Heterogeneity of apolipoprotein E epitope expression on human lipoproteins: importance for apolipoprotein E function

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Abstract The conformations of apolipoproteins on the surfaces of lipoprotein particles affect their physiologic functions. The conformations of apoE on plasma lipoproteins were examined using a panel of eight anti-apoE monoclonal antibodies (MAbs). The antibodies, which reacted with the major isoforms of apoE (E2, E3, and E4), defined at least five epitopes on apoE. Proteolytic fragments and synthetic peptides of apoE were used in binding assays to assign antibody epitopes; the epitopes were all localized to the middle third of the apoE molecule. The expression of apoE epitopes on isolated apoE and on lipoproteins was probed in competitive microtiter plate immunoassays using the anti-apoE MAbs, ^{125}I-labeled apoE as tracer, and isolated apoE, intermediate density (IDL), very low density ($VLDL_{1-3}$), and high density (HDL_2 and HDL_3) lipoproteins as competitors. The antibodies determined the patterns of competition exhibited by the lipoprotein preparations. Antibodies of the IgM class (WU E-1, WU E-2, WU E-3) defined two sets of conformation-dependent epitopes that were assigned towards the middle and the carboxyl terminal of the middle third of apoE. Competition curves using these antibodies, apoE, and lipoproteins showed a large variability in ED_{50} values. MAbs WU E-4, WU E-7, and WU E-10 defined epitopes near the receptor recognition site on apoE. Competition curves demonstrated small ranges of ED_{50} values. MAbs WU E-11 and WU E-12, which defined epitopes toward the amino-terminal region of apoE, exhibited competition curves for apoE and lipoproteins that had consistent, but wider ranges of ED_{50} values. There was no strict relationship between lipoprotein flotation rates and epitope expression for any of the MAbs. Immunoaffinity chromatography of VLDL subfractions on four different MAb columns indicated that the differences in the competitive abilities of VLDL subfractions were partly due to heterogeneity of apoE epitope expression within any population of particles. VLDL particles specifically retained on two different anti-apoE MAb columns were better competitors than unretained fractions for ^{125}I-labeled LDL binding to the apoB,E-receptor of cultured human fibroblasts, suggesting that increased accessibility of apoE on the surface of VLDL is associated with increased receptor recognition. These data suggest that individual epitopes of apoE can be modulated; epitope expressions are not determined solely by the sizes and/or densities of lipoprotein particles; and differences in apoE conformation have significant metabolic consequences. — **Krul, E. S., M. J. Tikkanen, and G. Schonfeld.** Heterogeneity of apolipoprotein E epitope expression on human lipoproteins: importance for apolipoprotein E function. *J. Lipid Res.* 1988. **29:** 1309–1325.

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Apolipoproteins stabilize lipoprotein structures, serve as recognition sites for cellular receptors, and modulate the activities of enzymes important in lipid metabolism. The expression of any given domain of an apolipoprotein may not be identical on the surfaces of different lipoproteins (1–5) and this may significantly influence apolipoprotein function (3, 5). Changes in the conformations and mobilities of apolipoproteins on lipoproteins could be produced frequently and rapidly during metabolic events that alter the sizes or compositions of lipoproteins (6–11).

The critical role of protein conformation in the function of apoE was demonstrated by Innerarity et al. (12, 13), who showed that apoE was bound to the B,E-receptor only when complexed to phospholipids (12). More subtle differences in the surface dispositions of specific domains of apoE probably account for the observation that the apoE molecules associated with the large $VLDL_1$ particles isolated from plasmas of hypertriglyceridemia patients interact more readily with cellular LDL (apoB,E) receptors (5, 14) and thrombin (3) than apoE associated with the smaller $VLDL_2$ or $VLDL_3$ particles (5, 14). The mutant apoE2 protein (cys for arg substitution at residue 158) exhibits poor binding to apoB,E-receptors apparently because the amino acid substitution distorts the receptor recognition domains of the mutant molecules relative to apoE3.

Abbreviations: apoE, apolipoprotein E; $VLDL_1$, very low density lipoprotein, S_f 120–400; $VLDL_2$, S_f 60–120; $VLDL_3$, S_f 20–60; IDL, intermediate density lipoprotein (d 1.006–1.019 g/ml); HDL_2 and HDL_3, high density lipoprotein, d 1.063–1.125 g/ml and d 1.125–1.21 g/ml, respectively; PBS, 0.01 M phosphate-buffered saline, pH 7.4; BSA, bovine serum albumin; BBS, 50 mM borate-buffered saline, pH 7.4; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean; MAbs, monoclonal antibodies; NIRS, non-immune rabbit serum.

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Gross changes in protein conformation are detectable by physical measurements such as circular dichroism, but discrete, localized alterations of protein structure can be detected best by sensitive immunochemical or functional assays. Immunochemical assays employing monoclonal antibodies are particularly useful for monitoring subtle conformational changes because they are capable of detecting alterations in the expression of specific and definable epitopes (1, 2, 4, 8, 15). To investigate apoE conformation on the surfaces of the major plasma lipoproteins, a panel of eight monoclonal antibodies was generated that defines five distinct epitopes on apoE. The epitopes probed with these antibodies exhibited varying degrees of heterogeneity of expression on the major classes of human lipoproteins. Lipoproteins expressing specific apoE epitopes that were bound by monoclonal antibodies on affinity columns showed increased apoB,E-receptor binding activity relative to unbound particles. This indicates that apoE epitope expression has distinct metabolic consequences.

MATERIALS AND METHODS

Materials

$Na^{125}I$ (IMS 30) was obtained from Amersham, Arlington Heights, IL; guanidine hydrochloride from Pierce Chemical Co., Rockford, IL; affinity purified goat anti-mouse IgG from Jackson Immunoresearch Laboratories, Avondale, PA; heparin-agarose (approx. 800 μg heparin from porcine intestinal mucosa/ml gel), cyanogen bromide-activated Sepharose 4B, and Sephadex G25-150 from Sigma Chemical Co., St. Louis, MO; urea (ultrapure) from Schwarz/Mann Biotech, Cleveland, OH.

Production and purification of monoclonal antibodies

Anti-human apoE monoclonal antibodies were produced by immunization of female Balb/c mice either with purified apoE (50–150 μg per subcutaneous injection) or with the major protein peak of apoHDL obtained after G-200 chromatography (100 μg per injection). Initial injections of antigen were made with complete Freund's adjuvant followed at 3–4-week intervals by injections in incomplete Freund's adjuvant. Injections were performed three times for apoE and seven times for the apoHDL fraction. Antibody titers of mouse sera were tested 5–10 days after the last subcutaneous injection. Approximately 2 weeks later those mice having strong titers were injected with either 50 μg apoE (intraperitoneally) or 10 μg apoHDL (intravenously). Splenic cell fusions were performed 3 days later using SP2/O-Ag14 mouse myeloma cells (16). Hybridoma culture supernatants were screened for immunoreactivity with their respective immunogens by either solid phase antigen or direct binding assays (16). Positive clones were subcloned, retested for activity, and expanded as ascites tumours in Pristane (2-6-10-14 tetramethyl-pentadecane, Sigma, St. Louis, MO) -treated Balb/c mice. Ascites fluids were cleared by centrifugation and stored in aliquots at −70°C. Immunoglobulin isotypes were determined in spent culture media by an ELISA inhibition assay using purified mouse immunoglobulins as positive controls (17). IgM monoclonal antibodies were purified from redissolved $(NH_4)_2SO_4$ (50% saturation) precipitates of ascites fluids either by sucrose density ultracentrifugation (18) or gel filtration on a FPLC Superose 6^R column (Pharmacia, Uppsala, Sweden). Monoclonal IgG antibodies were purified by ion exchange chromatography on the FPLC Mono Q^R column (19). These antibodies gave rise to three to five narrow bands upon isoelectric focusing in agarose. The monoclonal antibodies described in this study are listed in **Table 1.**

Preparation of apoE and apoE fragments

Blood was obtained from 10- to 12-hr fasted normolipidemic and hypertriglyceridemic donors in 0.1% (w/v) EDTA. Red blood cells were removed by centrifugation and phenylmethylsulfonylfluoride (PMSF) (10 μM), chloramphenicol (50 mg/l), gentamycin sulfate (50 mg/l), and sodium azide (0.02%) were added to the plasma. Lipoprotein isolations were begun within several hours of blood collection. Lipoproteins were isolated at the following densities: VLDL, d<1.006 g/ml; IDL, 1.006–1.019 g/ml; LDL, 1.019–1.063 g/ml; and HDL, 1.063–1.21 g/ml (20). Two ultracentrifugations were performed at each density.

ApoE was isolated from VLDL obtained from hypertriglyceridemic individuals. Delipidated VLDL was applied to either a Sephadex G-200 or an FPLC Superose 12 column and peaks corresponding to apoE were pooled, dialyzed against 5 mM NH_4HCO_3, lyophilized in aliquots, and stored at −70°C (21). In some cases apoVLDL was applied to heparin-agarose columns in 6 M urea, 5

TABLE 1. Anti-human apoE monoclonal antibodies

Monoclonal Antibody	Fusion Number	Immunogen	Isotype
WU E-1, 2, 3[a]	827	ApoE	IgM
WU E-4	1506	ApoHDL	IgG_1
WU E-7	LRC9	ApoE	IgG_1
WU E-10	LRC11	ApoE	IgG_1
WU E-11, 12[a]	LRC11	ApoE	IgG_1

[a]These two sets of monoclonal antibodies consist of distinct antibodies (WU E-1, WU E-2, WU E-3 and WU E-11, WU E-12, respectively) but are grouped together since the antibodies in each set exhibit very similar apoE binding characteristics (see text) and since each set of antibodies was obtained from a single fusion.

mM Tris, pH 8.0, and eluted with a gradient of 0–1 M NaCl to obtain apoE (22) prior to Sephadex G-200 or FPLC chromatography.

Cyanogen bromide-derived fragments of apoE were prepared as follows. ApoE was dissolved in 70% formic acid at a concentration of 1 mg/ml. A 30-fold excess (w/w) of cyanogen bromide was added and incubated for 24 hr at room temperature. The reaction mixture was then diluted with 10 volumes of deionized water and lyophilized. Cleavage products were dissolved in SDS-PAGE sample buffer for electrophoresis on 15–25% polyacrylamide gels under reducing conditions. Purified fragments of apoE obtained by proteolysis with *Staphylococcus aureus* V8 protease and synthetic fragments of apoE were kindly provided by Dr. Karl Weisgraber, Gladstone Foundation, San Francisco, CA (23).

Preparation of lipoprotein subfractions

VLDL and HDL subfractions were prepared by rate zonal ultracentrifugation (24, 25) from both hypertriglyceridemic and normolipidemic individuals. Subfractions of VLDL were isolated from d < 1.006 g/ml fractions of plasma and subfractions of HDL were isolated from whole plasma (24, 25). Fractions of VLDL isolated by zonal ultracentrifugation were pooled as follows: $VLDL_1$, (S_f 120–400); $VLDL_2$, (S_f 60–120); $VLDL_3$, (S_f 20–60). All pooled fractions were dialyzed against 1 mM EDTA, 0.15 M NaCl, pH 8.2, and concentrated by dry dialysis against Aquacide IIA (Calbiochem-Behring Corp., American Hoechst Corp., CA), sterilized by filtration (0.45-μm filters, Gelman, Ann Arbor, MI), stored under N_2 at 4°C, and used within 1–2 weeks of preparation.

Protein electrophoresis and Western blotting

Two-dimensional polyacrylamide gel electrophoresis was performed according to the methods of Zannis et al. (26). Protein was visualized on the gels with Coomassie Blue. Proteins on replicate, unstained gels were electrophoretically transferred to nitrocellulose (27) in a Trans-Blot cell (Bio-Rad, Richmond, VA) using 25 mM PO_4 buffer, pH 6.5, at 10°C for 2–3 hr at 27 V. After drying, nitrocellulose transfers were blocked with 3% BSA-PBS and incubated overnight (23°C) with the monoclonal antibodies in 3% BSA-PBS containing 3% nonimmune rabbit serum (NIRS). Transfers were then washed with PBS (50–100 ml per transfer) using an Omniblot System (American Bionetics, Emeryville, CA) and incubated with ^{125}I-labeled goat anti-mouse IgG (7.5×10^5 cpm/ml) in 3% BSA-PBS, 3% NIRS for 4 hr. Immunoblots were then washed with PBS and then PBS containing 0.02% Tween-20 as described above. Blots were dried and exposed to Kodak X-Omat AR X-ray film at −70°C.

Solid phase immunoassays

Antibody versus antibody competition assays. To enumerate the number of epitopes of apoE recognized by the monoclonal antibodies, the wells of polyvinylchloride microtiter plates were coated with 150 μl of VLDL (~5 μg protein). Inhibition of binding of ^{125}I-labeled antibodies to VLDL by unlabeled antibodies (1–100,000 ng/ml) was performed as previously described (16).

Solid phase assays using immobilized apoE or apoE fragments to determine the epitope specificities of monoclonal antibodies. Microtiter wells (Dynatech Laboratories Inc., Alexandria, VA) were coated with 150 μl of 1 μg/ml solutions of either apoE or peptide fragments of apoE in 5 mM glycine, pH 9.2, overnight at 23°C. The wells were washed with PBS and additional protein binding sites on the wells were blocked with 3% BSA-PBS for 1–2 hr at 23°C. Dilutions (1–50 μg/ml) of the various monoclonal antibodies in 1% BSA-PBS were added to the wells and incubated overnight at 23°C. After rinsing the wells with PBS, 150,000 cpm of affinity purified ^{125}I-labeled goat anti-mouse IgG in 1% BSA-PBS was added to each well and incubated for 4 hr. Plates were then washed, dried, and individual wells were counted for radioactivity.

Competition assays using ^{125}I-labeled apoE. Microtiter wells were coated with purified monoclonal antibodies (in 150 μl PBS, 3.75 μg for the IgM and 1.5 μg for the IgG antibodies). The competition between ^{125}I-labeled apoE and unlabeled apoE or intact apoE-containing lipoproteins was assessed in assays essentially the same as those performed for apoB monoclonal antibodies (15). Competition curves produced in a single monoclonal antibody assay were analyzed simultaneously using the four-parameter logistic model for multiple curve-fitting on a Digital VAX/VMS mainframe computer (ALLFIT) (28).

Immunoaffinity chromatography

Immunoglobulin from approximately 10 ml of ascites was precipitated with 50% saturated $(NH_4)SO_4$ and coupled to 4 ml of CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) according to the recommendations of the manufacturer. Columns containing the affinity matrix (1 bed volume) were placed above and joined in series to columns containing three bed-volumes of Sephadex G-25 (29). Before use, the columns were washed extensively with 50 mM borate-buffered saline (BBS), pH 7.5, "conditioned" with 1% BSA-BBS and washed with 3 M KSCN, 5 mM Tris-HCl, pH 7.2. Columns constructed with uncoupled Sepharose 4B, when treated similarly, did not bind lipoproteins nonspecifically. Finally, the columns were re-equilibrated with BBS. Lipoproteins were applied to the antibody-containing columns and allowed to penetrate the gel bed. The lipoproteins were then incubated with the affinity matrices overnight at 4°C. Unbound protein was eluted from the affinity and desalting columns

with BBS until no protein was detectable in the eluate as indicated by spectrophotometry at 280 nm. Bound proteins were eluted with 0.75–1.0 bed volumes of 3 M KSCN, 5 mM Tris-HCl, pH 7.2, followed by 4–5 bed volumes of BBS. Eluted proteins, essentially free of thiocyanate, were dialyzed against EDTA/saline, pH 7.4.

All affinity columns were run simultaneously by connecting the outlet tubing to a Technicon Autoanalyzer pump run at 0.4 ml/min. Samples were collected using an Isco Retriever IV fraction collector equipped for multicolumn collection.

Capacities of affinity columns (4-ml columns) were determined by applying increasing amounts of normolipidemic plasma onto the columns and determining the amount of protein maximally bound to the columns. All columns showed saturable binding characteristics, achieved between 2–5 ml of plasma, and recoveries of applied plasma proteins (flowthrough + retained) averaged 89.7 ± 2.0% (n=3). The maximum doses of total protein retained were 1035, 1400, 1510, 1480, 1190, and 9735 μg for antibody-containing columns R182, WU E-1, WU E-4, WU E-7, WU E-11 and the heparin-agarose column, respectively. The respective amounts of apoE retained [as determined by an RIA containing polyclonal antiapoE antiserum (21)] were 97, 105, 77, 121, 86, and 197 μg.

Inhibition of ^{125}I-labeled LDL cell association by affinity isolated VLDL subfractions

Human fibroblasts (GM0203) (30) were grown in culture dishes as previously described (5) and experiments were begun after growth in lipoprotein-deficient serum (LPDS) for 48 hr. VLDL subfractions isolated by affinity chromatography (retained or unretained) were added at various concentrations to the cell monolayers in media containing 10% LPDS, simultaneously with a constant amount (5 μg/ml) of ^{125}I-labeled LDL obtained from a normolipidemic donor. Incubations of the lipoproteins with cells were carried out at 37°C for 4 hr. At the end of the incubations, the fibroblasts were washed as described (31), dissolved in 0.1 M sodium hydroxide, and aliquots were taken to determine total cell protein and cell-associated radioactivity. Nonspecific binding (cell association) was determined as being the amount of cell-associated radioactivity after incubation of ^{125}I-labeled LDL in the presence of 50-fold excesses of nonlabeled homologous lipoprotein.

Miscellaneous methods

Proteins were determined by the method of Lowry et al. (32) using bovine serum albumin as standard. Phospholipids, free and esterified cholesterol, and triglycerides were determined enzymatically using commercially available kits (Wako Fine Chemicals, Dallas, TX). Lipid compositions of VLDL and HDL subfractions were comparable to those published previously (24, 25). ApoE contents of lipoproteins were quantified by a double antibody radioimmunoassay using a polyclonal anti-apoE antiserum in which the lipoproteins or apoE were pretreated with 4–8 M urea for 2 hr at 37°C to expose all apoE epitopes (21). Within assay %C.V. was 13.7%. SDS-polyacrylamide gel electrophoresis and amino acid compositional analyses were used to confirm the purity of the apoE preparations used as standards and tracers in these assays. ApoE was radiolabeled to specific radioactivities of 3000–8000 cpm/ng for use in the polyclonal radioimmunoassays as well as the monoclonal antibody immunoassays using lactoperoxidase as described previously (21). The apoE label had a trichloroacetic acid precipitability of 87.1 ± 5.8% (n=70) under these conditions. ^{125}I-Labeled apoE eluted as a single peak upon G-75 chromatography, indicating no major self-association or degradation (not shown).

RESULTS

ApoE isoform specificities of the anti-apoE monoclonal antibodies

Immunoblots were prepared from two-dimensional gel electrophoretic gels of VLDL freshly isolated from two subjects with phenotypes E3/E3 and E2/E2 (**Fig. 1**). All antibodies reacted with both apoE3 and apoE2 as well as with the sialylated isoforms of apoE in this qualitative analysis. All antibodies also recognized epitopes on apoE4 as determined by Western blot analyses after single-dimensional SDS gel electrophoresis (not shown) and reactivity in solid phase immunoassays (see Figs. 3, 4, 5). This indicates that the amino acid residue substitutions responsible for the genetic differences are not critical in determining the structures of the epitopes defined by our antibodies and that the epitopes do not interact directly with glycosylation sites.

Enumeration of epitopes on apoE

The number of distinct epitopes on apoE recognized by the panel of monoclonal antibodies was determined in antibody versus antibody cross-competition assays (**Table 2**). VLDL (rather than apoE) was coated on microtiter wells in order to assess any cross-reactivities of epitopes of apoE in the lipoprotein-associated, native conformation of apoE. Since antibodies WU E-1, WU E-2, and WU E-3, in a preliminary experiment (not shown), competed effectively with each other for binding to VLDL-apoE, indicating that they bind closely associated epitopes, only WU E-1 was included in the assays summarized in Table 2. In these experiments, five different epitopes appear to be defined by the monoclonal antibodies: a) WU E-1, WU E-2, WU E-3, b) WU E-4, c) WU E-7, d) WU E-10, and e) WU E-11, WU E-12. Group a) antibodies did not cross compete with the other groups. Although antibodies WU

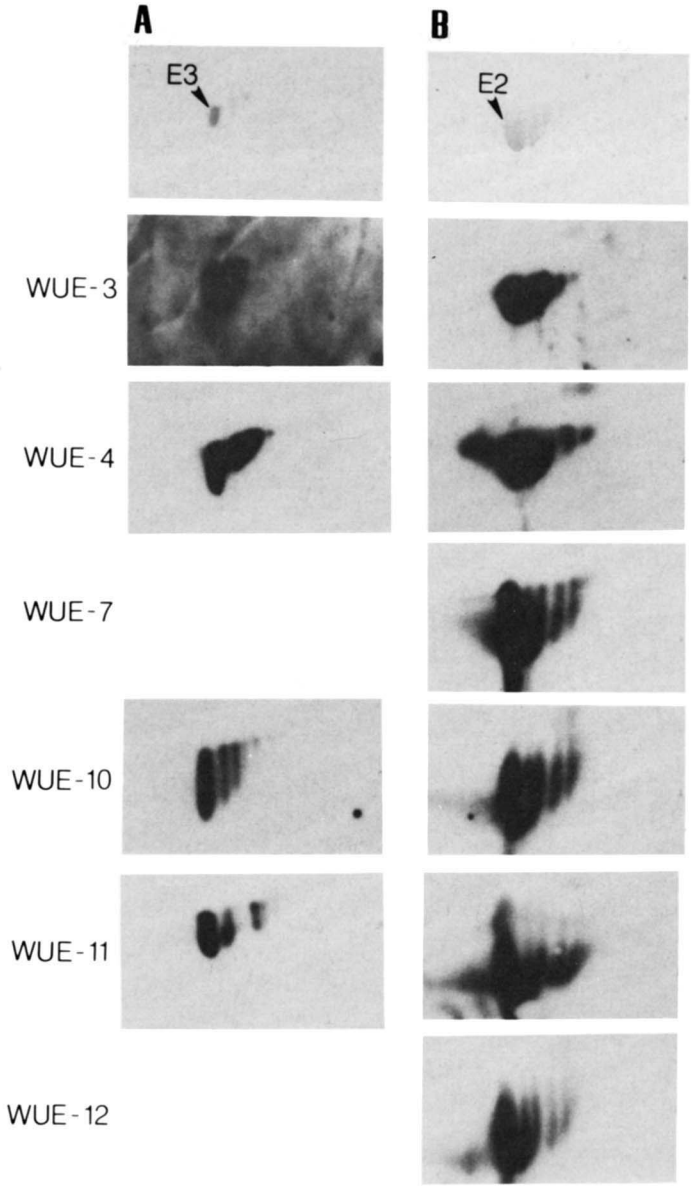

Fig. 1. Immunoblots of apoE. Two-dimensional gel electrophoresis of VLDL and electrotransfer to nitrocellulose were performed as described in Methods. The top panels in columns A and B are Coomassie blue-stained gels of the apoE region of VLDL proteins from two subjects having E3/E3 (A) and E2/E2 (B) phenotypes (first dimension cathode on the left, anode on the right). The major apoE3 or apoE2 isoforms are indicated by arrows. Below the stained gels are autoradiographs of the corresponding replicate transfers. The monoclonal antibodies incubated with the transfers are indicated on the left of each row. Monoclonal antibodies WU E-7 and WU E-12 were incubated with transfers of E3/E3 obtained from single-dimensional isoelectric focusing gels (not shown). Monoclonal antibodies WU E-1 and WU E-2 gave patterns with E3/E3 and E2/E2 apoE similar to those of WU E-3 and are not shown.

E-4, WU E-7, and WU E-10 (groups b, c, d) all cross-competed with each other they were not identical to each other (confirmed by isoelectric focusing analyses, not shown) and did not compete with antibodies from either groups a) or e). Antibody WU E-11 has an apparent higher affinity for its epitope than WU E-12 as it is better able to compete for ^{125}I-labeled WU E-12 binding than cold WU E-12 itself, suggesting that the two antibodies are not identical. Nonetheless, antibodies WU E-11 and WU E-12 exhibited very similar patterns of competition and thus may recognize sites on apoE that are very closely associated.

Assignment of epitopes on apoE

Protease-generated fragments and synthetic peptides of apoE were adsorbed onto microtiter wells and the abilities

TABLE 2. Cross competition of monoclonal antibody binding to normolipidemic VLDL apoE

Competitor Antibody	^{125}I-Labeled Monoclonal Antibody					
	WU E-1	WU E-4	WU E-7	WU E-10	WU E-11	WU E-12
WU E-1	+ +	±	−	±	±	±
WU E-4	−	+ +	+	±	±	±
WU E-7	±	+	+ +	+	±	−
WU E-10	−	±	+ +	+ +	±	±
WU E-11	−	±	−	±	+ +	+ + +
WU E-12	−	±	±	±	+	+ +

Microtiter plates were coated with VLDL (32 μg/ml) from a pool obtained from fasted normolipidemic donors. Increasing doses of competitor antibody (indicated vertically) were added to the wells (1–100,000 ng/ml) simultaneously with a constant amount of ^{125}I-labeled monoclonal antibody (indicated horizontally; specific radioactivities were approx. 4,000 cpm/ng). Symbols are as follows: −, no competition at any of the doses tested; ±, competition at high doses (dose to achieve 50% inhibition 10 times the dose of homologous antibody); +, competition at moderately high doses (dose to achieve 50% inhibition 2–10 times dose of homologous antibody); + +, competition at doses equivalent to those of homologous antibody); + + +, competition at doses less than that of homologous antibody.

of the monoclonal antibodies to bind to these peptides were assessed (**Table 3** and **Fig. 2**). Peptides 14-179/14-168 represent an unresolved mixture of these two peptides (roughly equal proportions) obtained after Sephadex G-100 SF chromatography of the protease-treated apoE (23). Monoclonal antibodies WU E-1, WU E-2, and WU E-3 were all bound by the carboxyl terminal thrombin-generated fragment (10K) and by the synthetic peptide 202–243. They were also bound by the amino terminal 22K fragment (residues 1–191) and synthetic peptide 139–169 but not by the mixture of fragments 14–179 and 14–168. The binding of the IgM antibodies to both the 22K and 10K thrombin-derived fragments of apoE was confirmed by Western blotting (**Table 4**) and was not due to contamination of one fragment by the other. It is possible that the binding to the 139–169 peptide but not the

TABLE 3. Relative binding of monoclonal antibodies to apoE and apoE fragments on microtiter wells

	WU E-1	WU E-4	WU E-7	WU E-10	WU E-11
ApoE	100.0	100.0	100.0	100.0	100.0
22K[1a]	29.5	111.6[3b]	98.1[3]	105.7[3]	123.6
22K[1]	—	121.5[3]	102.6[3]	104.5[3]	—
22K[1]	—	126.7[4]	100.1[4]	96.8[4]	—
22K[2]	—	87.1[3]	89.2[3]	85.8[3]	—
22K[2]	—	118.4[4]	98.6[4]	95.3[4]	—
10K	174.1	1.0	4.0	2.1	2.1
139–169	99.9	0.6	0.7	0.3	0.7
14–179/168[1]	0.0	73.4[3]	3.3[3,c]	53.2[3,c]	115.1
14–179/168[1]	—	86.3[3]	21.4[3,c]	69.8[3,c]	—
14–179/168[1]	—	110.6[4]	67.6[4,c]	51.0[4,c]	—
14–179[1]	—	91.7[3]	66.9[3]	84.4[3]	—
14–179[1]	—	113.7[4]	82.8[4]	64.5[4]	—
14–168[1]	—	93.1[3]	17.0[3,c]	82.0[3,c]	—
14–168[1]	—	124.7[4]	77.3[4,c]	61.7[4,c]	—
202–243	130.2	1.5	0.9	0.7	1.3

Results are expressed as a percent of the binding to apoE (E2/E2) for each monoclonal antibody measured using ^{125}I-labeled goat anti-mouse IgG. Calculations are based on a dose of 50 μg/ml of each purified monoclonal antibody. Values represent the means of duplicate determinations. Differences between duplicate counts averaged 6% for all the antibodies. Background counts obtained by incubating the monoclonal antibodies in BSA-coated wells alone have been subtracted from the counts obtained for apoE peptide-coated wells. Binding of antibodies WU E-2 and WU E-3 to the apoE/apoE fragments was similar to the results obtained for WU E-1, and the binding of WU E-12 was similar to that of WU E-11. The data are not shown for purposes of brevity; —, indicates experiment not performed.

[a1,2]Indicate two different preparations of peptide used to coat wells.

[b3,4]Indicate two different preparations of monoclonal antibodies used in assay.

[c]These antibodies did not exhibit saturable binding to the indicated peptide preparations.

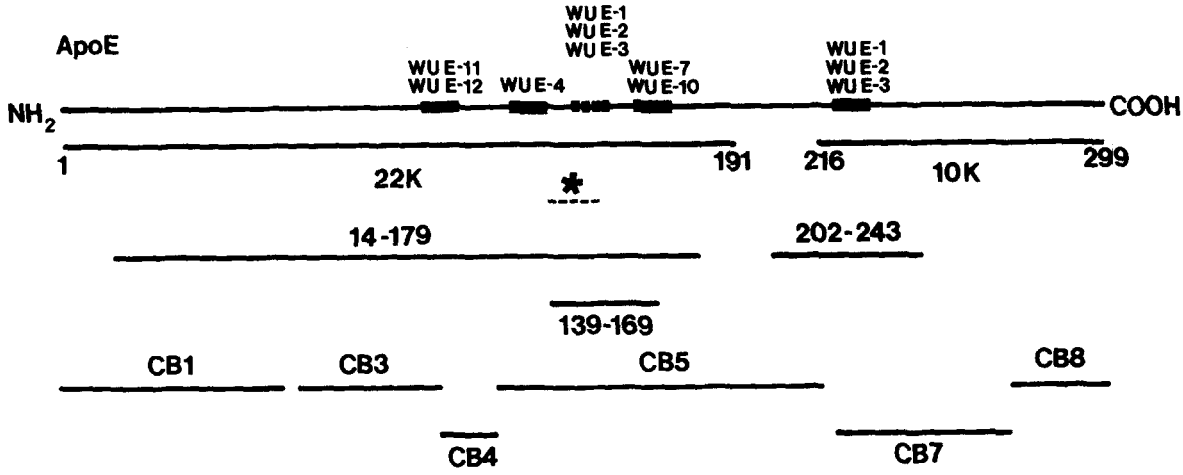

Fig. 2. Assignment of monoclonal antibody binding sites on apoE. The apoE molecule is represented as a linear sequence of amino acids from amino terminal residue 1 to carboxyl terminal residue 299. Peptides generated by thrombin cleavage are designated as 22K and 10K and other proteolytic or synthetic peptides of apoE are labeled with their corresponding amino and carboxyl terminal amino acid residues. Cyanogen bromide-cleaved fragments of apoE are indicated by CB and are as described in ref. 23. The apoB,E-receptor recognition region on apoE is indicated by the asterisk. Assignments of antibody epitopes (boxes) are based on antibody versus antibody cross-competition assays and on studies of binding of antibodies by the indicated peptides. Antibodies WU E-1, WU E-2, and WU E-3 recognize epitopes on two regions of apoE which share structural and functional similarities.

14–179/14–168 fragments is due to masking of the 139–169 region in the longer peptides. Perhaps residues 1–14 have a significant influence on the tertiary structural folding of the apoE molecule. This concept is supported by the observation that WU E-2 and WU E-3 antibodies bound to 14–168 electrotransferred to nitrocellulose after SDS-PAGE (Table 4), indicating that SDS may unfold the 14–168 peptide enough for the epitope 139–169 to become accessible.

Antibodies WU E-4, WU E-7, WU E-10, WU E-11, and WU E-12 all bound to the insolubilized amino terminal 22K thrombin-generated fragment of apoE and the mixture of peptides 14–179/14–168. Higher variability of binding was noted for antibody WU E-7 with peptides

TABLE 4. Immunoreactivity of apoE peptide fragments on Western blots

	Monoclonal Antibody				
Peptide	WU E-1	WU E-4	WU E-7	WU E-10	WU E-11
ApoE	+	+	+	+	+
22K	+	+	+	+	n.d.
14-168	+[a]	n.d.	n.d.	n.d.	+
14-179	–	n.d.	+	+	n.d.
10K	+	–	–	–	n.d.
202-243	–[a]	n.d.	n.d.	n.d.	–
CB1,2,3,4	–	+	+	+	–
CB3	–	–	–	–	–
CB3,4	–	–	–	–	+
CB3,4,5	–	+	+	+	+ +
CB4,5	–	+	+	+	–
CB5	–	+ +	+ +	+ +	+
CB7	–	–	–	–	–
CB7,8	–	–	–	–	–

Proteins were separated by electrophoresis on SDS-PAGE gels (15% acrylamide gels for the proteolytic or synthetic peptides of apoE; 15–25% linear gradient polyacrylamide gels for the cyanogen bromide fragments of apoE). Proteins were electrotransferred to nitrocellulose sheets and subsequently incubated with purified monoclonal antibodies at concentrations of 10 μg/ml overnight at 23°C. Monoclonal antibody binding was detected with freshly iodinated affinity-purified ^{125}I-labeled goat anti-mouse IgG. For the CNBr fragments, protein bands with the highest binding of monoclonal antibodies are indicated by (+ +). WU E-2 and WU E-3 gave patterns of reactivity with apoE fragments identical to that of WU E-1; however, these were not tested on Western blots with the 22K, 10K, or 14-179. WU E-12 gave a pattern of reactivity with apoE fragments identical to that of WU E-11. (–), No reactivity; n.d., not determined.

[a]Determined using WU E-2 and WU E-3 antibodies, not tested with WU E-1.

14–179/14–168 and 14–168 on microtiter wells. This may be due to *a*) the nonsaturable binding observed for this antibody and/or *b*) effects of storage on the antibody (preparations "a" versus "b"). Nonetheless, this antibody consistently demonstrated positive reactivity to these peptide preparations.

Western immunoblot analyses were also performed using apoE and its peptides (Table 4 and Fig. 2). All antibodies reacted with apoE and the 22K and 10K thrombin-derived fragments in this system as they had in the microtiter plate binding assays (Table 3). However, synthetic fragments 14–179 and 202–243 behaved differently on immunoblots than on microtiter plates. On immunoblots, fragment 14–179 did not react with WU E-1, although WU E-2 and WU E-3 bound 14–168 (WU E-1 was not tested). Antibodies WU E-1, E-2, and E-3 also did not bind to any CNBr peptides (Table 4) perhaps because the epitope may have been cleaved by CNBr or unfolded. Fragment 202–243 did not react with antibodies WU E-2 and WU E-3 on Western blots (WU E-1 was not tested). The epitopes recognized by the IgM antibodies are therefore expressed differently on microtiter wells than on Western blots, suggesting that the epitopes require optimal protein conformation for optimal antibody recognition.

Antibodies WU E-4, WU E-7, and WU E-10 bound CNBr fragment 5 (CB5) with highest affinity (Table 4). These results are consistent with reactions obtained with the protease-derived and synthetic fragments of apoE on microtiter wells. Antibodies WU E-11 and WU E-12 bound with highest selectivity to CB3,4,5 and not at all to CB3 and CB4,5. The need for the long fragment suggests that the epitope recognized by these antibodies may span the CB3–CB4 junction or requires the conformation imparted by longer sequences.

Assignments of antibody epitopes to regions of the apoE molecule (Fig. 2) were made based on the data shown in Tables 2–5. Epitopes defined by antibodies WU E-1, WU E-2, and WU E-3 were assigned to both the 10K and the 22K thrombin-generated fragments, one within residues 202–243 and the second within residues 139–169. Localization of epitopes was also aided by the abilities of antibodies to inhibit apoE-mediated binding to the fibroblast B,E-receptor. Antibody WU E-4 almost completely (5) and antibodies WU E-7 and WU E-10 partially inhibited apoE-mediated binding of VLDL to fibroblasts (not shown). Thus, the epitope recognized by WU E-4 was assigned closest to the receptor recognition site on apoE, and epitopes defined by antibodies WU E-7 and WU E-10 were assigned close to the receptor recognition site but within residues 168–179 (Table 3). Peptides representing shorter sequences of apoE in this region will be required to distinguish between the epitopes recognized by WU E-7 and WU E-10. Epitopes defined by antibodies WU E-11 and WU E-12 were assigned close to each other, towards the amino terminal of apoE because they *a*) both bound CB3,4,5 with high affinity (Table 4), *b*) cross-reacted strongly with each other but not with the other antibodies (Table 2), and *c*) did not inhibit binding of VLDL to fibroblasts (not shown).

Expression of apoE epitopes on isolated apoE and on native lipoproteins

Immunoassays containing antibodies WU E-1, WU E-2, and WU E-3 yielded similar patterns of competitive displacement by several apoE, $VLDL_{1\to3}$, and IDL preparations when a given preparation of apoE was labeled with ^{125}I and used as tracer. However, different patterns of displacement curves were obtained when different isolates of apoE labeled with ^{125}I were used (not shown). The differences seen were not related to ^{125}I-labeling, but rather to the isolation history of the apoE. Immunoassays employing the other antibodies (WU E-4→WU E-12), yielded more consistent patterns of competition curves, independent of the isolate of apoE used for tracer.

Since this anomalous behavior of the ^{125}I-labeled apoE with the IgM antibodies appeared to be related to the isolation of the apoE used as tracer, an experiment was designed to evaluate the effects of denaturants (some used in the isolation of apoE) on apoE immunoreactivity. An apoE preparation (E2/E2 obtained by Superose 6B chromatography in 4 M guanidine HCl) was radiolabeled and divided into five equal aliquots. Each aliquot was incubated with one of four different denaturants using deionized H_2O as control (**Table 5**). After removal of the denaturants by extensive dialysis in borate buffer, the ^{125}I-labeled apoE preparations were added to microtiter wells that had been precoated with anti-apoE monoclonal antibodies (Table 5). ^{125}I-Labeled apoE was bound to the IgM antibodies with significantly different avidity depending on which denaturant had been used. The nonionic detergent NP-40 totally disrupted binding of the ^{125}I-labeled apoE to the IgM antibodies, and urea treatment increased binding while guanidine HDl or SDS treatment of the apoE did not appreciably affect binding. (The lack of effect of SDS is in accord with the reactivity of apoE on Western blots of SDS-PAGE gels, see Fig. 1.) By contrast, the binding of ^{125}I-labeled apoE to the other monoclonal antibodies was not greatly altered by treatment with guanidine or urea, whereas SDS and NP-40 treatment generally reduced binding indicating that the epitopes recognized by the IgM and IgG antibodies reacted differently to perturbations of apoE structure. It is possible that some residual denaturant that was relatively resistant to exhaustive dialysis remained with the apoE (e.g., SDS) since this was not directly evaluated. In these cases, observed changes in apoE binding may be due to changes in the net charge of apoE epitopes.

TABLE 5. Treatment of apoE with various denaturants: effects on binding to monoclonal antibodies

Antibody	Control	Guanidine	Urea	SDS	NP-40
			% of control		
WU E-1	100	111	235	100	0.2
WU E-4	100	97	96	61	61
WU E-7	100	103	120	63	88
WU E-10	100	87	106	14	67
WU E-11	100	91	98	48	62
WU E-12	100	108	123	62	80

ApoE (E2/E2) was iodinated as described in Methods to a specific activity of 6820 cpm/ng. Five equal aliquots (5 μg ^{125}I-labeled apoE in BBS) were each incubated with *a*) deionized H_2O (control), *b*) 4 M guanidine, *c*) 4 M urea, *d*) 5% SDS, and *e*) 5% NP-40 (Non-idet) at room temperature for 1 hr. The mixtures of ^{125}I-labeled apoE and denaturant (and control) were then each dialyzed exhaustively against BBS (72 hr with six changes of buffer). Recoveries of ^{125}I-labeled apoE after dialysis were 28%, 30%, 29%, 60%, and 42% for the control-, urea-, SDS-, and NP-40-treated ^{125}I-labeled apoE, respectively. The ^{125}I-labeled apoE preparations were then diluted in 1% BSA-PBS to a specific activity of 1000 cpm/μl (± 2%). One hundred fifty μl of these preparations (in triplicate) were added to microtiter wellsl coated with the indicated antibodies and incubated with the antibodies for 5 hr. Plates were then washed, dried, and counted for radioactivity. Values represent the mean % cpm bound relative to *control* apoE. (100%). Nonspecific binding to non-antibody-coated wells has been subtracted from all values. The TCA precipitability of all ^{125}I-labeled apoE preparations after dialysis ranged from 90 to 94%. Control (100%) values (specific counts) were 9775, 59551, 40876, 33078, 52699, and 22706 for monoclonal antibodies WU E-1→WU E-12, respectively. WU E-2 and WU E-3 gave results very similar to those shown for WU E-1.

The patterns of lipoprotein immunoreactivities characteristic of antibodies WU E-4, WU E-7, and WU E-10 are shown in **Fig. 3.** Mean slopes of the lipoprotein displacement curves (± SEM) were −1.16 ± 0.04, −0.90 ± 0.06, and −1.24 ± 0.08 for WU E-4, WU E-7, and WU E-10, respectively. For isolated apoE the slopes were higher,

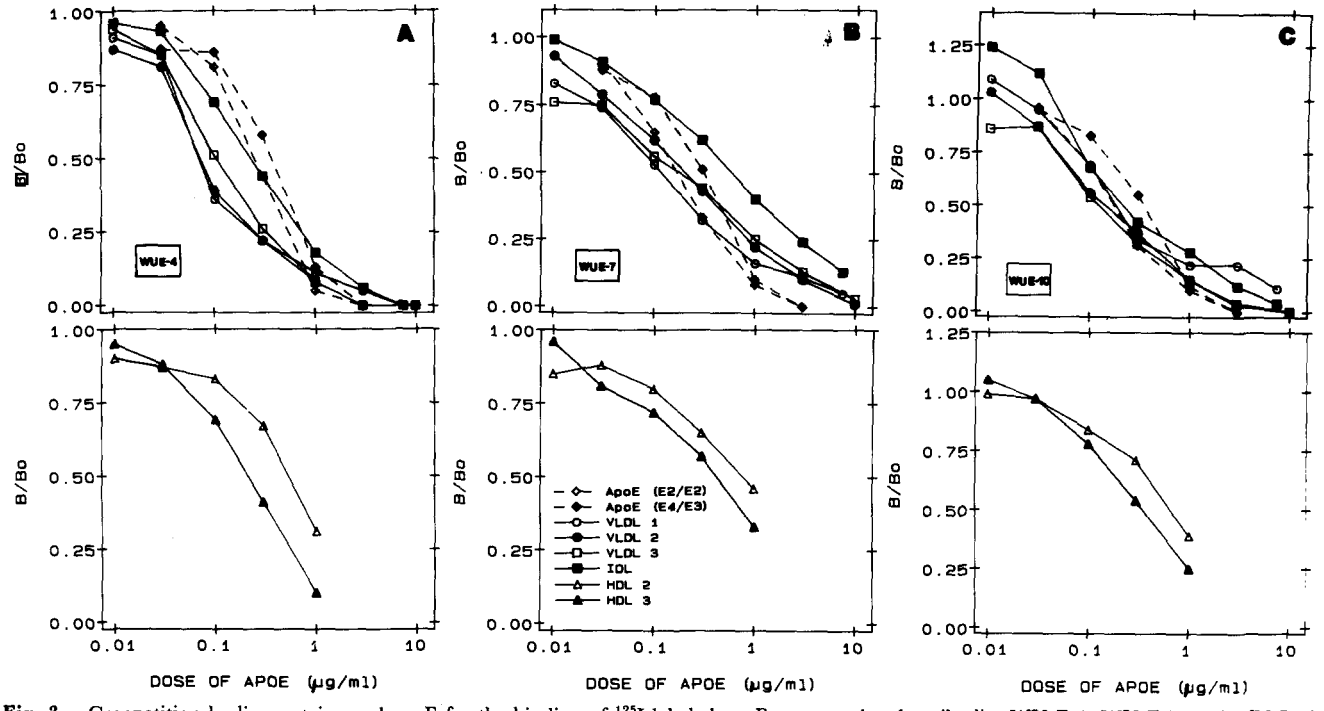

Fig. 3. Competition by lipoproteins and apoE for the binding of ^{125}I-labeled apoE to monoclonal antibodies WU E-4, WU E-7, and WU E-10. Doses of lipoprotein are expressed in terms of apoE mass. In all panels, the ^{125}I-labeled apoE was from a donor with E2/E2 phenotype. Lipoproteins were from E3/E3 donor GB (plasma triglyceride and cholesterol values were 191 and 172 mg/dl, respectively); and apoE was obtained from two donors, one with E2/E2 and the second with E4/E3 phenotype. VLDL and HDL subfractions were pooled as indicated in Methods. Symbols are as follows: (○), $VLDL_1$; (●), $VLDL_2$; (□), $VLDL_3$; (■), IDL; (△), HDL_2; (▲), HDL_3. Patterns of competition obtained using solid phase antibodies WU E-4, WU E-7, and WU E-10 are shown in panels (A), (B), and (C), respectively. B_o values for ^{125}I-labeled apoE binding to WU E-4, WU E-7, and WU E-10 were 30428 cpm, 33397 cpm, and 25320 cpm, respectively. The data depicted are representive of two to seven different apoE or lipoprotein subfraction preparations assayed with each monoclonal antibody. Mean ED_{50}s (± SEM) were 0.30 ± 0.04, 0.45 ± 0.09, and 0.45 ± 0.11, respectively for antibodies WU E-4, WU E-7, and WU E-10. Small differences in ED_{50}s between lipoprotein subfractions from a single individual were often statistically significant as determined using the ALLFIT program.

being −1.31 ± 0.06, −1.07 ± 0.01, and −1.63 ± 0.01, respectively, suggesting some positive cooperativity of antibody binding, since slopes were significantly greater than 1. The ED_{50}s for the lipoproteins or apoE showed little variability indicating that the epitopes recognized by these antibodies were uniformly expressed on apoE, whether or not it was associated with lipids. Small but significant differences in ED_{50} between lipoprotein fractions were noted with some VLDL subfractions and it was also noted that HDL_3 tended to be a better competitor than HDL_2 for ^{125}I-labeled apoE binding to these monoclonal antibodies based on the apoE mass in each fraction (Fig. 3).

The pattern of immunoreactivities observed with antibodies WU E-11 and WU E-12 is shown in **Fig. 4.** $VLDL_1$ was consistently the best competitor (smallest ED_{50}) and the competitive ability of the other lipoproteins appeared to diminish as particle sizes decreased. Mean slopes (± SEM) for lipoproteins were −1.07 ± 0.06 and −0.93 ± 0.06 for WU E-11 and WU E-12, respectively. Increases in slope were observed for apoE displacement curves as was seen for antibodies WU E-4, WU E-7, and WU E-10 (means were −1.49 and −1.66 for WU E-11 and WU E-12, respectively).

Immunoaffinity chromatography

As noted, in competition immunoassays, slopes of different lipoprotein competition curves were similar, while the ED_{50}s produced by lipoprotein subfractions of donors tended to differ, particularly with certain antibodies. These differences could be explained by one or both of the following: *a*) all lipoprotein particles express

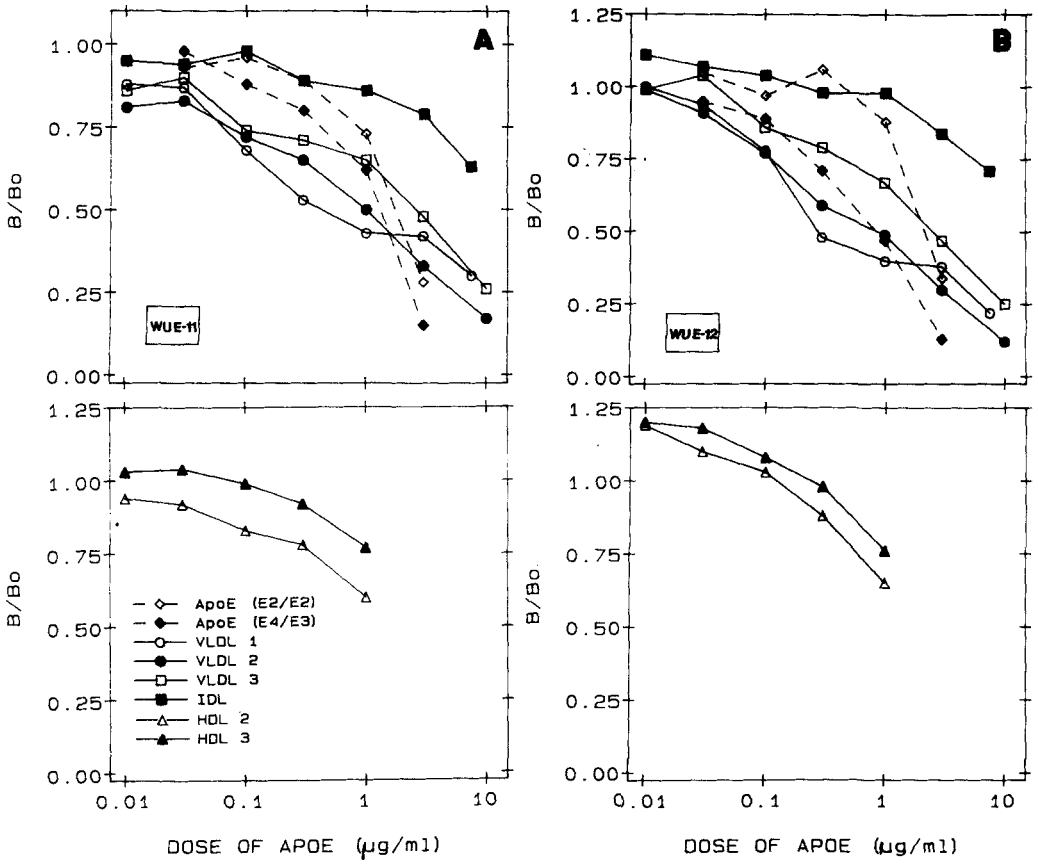

Fig. 4. Competition by lipoproteins and apoE for the binding of ^{125}I-labeled apoE to monoclonal antibodies WU E-11 and WU E-12. Doses of lipoprotein are expressed in terms of apoE mass. In all panels, the ^{125}I-labeled apoE was from a donor with E2/E2 phenotype. Lipoproteins and the two apoE preparations were isolated from the same E3/E3, E2/E2, and E4/E3 donors, respectively, as shown in Fig. 3. Symbols are as follows: (○), $VLDL_1$; (●), $VLDL_2$; (□), $VLDL_3$; (■), IDL; (△), HDL_2; (▲), HDL_3. Patterns of competition obtained using solid phase antibodies WU E-11 and WU E-12 are shown in panels (A) and (B), respectively. B_o values for ^{125}I-labeled apoE (E2/E2) binding to WU E-11 and WU E-12 were 40236 cpm and 23238 cpm, respectively. The data depicted are representative of two or three different apoE or lipoprotein preparations. Mean ED_{50}s (± SEM, where N > 2) for apoE, $VLDL_{1,2,3}$, IDL, HDL_2 or HDL_3 (in µg/ml apoE) were: a) for WU E-11, 1.6, 1.1 ± 0.1, 1.4 ± 0.5, 1.9 ± 0.9, 15.3, 2.1, and 4.9, respectively; and b), for WU E-12, 1.3, 1.5 ± 0.8, 2.3 ± 1.6, 1.4 ± 0.5, 11.4, 2.3, and 3.7, respectively. Differences between slopes and ED_{50}s of various lipoprotein preparations were not related to their apoE isoform components.

any specific epitope to some extent but the affinities of antibody binding are unequal, perhaps due to subtle differences in the conformation of apoE associated with the various lipoproteins, or *b*) only a subpopulation of particles within any subfraction expresses the particular epitope, but for those particles that do express the epitope the affinity of antibody binding is similar.

To explore these possibilities, affinity columns were prepared with four monoclonal antibodies: WU E-1, WU E-4, WU E-7, and WU E-11. Two columns, one constructed with a polyclonal anti-human apoE antibody (R182, purified by affinity chromatography on a human apoE-coupled column) coupled to Sepharose 4B, and another constructed with heparin-agarose beads (Sigma), were used for comparison. The binding capacities of the columns were determined in preliminary experiments and subsaturating quantities of lipoproteins were applied to the columns (**Fig. 5**). VLDL ($d<1.006$ or $d<1.019$ g/ml) or $VLDL_{1-3}$ zonal subfractions from several different individuals were applied to the affinity columns. The fractions of the total applied protein that were retained by each column varied for VLDL or VLDL subfractions from different individuals, indicating that VLDL from different donors had different proportions of particles that expressed the various apoE epitopes recognized by the monoclonal antibodies. However, the expression of four different epitopes on apoE could be compared when VLDL subfractions from single individuals were applied to four different monoclonal antibody columns. Shown in Fig. 5 are the results of such an experiment using VLDL subfractions from an E3/E3 donor. The affinity-purified polyclonal antibody and WU E-11 retained more E3/E3 VLDL as the particles became smaller in size (Fig. 5). Antibody WU E-7 bound about the same amount of $VLDL_1$, $VLDL_2$, and $VLDL_3$. Antibody WU E-4 bound 50% or more of applied VLDL protein but did not bind VLDL subfractions according to their flotation rates. Similar patterns of binding of VLDL subfractions were shared by WU E-1 and the heparin-agarose column. Both columns bound in the order $VLDL_1 > VLDL_2 > VLDL_3$.

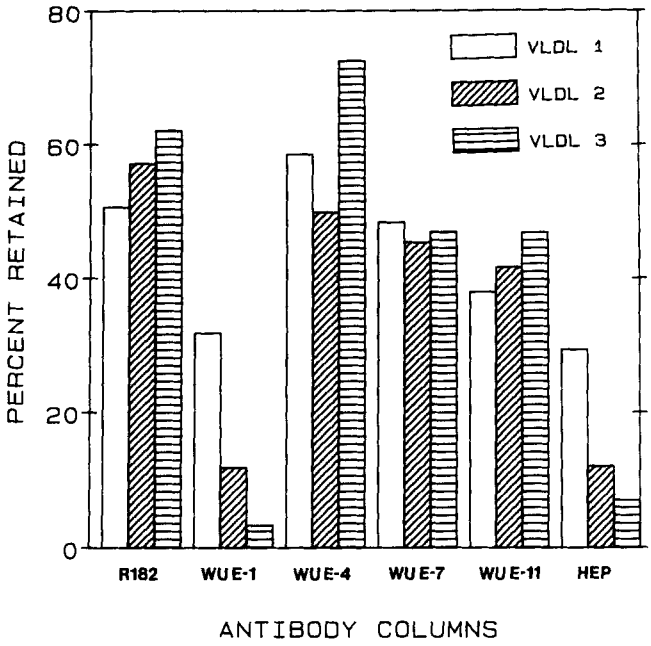

Fig. 5. Percent of VLDL protein retained by affinity columns. VLDL subfractions were isolated as described in Methods from an E3/E3 donor IZ (plasma triglyceride and cholesterol concentrations were 525 and 212 mg/dl, respectively). The VLDL subfractions (in 2 ml BBS) were applied and incubated with the affinity matrices overnight at 4°C. The unretained proteins were eluted with BBS. Retained proteins were eluted with 3 ml 3 M KSCN, pH 7.2, followed by BBS. To determine the percent protein retained by the affinity columns, tubes corresponding to the peaks of unretained or retained proteins were pooled and the total protein concentration in these fractions was determined by the method of Lowry et al. (32). Percent retained protein = (retained protein/unretained protein + retained protein) × 100. The same subsaturating amounts of VLDL protein were applied to each of the affinity matrices: VLDL 1, 205 μg; VLDL 2, 535 μg; VLDL 3, 600 μg as described in Methods. Recoveries of total lipoprotein protein (retained + unretained protein) were similar for each lipoprotein fraction on all columns and averaged 67.0 ± 10.7% (mean ± SD). R182, affinity purified polyclonal anti-apoE antibody column; HEP, heparin-agarose column.

The apoproteins of both the retained and unretained fractions were analyzed by SDS gel electrophoresis and densitometric scanning of the Coomassie blue-stained protein bands. All fractions contained some apoE (**Fig. 6**). As expected, the heparin column-retained VLDL particles had relatively higher apoE/apoC ratios than unretained particles which is consistent with the data of others (33). In general, VLDL particles that bound to the polyclonal antibody column had lower apoE to apoC ratios compared to those particles that were unbound. (This may reflect the fact that the antibody was affinity purified on a column coupled to delipidated apoE. The column may have a selected antibody population directed against apoE epitopes expressed in the absence of other apolipoproteins or lipids.) All monoclonal antibody columns bound $VLDL_1$ particles with higher apoE to apoC ratios compared to the unbound $VLDL_1$. However, only monoclonal antibodies WU E-4 and WU E-7 bound $VLDL_2$ and $VLDL_3$ particles with higher apoE to apoC ratios; monoclonal antibodies WU E-1 and WU E-11 retained $VLDL_2$ and $VLDL_3$ particles with lower apoE/apoC ratios. With antibodies WU E-1 and WU E-11, the apoE/apoC ratios of retained VLDL fell progressively from $VLDL_1$ to $VLDL_3$.

Compositional analyses of retained and applied VLDL particles revealed that the heparin column-retained VLDL particles had slightly higher proportions of triglyceride and decreased cholesterol (especially unesterified cholesterol) compared to the applied VLDL (**Table 6**). The polyclonal antibody column selectively retained $VLDL_1$ and $VLDL_2$ particles with lower triglyceride and higher cholesterol contents, whereas the lipid compositions of retained $VLDL_3$ were similar to applied $VLDL_3$.

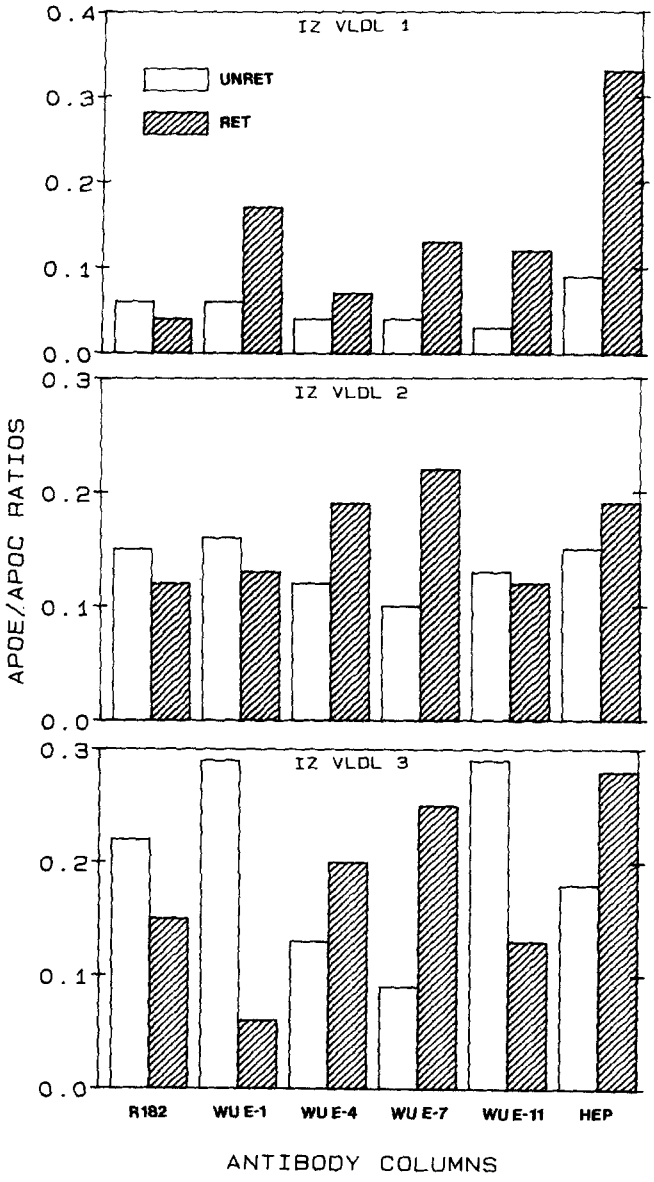

Fig. 6. ApoE/apoC ratios of retained and unretained lipoproteins separated by monoclonal antibody affinity columns. VLDL subfractions from the same E3/E3 donor (IZ) shown in Fig. 5 applied to the monoclonal, polyclonal, and heparin columns were analyzed by SDS polyacrylamide gel electrophoresis as follows. Unretained (UNRET) and retained (RET) fractions were dialyzed against 5 mM NH_4HCO_3, pH 8.2, lyophilized and delipidated with $CHCl_3$ MeOH 2:1 (v/v). The protein pellets were dissolved in SDS sample buffer overnight at 4°C, boiled for 2–3 min, and applied onto 15% SDS-PAGE slab gels for electrophoresis. Gels were stained with Coomassie Blue R250 for 18 hr and then destained for 3 days in 10% acetic acid with several changes of destain solution. After destaining, gels were soaked in 3% glycerol and scanned on an LKB Laser Densitometer. Areas under peaks of absorbance corresponding to apoE and the apoC proteins were calculated using a Planix 7 Digital Planimeter (Tamaya, Tokyo, Japan). Values represent the mean ratios of apoE peak area/apoC peak area for duplicate scans.

Antibodies WU E-1 and WU E-11 also retained VLDL particles with higher triglyceride and decreased cholesterol contents. In fact, the cholesterol contents of some WU E-1 bound particles were below the detectable range of the enzymatic assays employed (<5 μg/ml) (Table 6). Monoclonal antibodies WU E-4 and WU E-7 did not retain VLDL particles of significantly different lipid composition relative to the applied VLDL.

Cell interactions of affinity fractionated VLDL

To determine whether lipoproteins separated on the basis of apoE epitope expression represented metabolically distinct particles, hypertriglyceridemic $VLDL_1$ was applied to WU E-1 and WU E-7 columns and the abilities of bound and unbound $VLDL_1$ fractions to compete with ^{125}I-labeled LDL for binding to the LDL apoB,E-receptor on normal cultured human fibroblasts were tested (**Fig. 7**). $VLDL_1$ retained by both monoclonal antibody columns were more effective competitors of ^{125}I-labeled LDL binding than the unretained $VLDL_1$. In fact, the retained $VLDL_1$ could compete as effectively as unlabeled LDL itself, shown by the dashed line in Fig. 7.

DISCUSSION

The expression of apoE epitopes on isolated apoE and on lipoproteins was probed using eight monoclonal anti-

TABLE 6. Lipid compositions of lipoprotein particles fractionated by affinity columns

	TG	PL	UC	CE
		mole %		
$VLDL_1$ applied	74.1	14.0	7.6	4.3
Retained				
R182	57.3	19.3	8.7	14.7
WU E-1	80.7	19.3	<1	<1
WU E-4	67.5	17.4	6.3	8.9
WU E-7	71.3	16.7	4.8	7.2
WU E-11	69.6	21.4	<1	8.9
HEP	82.5	17.5	<1	<1
$VLDL_2$ applied	59.9	14.5	11.8	13.7
Retained				
R182	55.7	18.9	10.7	14.6
WU E-1	73.0	26.0	<1	0.9
WU E-4	57.3	18.2	10.9	13.7
WU E-7	58.3	18.3	11.1	12.3
WU E-11	57.0	21.6	7.8	13.7
HEP	61.9	21.0	3.9	13.2
$VLDL_3$ applied	41.1	19.3	16.4	23.1
Retained				
R182	43.0	21.3	14.3	21.4
WU E-1	58.0	31.0	<1	11.1
WU E-4	42.6	21.5	14.9	21.0
WU E-7	42.3	21.7	14.8	21.3
WU E-11	43.2	22.1	13.7	21.1
HEP	45.3	23.4	6.5	24.8

VLDL subfractions from a normolipidemic E3/E3 donor were applied to affinity columns as described in Methods. Lipid analyses were performed on the VLDL subfractions, as well as directly on the affinity column flow-through and retained fractions. Values are expressed as mole % of lipid; TG, triglyceride; PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl esters; <1, below detectable limits of the enzymatic lipid assays.

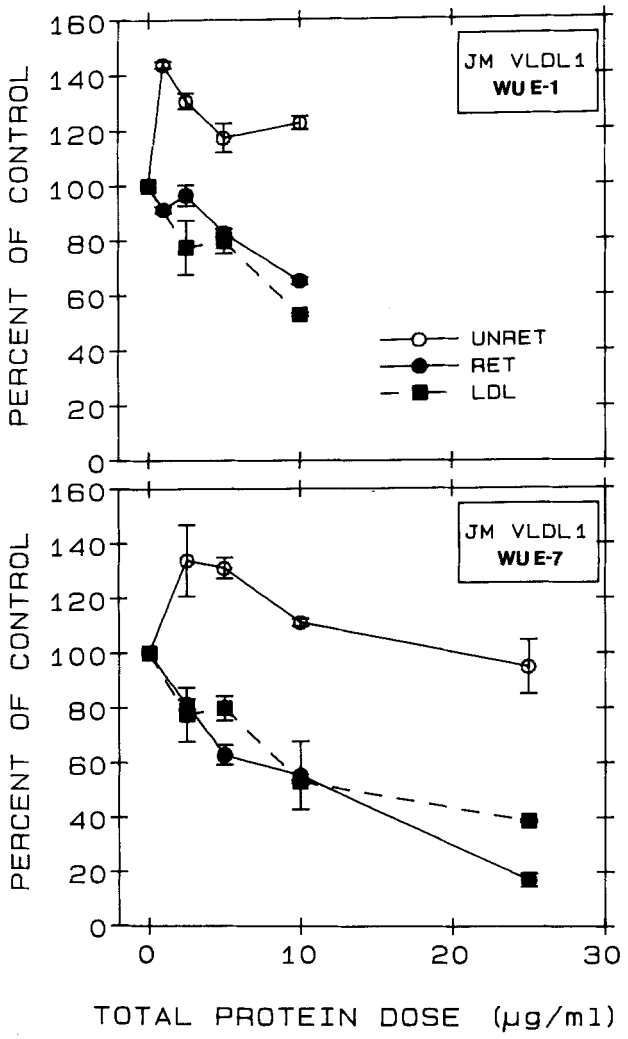

Fig. 7. Inhibition of ^{125}I-labeled LDL cell association to fibroblasts by lipoproteins fractionated by monoclonal antibody affinity chromatography. $VLDL_1$ from donor JM (plasma triglyceride and cholesterol concentrations were 1381 and 518, respectively) was applied onto WU E-1 and WU E-7 antibody columns as described. The unretained (UNRET) and retained (RET) lipoprotein fractions were pooled and protein concentrations were determined by the method of Lowry et al. (32). Human fibroblasts were grown in culture for 48 hr in media containing 10% lipoprotein-deficient serum (LPDS) to up-regulate the cell surface LDL receptors. The indicated amounts of the affinity column fractions were then added to the fibroblast monolayers along with 5 μg/ml ^{125}I-labeled normolipidemic LDL in media containing 10% LPDS; (○), unretained lipoprotein; (●), retained lipoprotein; (■), normolipidemic LDL. Cells and lipoproteins were incubated at 37°C for 4 hr. Values represent the percent of ^{125}I-labeled LDL binding to fibroblasts in the absence of any added unlabeled lipoproteins (100% value for binding was 116.7 ± 6.8 ng LDL protein bound/mg cell protein). Values obtained in the presence of added unlabeled lipoprotein correspond to the mean of duplicate determinations.

apoE antibodies (Table 1). The antibodies all bind the common genetic isoforms of apoE (Figs. 1, 3, 4) and by cross-competition assays define at least five distinct epitopes (Table 2). The epitopes defined by the three IgM antibodies (WU E-1, E-2, and E-3) were assigned to two regions, one epitope within residues 202–243 and the second within residues 139–169, as determined by solid phase immunoassay (Table 3, Fig. 2). Hydrophilicity profiles using a window setting of one amino acid reveal two very similar patterns residing between and including residues 145–156 and 226–237 (not shown). The IgM antibodies bound to each site independently of the presence of the other epitope (i.e., on separate peptide fragments), thereby discounting the possibility that these two determinants of apoE form a single discontinuous conformational epitope.

The epitope regions map closely to the heparin binding sites assigned between residues 211–218 and 142–147 (Fig. 2) (23,34). However, in a series of preliminary experiments (not shown), large, saturating amounts of soluble heparin (50,000:1, heparin:lipoprotein protein (w/w)) failed to inhibit the binding of WU E-1, WU E-2, and WU E-3 to VLDL or apoE and, reciprocally, these antibodies did not inhibit the binding of ^{125}I-labeled VLDL or ^{125}I-labeled apoE to heparin-coupled Sepharose beads. Thus, the epitopes are probably near to but do not coincide with the heparin binding sites on apoE.

The epitope defined by WU E-4, an antibody which effectively inhibits apoE-mediated binding of lipoproteins to the apoB,E-receptor on cells, was assigned close to the apoB,E-receptor recognition domain of apoE (amino acid residues 140–160) near the middle of the apoE molecule, and epitopes defined by WU E-7 and WU E-10 are located between residues 150–191, close enough to the center of apoE to inhibit VLDL-apoB,E receptor interactions partially and to cross-compete with WU E-4. Antibodies WU E-7 and WU E-10, unlike any other monoclonal antibodies, did not exhibit saturable binding (at doses up to 50 μg/ml) to peptides 14–168 or the mixture 14–168/14–179 in the experiments summarized in Table 3, even though the binding to 14–179 by these antibodies was saturable (data not shown). The observations with WU E-7 and WU E-10 are consistent with the following possibilities. *1*) The epitopes recognized by both WU E-7 and WU E-10 reside between residues 14–168, but residues 168–179 are required for optimal conformation, and in the absence of these residues, WU E-7 and WU E-10 are bound with much reduced affinity. *2*) The epitopes are localized between residues 168–179 and are partially cross-reactive with another site on 14–168. *3*) The epitopes recognized by WU E-7 and WU E-10 span residue 168; therefore, in fragment 14–168 only a partial epitope is expressed. The last possibility is most likely in view of the antibody competition data. Epitopes defined by WU E-11 and WU E-12 were assigned between amino acid residues 69–125.

Antibodies WU E-1, WU E-2, and WU E-3 defined epitopes whose expressions were affected by some denaturants, indicating that these epitopes are probably highly conformation-dependent. Furthermore, the patterns of immunoreactivities of lipoproteins also were greatly affected by the nature of the apoE preparations

used as tracer in the assay. It is possible that chemical modifications of apoE (i.e., deamidation, oxidation, or reaction with lipid peroxides (35)) artifactually resulting from the isolation procedures or from storage may also account for some of the heterogeneity of the IgM antibody binding. These possibilities were not directly assessed in this study. Finally, because the IgM antibodies appeared to define two epitopes, it is difficult to know with certainty which of the epitopes on isolated apoE and on apoE associated with lipoproteins were being probed. These facts limit the utility of these antibodies for some applications.

The more centrally localized epitopes (WU E-4, WU E-7, WU E-10) appeared to be more homogeneously expressed on lipoproteins of different sizes and densities compared to other epitopes and to be less affected by some denaturing agents (Fig. 4). The range of ED_{50}s was similar and relatively narrow for displacement curves produced by VLDL and HDL subfractions obtained from normolipidemic or hypertriglyceridemic donors, who between them possessed all of the major apoE isotopes (not shown), suggesting that differences in lipoprotein size, apoE isotype, and donor lipid levels did not greatly affect the expression of these epitopes. For these reasons, these antibodies have the potential of being useful in immunoassays for determining total apoE concentrations in plasma. Small but consistent differences between HDL_2 versus HDL_3 competition were noted with antibodies WU E-4, WU E-7, and WU E-10 (Fig. 3) and WU E-11 and WU E-12 (Fig. 4). Since the HDL used in these studies was obtained from an E3/E3 donor, the possibility exists that the HDL_2 (Figs. 3, 4) may have contained apoE-A-II complexes (36). This could account for some of the difference in apoE immunoreactivity between HDL_2 and HDL_3. The amount of apoE-A-II complex that may be present in the HDL subfractions was not quantified however.

Surprisingly, the expressions of the WU E-4, WU E-7, and WU E-10 epitopes on isolated apoE and on intact lipoproteins were similar (Fig. 3). More modulation of these epitopes was expected in view of the absence of high affinity binding of isolated apoE to the cellular apoB,E receptor and the restoration of the high affinity binding when apoE is reconstituted with lipids (12). Greater differences between ED_{50}s of $VLDL_{1\text{-}3}$ subfractions also may have been expected, particularly with antibody WU E-4 because the interactions of hypertriglyceridemic $VLDL_1$ subfractions with fibroblast apoB,E receptors were inhibited by WU E-4 in the order of $VLDL_1 > VLDL_3$ (5). One explanation for the apparent dissociation between cell-binding and immunochemical data is that the interaction of apoE with the apoB,E-receptor is more sensitive to conformational alterations of the receptor recognition region of apoE than is the interaction of apoE with antibody WU E-4, i.e., although the antibody is an effective inhibitor of lipoprotein–cell interactions, it may not be a very sensitive reporter of the conformation of the cellular receptor recognition region itself.

The rank order of reactivities of apoE on VLDL and HDL subfractions with the WU E-11 and WU E-12 antibodies was different from the reactivities with the other antibodies (Fig. 5). Apparently, regions further away from the middle region of apoE differ in their responses to changes in lipoprotein composition. Here, too, the lipid levels and apoE isoform phenotypes of the donors did not appear to be relatable to the immunoreactivities of the lipoproteins (data not shown).

Results of the immunoaffinity column experiments suggested that some VLDL particles express certain apoE epitopes and others do not (Fig. 5). Since WU E-4 and WU E-7 recognize epitopes on apoE close to the receptor recognition site of apoE, the higher apoE/apoC ratios of VLDL retained on affinity columns prepared with these antibodies (Fig. 6) may indicate that apoC, which is known to interfere with apoE-mediated binding to cellular receptors (37), may also negatively affect the recognition of antibody epitopes close to this region. The observation that WU E-1 and WU E-11 retained $VLDL_2$ and $VLDL_3$ particles with relatively low apoE/apoC ratios suggests that only a small percentage of the apoE molecules on these lipoproteins expresses the appropriate epitopes. The progressive fall of apoE/apoC ratios from $VLDL_1$ to $VLDL_3$ may indicate that antibodies WU E-1 and WU E-11 react with "less lipolyzed" (higher apoC) particles contained within each VLDL zonal subfraction. The less lipolyzed particles would be expected to form smaller percentages of the VLDL populations progressively from $VLDL_1$ to $VLDL_3$. Also of interest were the similar patterns of binding of VLDL subfractions shared by WU E-1 and the heparin-agarose column, suggesting that these two antibodies may be binding the same subset of particles, namely, those expressing the apoE epitopes for WU E-1 and heparin binding concurrently.

The relative inaccessibility of apoE in the unretained VLDL fractions to bind antibody (Fig. 5) or LDL receptor (Fig. 7) may also be due to apoE existing in a buried form on these particles (3, 38). Rubinstein et al. (39) demonstrated that apoE on VLDL consisted of a spontaneously exchangeable and nonexchangeable pool. The inaccessible apoE may consist of a population of apoE molecules associated more intimately with lipid, as hepatic lipase appears to convert this apoE to a more accessible form (39). This inaccessible apoE may be the apoE secreted with nascent VLDL, whereas the apoE that transfers readily from HDL to VLDL in plasma, may be the form recognized by receptors and antibodies (40).

Whether evaluated by competition assay or affinity chromatography, the expressions of apoE epitopes within given lipoprotein preparations were not strictly related to

lipoprotein size or the percentage of apoE mass relative to total protein. The lack of a relationship to sizes most likely reflects the metabolic complexities of lipoproteins: *a*) liver and intestine secrete particles ranging greatly in sizes and compositions; *b*) multiple interactions occur in plasma that alter sizes and compositions; and *c*) lipoproteins are cleared at varying stages of their intravascular degradation, primarily by liver. The results of the cell experiments (Fig. 7) also illustrate that physical methods used for isolating and separating lipoproteins may not necessarily yield fractions that are related to each other in physiologically meaningful ways. In contrast, affinity isolation of $VLDL_1$ (S_f 120–400) on two anti-apoE monoclonal antibody columns separated particles into two populations: *a*) the unretained $VLDL_1$, which did not inhibit ^{125}I-labeled LDL cell association, and *b*) the retained $VLDL_1$, which effectively competed for binding to the LDL receptor. In an earlier study, it was demonstrated that $VLDL_1$ uptake was mediated almost exclusively by apoE (5). The current study suggests that the overall "exposure" of apoE, rather than the expression of specific epitopes, on the surface of the VLDL particle may be most important in mediating cellular recognition.

It was noted that in incubations of ^{125}I-labeled LDL with unretained $VLDL_1$ from both affinity columns, the ^{125}I-labeled LDL cell association actually increased by 20–40% at the lower doses of $VLDL_1$. This may have been due to modification of the composition of ^{125}I-labeled LDL by net exchange and/or transfer between $VLDL_1$ and ^{125}I-labeled LDL core lipid and/or apolipoprotein components (41, 42), leading to altered biological activity of the ^{125}I-labeled LDL (42). This possibility was not directly addressed in the current study. Similar exchanges could have occurred between the retained $VLDL_1$ and ^{125}I-labeled LDL, nevertheless the retained $VLDL_1$ still competed more effectively for binding and uptake by the LDL receptor than did ^{125}I-labeled LDL or unretained $VLDL_1$.

The fact that particles can be separated based on the accessibilities of individual apoE epitopes makes it possible to study lipoprotein metabolism from a new perspective. Such studies are likely to be fruitful because apoE serves several different metabolic and structural functions, some of which depend on conformations that exist when apoE is associated with lipid. Examples of this are cell receptor recognition (12, 43) and modulation of the lipolysis of triglyceride-rich particles (44). Other functions may be less dependent on lipid association such as heparin binding (22), cellular cholesterol efflux (45, 46), and inhibition of mitogen-induced lymphocyte proliferation (47). The transferability of apolipoproteins between lipoprotein particles may depend on altered conformations acquired during metabolic events. Therefore, future studies of apoE function will have to employ methods that can accurately monitor the conformations of apoE, such as those illustrated here, using monoclonal antibodies as probes of subtle conformational alterations of apoE.

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