Differential reactivity of human low density lipoproteins with monoclonal antibodies

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Abstract The immunoreactivities of LDL (low density lipoprotein) samples obtained from a variety of subjects were analyzed by comparing their capacities to compete with ^{125}I-labeled LDL for binding to various monoclonal anti-LDL antibodies in competitive binding assays. A marked variation in epitope expression was observed. In comparison to an LDL standard, different preparations exhibited immunoreactivities (expressed as apparent apoB content) ranging from 30 to 400% of the LDL standard. Some epitopes were much more uniformly expressed than others. The number of epitopes expressed in different LDL preparations appeared to be related to the percentage composition of various lipid constituents in LDL. The results support the hypothesis that the epitope expression of apoB is modulated by the composition of the lipids associated with it.—**Tikkanen, M. J., T. G. Cole, and G. Schonfeld.** Differential reactivity of human low density lipoproteins with monoclonal antibodies. *J. Lipid Res.* 1983. **24:** 1494–1499.

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The immunogenetic polymorphism of low density lipoprotein (LDL) was discovered two decades ago (1). The possible clinical significance of this heterogeneity has not been established in man, but an intriguing relationship between certain genetically determined antigenic types of LDL and aortic intimal lipidosis in swine has been reported (2, 3). Swine carrying the Lpp^5 allele at the main LDL locus showed a greater tendency to develop fatty streaking than swine with Lpp^8 when fed a high-fat ration (2). The possible analogy that human apoB should consist of a family of genetically determined isoforms, some of which could be related to the development of hyperlipidemia and/or atherosclerosis, has not been evaluated. The generation of libraries of monoclonal antibodies against LDL by us (4) and others (5–8) has provided the tools for large-scale screening of LDL samples for apoB variants. However, the immunoreactivity of LDL may be determined not only by the structure of the protein moiety itself but also by other lipoprotein constituents. Studies utilizing both polyclonal (9) and monoclonal (10) anti-apoB antibodies have shown that apoB reacts differently in different types of lipoprotein particles and that immunochemical differentiation between apoB in VLDL, IDL, and LDL is possible. In the present report we studied the immunological reactivities of apoB within the LDL class. The results showed that the expression of epitopes in different LDL preparations indeed varied, and that the number of epitopes expressed appeared to be influenced by the lipid composition of LDL.

MATERIALS AND METHODS

Subjects

To obtain a random selection, LDL was isolated from blood samples from 17 consecutive patients visiting the Lipid Clinic. Eight of the patients (one woman, seven men) initially had type IV hyperlipoproteinemia, five had the IIA phenotype (three men and two women, one of whom had familiar hypercholesterolemia (heterozygote)), three men had the type IIB phenotype, and one woman had normal lipid levels. The clinical characteristics of the subjects are summarized in **Table 1.**

Production and purification of monoclonal antibodies

Monoclonal IgG class antibodies directed against intact LDL were produced in a mouse-spleen myeloma system and purified by chromatography on an LDL immunoaffinity column (4). The purified antibodies migrated as single sharp bands on cellulose acetate electrophoresis (Microzone electrophoresis system, Beckman Instruments, Inc., Fullerton, CA), and formed

Abbreviations: LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; apoB, apolipoprotein B; IgG, immunoglobulin G; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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TABLE 1. Clinical characteristics of 17 subjects participating in the study

	Age	Height	Weight	Serum TG	Serum Chol
	yr	*cm*	*kg*	*mg/dl*	*mg/dl*
Mean ± SD	55.5 ± 11.6	170.7 ± 11.5	77.2 ± 15.8	218 ± 114	289 ± 74
Range	(28–72)	(143–188)	(51.7–110.8)	(72–527)	(121–454)

three or four closely spaced bands on isoelectric focusing on 1% agarose (Isogel Agarose, Marine Colloids Div., FMC Corporation, Rockland, ME). The six antibodies studied here were: 457C4D1 [1], 464B1B3 [2a], 464B1B6 [2b], 465B6C3 [3], 465C3D1 [4], and 465D3D5 [5]. Competitive displacement assays demonstrated that antibodies 2a and 2b were directed against the same or two closely related epitopes, whereas the epitopes defined by the other antibodies were different from each other as well as from epitope 2 (4).

Solid phase competitive assays

Different LDL preparations were assayed in competitive displacement assays on microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) as follows. Microtiter wells were coated with 150 μl of monoclonal antibody solution (5 μg/ml of PBS) overnight at 4°C. Wells then were incubated with 3% BSA-PBS (3 hr, 23°C) in order to saturate extra binding sites on the plastic. Wells then received serial dilutions (in 150 μl) of unknown LDL samples (highest dose 12.5 μg LDL protein/ml), and a constant amount of ^{125}I-labeled LDL (100,000 cpm in 20 μl of 3% BSA-PBS). All dilutions were carried out in 3% BSA-PBS. After incubation overnight at 4°C, wells were rinsed three times with PBS, sliced, and counted in a gamma counter. The results were expressed as percent of the immunizing LDL which was used as a standard (see below) and also as label.

Iodination of LDL was carried out with lactoperoxidase (Calbiochem, La Jolla, CA) (11). Twenty-five μg of lipoprotein were mixed with Na^{125}I (0.5 mCi) and lactoperoxidase (225 ng) in 0.05 M sodium phosphate buffer, pH 7.6 (total volume 50 μl). The reaction was initiated by the addition of hydrogen peroxide (300 ng) and terminated after 4 min by dilution with 0.05 M Na phosphate buffer. ^{125}I-labeled LDL was separated from unreacted [^{125}I]iodide by gel filtration on Sephadex G-50. More than 95% of purified LDL was precipitable by 10% trichloroacetic acid and by polyclonal rabbit antihuman LDL antisera in a double antibody test tube assay system (12). Approximately 100,000 cpm per well (in 20 μl) was used as label in competitive displacement assays on microtiter plates. The binding of the label in the absence of competitor (Bo) varied as follows: antibody 1, ~15,800 cpm; Ab 2a, ~15,100 cpm; Ab 2b, ~15,000 cpm; Ab 3, ~4,100 cpm; Ab 4, ~5,200 cpm; Ab 5, ~6,100 cpm. The background varied between 200 to 400 cpm. All assays were carried out in duplicate. The within-assay and between-assay variations were estimated according to Rodbard (13) from duplicate determinations of an LDL sample in each of ten assays. The coefficient of variation for within-assay variability was 6.95% and that for between-assay variability was 9.3%.

Statistical procedures

The displacement curves produced in competitive binding assays were usually curvilinear or sigmoidal. In order to compare the slopes of different curves with that of the LDL standard curve, curves were linearized by logit-log transformation of the data (13, 14), and differences between slopes were assessed by analysis of covariance using an SAS General Linear Model procedure (ref. 15, pp. 139–199). The LDL samples producing displacement curves with significantly different ($P < 0.05$) slopes were excluded from subsequent calculations of correlations. Accurate computation of apparent apoB contents was possible from the parallel curves. The results were expressed as percent of the LDL standard (=100). Correlations were tested by calculating the Pearson and Spearman rank correlation coefficients.

Isolation of LDL

Erythrocytes were immediately separated from plasma. Sodium azide (0.02%), EDTA (1 mM), chloramphenicol (50 mg/l), and gentamycin sulfate (100 mg/l) were added to the plasma (16). Isolation of LDL was carried out between densities 1.025 and 1.050 g/ml with two ultracentrifugations at each density. The density range 1.025–1.050 g/ml was chosen because it has been found, in previous studies in this laboratory, to exclude VLDL and HDL, as well as Lp(a) (12). The purity of the isolated LDL preparations was assessed by 3% SDS polyacrylamide gel electrophoresis of the individual samples (16). Three bands corresponding to apoB-100, B-74, and B-26 were found by Coomassie blue staining, but none of the samples contained detectable amounts of B-48, serum albumin, or non-apoB apolipoproteins. After dialysis against sodium chloride (0.15 M) and EDTA (1 mM, pH 8.2), the LDL

TABLE 2. Composition of LDL

	N	Protein	Phospholipid	Cholesteryl Ester	Free Cholesterol	Triglycerides
% of LDL mass	17	22.5 ± 1.6	22.5 ± 1.1	39.1 ± 4.9	8.9 ± 1.9	6.5 ± 2.0
Range		(20.8–26.0)	(20.6–24.4)	(32.3–45.9)	(7.1–15.0)	(3.9–10.4)

Results are means ± SD.

was sterilized by filtration through a 0.2-μm filter (Acrodisc®, Gelman, Ann Arbor, MI) and stored in the EDTA-saline containing the above-mentioned concentrations of antibacterial agents at 4°C. LDL preparations were analyzed for protein (17, 18), phospholipid (19), triglyceride, and free and esterified cholesterol (Triglyceride and Cholesterol Kits, Biodynamics, Indianapolis, IN). Each of the chemical determinations was carried out for all 17 LDL samples on the same day. Within-assay coefficients of variation for LDL protein were 3.2% and for the lipids 4–6%.

RESULTS

Chemical composition of LDL

The average chemical composition of the LDL preparations is shown in **Table 2.** The percentage of triglyceride correlated positively with percent protein ($r = 0.72$, Pearson correlation coefficient; $P = 0.0011$) and inversely with percent total cholesterol ($r = -0.89$, $P = 0.0001$) as well as with percent esterified cholesterol ($r = -0.68$, $P = 0.0023$) but not with free cholesterol. In addition, the percentage of total cholesterol correlated negatively with percent protein ($r = -0.84$, $P = 0.0001$) but percent phospholipid did not correlate significantly with any of the other LDL constituents.

Immunoreactivity of LDL preparations

The immunoreactivities of LDL samples were analyzed by comparing their capacities to displace ^{125}I-labeled LDL from binding to different monoclonal antibodies (example displacement curves are in **Fig. 1**). Statistical analysis of the slopes of the displacement curves (after linearization of the curves by logit-log transformation of data) revealed that one of the curves produced with antibody 2a, two of those produced with antibody 4, as well as nine of those produced with antibody 5 had slopes that were significantly different ($P < 0.05$) from the slope of the LDL standard curve. The apparent apoB contents calculated from all assays are compiled in **Fig. 2.** The apparent apoB contents obtained from curves that were significantly nonparallel with the LDL standard (identified in Fig. 2) were excluded from all subsequent calculations. Since the majority of curves produced with antibody 5 were nonparallel, this antibody was omitted altogether from calculations of correlations.

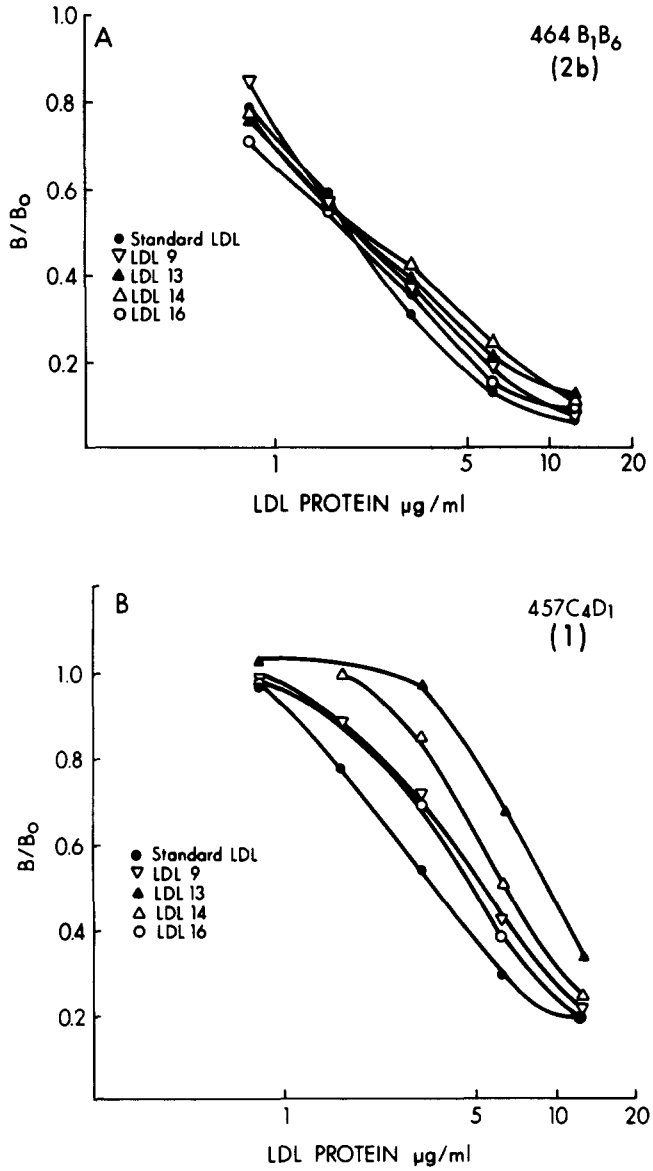

Fig. 1. Competitive displacement curves produced by different LDL preparations with antibodies 464B1B6 (A) and 457C4D1 (B). The immunizing LDL preparation was used as LDL standard. The results (expressed as percent of LDL standard) were calculated from the curves using three or four points from the parallel parts of the curves.

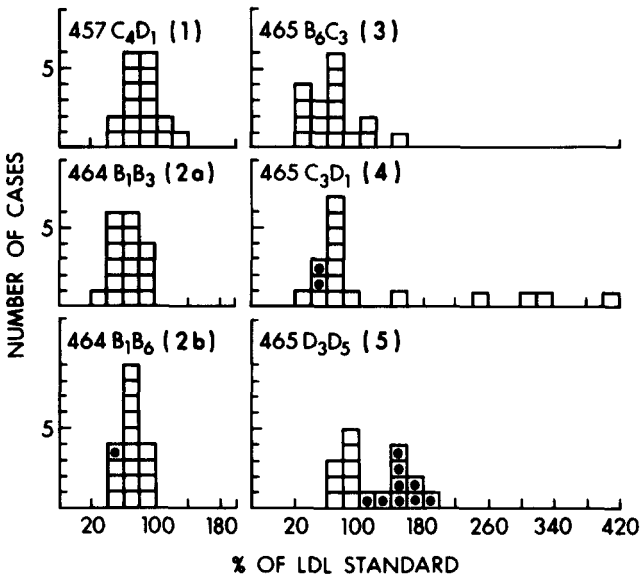

Fig. 2. Distribution of apparent apoB contents in 17 different LDL preparations. The apparent apoB contents were calculated from competitive displacement curves (see Fig. 1). The following means ± SD and ranges of values (in parentheses) as % of LDL standard were obtained: [1] 457C4D1: 82.5 ± 19.4 (50–127), [2a] 464B1B3: 66.7 ± 14.6 (44–96), [2b] 464B1B6: 68.8 ± 14.4 (44–95), [3] 465B6C3: 65.9 ± 32.3 (29–143), [4] 465C3D1: 136.9 ± 125.2 (35–413). The cases marked with black dots indicate apparent apoB content values estimated from displacement curves that were nonparallel with the curve produced by the LDL standard and were not included in the calculation of mean ± SD and range.

Relation of LDL lipid composition to immunoreactivity

The immunoreactivities of LDL preparations as determined by antibodies 2a, 2b, 3, and 4 correlated significantly and positively with their percentages of phospholipids (**Fig. 3B** and **Table 3**). On the other hand, immunoreactivity as determined by antibody 1 correlated inversely with percent triglyceride (Fig. 3A) and positively with percent total cholesterol. Calculations of the Spearman coefficients of rank correlation preserved the above-mentioned significant correlations, with the exception of the correlations between phospholipid percentages and immunoreactivities as determined by antibody 2b ($P = 0.085$) and antibody 4 ($P = 0.067$). The percentages of esterified or free cholesterol alone did not correlate significantly with immunoreactivity as determined by antibody 1. No apparent correlations between lipoprotein phenotype and LDL immunoreactivity were observed.

DISCUSSION

In competitive displacement assays in which antibodies were made to compete with each other for binding sites on LDL, we showed that at least five spatially independent epitopes on apoB could be defined with the antibodies used here (4). These epitopes could be selectively perturbed by different chemical modifications of lysyl or arginyl residues (4) and by limited proteolysis of LDL with trypsin, staphylococcal protease, and pronase (20). The results reported here indicate that even freshly isolated LDL particles may differ from each other in their immunoreactivities. Thus, with some antibodies (Fig. 2), the number of epitopes expressed varied from 30 to 400% of the LDL standard. With the exception of antibody 5, with which most LDL prepa-

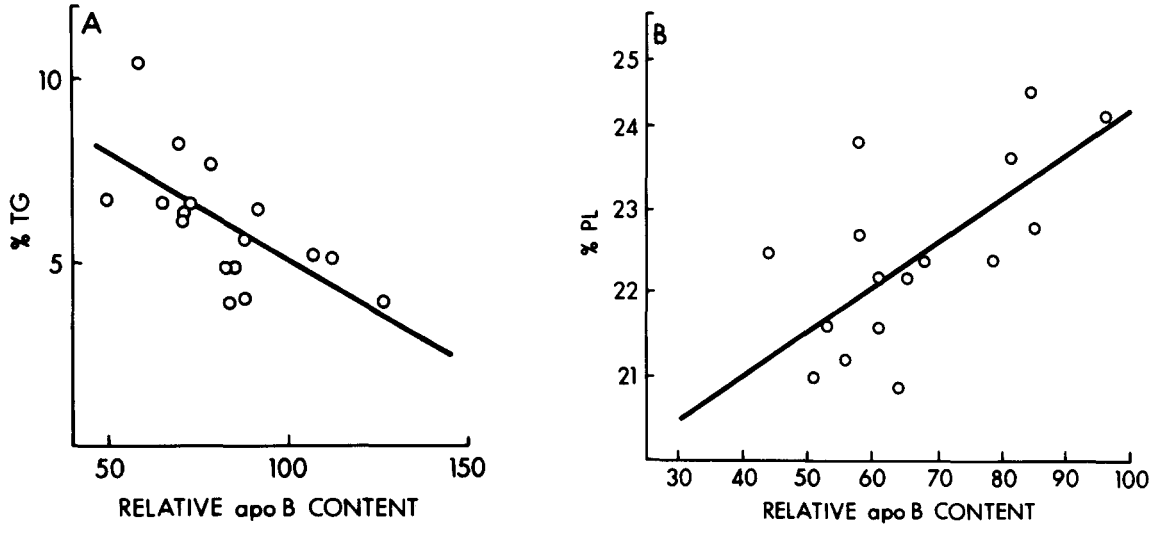

Fig. 3. Relations between relative apparent apoB content and percent composition in LDL preparations. A, Apparent apoB content as determined by antibody 475C4D1 [1] vs. percentage of triglyceride (n = 17): $y = -0.056 \times +10.62$. B, Apparent apoB content as determined by antibody 464B1B3 [2a] vs. percentage of phospholipid (n = 16): $y = 0.051 \times +18.98$. Correlation coefficients: see Table 3.

TABLE 3. Relation of apparent apoB content to lipid composition in 17 LDL preparations

Code	N	Identifying Number	%PL	%CHOL	%TG
1	17	457C4D1	0.00	0.65***	−0.63**
2a	16[a]	464B1B3	0.65**	0.03	−0.14
2b	17	464B1B6	0.51*	−0.16	0.02
3	17	465B6C3	0.71***	−0.25	−0.01
4	15[a]	465C3D1	0.54*	0.03	−0.10

Values are Pearson correlation coefficients. PL, phospholipid; CHOl, cholesterol; TG, triglyceride.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

[a] Data from nonparallel curves have been excluded.

rations produced nonparallel curves, and three other exceptions, the differences in immunoreactivity probably were not caused by altered affinities of interaction between LDL and antibodies but by altered number of epitopes expressed in the LDL population.

We sought to ascertain whether immunologic difference could be related to the compositions of the particles. With antibodies 2a, 2b, 3, and 4, the number of epitopes expressed correlated positively with the percentage of phospholipid in LDL (Table 3), whereas with antibody 1, it correlated inversely with percent triglyceride and positively with the percentage of cholesterol. Perhaps it should not be surprising that these correlations were found. Phospholipids are constituents of the surface layer of LDL and may be important in the "presentation" of epitopes for interaction with monoclonal antibodies; phospholipids indeed have been suggested to play an important role in the antigenicity of human LDL towards polyclonal antibodies (21). If the percentage of phospholipid is a measure of the particle surface area in the LDL population, the results could reflect the possibility that smaller particles with relatively greater surface to core ratios express a greater number of epitopes than do larger particles. Since the percentage of triglyceride and cholesterol were inversely related to each other, it is not clear which of the two influenced the immunoreactivity of LDL towards antibody 1. Nevertheless, it appears that changes in core constituents also may modify apoB epitopes. It may be important that some of the LDL displacement curves produced had slopes which differed from those produced by other LDL samples. Such differences may indicate that LDL samples may differ from each other not only in the number of epitopes expressed but also in their affinity. Subsequent studies will deal with the possible importance of this finding.

Since LDL is a complex of apoB and various lipid constituents, it is not possible from the above experiments to differentiate between apoB heterogeneity due to differences in protein structure and heterogeneity secondary to the influence of the lipids associated with apoB, although the profound effects on LDL immunoreactivity produced by chemical modification of amino acid residues and proteolytic cleavage of peptides (4, 20) are consistent with a central role for the protein moiety in the formation of epitopes. The potential effects of LDL lipids on epitope expression need to be studied by selective perturbation of the lipids of LDL.

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