefficients of variation <8%.

potent, on a protein mass basis, in competing against  $^{125}\text{I}$ -LDL for occupancy of the LDL receptor, suggesting that the VLDL-cell interactions indeed were occurring at that receptor.

**Immunoaffinity chromatography.** In previous work using monoclonal antibodies 2a or 2b, the relative potencies of VLDL subfractions and LDL in competing against  $^{125}\text{I}$ -LDL for antibody binding was assessed in solid-phase plate assays (36). Competitive abilities of VLDL were inversely related to their sizes, i.e., LDL > VLDL<sub>3</sub> > VLDL<sub>2</sub> > VLDL<sub>1</sub>. To confirm the results obtained with VLDL subfractions in the competitive RIAs, the VLDL subfractions and LDL of donor

Table III. Inhibition by Antibodies of  $^{125}\text{I}$ -Lipoprotein Degradation by Cultured Fibroblasts

$^{125}\text{I}$ -tracers	Subject 6			Subject 5					
	Aby 2a†	Aby 1a	LDL	Aby 2a	Fab 2a*	Aby 1a	Irr§	LDL	VLDL
VLDL <sub>1</sub>	7	18	<5	11	<5	<5	<5	<5	40±8
VLDL <sub>2</sub>	25	22	9	32	28	20	<5	27	50±19
VLDL <sub>3</sub>	46	21	47	35	52	46	<5	24	64±12
LDL	89	<5	93	88	92	38	<5	78	—

Cells were grown in Eagle's minimal essential medium (MEM)-10% LPDS for 48 h before experiments. To MEM-10% LPDS were added 10  $\mu\text{g/ml}$  of the  $^{125}\text{I}$ -VLDL fractions or 5  $\mu\text{g/ml}$   $^{125}\text{I}$ -LDL (see tracers) with the indicated inhibitors. Antibodies 2a, 1a, or Irr (25 or 50  $\mu\text{g/ml}$ ) were added to cells 5 min before addition of lipoproteins. To test effects of longer exposure of lipoproteins to antibody, Fab 2a and lipoproteins were mixed in a test tube in proportions as outlined and incubated at 37°C for 30 min. The mixtures were then added to cell cultures. This experiment is indicated by an asterisk. Homologous VLDL subfractions (i.e.,  $^{125}\text{I}$ -VLDL<sub>1</sub> vs. VLDL<sub>1</sub>;  $^{125}\text{I}$ -VLDL<sub>2</sub> vs. VLDL<sub>2</sub>, etc.) or LDL (each in 20-fold excess of tracer) were added to test their effectiveness in inhibiting tracer uptake. Incubations with cells were for 4 h at 37°C for all ligands and inhibitors tested. Results are percent of inhibition produced by the various inhibitors. In the case of inhibition by homologous VLDL subfractions, percent±SEMs are given for 4 separate experiments with VLDLs from subjects 4 ( $n = 2$ ), 5, and 6. Each point was performed in duplicate dishes with an average coefficient of variation of <8%. 100% values for subject 6 were 525, 224, 448, and 631 ng/mg cell protein for VLDL<sub>1</sub>, VLDL<sub>2</sub>, VLDL<sub>3</sub>, and LDL, respectively. For subject 5, the values were 1,173, 756, 1,595, and 1,393 ng/mg cell protein. Nonspecific degradation has been subtracted from all values. † Aby, antibody. § Irr, a nonimmune mouse IgG fraction isolated from mouse serum on Staphylococcal Protein A Sepharose CL4B, added at equivalent doses.

Table V. Binding of  $^{125}\text{I}$ -Lipoproteins to an Anti-LDL Immunoaffinity Column

$^{125}\text{I}$ -ligands	Counts bound to column	Counts precipitable by TMU
	%	%
VLDL ( $d < 1.006$ )	34	50
VLDL <sub>1</sub>	23	37
VLDL <sub>2</sub>	42	49
VLDL <sub>3</sub>	53	69
LDL	96	97
HDL	<0.5	<0.1

$\sim 2.5 \times 10^6$  of  $^{125}\text{I}$ -labeled lipoprotein (10–25  $\mu\text{g}$  protein) of donor 5 were loaded in 3% BSA-PBS buffer, pH 7.4, onto an immunoaffinity column containing monoclonal anti LDL antibody 2b coupled to Sepharose 4B.

5 were iodinated and subjected to affinity chromatography on a column containing antibody 2b (Table V). 23% of VLDL<sub>1</sub>, 53% of VLDL<sub>3</sub>, and 34% of the whole VLDL ( $d < 1.006$ ) were bound. In control experiments, iodinated HDL was passed through the same column, and the  $^{125}\text{I}$ -VLDL fractions and  $^{125}\text{I}$ -LDL were also passed through a column containing no antibodies. Virtually all of  $^{125}\text{I}$ -LDL, but none of  $^{125}\text{I}$ -HDL, were bound to the antibody-containing column. <1.0% of any of the lipoproteins was retained on the control column. To ascertain that there was no selective retention of apo B on the column, the proportion of TMU-precipitable counts in the starting whole  $^{125}\text{I}$ -VLDL and in nonbound  $^{125}\text{I}$ -VLDL fractions were compared. They were found to be indistinguishable (50 and 53%, respectively), suggesting that the apo B and non-apo B proteins of VLDL were not dissociated on the column. The retention of VLDL fractions by the column was similar to the rank order of competition potencies of the fractions in the competitive immunoassays. Also, the inhibition by antibody 2b of VLDL-dependent cholesterol esterification was compatible with the affinity column data (Table II). The data strongly

suggest that the expression of the 2a and 2b epitopes on VLDL is inversely related to VLDL size.

**Inhibition by anti-apo E antibodies of the interaction of VLDL and LDL fractions with cultured human fibroblasts.** Since a significant proportion of HTG-VLDL<sub>1</sub> and VLDL<sub>2</sub> cellular uptake could not be inhibited by anti-apo B antibodies 2a or 2b, it followed that other apolipoproteins on the surface of these lipoproteins could be responsible for binding to cells. Therefore, antibodies directed against apo E were tested for their abilities to block VLDL-cell interactions (Figs. 5 and 6). Polyclonal anti-apo E antiserum (R224-3) inhibited binding (not shown) and degradation of VLDL<sub>1</sub> by  $\sim 25\%$  (Fig. 6), and VLDL<sub>1</sub>-dependent cholesterol esterification by  $\sim 45\%$  (Fig. 5). On the other hand, VLDL<sub>3</sub> was poorly inhibited if at all under the same conditions. Two anti-apo E monoclonal antibodies were even more effective inhibitors of VLDL<sub>1</sub>-cell interactions (Figs. 5 and 6, B and C). These antibodies did not inhibit VLDL<sub>3</sub>. Differences in apo E content of the VLDL fractions cannot account for the difference in antibody inhibition (Fig. 6), since VLDL<sub>3</sub> generally has less apo E than VLDL<sub>1</sub> and therefore antibody was clearly in excess. A third monoclonal anti-apo E antibody, 1363 C3A10, did not prevent cellular uptake and processing of either normolipidemic (Fig. 4 D) or HTG-VLDL subfractions (Figs. 5 D and 6 D). Six other monoclonal IgM anti-apo E antibodies also were equally ineffective in inhibiting VLDL uptake (not shown).

Interestingly, in several cases the anti-apo E antibodies seemed to enhance the cellular processing of the HTG-VLDL<sub>3</sub> (Figs. 5 and 6). This effect may be due to a conformational change in the lipoprotein structure induced by antibody-binding to a determinant distant from the cellular recognition site (cooperativity) such that cellular uptake is enhanced. Another explanation of the enhanced uptake is the possible interaction of the antibody-VLDL<sub>3</sub> immune complex with a purported Fc receptor on human fibroblast (37). As the Fc receptor binds aggregated IgG or immune complexes preferentially over monomeric IgG, this may explain the increased stimulation of VLDL<sub>3</sub> uptake at higher antibody/antigen ratios (Figs. 5 and 6).

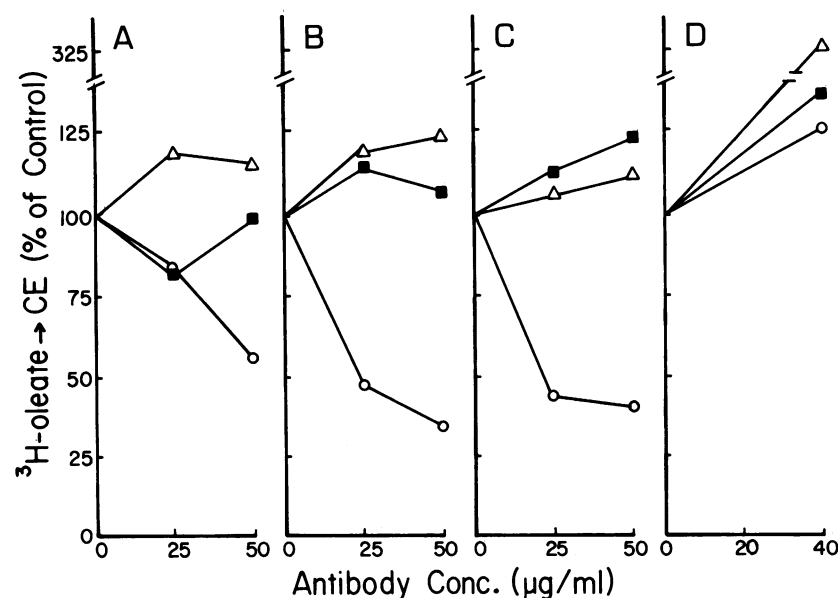
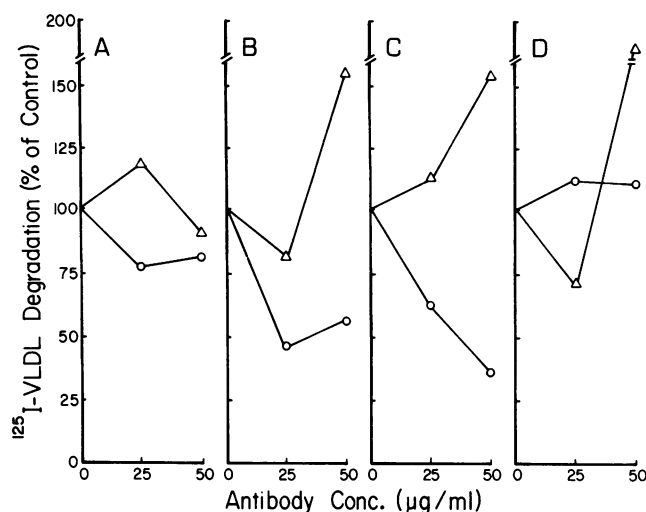


Figure 5. Inhibition by polyclonal or monoclonal anti-apo E antibodies of the HTG lipoprotein-dependent esterification of cholesterol in cultured human fibroblasts. Lipoproteins were isolated from HTG subject 5 (A–C) or subject 4 (D). Indicated doses of antibodies were incubated with 25  $\mu\text{g}/\text{ml}$  (A–C) or 20  $\mu\text{g}/\text{ml}$  lipoprotein (D) for 30 min at  $37^\circ\text{C}$  before being added to the cells. Basal rates of cholesterol esterification of  $0.48 \pm 0.09$  ( $n = 3$ , A–C) and  $0.31 \pm 0.05$  ( $n = 5$ , D) nmol CE/mg cell protein/18 h, respectively, have been subtracted to obtain net esterification rates in the presence of lipoproteins. In panels A–C, net rates for VLDL<sub>1</sub>, VLDL<sub>3</sub>, and LDL were 1.12, 1.02, and 17.1. Respective net rates in D were 0.27, 1.18, and 7.88 nmol CE/mg cell protein/18 h. The following antibodies were used: A, polyclonal R224-3; B, monoclonal 1506 A1.4; C, monoclonal 1907 F6.4; and D, monoclonal 1363 C3A10. Mean coefficients of variation were 7% (A–C) and 18% (D) for points determined in duplicate dishes.  $\circ$ —, VLDL<sub>1</sub>;  $\triangle$ —, VLDL<sub>3</sub>;  $\blacksquare$ —, LDL.



**Figure 6.** Inhibition by polyclonal or monoclonal anti-apo E antibodies of the degradation of  $^{125}\text{I}$ -lipoproteins by cultured human fibroblasts. VLDL subfractions were isolated from HTG subjects 5 (A–C) or 4 (D) and iodinated as described in Methods. Indicated doses of antibodies were incubated with 25  $\mu\text{g}/\text{ml}$  of  $^{125}\text{I}$ -VLDL<sub>1</sub> or  $^{125}\text{I}$ -VLDL<sub>3</sub> for 30 min at 37°C before being added to the cells. 100% values (nanograms per milligram of cell protein, 4 h) for degradation of VLDL<sub>1</sub> and VLDL<sub>3</sub> were 186 and 99 for A–C and 745 and 68 for D. Mean coefficients of variation were 14% (A–C) and 8% (B) for points determined in duplicate dishes. The antibodies used are described in the legend to Fig. 5. VLDL-apo E contents were determined by conventional RIA. For VLDL<sub>1</sub> and VLDL<sub>3</sub> in A–C, apo E was 3.2% and 1.2% of total VLDL protein. In D the values were 8.9% and 2.7%. —○—, VLDL<sub>1</sub>; —△—, VLDL<sub>3</sub>.

## Discussion

Triglyceride-rich lipoproteins isolated from plasma are bound, internalized, and degraded by cultured fibroblasts and aortic smooth muscle cells via the LDL (apo B,E) receptor in a metabolic sequence that resembles the cellular processing of LDL (38). But not all VLDL or chylomicrons isolated from plasma seem to be taken up at identical rates. Chylomicrons and large VLDL particles are taken up more slowly than the smaller chylomicrons or VLDL remnants produced by lipoprotein lipase catalyzed lipolysis (17, 39). Larger  $\alpha$ -migrating VLDL isolated from plasmas of subjects with various forms of hypertriglyceridemia are taken up much more rapidly than seemingly similar  $\alpha$ -VLDL particles isolated under identical conditions from normal plasma (2, 16).  $\beta$ -VLDL induced by high fat, high carbohydrate diets are processed more rapidly than  $\alpha$ -VLDL (38). Presumably, all of these lipoprotein-cell interactions are mediated by apoproteins, but the alterations in lipoprotein-apoprotein structure responsible for the differences are not known.

Four general approaches have been used to ascertain which apoproteins play roles in the recognition of lipoproteins by cellular receptors; a) studies of interactions of apoprotein-lipid recombinants with cultured cells or perfused organs, b) studies of effects of alterations of compositions of hololipoproteins on cell interactions, c) studies of consequences of cleavage of apoproteins in lipoproteins on cell interactions, and d) studies relating immunologic activities of apoproteins to cellular binding. The role of apo E in cell binding was recognized in experiments with apo E-phospholipid recombinants using both

wild type ( $E_3$ ) and mutant ( $E_2$ ) forms of the protein (9–11). The compositional approach provided information on the opposing roles of apo E and apolipoprotein CIII in VLDL binding (40, 41). Thrombin-induced cleavage of apo E abolished the binding of large HTG-VLDL<sub>1</sub> to fibroblasts, implying that apo E mediates the interactions (16). The immunologic approach uncovered that the size of VLDL affects the dispositions of apo B on the surfaces of lipoproteins (17, 42–46) and also that the cellular binding of LDL can be inhibited only by selected monoclonal anti-LDL antibodies, i.e., many antibodies did not inhibit (17, 18, 42). Therefore, it was possible to connect single epitopes of LDL with cellular binding.

In the present experiments, each of the HTG  $^{125}\text{I}$ -VLDL subfractions was appreciably taken up and degraded by the cultured normal fibroblasts, and each fraction also stimulated the esterification of [ $^3\text{H}$ ]oleate into cholesteryl-esters. Normal VLDL interacted much less effectively with cells. Antibodies 2a and 2b and Fab 2a inhibited these processes in a progressive fashion, with inhibition increasing from VLDL<sub>1</sub> to VLDL<sub>2</sub> to VLDL<sub>3</sub>, and finally to LDL. A similar pattern of inhibition was produced by excess LDL. Antibodies 1a or 6 produced much less inhibition, and nonimmune mouse IgG produced none at all. Therefore, the inhibition by antibodies 2a and 2b was specific. The ability of Fab 2a at equimolar doses to produce similar degrees of inhibition further confirms the specificity of the inhibition. Since lower doses of the inhibiting antibodies and Fab fragments than were used for most experiments were shown to inhibit to the same extent (Fig. 2), all available epitopes must have been saturated, yet some proportion of each VLDL subfraction was still taken up by the cells. If epitopes 2a and 2b are involved in cellular recognition and for the reasons enumerated in the Introduction, we believe they are, cellular recognition of VLDL must have occurred not only via apo B, but also at alternate domains, probably on apo E. This hypothesis was tested by incubating lipoproteins before their addition to cells with anti-apo E antibodies. The experiments clearly showed a preferential inhibition of VLDL<sub>1</sub> uptake and cellular processing by these antibodies when compared with the smallest VLDL<sub>3</sub> fraction. From the data, it seems that the size heterogeneity introduced either during VLDL catabolism (47) or during secretion of nascent VLDL particles (48) is accompanied by heterogeneity of cell binding characteristics. While the vast majority of the largest VLDL<sub>1</sub> particles interacts with the apo B,E-receptor almost solely via apo E, a progressively larger proportion of the smaller particles of VLDL<sub>2</sub> and VLDL<sub>3</sub> interact via apo B, and the end product of the "cascade," LDL, interacts almost completely via apo B. Similar conclusions based on thrombin-induced proteolysis of apo E in VLDL subfractions have been reported in abstract form (49).

What accounts for the initial preference for apo E and the gradual changeover to apo B? Several possibilities exist. The relevant binding sites on apo B may not be available on the largest VLDL, either because they are buried in lipid or are masked by other apoproteins. Alternatively, perhaps the domains are not masked, but the organization of apo B in larger VLDL is such that the appropriate domains interact with cellular receptors very weakly. The experiments in which the inhibition of  $^{125}\text{I}$ -lipoprotein binding to antibody 2a, but not to antibody 1a, varied inversely with the flotation rate of VLDL (Table II), demonstrated that the expression of relevant regions of apo B vary with VLDL size and/or density. These



results support the hypothesis that the disposition of apo B may play a role in the interaction of VLDL with cells. However, these experimental results do not distinguish whether the relevant domain is buried or weakly reactive for other reasons. Another possibility for the gradual changeover from apo E to apo B may be simply that there are more molecules of apo E per particle on large than on small VLDL, and virtually none on LDL. This idea is supported by the observation that apo E contents of VLDL<sub>1</sub> tend to be higher than those for VLDL<sub>3</sub> (Krul, E. S., M. J. Tikkanen, and G. Schonfeld, manuscript in preparation) (Fig. 6), and by the direct relationship between binding of phospholipid vesicles to fibroblasts and the amounts of functional apo E associated with the vesicles (10, 11). The effective interactions of HTG-VLDL with cells and the lack of such interactions by normal VLDL<sub>1</sub> also may be due to the presence of greater amounts of apo E on the former (50–52). A third alternative is that the conformation of apo E in VLDL<sub>1</sub> favors cellular interaction but apo E conformations may change during lipolysis and catabolism of VLDL in an unfavorable direction. These three alternatives are not mutually exclusive, and one or more of them may be operating at the same time, but more work is needed to ascertain which are operative and to what extent.

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