Hypolipidemic therapy modulates expression of apolipoprotein B epitopes on low density lipoproteins. Studies in mild to moderate hypertriglyceridemic patients

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Abstract Low density lipoproteins (LDL) of untreated moderate to severe hypertriglyceridemic patients (HTG-LDL) are smaller in size and are relatively enriched in triglycerides and proteins compared with normal LDL (N-LDL). HTG-LDL also manifest defective binding to the LDL receptors of normal cultured human fibroblasts. These structural and functional defects are reversible by effective hypolipidemic therapy. The aims of the present study were to confirm the reversibility of the structural and functional defects in mild to moderate hypertriglyceridemic patients and also to test the hypothesis that therapy improved the binding of HTG-LDL to cells by modulating epitopes of apolipoprotein B (apoB-100) on the surfaces of LDL particles. Fasting plasma samples were obtained from five mild to moderate hypertriglyceridemic patients before and 3 weeks after bezafibrate therapy when mean triglyceride levels were 436 and 157 mg/dl ($P < 0.01$), respectively. LDL particles were isolated by zonal ultracentrifugation, characterized chemically, and assayed for cell association and proteolytic degradation in-up regulated normal human skin fibroblasts. LDL immunoreactivity was tested in solid phase competitive binding radioimmunoassays (RIA) using three monoclonal antiLDL antibodies (Mab). Mab 464B1B3 and Mab 465B6C3 react against epitopes in the COOH-terminal (T_2/K_4) fragment of apoB-100. Mab D7.1 reacts with an epitope in the midportion (T_3/K_3) fragment. Mab 464B1B3 inhibits the binding of LDL to the LDL receptor. Hypolipidemic treatment altered the composition of LDL. Mean LDL triglycerides fell from 9.4 to 5.8% of LDL mass ($P < 0.025$). LDL protein fell from 29.4 to 24.8% ($P < 0.025$), while free and esterified cholesterol increased from 6.1 to 8.5 and from 35.2 to 39.5%, respectively ($P < 0.025$ for both comparisons). Phospholipids increased as well from 19.9 to 22.7% ($P < 0.05$). In cell culture experiments, mean cell-associated LDL rose from 277 to 400 ng of LDL/mg of cell protein ($P < 0.015$) and proteolytic degradation rose from 596 to 957 ng/mg ($P < 0.025$). In RIAs, mean ED_{50} values of LDL with Mab 464B1B3 fell from 6.0 to 3.2 μg of LDL protein ($P < 0.005$) and with Mab 465B6C3 from 17.3 to 7.5 ($P < 0.05$). In contrast, mean ED_{50} with Mab D7.1 was unaltered (2.6 to 2.9, $p > 0.1$). Thus improvement of LDL activity in tissue culture could be achieved in mild to moderate hypertriglyceridemic patients by reducing their plasma triglyceride levels to normal. Moreover, the improved interaction of LDL with the LDL receptor in fibroblasts after therapy is related to altered disposition of apoB epitopes on LDL.—**Kleinman, Y., G. Schonfeld, D. Gavish, Y. Oschry, and S. Eisenberg.** Hypolipidemic therapy modulates expression of apolipoprotein B epitopes on low density lipoproteins. Studies in mild to moderate hypertriglyceridemic patients. *J. Lipid Res.* 1987. **28:** 540–548.

Supplementary key words apoB • epitopes • LDL • hypertriglyceridemia

We have shown previously that LDL isolated from plasmas of moderate to severe hypertriglyceridemic subjects (HTG-LDL) manifest defective binding to the LDL receptor in up-regulated normal human skin fibroblasts and that the interaction can be improved by lowering the plasma triglyceride levels (1). The HTG-LDL and LDL of normal subjects (N-LDL) used in those studies differed from each other in several respects. HTG-LDL floated more slowly on zonal ultracentrifugation, was smaller in diameter, and contained more triglycerides and proteins relative to cholesterol and phospholipids (1–7). Virtually all of the structural and compositional abnormalities of HTG-LDL returned towards normal with hypolipidemic therapy (1, 7) which although effective, still left the treated triglyceride levels far above normal. From the reversibility of the cellular binding defect it was concluded that the

Abbreviations: TG, triglyceride; LPDS, lipoprotein-deficient serum; RIA, radioimmunoassay; VLDL, very low density lipoproteins; LDL, low density lipoproteins; N-LDL, normal LDL; HTG-LDL, LDL from untreated moderate to severe hypertriglyceridemic patients; BZ, bezafibrate; Mab, monoclonal antibody; FCS, fetal calf serum.

defective binding was not due to some intrinsic structural defect in the apoB moiety of LDL, but that other structural and/or compositional features of LDL were responsible for the less efficient binding.

Immunologic techniques have proved to be useful for studying lipoprotein structure, in particular for detecting subtle alterations of the "availability" of expression of domains of apoproteins on holo-lipoproteins (8–12). For instance, the availability of the LDL receptor recognition domain on VLDL subfractions, as determined by binding affinity to cellular LDL receptors, and apoB epitope expression, as assayed by relative ^{125}I-labeled LDL displacing potency in immunoassays, decreased with increasing flotation rate. The binding to antibody was least avid for the largest VLDL subfraction ($VLDL_1$) and avidity progressively increased as VLDL decreased in size ($VLDL_1 < VLDL_2 < VLDL_3$). The binding of VLDL subfractions to cultured fibroblasts follows the same rank order ($VLDL_1 < VLDL_2 < VLDL_3$), suggesting that cellular binding may be related to the disposition of apoB on the surfaces of VLDL (13, 14). The aims of the present experiments were first, to confirm that the differences in binding of HTG-LDL before and after hypolipidemic therapy also occur in mild to moderate hypertriglyceridemic patients, i.e., to exclude the possibility of a low threshold value below which no beneficial effect on cellular binding could be detected; and second, to relate LDL-cell interactions to changes in the disposition of specific domains of the apoB moiety on the LDL particles.

MATERIALS AND METHODS

Subjects

Five new patients with primary endogenous hypertriglyceridemia (HTG) (type IV) as defined by Fredrickson, Levy, and Lees (15) were studied. The patients were attending the lipid clinic at the Hadassah University Hospital, Jerusalem. In contrast with patients reported previously (1) who had severe hypertriglyceridemia, these patients had mild to moderate triglyceride elevations. Informed consent was obtained in all cases. All patients were normoglycemic and their hepatic and thyroid functions were normal. None was receiving drug treatment for other conditions. All patients achieved normal triglyceride levels after institution of bezafibrate therapy.

Experimental protocol

Fasting plasma samples were obtained from an antecubital vein in the morning hours after a 14–16-hr fast. In three patients (1, 2, and 4), an HTG plasma sample was drawn before therapy, on the morning of the day bezafibrate (BZ) treatment (0.2 g three times a day) was instituted. A second plasma sample was obtained after 3 weeks of therapy (BZ sample). In two patients (3 and 5), who had been on BZ therapy for several months, a blood sample was drawn (BZ sample) and on the same day therapy was discontinued for 3 weeks. Then a second blood sample was obtained (HTG sample). LDLs were isolated from plasma, characterized with respect to their diameters and chemical compositions, and their cell association and proteolytic degradations by cultured normal human skin fibroblasts were assessed. Epitope expression was determined by competitive binding assays, using several monoclonal antibodies (Mabs). In each experiment HTG-LDL and BZ-LDL from the same patient were examined together.

Isolation and characterization of LDL

Blood was drawn into tubes containing EDTA (1 mg/ml). LDL was isolated by zonal ultracentrifugation according to the method published by Patsch et al. (16). A NaBr gradient of 1.0–1.3 g/ml was established using a high capacity gradient pump (Model 141; Beckman Instruments, Inc., Fullerton, CA). The rotor was loaded with a plasma fraction of d 1.006–1.085 g/ml (isolated from 80–140 ml of plasma) and the LDL was eluted after 140 min centrifugation at 42,000 rpm. The eluate was monitored continuously at 280 nm by a multiwavelength absorbance monitor (Model VA-5; Isco, Inc., Lincoln, NE). The major LDL fraction (7) was isolated for each patient and used in the tissue culture and immunological studies. All preparations were sterilized by passage through a 0.45-μm Millipore filter. Electron microscopy was used to determine the size of the lipoproteins (300; Philips Electronic Instruments, Inc., Mahwah, NJ) as described previously (7). Triglycerides were determined according to the Lipid Research Clinic's protocol using an AutoAnalyzer AA-II (Technicon Corp., Tarrytown, NY) (17). Cholesteryl ester and free cholesterol contents of lipoproteins were determined enzymatically (18) with a commercial kit (Boehringer Mannheim GmbH Diagnostics, Mannheim, West Germany). Phospholipids were determined by the Bartlett procedure (19). To assess the apoprotein contents of LDL, sodium dodecyl sulfate polyacrylamide gel electrophoresis of apolipoproteins on 10% gels was performed after the method described by Weber and Osborn (20). Only LDLs in which apoB was the only apoprotein were used. LDL protein was measured by the method of Lowry et al. (21) using bovine serum albumin (BSA) as a standard.

Iodination of LDL

^{125}I-labeled LDL was prepared by the iodine monochloride method of MacFarlane (22) as modified by Bilheimer, Eisenberg, and Levy (23). The iodinated preparations were dialyzed extensively against 0.15 M NaCl, 0.001 M EDTA, pH 7.4. The final specific activity varied between 50 and 500 cpm/ng protein. In all

preparations, >95% of the radioactivity was precipitated by trichloroacetic acid and <5% was extractable by chloroform–methanol. All the iodinated and noniodinated LDL preparations were used within 3 weeks and sterilized by passage through a 0.45-μm milipore filter. They were kept sterile and tightly closed at 4°C.

Cultures of human skin fibroblasts

Biopsies were obtained from the medial part of the forearm of a normal male adult donor. The cells were cultured in plastic flasks (Falcon Labware, Div. of Becton-Dickinson and Co., Oxnard, CA) in modified Dulbecco-Vogt medium (24) containing 10% fetal calf serum (FCS), and maintained in a humidified incubator (5% CO_2) at 37°C. Fibroblasts from third to fifteenth subcultures were trypsinized and 3×10^4 cells were plated (day 0) in 35-mm dishes (Falcon Labware) containing 2 ml of medium with 10% FCS. The medium was changed on days 3 and 5, before the cells became confluent. On day 6, monolayers were washed with Dulbecco's phosphate-buffered saline (PBS) and 2 ml of fresh medium containing human lipoprotein-deficient serum (LPDS) at a final concentration of 5 mg/ml was added. The cells were incubated for another 48 hr. All experiments were initiated on day 8.

Assays of cell association and proteolytic degradation of ^{125}I-labeled LDL

On the day of the experiment, the serum-containing medium was removed, the cell layer was washed with PBS, and the cells were incubated with ^{125}I-labeled LDL, 15 μg of protein/ml, in lipoprotein-deficient medium at 37°C. After 4 hr of incubation, the medium was examined for noniodide ^{125}I-labeled LDL degradation products according to Bierman, Stein, and Stein (25).The results were expressed as nanograms of ^{125}I-labeled LDL protein degraded per milligram cell protein. Dishes with ^{125}I-labeled LDL in the presence of unlabeled LDL (500 μg of protein/ml) were processed simultaneously and the results were subtracted from the total degradation values. After removal of the medium, the cells were washed three times with PBS, 0.2% BSA, three times with PBS, and then dissolved in 1 ml of 0.5 N NaOH. The ^{125}I-labeled LDL radioactivity counted in the dissolved cells after subtracting the radioactivity in the dishes with excess of cold LDL represents the cell-associated LDL. The nonspecific degradation and cell association were 5–10% of the total amounts.

Monoclonal antiLDL antibodies

AntiLDL mouse Mabs were produced by using an intact human holo-LDL preparation as immunogen. The apoB of this LDL was virtually all apoB-100 (26). The antiapoB antibodies used in the present study were 464B1B3, which appears to be directed against an epitope that is spatially related to the domain on apoB recognized by cellular LDL receptor; Mabs 464B1B3 and 465B6C3, which both define epitopes on the COOH-terminal (T_2/K_4) region of apoB-100; and D7.1 which defines an epitope on the middle portion of the molecule (T_3/K_3) fragment (27). The preparation of the first two Mabs was described previously (10, 11, 28). D7.1 is a new antiapoB Mab prepared by immunizing Balb/c mice with human VLDL fractions isolated from a single donor. Screening was done with solid phase RIA in microtiter plates coated with human LDL.

Solid phase competitive binding immunoassays

HTG-LDL and BZ-LDL from the same patient were assayed in competitive displacement assays on microtiter plates (12) (Dynatech Laboratories, Inc., Alexandria, VA) as follows. Microtiter wells were coated with 150 μl of monoclonal antibody solution (5 μg/ml of PBS) overnight. Wells then were incubated with 3% BSA-PBS for 3 hr at room temperature in order to saturate extra binding sites on the plastic. Wells then received serial dilutions (in 100 μl) of the LDL preparations (highest dose, 64 μg of LDL protein/ml) and a constant amount of ^{125}I-labeled LDL (100,000 cpm in 50 μl of 3% BSA-PBS). After incubation

TABLE 1. Total plasma and lipoprotein lipid concentrations of HTG patients in the hypertriglyceridemic state and during bezafibrate therapy

	Values before BZ Therapy					Values during BZ Therapy				
		Cholesterol					Cholesterol			
Patients	TG	Plasma	VLDL	LDL	HDL	TG	Plasma	VLDL	LDL	HDL
	mg/dl plasma					*mg/dl plasma*				
1	575	223	88	134	25	164	229	15	182	22
2	336	224	72	106	20	117	213	17	159	29
3[a]	217	204	52	156	31	89	213	19	180	37
4	778	286	212	48	20	241	186	52	98	22
5[a]	275	277	49	179	23	176	256	23	217	24
Mean ± SEM	436 ± 105*	243 ± 16	95 ± 30**	125 ± 23***	24 ± 2.0	157 ± 26.2	219 ± 11.5	25.2 ± 6.83	167 ± 19.6	26.8 ± 2.85

Differences between HTG and BZ samples are significant at level of $^*P < 0.01$, $^{**}P < 0.025$ and $^{***}P < 0.0005$ by paired t-test.
[a] Studied in reverse order.

TABLE 2. Chemical compositions and diameters of HTG-LDL and BZ-LDL

Source of LDL	Protein	CE	TG	FC	PL	Diameter
	ng/100 mg lipoprotein					Å
HTG	29.4 ± 1.70	35.2 ± 1.94	9.4 ± 1.42	6.1 ± 1.05	19.9 ± 1.50	205 ± 3.35
BZ	24.8 ± 2.36*	39.5 ± 1.45*	5.8 ± 0.53*	8.5 ± 0.61*	22.7 ± 0.61**	227 ± 6.86*

Data are means ± SEM of five hypertriglyceridemic patients who participated in the study. Differences between HTG and BZ samples are significant at level of $^{*}P < 0.025$ or $^{**}P < 0.05$ by paired *t*-test. CE, cholesteryl ester, TG, triglyceride, FC, free cholesterol, PL, phospholipids.

overnight the wells were rinsed three times, cut, and counted in a Packard gamma spectrometer. All assays were carried out in triplicate. B/Bo versus LDL concentrations were plotted and all the data were computed by the "Program to calculate parameters for the four parametric logistic function, using 2 + 2 linear regression approach" as developed by P. Munson, D. Rodbard, and M. L. Jaffe, NIH, Bethesda, MD, USA, 20014, IBM PC version 1985. Slopes and midrange (ED_{50}) values were calculated for the various curves and compared.

Materials

$Na^{125}I$ was purchased from the Radiochemical Centre, Amersham, England. Culture flasks and dishes were purchased from Falcon Labware. Medium and FCS were obtained from Gibco Laboratories, Grand Island, NY. Ninety six-well microtiter plates were purchased from Dynatech Laboratories, Inc., Alexandria, VA. All other chemicals and reagents were of analytical grade.

Statistical Analysis

Significant differences between HTG-LDL and BZ-LDL were evaluated by paired *t*-tests. Linear regression analysis was used to determine correlation between ratios of HTG/BZ compositional parameters of LDL (e.g.,

$$\frac{\text{HTG-LDL cholesteryl ester/protein ratio}}{\text{BZ-LDL cholesteryl ester/protein ratio}},$$

$$\frac{\text{HTG-LDL cholesteryl ester/triglyceride ratio}}{\text{BZ-LDL cholesteryl ester/triglyceride ratio}},$$

and ratios of HTG/BZ cell reactivity (proteolytic degradation and cell association) and ratios of HTG/BZ immunoreactivity (ED_{50} values). Significance levels were obtained from statistical tables (29, 30).

RESULTS

Plasma and lipoprotein lipids

The patients' plasma lipid levels in the hypertriglyceridemic state and during BZ therapy are shown in **Table 1.**

TABLE 3. Cell association and proteolytic degradation of HTG-LDL and BZ-LDL by cultured normal human skin fibroblasts

	Cell Association		Proteolytic Degradation	
Patients	HTG	BZ	HTG	BZ
	ng LDL protein/mg cell protein			
1	169 ± 14.2	325 ± 37.4	334 ± 64.9	919 ± 185
2	570 ± 19.6	716 ± 56.1	1007 ± 84.9	1270 ± 142
3	194 ± 19.9	402 ± 28.7	438 ± 65.7	1139 ± 109
4	266 ± 24.2	378 ± 7.4	636 ± 53.6	863 ± 43.3
5	187 ± 26.6	211 ± 30.3	564 ± 12.0	595 ± 28.6
Mean ± SEM	277 ± 75.0	400 ± 85.8**	596 ± 115	957 ± 117*

Normal fibroblasts were preincubated for 48 hr with lipoprotein-deficient serum. They were then incubated for 4 hr at 37°C either with ^{125}I-labeled HTG-LDL or ^{125}I-labeled BZ-LDL (each at 15 μg of protein/ml) in triplicate dishes. Nonspecific association and degradation were determined on plates containing ^{125}I-labeled LDL in the presence of unlabeled LDL (500 μg of protein/ml). Specific cell association and degradation were obtained by subtraction of nonspecific from total counts. Data are from five patients. Differences between HTG and BZ are significant at levels of $^{*}P < 0.025$ or $^{**}P < 0.015$ by paired *t*-test.

All the patients achieved normal triglyceride values after institution of bezafibrate. The mean TG level decreased by 64%. The same trend was found for VLDL cholesterol which decreased by 73%, while the mean LDL cholesterol level increased by 34%. The chemical composition and size of LDL particles before and after BZ therapy are shown in **Table 2.** The HTG-LDL particles were smaller and contained more triglyceride and protein and less cholesterol. The general trend of these data is in accord with our earlier publications (1, 7).

Proteolytic degradation and cell association

Table 3 presents the cell association and degradation of HTG-LDL and BZ-LDL as assayed in normal human skin fibroblasts with up-regulated LDL receptors with ^{125}I-labeled LDL concentrations in the high affinity range of the LDL receptor (31). The cell association and degradation of HTG-LDL were substantially and consistently lower as compared to paired BZ-LDL ($P < 0.005$).

Immunoreactivities of HTG-LDL and BZ-LDL with monoclonal antiLDL antibodies

The binding of HTG-LDL and BZ-LDL to LDL Mabs was tested in a solid phase competitive binding assay **(Fig. 1).** The competitive displacement curves produced by HTG-LDL were consistently to the right of the BZ-LDL curves in all the patients, in assays containing either the 464B1B3 or 465B6C3 antibodies, while the relative positions of the HTG-LDL and BZ-LDL curves with D7.1 were inconstant. Computer analysis of the curves showed slopes close to 1 in most cases with Mab 464B1B3 but the slopes varied considerably with the other Mabs **(Table 4).** ED_{50} values were also computed for each LDL and each antibody according to Munson et al. (see Methods), and De Lean, Munson, and Rodbard (32) (Table 4). Mean ED_{50} values were significantly larger for HTG-LDL versus BZ-LDL in assays using either Mabs 464B1B3 or 465B6C3, but with Mab D7.1, ED_{50} values were about the same for HTG-LDL and BZ-LDL.

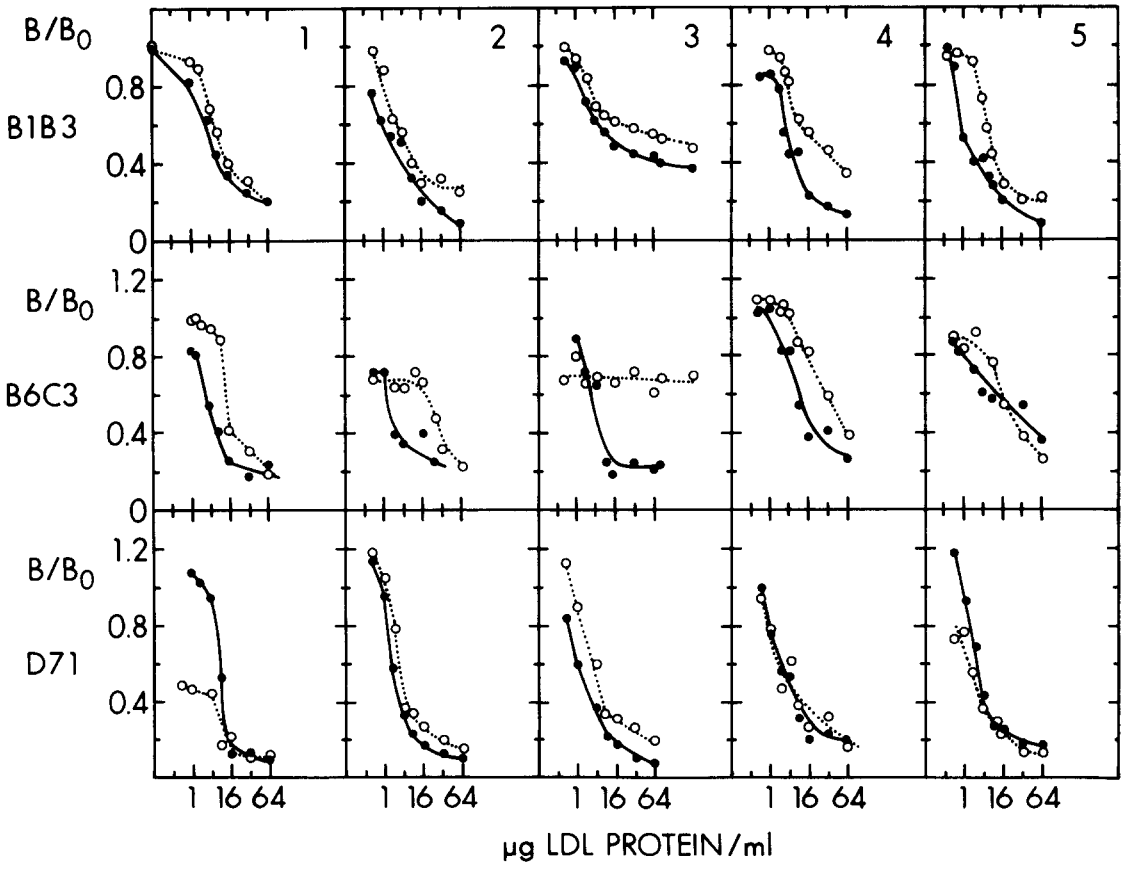

Fig. 1. Displacement curves obtained in solid phase competitive radioimmunoassay using normal ^{125}I-labeled LDL as tracer, three monoclonal antibodies (464B1B3, 465B6C3, D7.1), and either HTG-LDL (○---○) or BZ-LDL (●—●) from five hypertriglyceridemic patients as competitors at doses indicated on the abscissa.

TABLE 4. Slopes and midrange (ED_{50}) values from competitive binding assays of HTG-LDL and BZ-LDL with three different LDL monoclonal antibodies

	Mab 464B1B3				Mab 465B6C3				Mab D7.1			
	Slope		ED_{50}		Slope		ED_{50}		Slope		ED_{50}	
Patients	HTG	BZ	HTG	BZ	HTG	BZ	HTG	BZ	HTG	BZ	HTG	BZ
1	1.07	1.14	6.76	4.49	4.11	2.47	12.22	4.65	6.40	4.01	5.56	6.80
2	0.95	1.08	5.53	1.68	1.25	2.06	25.75	5.70	2.12	1.63	2.16	1.56
3	2.99	0.88	3.10	1.33	[a]	5.92	[a]	4.75	1.00	2.41	1.17	2.93
4	0.86	1.25	11.99	4.23	1.07	1.35	16.45	5.76	0.65	0.99	1.03	1.45
5	2.85	1.24	5.33	0.71	2.50	0.53	14.66	13.73	1.85	1.42	2.91	1.59
Mean ± SEM	1.74	1.12	6.02	3.21	2.23	2.47	17.27	7.46	2.40	2.09	2.57	2.87
	0.48	0.067 ns	0.41	0.96*	0.70	1.03 ns	2.96**	2.11	1.03	0.53 ns	0.87	1.02 ns

Data are from five patients. The slopes and midrange (ED_{50}) values (in μg LDL) were computed by the "Program to calculate parameters for the four parameter logistic function, using 2 ± 2 linear regression approach" developed by P. Munson, D. Rodbard, and M. L. Jaffe, National Institute of Child Health and Human Development, Bethesda, MD, USA, 20014, IBM PC version 1985. Differences between HTG and BZ are significant at levels of $^{*}P < 0.005$ or $^{**}P < 0.05$ or ns (not significant) by paired t-test.

[a] No significant competition obtained (see Fig. 1).

Relationship between LDL composition, cell reactivity and immunoreactivity

Statistical relationships were sought between ratios of indexes of HTG/BZ-LDL composition (CE/Pr, cholesteryl ester/protein and CE/TG, cholesteryl ester/triglyceride), HTG/BZ cell reactivity (proteolytic degradation and cell association) and HTG/BZ immunoreactivity (ED_{50} values as described in Methods for all the antibodies). Linear regression analyses were carried out on five paired (HTG-LDL and BZ-LDL) studies, and the values of the correlation coefficients (r) and their significance (P values) are presented in **Table 5.**

$$\frac{\text{HTG-LDL-CE/Pr}}{\text{BZ-LDL-CE/Pr}}$$

ratios were positively correlated with the HTG/BZ ratios of immunoreactivity of LDL in 464B1B3 assays. Ratios of HTG/BZ proteolytic degradation and ratios of cell association were strongly correlated with the ratios of HTG-ED_{50}/BZ-ED_{50} of LDL in 464B1B3 and 465B6C3 assays, respectively, but ratios of cell reactivity were not correlated with ratios of LDL-HTG-ED_{50}/BZ-ED_{50} in D7.1 assays.

DISCUSSION

The present data indicate that the hypolipidemic treatment-related changes in cell reactivity are apparent not only in patients who have severe hypertriglyceridemia, such as those we studied before (1), but also in the new patients with mild to moderate hypertriglyceridemia who achieved normal triglyceride values on therapy. As the LDL-cell interaction experiments were conducted at concentrations of LDL in the high affinity range of the LDL receptor activity, the changes in cell association and degradation probably reflect changes in receptor binding similar to those previously noted in the severe HTG patients.

The BZ-induced differences in immunoreactivity of LDL with the three Mabs deserve particular consideration. As the same Mabs and radioligand were used throughout all the experiments, differences between the unlabeled competitors (HTG-LDL and BZ-LDL) must have accounted for the diversity of results. Visual examination of the RIA curves showed consistent changes in the same directions for two of the antibodies and inconsistent changes for the third. More quantitative methods of curve analysis supported these impressions.

When slope values obtained from the computer analysis are close to 1, the reaction probably involves the binding of a single ligand (an epitope on LDL-apoB-100) to a homogeneous population of binding sites (a Mab), i.e., the reaction behaves according to the "simple mass action law" (33), and the ED_{50} values represent the equilibrium constant. However, where the slopes depart from 1.0, probably multiple LDL populations with different affinities and kinetic constants for the Mabs are bound to the homogeneous antibodies. The heterogeneity of LDL by immunologic criteria noted here has been documented also by other techniques particularly in patients with HTG (34–36). When slopes do not equal 1, ED_{50} values do not, strictly speaking, represent equilibrium constants. Nevertheless, ED_{50}s can be used to describe the mid-point positions of binding isotherms and, as empirical parameters, can be used to compare biological systems under differing experimental conditions (33).

TABLE 5. Correlation between ratio of HTG/BZ of LDL composition, cell reactivity, and immunoreactivity values

		LDL Composition	LDL Cell Reactivity		LDL Immunoreactivity, ED_{50}		
		CE/TG	Proteolytic Degradation	Cell Association	B1B3	B6C3	D7.1
LDL composition							
CE/Pr	*r*	0.68	0.7	0.61	0.84	−0.16	−0.72
CE/Pr	*P*	ns	ns	ns	<0.05	ns	ns
CE/TG	*r*		0.801	0.811	0.55	0.47	−0.81
CE/TG	*P*		<0.8	<0.1	ns	ns	<0.1
LDL cell reactivity							
Proteolytic degradation	*r*			0.96	0.87	0.81	−0.54
Proteolytic degradation	*P*			<0.01	<0.05	<0.1	ns
Cell association	*r*				0.81	0.95	0.61
Cell association	*P*				<0.1	<0.05	ns

Linear regression analysis between ratios of the indices of HTG-LDL and BZ-LDL composition (HTG-LDL-CE-Pr/BZ-LDL-CE-Pr; HTG-LDL-CE-TG/BZ-LDL-CE-TG); ratios of LDL cell reactivity (HTG-proteolytic degradation/BZ-proteolytic degradation); HTG-cell association/BZ-cell association, and to ratios of ED_{50} values were determined. The correlation coefficients (*r* values) and the *P* values (derived from a statistical table (30)) are presented. The linear regression analysis data are from five paired studies in five patients. CE, cholesteryl ester; Pr, protein; TG, triglyceride; ns, nonsignificant $P > 0.2$.

The slope data indicate that LDL was least heterogeneous with respect to the epitope defined by Mab 464B1B3 (slopes close to 1.0 in most experiments) and that the degree of heterogeneity was altered relatively little compared to the changes experienced by other epitopes. This is consistent with the importance of the domain represented by this epitope of apoB-100 in binding to the LDL receptor. Nevertheless, the affinity of the epitope for Mab 464B1B3 was improved by BZ treatment, i.e., ED_{50} values for BZ-LDL were less than for HGT-LDL. The heterogeneities of the other epitopes (465B6C3 and D7.1) were greater before therapy (slopes departed from 1.0) and were more variably affected by therapy than epitope 464B1B3. The greater heterogeneity of expression of the 465B6C3 epitope on LDL compared with the 464B1B3 epitope was also noted previously when LDLs isolated from different human (28) and nonhuman primates were studied (11). However, treatment affected the 465B6C3 and D7.1 epitopes differently because the ED_{50} values for the 465B6C3 epitope were consistently decreased, whereas the alteration of the D7.1 epitope was inconsistent from patient to patient. Thus, BZ therapy affected the expression of the three epitopes probed with our antibodies in different ways.

Parallel changes were observed in the cell reactivities and immunoreactivities of the LDL preparations isolated before and after treatment. The increased degradation and cell association of the larger BZ-LDL by fibroblasts and the enhanced expression of the epitopes defined by the antiLDL Mab 464B1B3 were consistently seen in the LDL of all patients after treatment. These results suggest that the post-treatment enhancement of cellular reactivity was due to the enhanced expression of the recognition region of apoB. The significant correlation between the CE/Pr ratio and 464B1B3 immunoreactivity suggests that cholesteryl ester is important for the expression of the receptor recognition determinant of apoB as suggested also by Marcel et al. (37).

The changes in expression of the 465B6C3 epitope, which is not related to cellular binding, indicate that the disposition of regions larger than just the cellular binding domain itself was changed by hypolipidemic therapy. However, the absence of correlation of cell interaction indexes or indexes of composition to the D7.1 expression of the epitope implies that the dispositions of some regions of apoB were not always altered in parallel with changes in composition. Further studies have shown that both Mabs 464B1B3 and 465B6C3 were directed to the carboxyterminus (T_2 thrombin digestion fragment) of the molecule and Mab D7.1 was directed towards the middle (T_3) fragment (27) implying that the mid-region and carboxyterminal of apoB-100 have different susceptibilities to the changes produced by this protocol.

Consonant with the present experiments, is the report of Teng et al. (38) who found large LDL subfractions to be more immunoreactive than the smaller and denser LDL subfractions. Also, turnover studies in humans (39) of apolipoprotein B in LDL subfractions showed that light LDL has a higher fractional catabolic rate than heavy LDL in normal subjects. These data could be explained if light LDL were removed from the circulation more effectively than heavy LDL through processes mediated by the LDL receptor. Conflicting results were published by Knight, Thompson, and Sontar (40). In their hands the heavy and light subfractions of low density lipoproteins were bound to the same extent and with the same affinity by the LDL receptors of cultured human fibroblasts. The reasons for the different results are not clear, but it is possible that the different isolation procedures for LDL could be responsible. In addition, drug-induced modula-

tion may cause changes in epitope expression, while epitope expression may be similar in the LDL subfractions of hyperapobetalipoproteinemic patients.

The sizes of LDL are altered not only during the treatment of hypertriglyceridemia but also during treatment of hypercholesterolemia (41). However, the changes are in the opposite directions. While lowering of triglycerides by bezafibrate is associated with the disappearance of smaller (~20 nm) LDL particles and the appearance of larger ones (~22 nm), the treatment of hypercholesterolemia in guinea pigs (41) and in man (J. Witztum, personal communications) with colestipol is followed by the appearance of smaller particles and the disappearance of the normal-sized larger particles. When pre- and post-colestipol LDL are radiolabeled and simultaneously injected into experimental subjects, the larger particles are cleared more rapidly than are the smaller particles and the difference is due to the LDL receptor - mediated clearance of the particles, suggesting that smaller LDL particles are less well cleared by the LDL receptor-mediated pathways. These results also suggest that the receptor region of apoB on LDL may be susceptible to modulation by changes in LDL size.

The excellent technical help of Ms. A. Mendeles, M. Ben-Nain, R. Avner, and H. Lefkovitz is greatly appreciated. Phyllis Anderson typed the manuscript.

Manuscript received 27 May 1986 and in revised form 22 October 1986.

REFERENCES

1. Kleinman, Y., S. Eisenberg, Y. Oschry, D. Gavish, O. Stein, and Y. Stein. 1985. Defective metabolism of hypertriglyceridemic low density lipoprotein in cultured human skin fibroblasts. *J. Clin. Invest.* **75:** 1796–1803.
2. Vakakis, N., T. G. Redgrave, D. M. Small, and W. P. Castelli. 1983. Cholesterol content of red blood cells and low density lipoproteins in hypertriglyceridemia. *Biochim. Biophys. Acta.* **751:** 280–285.
3. Nelson, C. A., and M. D. Morris. 1983. Human low density lipoprotein structure: correlations with serum lipoprotein concentrations. *Lipids.* **18:** 553–557.
4. Breckenridge, W. C., J. A. Little, G. Steiner, A. Chow, and M. Poapst. 1978. Hypertriglyceridemia associated with deficiency of apolipoprotein C-II. *N. Engl. J. Med.* **298:** 1265–1273.
5. Miller, N. E., S. N. Rao, R. Alaupovic, N. Noble, J. Slack, J. D. Brunzell, and B. Lewis. 1981. Familial apolipoprotein CII deficiency: plasma lipoproteins and apolipoproteins in heterozygous and homozygous subjects and the effects of plasma infusion. *Eur. J. Clin. Invest.* **11:** 69–76.
6. Deckelbaum, R. J., E. Granot, Y. Oschry, L. Rose, and S. Eisenberg. 1984. Plasma triglyceride determines structure–composition in low and high density lipoproteins. *Arteriosclerosis.* **4:** 224–231.
7. Eisenberg, S., D. Gavish, Y. Oschry, M. Fainaru, and R. J. Deckelbaum. 1984. Abnormalities in very low, low, and high density lipoproteins in hypertriglyceridemia. Reversal toward normal with bezafibrate treatment. *J. Clin. Invest.* **74:** 470–482.
8. Betty, P. T., L. K. Curtiss, and T. S. Edgington. 1982. Immunochemical heterogeneity of human plasma apolipoprotein B. II. Expression of apolipoprotein B epitopes on native lipoproteins. *J. Biol. Chem.* **257:** 15222–15228.
9. Marcel, Y. L., M. Hogue, R. Theolis, and R. W. Milne. 1982. Mapping of antigenic determinants of human apolipoprotein B using monoclonal antibodies against low density lipoproteins. *J. Biol. Chem.* **257:** 13165–13168.
10. Tikkanen, M. J., R. Dargar, B. Pfleger, B. Gonen, J. M. Davie, and G. Schonfeld. 1982. Antigenic mapping of human low density lipoprotein with monoclonal antibodies. *J. Lipid Res.* **23:** 1032–1038.
11. Nelson, C. A., M. A. Tasch, M. J. Tikkanen, R. Dargar, and G. Schonfeld. 1984. Evolution of low density lipoprotein structure probed with monoclonal antibodies. *J. Lipid Res.* **25:** 821–830.
12. Krul, E. S., and G. Schonfeld. 1986. Immunochemical method for studying lipoprotein structure. *Methods Enzymol.* **128:** 527–553.
13. Tikkanen, M. J., T. G. Cole, K. S. Hahm, E. S. Krul, and G. Schonfeld. 1984. Expression of apolipoprotein B epitopes in very low density lipoprotein subfractions. Studies with monoclonal antibodies. *Arteriosclerosis.* **4:** 138–146.
14. Krul, E. S., M. J. Tikkanen, T. G. Cole, J. M. Davie, and G. Schonfeld. 1985. Roles of apolipoprotein B and E in the cellular binding of very low density lipoproteins. *J. Clin. Invest.* **75:** 361–369.
15. Fredrickson, D. S., R. I. Levy, and R. S. Lees. 1967. Fat transport in lipoproteins—an integrated approach to mechanisms and disorders. *N. Engl. J. Med.* **276:** 36–44, 94–103, 148–156, 215–225, 273–281.
16. Patsch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holasek, and H. Braunsteiner. 1979. Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation. *J. Lipid Res.* **15:** 356–366.
17. Lipid Research Clinics Program: Manual of Laboratory Operations. 1976. Vol. I. Lipid and Lipoprotein Analysis. Department of Health, Education and Welfare, National Institutes of Health Publication No. 76-628. U. S. Government Printing Office, Washington, DC.
18. Patsch, W., S. Sailer, and H. Braunsteiner. 1976. An enzymatic method for the determination of the initial rate of cholesterol esterification in human plasma. *J. Lipid Res.* **17:** 182–185.
19. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
20. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
22. MacFarlane, A. S. 1958. Efficient trace labeling of proteins with iodine. *Nature* (London) **182:** 53–57.
23. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. *Biochim. Biophys. Acta.* **260:** 212–221.
24. Stein, O., J. Vanderhoek, G. Friedman, and Y. Stein. 1976. Deposition and mobilization of cholesterol ester in cultured human skin fibroblasts. *Biochim. Biophys. Acta.* **450:** 367–378.
25. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein

uptake and metabolism by rat aortic smooth muscle cells in tissue culture. *Circ. Res.* **35:** 136–150.
26. Kane, J. P., A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* **77:** 2465–2469.
27. Kleinman, Y., E. S. Krul, M. Burnes, S. Eisenberg, and G. Schonfeld. 1986. Experimental modulation of epitope expression of the carboxyterminal (T_2) region of apoB. *Circulation.* **74:** 113.
28. Tikkanen, M. J., T. G. Cole, and G. Schonfeld. 1983. Differential reactivity of human low density lipoproteins with monoclonal antibodies. *J. Lipid Res.* **24:** 1494–1499.
29. Hill, A. B. 1971. Principles of Medical Statistics. 9th edition. Lancet Ltd., London. 143–151; 317; 376.
30. Goldstone, L. A. 1983. Understanding Medical Statistics. 1st edition. William Heinemann Medical Books Limited. London, Appendix 3e.
31. Goldstein, J. L., S. K. Basu, and M. Brown. 1983. Receptor-mediated endocytosis of low density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
32. De Lean, A., P. J. Munson, and D. Rodbard. 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **235(2):** E94–E102.
33. Limbird, L. E. 1986. Cell Surface Receptors: A Short Course on Theory and Methods. Martinus Nijhoff Publishing, Boston. 51; 110.
34. Krauss, R. M., and D. J. Burke. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23:** 97–104.
35. Fisher, W. R. 1983. Heterogeneity of plasma low density lipoproteins manifestations of the physiologic phenomenon in man. *Metabolism.* **32:** 283–291.
36. Foster, D. M., A. Chait, J. J. Albers, R. A. Failor, C. Harris, and J. D. Brunzell. 1986. Evidence for kinetic heterogeneity among human low density lipoproteins. *Metabolism.* **35:** 685–696.
37. Marcel, Y. L., P. K. Weech, P. Milthorp, F. Terce, L. Vezina and R. W. Milne. 1985. Monoclonal antibodies and the characterization of apolipoprotein structure and function. *Prog. Lipid Res.* **23:** 163–195.
38. Teng, B., A. Sniderman, R. M. Krauss, P. O. Kwiterovich, J. B. W. Milne, and Y. L. Marcell. 1985. Modulation of apolipoprotein B antigenic determinants in human low density lipoprotein subclasses. *J. Biol. Chem.* **260:** 5067–5072.
39. Teng, B., A. D. Sniderman, A. K. Soutar, and G. R. Thompson. 1986. Metabolic basis of hyperapobetalipoproteinemia. *J. Clin. Invest.* **77:** 663–672.
40. Knight, B. L., G. R. Thompson, and A. K. Soutar. 1986. Binding and degradation of heavy and light subfractions of low density lipoprotein by cultured fibroblasts and macrophages. *Atherosclerosis.* **59:** 301–306.
41. Witztum, J. L., S. G. Young, R. L. Elam, T. E. Carew, and M. Fisher. Cholestyramine-induced changes in low density lipoprotein composition and metabolism. I. Studies in the guinea pig. *J. Lipid Res.* **26:** 92–103.