Expression of a monoclonal antibody-defined aminoterminal epitope of human apoC-I on native and reconstituted lipoproteins

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Abstract Nine distinct mouse monoclonal antibodies were produced in two fusions using holo-human very low density lipoprotein (VLDL) as antigen. On immunoblotting first with human VLDL and then with isolated human apoC-I, seven of the antibodies, representing three isotypes, manifested specificity for apoC-I. Two antibodies were directed against apoB. To assess whether the seven anti-apoC-I antibodies were directed against the same or distinctively different epitopes, cross-competition assays were performed wherein ^{125}I-labeled monoclonal antibodies were made to compete with unlabeled antibodies for occupancy on immobilized VLDL-associated apoC-I. All antibodies cross-competed to varying extents implying that they were directed against closely spaced epitopes, but based on these experiments three different epitopes were defined. On immunoblotting with CNBr fragments, all of the epitopes were assigned to the CNBr I fragment of human apoC-I (amino acids 1–38) suggesting that the NH_2-terminal region of apoC-I is more immunogenic in mice than other parts of the molecule when apoC-I is associated with VLDL. A competitive solid-phase radioimmunoassay (RIA) was developed employing one of the anti-apoC-I antibodies (A3–4). VLDL was adsorbed to plastic microtiter wells, and a limiting amount of the antibody was reacted with the adsorbed VLDL. The amount of monoclonal antibody that bound to the immobilized VLDL-apoC-I was determined with a ^{125}I-labeled goat anti-mouse IgG antibody. The addition of competitor apoC-I complexed with lipids resulted in reduced binding of the anti-apoC-I antibody to the immobilized VLDL-apoC-I. Competitor complexes consisted of an artificial lipid emulsion (Intralipid^{R}) incubated with apoC-I at phospholipid/apoC-I ratios of 1:1 to 60:1 (w/w). As the lipid/protein ratios were increased, the competitive displacement curves produced by the complexes become progressively steeper, while isolated lipid-free apoC-I produced curves with very shallow slopes, suggesting that a conformation-dependent epitope was being probed. Other apoproteins (C-II, C-III, A-I, A-II, and E) whether lipid-free or complexed with lipids did not compete. Fractionation of the 30:1 apoC-I-Intralipid complex by gel permeation chromatography suggested that apoC-I bound to phospholipids was the most effective competitor. This was confirmed by testing of apoC-I-DMPC complexes, which yielded curves that paralleled those produced by apoC-I-Intralipid. VLDL and high density lipoprotein isolated from plasma by ultracentrifugation produced competiton curves that were qualitatively different from those produced by the reconstituted lipoproteins. These results suggest that the structure and composition of lipid particles exert a major effect on the expression of the aminoterminal epitope of the lipid-associated apoC-I recognized by monoclonal antibody A3–4.—**Krul, E. S., K. Oida, and G. Schonfeld.** Expression of a monoclonal antibody-defined aminoterminal epitope of human apoC-I on native and reconstituted lipoproteins. *J. Lipid Res.* 1987. **28:** 818–827.

Supplementary key words monoclonal antibodies • apoC-I • solid phase radioimmunoassay • phospholipid

Most apolipoproteins associated with lipoproteins are believed to interact with phospholipids on the surfaces of the particles. Apolipoprotein C-I (apoC-I), one of the low molecular weight apolipoproteins of VLDL and HDL, is synthesized as an 83-amino acid pre-protein that contains a 26-amino acid signal sequence (1). Mature apoC-I, which contains 57 amino acids (2, 3), has been reported to activate lecithin:cholesterol acyltransferase (4, 5), inhibit hepatic lipase (6, 7), and to affect cell growth (8). Thus much is known about the biochemistry of apoC-I, but little is known about its disposition on lipoproteins.

Monoclonal antibodies are useful in studying the structure–function relationships of many proteins including several apolipoproteins (9–16). Our aim was to study the epitope expression of apoC-I on lipoproteins using monoclonal antibodies. We report on the generation of seven monoclonal antibodies against apoC-I that define three distinct epitopes, assign the epitopes defined by these antibodies to the aminoterminal CNBr fragment of apoC-I, and describe a competitive solid-phase radiometric assay, employing one of the monoclonal antibodies. This assay sys-

Abbreviations: VLDL, very low density lipoproteins; PBS, phosphate-buffered saline; DMPC, dimyristoylphosphatidylcholine; IL, Intralipid^{R}; RIA, radioimmunoassay; HDL, high density lipoproteins.

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tem was used to probe the conformation of apoC-I on reconstituted lipid-protein complexes and on human lipoproteins isolated from plasma.

MATERIALS AND METHODS

Production of monoclonal antibodies against native VLDL

Female Balb/C mice (Jackson Memorial Laboratories, Bar Harbor, ME) were immunized with human VLDL ($d < 1.006$ g/ml), isolated by fixed angle rotor ultracentrifugation, from the plasma of a donor with homozygous familial hypercholesterolemia. One hundred μg of VLDL (as protein in 1 mM EDTA, 0.15 mM NaCl, pH 8.2, emulsified in complete Freund's adjuvant) were given subcutaneously, followed by two booster injections (in complete and incomplete adjuvant) at intervals of 2 weeks. The techniques for cell fusion and cloning have been previously described (9). Clones were expanded as ascites tumors. Antibody isotypes were determined on cell culture supernatants using isotype-specific antibodies (17).

Purification of antibodies

IgG_1 subclass monoclonal antibodies were purified from ascites fluid on a column (1 ml) of Protein A-Sepharose (Sigma, St. Louis, MO) with a step-wise pH gradient (18, 19). IgM subclass monoclonal antibodies were purified by sucrose density gradient ultracentrifugation (20). Isolated monoclonal antibodies showed single heavy and light chain bands on SDS-polyacrylamide gel electrophoresis. IgG_3 subclass antibody (1888 A5-1) was used as a 50% $(NH_4)_2SO_4$ precipitate dissolved in PBS.

Immunoassays on microtiter plates

Screening of hybridoma fluids for antibody activity was done by a sandwich binding assay on 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). First, 150 μl of VLDL (20 μg/ml) was added to each well. After 16 hr at 23°C, wells were rinsed with PBS (0.16 M NaCl and 0.05 M phosphate, pH 7.4), filled with PBS-3% bovine serum albumin (BSA) for 3 hr, and rinsed again with PBS. Then each well received 150 μl of hybridoma culture media or an antibody containing solution obtained by ammonium sulfate precipitation (50% of saturation) of ascitic fluid. After 16 hr at 23°C, each well was washed with PBS and incubated with ^{125}I-radiolabeled goat anti-mouse IgG (100,000 cpm in PBS-1% BSA well) for 4 hr at 23°C. Microtiter wells were rinsed with PBS, sliced, and counted in a Packard gamma spectrometer.

To determine whether the different antibodies bound to the same or different regions of apoC-I, ^{125}I-radiolabeled and nonlabeled antibodies were made to compete against each other for binding to insolubilized VLDL-apoC-I. Microtiter plates were coated with 150 μl of VLDL (20 μg/ml) overnight at 23°C. After rinsing with PBS and blocking with PBS-3% BSA, wells received increasing amounts of a nonlabeled antibody and a constant amount (150,000 cpm) of a ^{125}I-labeled antibody. Plates were then rinsed, sliced, and counted.

Gel electrophoresis and protein transfer blots

SDS-polyacrylamide gel electrophoresis was performed in 4 to 20% gradient gels using a vertical slab gel apparatus (14 × 12 × 0.15 cm) (Bio-Rad, Richmond, CA) as previously described (12). The lipoproteins were boiled at 100°C for 3 min in an electrophoresis sample buffer that contained 5.6 mM $CaCl_2$ to promote electrophoretic transfer of apoB to nitrocellulose paper as suggested by Curtiss and Edgington (10). Molecular weight markers were: bovine serum albumin, 66,000; ovalbumin, 45,000; α-chymotrypsinogen, 25,000; cytochrome c, 12,000; and porcine insulin, 6,000. After running, the gels were cut longitudinally into series of replicate lanes and one piece was stained with Coomassie Brilliant Blue R-250. Other gel strips were washed in a transfer buffer (25 mM sodium phosphate, pH 6.5), mounted in a Trans-Blot Cell (Bio-Rad) (21). Transfer to nitrocellulose (0.45 μm, Schleicher and Schuell, Keene, NH) was accomplished by electrophoresis at 27 V for 3 hr. Extra protein binding sites on the nitrocellulose papers were blocked with PBS-3% BSA plus 3% nonimmune rabbit serum for 1 hr. The nitrocellulose transfers were then incubated with an antibody-containing solution diluted in PBS-3% BSA overnight at 23°C followed by extensive washing with PBS. Antibody binding was localized by a second 4-hr incubation at 23°C with ^{125}I-labeled goat anti-mouse IgG (750,000 cpm/ml) in PBS-3% BSA followed by extensive washing with PBS containing 0.02% Tween-20. ^{125}I radioactivity was detected by autoradiography (X-Omat, Eastman Kodak) at −70°C.

Isolation of human apoC-I

Plasmas were obtained from fasting patients with types IV and V hyperlipoproteinemia. The VLDL were isolated by ultracentrifugal flotation (50.3 rotor, 45,000 rpm, 16 hr, 10 C, Beckman model L8-65 ultracentrifuge) at plasma density, washed once at d 1.006 g/ml (0.16 M NaCl, mM EDTA, pH 8.2), delipidated with ethanol–ether 3:1 (v/v) at 4°C and dissolved in 6 M urea, 100 mM Tris, pH 8.5. The apoproteins were separated by ion-exchange chromatography on a Mono Q^R column in the FPLC system (Pharmacia, Uppsala, Sweden) (22). The initial buffer consisted of 100 mM Tris, 6 M urea, pH 8.5; the terminal buffer was the same but it also contained 2 M NaCl. The shape of the gradient is shown in **Fig. 1.** This column procedure provided rapid (20 min/run), highly reproducible separations of the C-apolipoproteins on a preparative scale. This procedure also made it possible to quantify the apoC-I

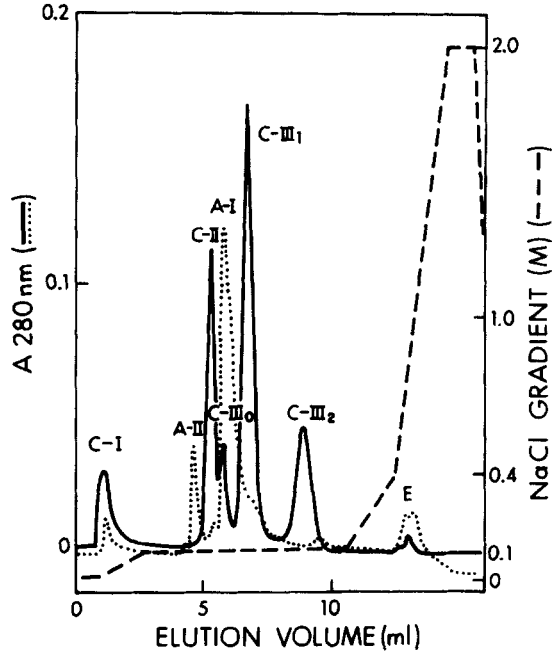

Fig. 1. Mono Q^R column chromatography of urea-soluble proteins of VLDL and HDL. VLDL and HDL were lyophilized, delipidated, and apoproteins were solubilized in 6 M urea, 100 mM Tris, pH 8.5. One hundred μg of total urea-soluble apo-VLDL (---) and apoHDL (···) were applied onto the column. Identification of each peak was made by isoelectric focusing polyacrylamide gel electrophoresis. Amino acid compositional analysis was also carried out for apoC-I as described in Methods.

contents of VLDL. The fractions containing apoC-I were pooled, dialyzed against 1 mM EDTA, pH 8.2, and lyophilized. The purity of the isolated apoprotein was confirmed by isoelectric focusing on polyacrylamide gels (stained with Coomassie Brilliant Blue-R250 or silver) and amino acid compositional analysis (3). To obtain the apoC-I contents of HDL, plasmas of normal subjects were subjected to ultracentrifugation twice at d 1.21 g/ml and once at d 1.063 g/ml. Conditions of delipidation of HDL and column separation of apoC-I were the same as for VLDL (Fig. 1).

Cleavage of apoC-I into two fragments, CNBr (amino acids 1–38) and CNBr II (amino acids 39–57), was performed according to the procedure described by Jackson et al. (2). After cleavage, the fragments were separated by electrophoresis in highly cross-linked polyacrylamide gels containing 0.1% SDS and 8 M urea as described by Swank and Munkres (23).

ApoC-I-lipid complexes

Lipid-protein complexes were made using Intralipid^R (IL) a commercially available phospholipid-triglyceride emulsion (Cutter Laboratories, Berkeley, CA), or dimyristoylphosphatidylcholine (DMPC, Sigma-Aldrich, St. Louis, MO). Intralipid was used neat or the triglyceride emulsion (WIL, see Fig. 7B) was washed by ultracentrifugation at d 1.006 g/ml as described by Granot et al. (24). DMPC vesicles were prepared by sonication as described by Roth et al. (25) using a buffer consisting of 0.05 M phosphate, 0.10 M NaCl, pH 7.4. To ascertain optimal apoC-I-Intralipid ratios, fixed amounts of isolated apoC-I (5–10 μg) were incubated with various concentrations of the lipid emulsion or DMPC vesicles for 16 hr at 4°C, then 2 hr at 37°C. Complexes then were diluted as necessary for assay. To obtain complexes for characterization ca. 100 μg of apoC-I was incubated with Intralipid or DMPC vesicles. The complexes then were loaded onto a 0.9 cm × 60 cm Sepharose CL 4B column. Eluted fractions were analyzed by photometry (absorbance at 210 nm or 280 nm) and for proteins and lipids.

Competitive solid-phase radioimmunoassay

Microtiter wells were coated with 100 μl of VLDL solution in PBS for 3 hr at 23°C. After rinsing twice with PBS, wells were filled with PBS–3% BSA for 30 min and rinsed again twice with PBS. Next, each well received 50 μl of apoC-I/DMPC complex, apoC-I/Intralipid complex, plasma, or lipoprotein solution, and then 50 μl of anti-apoC-I monoclonal antibody solution diluted to appropriate concentrations in PBS–1% BSA. After incubation for 2 hr at 23°C, plates were washed twice with PBS. Finally, ^{125}I-labeled goat anti-mouse IgG antibody (200,000 cpm/well) was added and incubated for 3 hr at 23°C. The wells then were rinsed with PBS containing 0.05% Tween-20, dried, sliced, and counted. Each data point was performed in duplicate or triplicate with coefficients of variation ranging 1–9% and averaging 4%. Where indicated, slopes and ED_{50} s were calculated by a four parameter logistic model method (26).

Other methods

Concentrations of proteins were determined by the procedure of Lowry et al. (27) using bovine serum albumin as a standard. Protein determination on column fractions was carried out using a modification of the Lowry method (27) as described by Bensadoun and Weinstein (28). Triglyceride and phospholipid were determined enzymatically using kits commercially available from Wako Fine Chemicals, Dallas, TX. ^{125}I-Labeling of lipoproteins, apoproteins, and antibodies was carried out using lactoperoxidase (29).

RESULTS

Immunological specificites of the antibodies

Nine cloned hybridoma cell lines that produced antibodies to human VLDL were obtained from two fusions (**Table 1**). Five clones produced IgG_1, one IgG_3, one IgG_{2b}, and two IgM antibodies.

On immunoblotting with VLDL and HDL, seven antibodies reacted only with a ~7,000 mol wt protein (**Fig. 2**).

TABLE 1. Specificities of monoclonal anti-VLDL antibodies

Hybridoma (Antibody)	Immunizing Antigen	Isotype	Apoprotein Specificity
1888 A3-4	VLDL	IgG_1	apoC-I
1888 A5-1	VLDL	IgG_3	apoC-I
1888 C7-3	VLDL	IgG_1	apoC-I
1888 D2-6	VLDL	IgG_1	apoC-I
1888 E2-5	VLDL	IgM	apoC-I
1888 F4-6	VLDL	IgM	apoC-I
1889 E6-4	VLDL	IgG_1	apoC-I
1888 C1-1	VLDL	IgG_1	apoB
1888 D7-1	VLDL	IgG_{2b}	apoB

Immunoglobulin chain types were determined immunochemically by an enzyme-linked immunosorbent assay using isotype specific antisera as described in the Methods.

Two reacted with apoB. On immunoblotting with purified apoC-I, each of the seven antibodies showed a positive reaction.

Enumeration and assignment of epitopes

To test whether the seven anti-apoC-I antibodies bound to the same or different epitopes on apoC-I, the ability of each antibody to compete with the other antibodies for binding to VLDL was assessed (**Table 2**). After VLDL was immobilized on microtiter plates, increasing amounts of unlabeled antibodies and a constant amount of labeled antibody were added to each well. Antibody 1888 A5-1 was not used in this experiment because it lost immunological activity upon purification on affinity columns. Every antibody competed against itself and with the other antibodies used at high doses, suggesting that the epitopes defined by the six antibodies tested are located close to each other. Patterns of reactivity for antibodies 1888 A3-1 and 1888 E2-6 were shown as identical with antibodies 1888 A3-4 and 1888 E2-5, respectively. Based on the antibody competition assays, at least three different epitopes appeared to be present on the CNBr fragment. Epitope 1 defined by antibodies A3-4 and D2-6; epitope 2 defined by C7-3 and E6-4; and epitope 3 defined by E2-5 and F4-6. The results of cotitration experiments (not shown) supported the conclusion that three different epitopes were being probed by anti-apoC-I-monoclonal antibodies. Immunoblotting studies using CNBr fragments of apoC-I confirmed that all epitopes were located close to each other, i.e., the epitopes defined by all

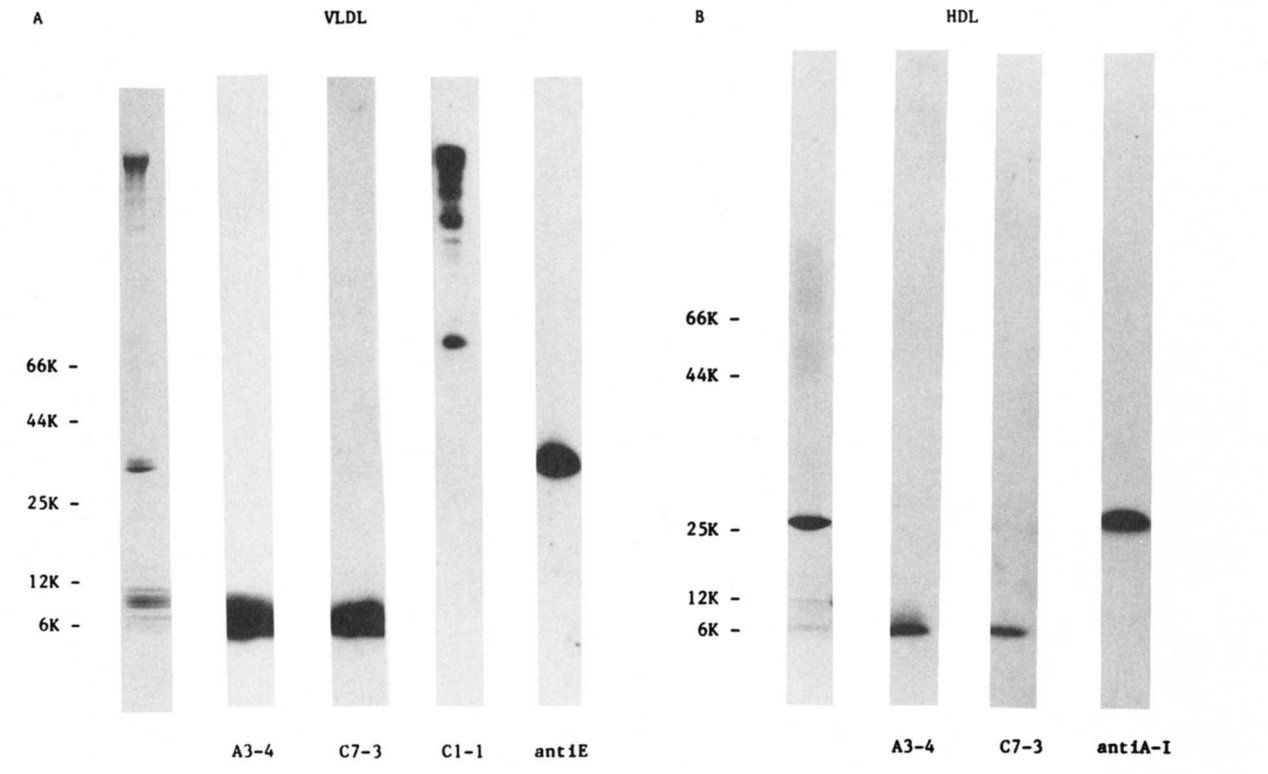

Fig. 2. Assessment of apoprotein chain specificity. A: VLDL (20 μg of protein) was electrophoresed in 4–20% polyacrylamide gradient slab gels containing 0.1% SDS. On the left is a gel stained with Coomassie Brilliant Blue R-250. The remaining panels are autoradiographs of identical nitrocellulose paper transfers that had been incubated with the indicated antibody-containing ascites fluids and ^{125}I-labeled goat anti-mouse IgG. The A5-1, D2-6, E2-5, F4-6, and E6-4 antibodies demonstrated similar patterns of reactivity on autoradiography as did antibodies A3-4 and C7-3 shown here. Autoradiographs obtained by incubation with monoclonal anti-apoB (Cl-1) and anti-apoE (1506A1-4) antibody are also shown for contrast. B: Experimental conditions were the same as Fig. 2A except HDL (20 μg of protein) was electrophoresed and transferred. The autoradiograph obtained by incubation with a monoclonal anti-apoA-I antibody (2451F3-6) antibody is shown for contrast.

TABLE 2. Cross-competition between anti-apoC-I antibodies

Competitor Antibody	Radiolabeled Antibody						
	A3-4	D2-6	C7-3	E6-4	E2-5	F4-6	
1888 A3-4 (A3-1)	3 +	3 +	1 +	3 +	±	1 +	Epitope 1
1888 D2-6	3 +	3 +	1 +	3 +	1 +	1 +	
1888 C7-3	±	2 +	3 +	2 +	-	-	Epitope 2
1889 E6-4	1 +	2 +	2 +	3 +	1 +	+	
1888 E2-5 (E2-6)	3 +	3 +	3 +	3 +	3 +	2 +	Epitope 3
1888 F4-6	3 +	3 +	3 +	3 +	3 +	3 +	

Competitive antibody binding assay using insolubilized VLDL and ^{125}I-labeled and unlabeled monoclonal anti-apoC-I antibodies as mutual competitors. Microtiter wells were coated with human VLDL. The unlabeled competitor antibodies were used in amounts of 0.1 ng to 10 μg/well. Numbers in parentheses (A3-1 and E2-6) are monoclonal antibodies derived from the same fusion and are probably subclones of the same parental cell line giving rise to 1888 A3-4 and 1888 E2-5, respectively. Results are expressed according to the extent of competition at the highest dose of the competitors; 3 + = > 80% displacement of Bo counts; 2 + = 60–80%; 1 + = 40–60%; ± = 20–40%; - = < 20%.

of these antibodies were located on the aminoterminal CNBr I fragment (amino acids 1–38) (**Fig. 3**).

Expression of an apoC-I epitope

We next wished to examine the expression of the apoC-I epitopes on lipoproteins. This is usually done in competitive assays using radioiodinated antigens (9, 15). However, because apoC-I contains no tyrosyl residues, it is difficult to label it with radioiodine. Therefore, a different assay configuration was developed in which radioiodinated antibodies could be used. In initial experiments, the optimal concentrations of VLDL (antigen) and antibody to be used in such an assay were determined (**Fig. 4**). Individual wells were coated with different concentrations of VLDL and incubated with a constant concentration (1.0 μg/ml) of purified antibodies. After incubation with ^{125}I-radiolabeled goat anti-mouse IgG, the radioactive bound counts were plotted against the concentration (μg/ml) of VLDL pro-

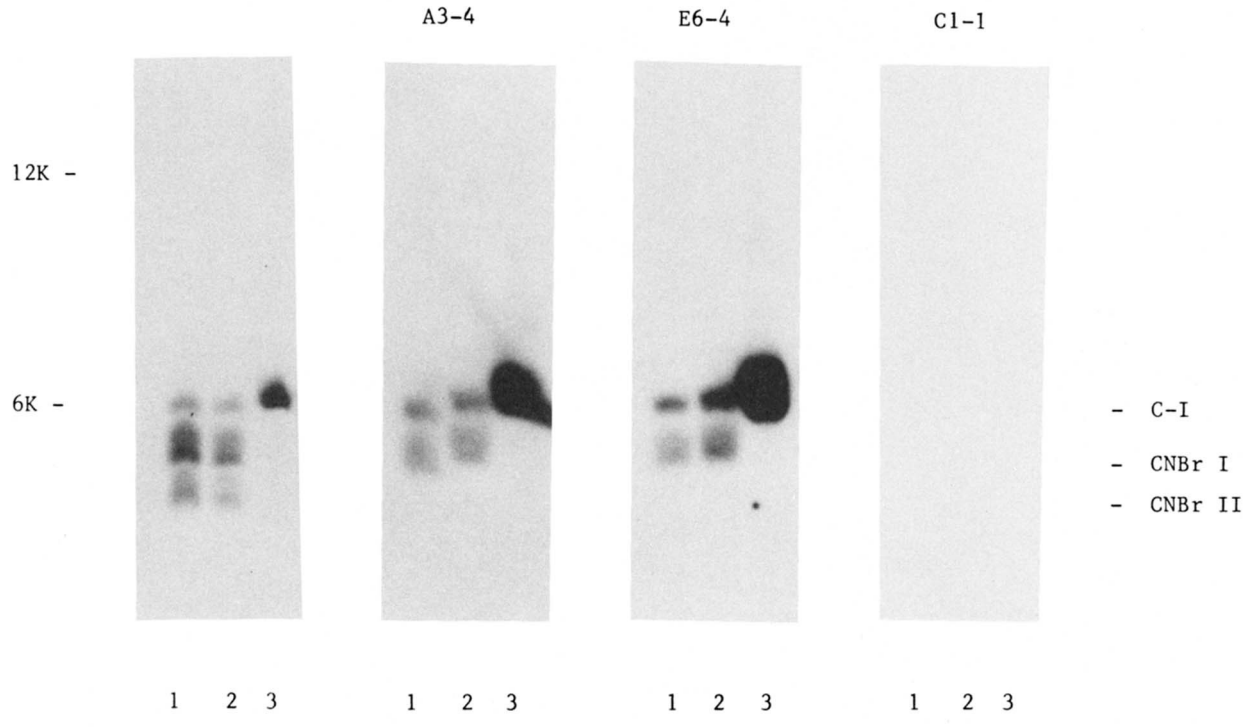

Fig. 3. Assignment of apoC-I epitopes. Isolated apoC-I (lane 3, 3 μg) and its cyanogen bromide cleavage fragments (lanes 1 and 2, 3 μg) were electrophoresed in 12.5% polyacrylamide (acrylamide–bisacrylamide 15:1) slab gels containing 0.1% SDS and 8 M urea. On the left is a photograph of a Coomassie Brilliant Blue R-250-stained gel. On the right are autoradiographs of identical nitrocellulose paper transfers that had been incubated with the indicated antibody containing ascites fluids and ^{125}I-labeled goat anti-mouse IgG. Each of the anti-apoC-I antibodies reacted with CNBr I (amino acids 1–38) as shown here. The negative autoradiograph obtained by incubation with a monoclonal anti-apoB antibody (Cl-1) is shown for contrast.

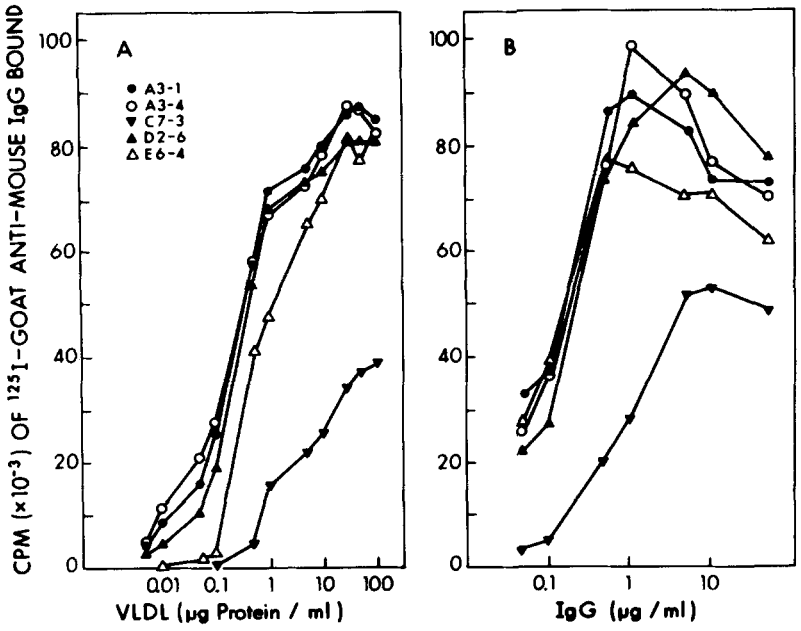

Fig. 4. Determination of optimal concentrations of VLDL and anti-apoC-I antibodies to be used in the competitive assays. A: Binding of single concentrations of anti-apoC-I antibodies (1.0 μg/ml) to polyvinylchloride plastic wells coated with the indicated concentrations of VLDL (as VLDL-protein). Binding was detected with ^{125}I-labeled anti-mouse IgG antibody. B: Binding of anti-apoC-I antibodies added at the indicated concentrations to wells coated with 30 μg/ml VLDL.

tein used for coating (Fig. 4A). The optimal coating concentration (30 μg/ml) was selected for further use. Next, several concentrations of the antibodies were incubated with a fixed concentration of VLDL (30 μg/ml) coated on wells and the samples were processed as described in Methods (Fig. 4B). The concentration of antibody A3-4 selected to be used in the radioimmunoassay was that at which 50–80% of maximal binding of antibody to insolubilized VLDL was obtained (e.g., 0.5 μg/ml). Antibody A3-4 was chosen for further use because it was one of the antibodies that appeared to bind to VLDL apoC-I with high avidity.

The specificity of antibody A3-4 versus apoC-I was reexamined using this RIA system. VLDL-coated wells were incubated with varying concentrations of competing purified apoA-I, A-II, C-II, C-III, E, or LDL ($1.025 < d < 1.050$ g/ml). None competed effectively (< 10% decrease in B/B_o) up to doses of 10 μg of total protein (data not shown). Purified lipid-free apoC-I also competed relatively poorly (**Fig. 5B**), but whole plasma and various density fractions did produce competition curves (Fig. 5A), suggesting that the A3-4 epitope was conformation-dependent. It should be noted that no apoC-I immunoreactivity was detectable in the $d > 1.21$ g/ml fraction of plasma.

To test the role of lipids in modulating the apparently conformational-dependent epitope on apoC-I recognized by monoclonal antibody A3-4, apoC-I-IL complexes were prepared. Virtually all lipid-apoC-I complexes yielded competition curves. The steepness of the slopes of the curves increased as the mass ratios of Intralipid-phospholipid/apoC-I rose from 1/1 to 60/1 (Fig. 5B). Neither IL itself nor IL complexed with purified apoproteins A-I, A-II, C-III$_o$, and E competed (< 10% decrease in B/B_o) at doses of protein up to 10 μg (data not shown). These results confirm that the antibody was directed only against apoC-I, that apoC-I and the other apoproteins tested did not share this epitope, and that the expression of the A3-4 epitope was increased by lipid binding.

In order to characterize the binding of apoC-I to Intralipid more completely, a 30/1 complex was applied to a gel permeation chromatography column (**Fig. 6A**). Virtually all of the triglycerides eluted in the void volume between fractions 16 and 24. Phospholipids eluted as a large peak (fractions 16–25) followed by a descending shoulder (fractions 25–35). Approximately 80% of the recovered protein was found in the lipid-containing peaks and the rest between fractions 47–53 apparently uncomplexed with lipids. Three pools were combined as indicated (Fig. 6A). They were assumed to contain apoC-I complexed primarily to triglyceride-rich particles and phospholipid-rich particles, and uncomplexed apoC-I, respectively. When these complexes were used as competitors, apoC-I associated with the phospholipid-rich particles was the most effective competitor (Fig. 6B), suggesting that apoC-I bound primarily to phospholipids may be more immunoreactive than apoC-I bound to triglyceride-rich particles. To test this more directly, DMPC vesicle-apoC-I complexes were formed and used in the immunoassay (**Fig. 7B**). Upon chromatography of the apoC-I-DMPC complex on the Sepharose CL4B

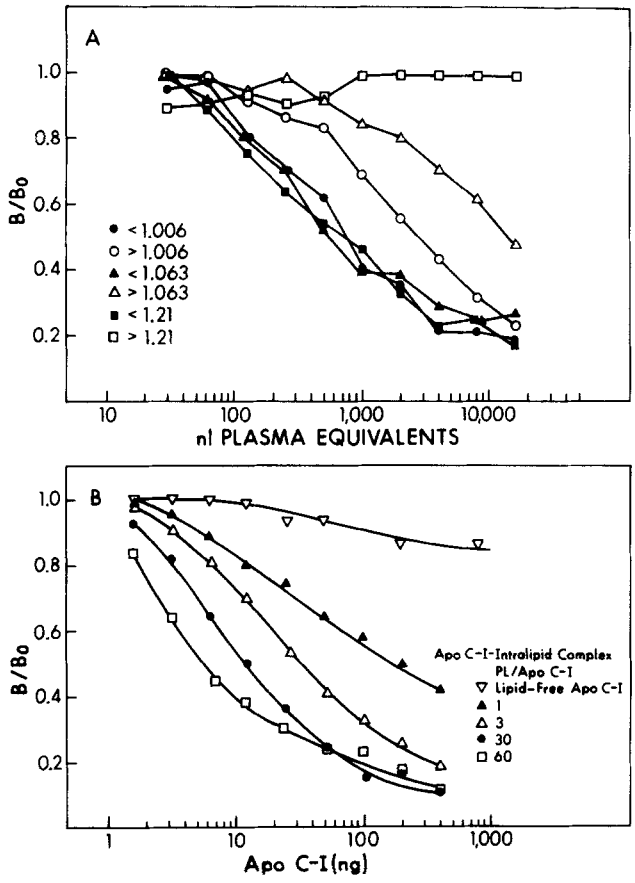

Fig. 5. Competition curves produced by plasma density fractions (panel A) and by lipid-free apoC-I and by apoC-I-Intralipid complexes (panel B). Antibody 1888 A3-4 was used. Plasma fractions were obtained by ultracentrifugation at the indicated densities (g/ml). Fractions then were diluted and added to the assay. Doses are expressed in relation to original plasma volumes. ApoC-I-Intralipid complexes prepared as described under Methods were added in doses related to apoC-I mass determined by Lowry protein assay. The numbers 1, 3, 10, and 60 indicate the mass ratios of Intralipid-phospholipid to apoC-I used in the preparation of the complexes.

column, virtually all of the phospholipids and apoC-I eluted between fractions 16 and 25, i.e., all of the apoC-I was complexed to lipid (Fig. 7A). The apoC-I-DMPC complexes yielded displacement curves that were similar in slope to those produced by the apoC-I-Intralipid complexes (Fig. 7B). The neutral detergent Tween-20 could not substitute for phospholipids. In fact, Tween-20 abolished the immunoreactivity of the apoC-I-lipid complexes (Fig. 6B).

Finally, in order to assess whether the expression of the A3-4 epitope on native lipoproteins and reconstituted particles was similar, the competition curves produced by isolated VLDL and HDL were compared to curves produced by the lipid-apoC-I complexes (Fig. 7B). The curves produced by the two native lipoproteins had slopes that were similar to each other but not to the slopes of the curves generated by the apoC-I-lipid complexes. Furthermore, apoC-I associated with VLDL was a more effective competitor in the assay than apoC-I associated with HDL, indicating a difference in apoC-I epitope expression on these particles.

DISCUSSION

This report represents one of a series of studies on the expression of epitopes of apoproteins on lipoproteins. To examine epitopes of apoC-I, seven monoclonal antibodies were produced which were found to be directed against at least three distinct epitopes located on the NH_2-terminal CNBr fragment of the molecule, representing amino acids 1–38 (Table 1 and Figs. 2 and 3). Since VLDL was the immunogen in all these cases, it appears that this portion of apoC-I is more immunogenic in Balb/C mice than is the COOH-terminal portion. Although it was not the prime aim of this study, we adapted existing methods for isolating apoC-I to the FPLC system and found the method suitable for both analytic and preparative scale isolation of VLDL or HDL apoC-I (Fig. 1).

Our original aims, in addition to probing the expression of apoC-I epitopes on lipoproteins, also included the development of an immunoassay for quantifying apoC-I in biologic fluids. ApoC-I has been quantified by radial immunodiffusion (30), electroimmunoassay (31), or enzyme linked immunoassay (ELISA) using *p*-nitrophenylphosphate-conjugated apoC-I as probe (32). Others have found ELISA difficult because conjugation of enzymes to low molecular weight proteins such as apoC-I may change the immunological activity. Total loss of immunological activity of apoC-I by coupling with alkaline phosphatase was observed by Holmquist (33). An RIA for human apoC-I has not been reported even though electroimmunoassay and radial immunodiffusion are generally less sensitive than the RIA and require relatively large amounts of antibody, probably because for competitive RIAs protein antigens are most frequently iodinated in their tyrosine residues and apoC-I contains no tyrosines (2). Since other methods of iodination require the covalent coupling of radiolabeled adducts that could interfere with the immunoreactivity of a protein as small as apoC-I, we chose to use an assay in which antibodies are labeled. When two monoclonal antibodies that define distinctively different epitopes are available, a a noncompetitive two-site immunometric assay is a good choice for protein assays (34). However, the epitopes defined by our seven anti-apoC-I monoclonal antibodies seem to be located close to each other. For all these reasons, it seemed that a two-step immunoradiometric assay (35) would be a suitable technique for quantifying apoC-I. In this method, neither the antigen nor the antibody need be modified, and although large amounts of antigen are required for immobilization on plates, relatively crude preparations (e.g., VLDL rather than pure apoC-I) can be used. We chose initially to use only one antibody and selected one that appeared to bind VLDL with high avidity (Fig. 4B).

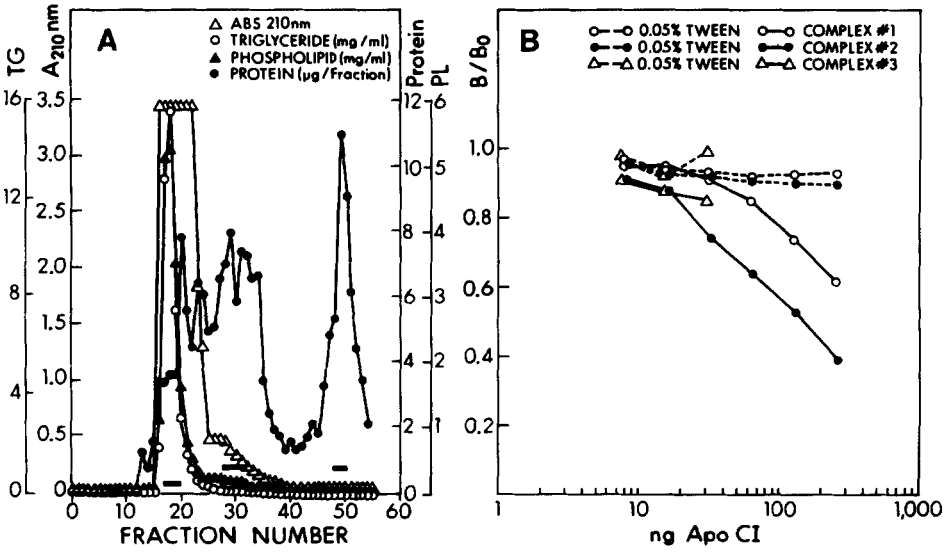

Fig. 6. Gel permeation chromatography of the 30/1 apoC-I-Intralipid complex. Three mg of Intralipid (as phospholipid) was incubated with 0.1 mg of apoC-I and then loaded onto a 0.90 × 60 cm column of Sepharose CL 4B. Ninety-one percent of the loaded protein was recovered in the fractions. The elution fractions indicated by the horizontal bars were pooled as complex #1 (fractions 16–20), complex #2 (fractions 28–32), and complex #3 (fractions 48–51). The mass ratios of phospholipid to protein in the three pooled fractions were 42, 11, and 16, respectively. B: Competition curves produced by apoC-I-lipid complexes #1, #2, and #3 obtained by gel permeation chromatography (see Fig. 6A) in the absence or presence of 0.05% Tween-20 (indicated by dashed lines). Antibody 1888 A3-4 was used. Complex #2 is the most effective competitor of antibody binding to the insolubilized VLDL apoC-I. Addition of Tween-20 (final concentration 0.05% w/v) abolished the ability of apoC-I on the complex to compete. At 0.05% Tween-20 did not inhibit the binding of ^{125}I-labeled anti-mouse IgG to mouse IgG (not shown, i.e., Bo values were nearly identical in the presence and absence of Tween-20).

When this assay was employed it became clear that lipid-free apoC-I added to the assay was a very poor competitor versus immobilized VLDL for binding of limiting amounts of antibody A3-4 (Fig. 6B), whereas the apoC-I present in lipoprotein fractions of plasma did compete (Fig. 6A). Compatible results were obtained by Wong (36) and Wong et al. (37) in an anti-rat apoC-I monoclonal antibody system. Why lipid-free apoC-I transferred to nitrocellulose

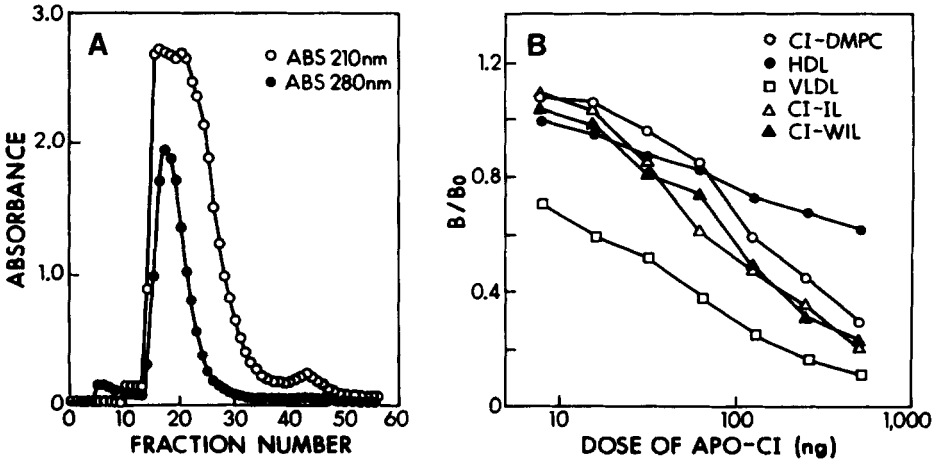

Fig. 7. A: Gel permeation chromatography of apoC-I-DMPC mixture. For conditions see Fig. 6A and Methods. Fractions 15–23 were pooled and had a mass ratio of phospholipid to apoC-I of 30/1. B: Competition curves produced by apoC-I-DMPC, apoC-I-Intralipid (CI-IL), and "washed" Intralipid (CI-WIL). Antibody 1888 A3-4 was used. ApoC-I was incubated with each of the lipid preparations at a final phospholipid to protein ratio of 30/1. The apoC-I contents of VLDL and HDL were determined after separation of the soluble apoproteins on the FPLC Mono Q^R column as described in Methods. The displacement curves produced by the apoC-I-lipid complexes were similar to each other. (Slopes were −1.21 ± 0.09, −1.21 ± 0.10, and −1.13 ± 0.09 for apoC-I-DMPC, CI-IL, and CI-WIL, respectively.) However, VLDL and HDL curves had shallower slopes (−0.82 ± 0.06 and −0.75 ± 0.05 for VLDL and HDL, respectively) and the apoC-I on HDL competed less effectively than the apoC-I on VLDL. The calculated ED_{50}s for HDL and VLDL, respectively, were 1158 ± 134 ng and 33.1 ± 3.0 ng of apoC-I.

membranes did react with the anti-apoC-I antibodies in our studies (Figs. 2 and 3) is not entirely clear. A possible explanation is that lipid-free apoC-I exists in an oligomeric association (38) and thus has a conformation unsuitable for antibody binding, whereas in the presence of SDS and reducing agents in polyacrylamide gels the apoC-I may have been transformed to a monomeric, accessible configuration which then bound to the nitrocellulose. However, this observation may simply reflect differences in the sensitivity between the immunoblotting and solid phase radioimmunoassay technique. Nevertheless, these results are consistent with the concept that the epitope defined by antibody A3-4 is conformation-dependent. Since VLDL-associated apoC-I demonstrated significant immunoreactivity and VLDL are triglyceride-rich lipoproteins, Intralipid, a triglyceride-rich phospholipid emulsion was used initially to form apoC-I-lipid complexes. As the lipid to protein ratio of the Intralipid and apoC-I mixtures was increased, competitive curves became progressively steeper, indicating that the apoC-I in the mixture was becoming increasingly homogeneous in its reactivity, i.e., increasing proportions of apoC-I in the solutions were becoming effective competitors for antibody binding. To assess the lipid associations of apoC-I, the 30/1 complex was subjected to gel permeation chromatography. Virtually all of the triglycerides and the vast majority of the phospholipids eluted in the void volume, but a small peak of phospholipids eluted as a shoulder, indicating a population of smaller phospholipid-rich vesicles present in the Intralipid emulsion. About 80% of apoC-I was associated with lipids. Of interest was that, on a mass basis, the apoC-I associated with the phospholipid-rich fraction was the most immunoreactive (Fig. 6B). The effectiveness of phospholipid in enhancing the immunoreactivity of apoC-I was confirmed by the competitiveness of the apoC-I-DMPC complexes (Fig. 7). Thus, the competition curves yielded by the whole apoC-I-Intralipid mixtures probably represented mean curves produced by the heterogeneous mixture of apoC-I-Intralipid particles (shown in Fig. 6B), with the phospholipid-rich particles having the highest competitive ability, the triglyceride-rich particles being 3–4 times less effective, and the lipid-free apoC-I being noncompetitive.

ApoC-I is largely localized on the surfaces of lipid complexes, at the phospholipid head group region (39, 40) and apoC-I associated with lipids is less flexible than lipid-free apoC-I (40). The CNBr I fragment, which contains the epitopes recognized by our antibodies, is the fragment largely responsible for phospholipid binding (41). Our data suggest that interaction of apoC-I with lipids renders the CNBr I region both immunogenic and antigenic.

Finally, one requirement for assays of apoproteins in biologic solutions by competitive immunoassay is that the competition curves of standards and lipoprotein (or plasma) samples be parallel. The curves for the apoC-I-Intralipid and apoC-I-DMPC complexes on the one hand, and VLDL and HDL on the other were found not to be parallel (Fig. 7B) suggesting that the range of heterogeneities of epitope expression on the reconstituted and native particles was not the same. It is interesting to note that apoC-I on HDL appears to be less available to bind to antibody than the apoC-I on VLDL (Fig. 7B). It would be interesting to determine whether apoC-I on HDL is "masked" by *a)* having a very different conformation due to a smaller particle diameter of HDL versus VLDL, i.e., phospholipid head group packing is altered; or *b)* the presence of other apoproteins that interfere with the antibody-binding epitopes. More work is needed in reconstituting particles that produce competition curves consistently paralleling those of native lipoproteins. This will help us to discover the compositional and structural determinants of lipid particles that may result in a more "native" disposition of apoC-I on their surfaces and perhaps provide suitable standards for assays.

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