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Monoclonal Antibodies as Probes of High-Density Lipoprotein Structure: Identification and Localization of a Lipid-Dependent Epitope†

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ABSTRACT: Eight stable murine monoclonal antibodies (mabs) were raised against human high-density lipoproteins (HDL). Three different antibody reactivities were demonstrated by immunoblotting. A group of five antibodies were specific for apolipoprotein A-I (apoA-I) and bound to similar or overlapping epitopes. The second type of reactivity, shown by mab-32, was specific for apoA-II. In the third group, two antibodies showed high reactivity with apoA-II and slight cross-reactivity with apoA-I. The properties of two antibodies, mab M-30 specific for apoA-I and mab M-32 specific for apoA-II, were characterized in detail as probes of HDL structure. The association of ^{125}I-labeled HDL or synthetic complexes of apoA-I and phosphatidylcholine with mab M-30 was lipid dependent. Mab M-32 binding to apoA-II was independent of lipid. The lipid-dependent epitope bound by mab M-30 has been localized to an 18 amino acid synthetic apoA-I peptide. Moreover, studies with HDL_2, HDL_3, and immunoadsorbed HDL subfractions indicate that binding of mab M-30 to HDL is influenced by some component within the microenvironment of individual HDL particles. These lines of evidence suggest that the molar ratio of apoA-I to apoA-II is the critical determinant. Binding of mab M-32 to HDL increased the reactivity of HDL to mab M-30 in a dose-dependent manner, indicating an unusual form of cooperativity between two mabs that recognize different proteins in HDL. These monoclonal antibodies will be valuable in studies of the metabolic significance of protein–protein and lipid–protein interactions in HDL.

The physiological functions of HDL include regulation of cholesterol transport in the blood and cellular maintenance of cholesterol homeostasis. The two major proteins of human plasma high-density lipoproteins (HDL)[1] apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II), have been investigated intensely since 1975, when HDL was rediscovered as a negative predictor of atherosclerosis. Some studies have suggested that the concentrations of these apolipoproteins are better discriminators of coronary disease than is HDL cholesterol (Maciejko et al., 1983; Brunzell et al., 1983; Sniderman et al., 1980). ApoA-I and apoA-II comprise more than 90%

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[1] Abbreviations: HDL, high-density lipoprotein(s); apoA-I, the major protein component of HDL; apoA-II, the disulfide-containing protein component of HDL; sHDL, synthetic HDL; mab, monoclonal antibody; VLDL, very low density lipoprotein(s); LDL, low-density lipoprotein(s); BSA, bovine serum albumin; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; RIA, radioimmunoassay; Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase.

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of HDL protein. A major role of apoA-I is to regulate lipoprotein composition and structure by increasing the activity of the enzyme lecithin:cholesterol acyltransferase [LCAT] (Fielding et al., 1972; Chung et al., 1979). No function of apoA-II has been definitely established. The metabolism of apoA-I and apoA-II has been the subject of several reviews (Schafer et al., 1978; Morrisett et al., 1977; Eisenberg, 1984).

The antigenic structure of HDL has been studied with a variety of polyvalent immune serums and more recently with monoclonal antibodies. HDL is immunochemically heterogeneous (Curtiss & Edgington, 1985; Cheung & Albers, 1984). Certain apoA-I antigenic domains are masked by lipid, while the antigenicity of apoA-II is most often the same when free of lipid in solution or when bound to HDL lipids (Schonfeld et al., 1977; Mao et al., 1975, 1980; Fainaru et al., 1975). In this report, we describe the characterization of five monoclonal antibodies (mabs) that are specific for human apoA-I and three that are specific for human apoA-II. In these studies, we have developed a more complete immunochemical description of HDL, including the demonstration and localization of a lipid-dependent apoA-I epitope. In addition, we have shown an unusual cooperativity between mabs which bind to apoA-I and apoA-II. From the higher apoA-I:apoA-II ratio of HDL immunoadsorbed to mab M-30, and a differential affinity of mab M-30 for HDL_2 and HDL_3, we postulate that apoA-II functions as a regulator of the expression of lipid-dependent apoA-I epitopes in HDL. A preliminary report of our findings has been published (Siberman et al., 1986).

Materials and Methods

Lipoproteins and Apolipoproteins. Sequential ultracentrifugation in KBr (Havel et al., 1955) of pooled normolipemic plasma obtained from the Methodist Hospital Blood Donor Center gave VLDL (ρ < 1.006 g mL^{-1}), LDL (ρ = 1.019–1.063 g mL^{-1}), HDL (ρ = 1.063–1.21 g mL^{-1}), and lipoprotein-deficient serum (ρ > 1.21 g mL^{-1}). Isolated lipoproteins were dialyzed extensively against standard buffer (0.15 M NaCl containing 20 mM Tris and 0.3 mM EDTA, pH 7.4), sterilized by filtration (0.45 μm Millex-HA, Millipore), and stored at 4 °C for up to 5 weeks with no loss of immunoreactivity detected. Purified apoA-I and apoA-II were kindly provided by Dr. Henry J. Pownall of this department and stored in 3 M guanidine hydrochloride at −20 °C for up to 3 months with no detectable loss of immunoreactivity. Criteria for homogeneity of the apoproteins was a single band on SDS–polyacrylamide gel electrophoresis. For a 10-μg sample of each protein stained with Coomassie blue, 0.1 μg of other proteins would have been detected by gel scanning densitometry. Lipoproteins were analyzed for protein content by a modification (Markwell et al., 1978) of the method of Lowry using bovine serum albumin (BSA) as a standard. The purity and apoprotein composition of isolated lipoproteins were assessed by 1% agarose gel electrophoresis and SDS–polyacrylamide gel electrophoresis.

Preparation of Monoclonal Antibodies. Balb/c mice were used as the source of splenocytes and thymocytes and for the production of antibody-containing ascites. For initial immunizations, 200 μg of HDL was emulsified in complete Freund's adjuvant for intraperitoneal injection, followed 3 weeks later with a boost of 100 μg of HDL in saline. On the fifth day after the booster injection, single cell suspensions of splenocytes and hypoxanthine/aminopterin/thymidine-sensitive myeloma cells P3X63Ag8.653, a nonproducer of immunoglobulins (Frazekas & Schneideger, 1980), were mixed at a ratio of 7:1 and centrifuged. After the suspension was washed to remove serum components, 1 mL of 50% poly(ethylene glycol) was added to the cell pellet to achieve fusion (Kearney et al., 1979). Cells were dispensed dropwise into the wells of five 96-well tissue culture plates and maintained for 3 weeks in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 13.6 μg mL^{-1} hypoxanthine, 0.4 μM aminopterin, 7.6 μg mL^{-1} thymidine, 0.3 mg mL^{-1} glutamine, 1 mM pyruvate, and 50 μg mL^{-1} garamycin. Hybridomas were screened for the secretion of HDL-specific antibody by ELISA (Voyta et al., 1985). A solution of 20 μg mL^{-1} HDL was used to coat plastic microtiter plates (Linbro, Flow Labs, Inc.). Horseradish peroxidase conjugated anti-mouse IgG (Cappell) was used for detection of positive wells. Positive high titer (100–1000–fold diluted spent medium) wells were cloned once by limit dilution and were finally subcloned on a fluorescence-activated cell sorter with a single cell deposition system (Parks et al., 1979).

Antibody Purification. For the purification of large quantities (3–20 mg) of antibody from antibody containing murine ascites fluid, HDL–Sepharose 4B was prepared with 40 mg of HDL and 3 g of CNBr-activated Sepharose 4B according to the instructions of the manufacturer (Pharmacia). Ascites fluid (3–5 mL) was diluted 10-fold with 0.14 M sodium phosphate buffer, pH 8.0, and applied to a 1.0 × 5.0 cm column of HDL–Sepharose at a flow rate of 10 mL h^{-1}. The column was washed with PBS until the absorbance at 280 nm was negligible. The antibody was eluted with 0.2 M glycine, pH 2.8, and neutralized immediately with 1 M Tris, pH 8.0. Peak fractions were dialyzed against PBS and stored at −20 °C. The preparation after affinity purification was greater than 95% IgG heavy and light chains, as determined by SDS gel electrophoresis. The IgG content of these purified antibodies was determined from its A_{280} value (1.0 mg mL^{-1} solution = 1.4). Murine IgG heavy and light chains, subtyped with the Mono Ab-IDEISA kit (Zymed Laboratories), were of the IgG_1 subtype with κ light chains. All mabs used in these studies were affinity purified on HDL–Sepharose 4B.

Immunoblot Analysis. HDL, 20 μg per lane, were electrophoresed on 12.5% SDS–polyacrylamide slab gels (Laemmli, 1970) and were then transferred by electrophoresis to nitrocellulose sheets (0.45-μm pore size, Schleicher & Schuell) by the method of Towbin et al. (1979) in a Hoeffer transfer apparatus at 600 mA for 3 h at 4 °C. Complete protein transfer was confirmed by staining of the gel with Coomassie blue. The position of the transferred bands was established by Amino-black staining of one lane cut from the nitrocellulose. The unstained nitrocellulose was treated for 1 h at room temperature in a solution of 3% BSA in 150 mM NaCl, 20 mM Tris, pH 7.5, and 267 μM thimerosol for 1 h at room temperature. Individual lanes were cut from the nitrocellulose and incubated separately with 30 μg of each purified mab for 18 h at 4 °C on a shaker. Unbound antibody was removed by washing each strip 5 times with washing buffer which was 0.15 M NaCl containing 0.5% Triton X-100 (v/v), 0.2% SDS (w/v), 20 mM Tris-HCl, pH 7.5, and 1.0% BSA. The nitrocellulose lanes were then incubated for 3–4 h at room temperature with a 1:1000 dilution of an affinity-purified preparation of horseradish peroxidase conjugated to anti-mouse IgG (Cappel). After a second washing, the immunoblot was visualized after development with 0.075 mg mL^{-1} *o*-dianisidine in 150 mM NaCl, containing 20.0 mM Tris, pH 7.5, and 0.015% H_2O_2.

Relative Association Constants of HDL Mabs. The relative titers of the HDL mabs for HDL, LDL, VLDL bound to microtiter plates were determined by ELISA. A solution of 10 μg mL^{-1} of the respective lipoprotein in 50 μL of PBS, pH

7.2, was incubated in Linbro/Titertek 96-well microtiter plates (Flow Laboratories, Inc.) for 18 h at 4 °C. Approximately 42 ng of HDL was bound to each well under these conditions, as determined with ^{125}I-HDL. Wells were washed first with PBS containing 0.25% BSA and then treated with 1% BSA in PBS before incubation with 50 μL of 1:5 serial dilutions of purified mabs. After five more washings, the plates were incubated for 3–4 h at room temperature with a 1:1000 dilution of an affinity-purified preparation of horseradish peroxidase conjugated goat anti-mouse IgG (Cappel). After the wells were washed, 100 μL of substrate, which was 0.02% 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.02% H_2O_2 in 0.1 M citrate, pH 4.0, was added to each well. After 30 min, the absorbance at 414 nm was determined with a Titertek Multiscan (Flow Laboratories, Inc.). The reciprocal of the concentration (M^{-1}) of mab which gave 50% of the maximal response is used only as a relative measurement of antibody affinity and is designated the apparent K_a value.

Cotitration Analysis of HDL Mabs. Cotitration (Milne et al., 1983) was employed to determine the relationship of the antigenic determinants bound by the HDL mabs. Each mab was titered to determine the necessary dilution for antibody excess, based on a constant amount of plastic-bound HDL, in plates coated with 3 μg mL^{-1} HDL. At a concentration 2-fold higher than this predetermined dilution, 25 μL of each purified mab pair was incubated in a 1:1 (v/v) ratio (50 μL total) for 18 h at 4 °C in each well of a microtiter plate precoated with 3 μg mL^{-1} HDL. After the wells were washed, the plates were incubated sequentially with horseradish peroxidase conjugated anti-mouse IgG (Cappel) and substrate solution as described above. An increase in the A_{414} obtained with a 1:1 (v/v) ratio of two different mab clones (25 μL of each) compared to that with a single mab (50 μL) was used as an index of the extent to which the mabs can recognize different antigenic determinants.

Preparation of Synthetic HDL, POPC–ApoA-I, and POPC–ApoA-II. To establish the specificity of the mabs with direct binding studies, ^{125}I-labeled apoprotein–lipid complexes in a molar ratio of 1:100 were prepared from apoprotein and POPC (1-palmitoyl-2-oleoylphosphatidylcholine) using the cholate dialysis method (Pownall et al., 1982). ApoA-I and apoA-II were iodinated, and free iodine was removed as described for HDL above. After addition of labeled apoprotein, POPC–^{125}I-apoA-I and POPC–^{125}I-apoA-II were dialyzed extensively agaisnt PBS containing 0.02% NaN_3 (w/v) and could be stored for as long as 4 weeks with no loss in immunoreactivity. Specific activities of both synthetic HDL (sHDL) complexes averaged about 45.0 μCi mg^{-1}. Complexes of POPC–apoA-I, POPC–apoA-II, or POPC–apoA-I synthetic peptides were prepared by the same procedure with unlabeled proteins.

Several lipid–apoA-I vesicles, including those containing POPC, have been described in detail (Pownall et al., 1982; Jonas et al., 1984; Zorich et al., 1985; Reynolds, 1984). Under the conditions of this study, free apoprotein was not detectable at the concentrations of the complexes used.

Solid Phase Competitive Radioimmunoassays (RIAs). Radioiodination of HDL was performed by the iodine monochloride method of McFarlane (1958). Free I_2 was removed by chromatography on Sephadex G-25 and by extensive dialysis in PBS containing 0.02% NaN_3. Specific activities ranged from 0.13 to 0.32 μCi μg^{-1}. Greater than 96% of the radioactivity was precipitable with trichloroacetic acid. Assays were performed in flat-bottomed Immulon II Removawells strips (Dynatech Labs, Inc., Alexandria, VA) using a 96-well plate holder. The wells were precoated with 50 μL of a solution of affinity-purified mab, routinely 25–50 μg mL^{-1} in PBS, by an overnight incubation of the plates at 4 °C with constant shaking. After the antibody solution was removed, the plates were washed 3 times with PBS containing 0.25% BSA (w/v) and 0.02% NaN_3 (w/v) and then with a solution of 3% BSA (w/v) in PBS for at least 1 h at 23 °C. After this blocking step, 35 μL of competing antigen (HDL), apoA-I, apoA-II, POPC–apoA-I, POPC–apoA-II, LDL, or VLDL) in serial 1:2 dilutions was added to two rows of a 96-well plate. A second overnight incubation was performed at 4 °C with 15 μL of ^{125}I-HDL diluted in 1% BSA (w/v) in PBS. For most RIAs, the concentration of the competing antigen was adjusted so that the final concentration of the solution in the first well of a dilution row was 25 μg mL^{-1}. Wells were finally washed 4 times, separated, and counted in 12 × 75 mm tubes in a Micromedic automatic γ counter. Background values for nonspecific binding were the cpm bound in the absence of mab. Maximal binding (B_0) was determined from the cpm bound in the absence of competitor, which was replaced by BSA, minus background values. Bound values (B) were calculated from the cpm minus background. All experiments were performed in duplicate, and the average was used for all calculations. Logit transformation of displacement data was performed according to the standard formula logit $y = \ln [y/(1 - y)]$ where $y = B/B_0$ (Rodbard, 1974). All logit-transformed lines had a variance of less than 0.2 from linearity. Maximal binding of ^{125}I-IgG to the plates occurred at 25–50 μg mL^{-1} IgG under these conditions. Titrations with mab M-30 and mab M-32 showed that maximal HDL binding was also achieved at a coating concentration of 25–50 μg mL^{-1}.

Peptide Synthesis and Purification. Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer using the programs supplied with the instrument which utilize polystyrene resin (Applied Biosystems) and preformed Boc-amino acid symmetric anhydrides (Boc-amino acids, Applied Biosystems). The peptide was cleaved from the resin and deprotected at 0 °C with anhydrous HF containing 10% anisole (Aldrich) and 1% ethanedithiol (Aldrich). The peptide was precipitated with ether and extracted from the resin with trifluoroacetic acid (TFA, halocarbon redistilled). The TFA was evaporated in vacuo and the peptide again precipitated with ether. The peptide was dissolved in 1 M Tris containing 6 M guanidine hydrochloride (Schwarz/Mann Biotech). For those peptides containing tryptophan, the solution was cooled to 4 °C and ethanolamine added to remove the formyl protecting group from tryptophan. In all cases, the pH was adjusted to 3 and the peptide desalted on Bio-Gel P-2 equilibrated in 5% acetic acid. The acetic acid was removed by lyophilization and the peptide purified by reversed-phase HPLC on a 1 × 25 cm Vydac C_4 column eluted with a linear gradient of 0.1 M $(NH_4)H_2PO_4$ (Fisher), pH 3.0, and 2-propanol (Burdick and Jackson). The peptide was removed from the propanol buffer by desalting on a P-2 column in 5% acetic acid, followed by lyophilization. Analytical reversed-phase HPLC on a 0.46 × 25 cm Vydac C_4 column indicated the purity of the peptide was greater than 99%. Amino acid analysis confirmed the composition of each peptide.

Results

Monoclonal Antibody Antigen Specificity. Immunization with human HDL followed by standard cell fusion and hybridoma cloning produced eight IgG-secreting monoclonal cell lines with similar reactivity. To ascertain the nature of mab binding to HDL, the antigen specificity was examined with HDL apoproteins bound to nitrocellulose (Figure 1). HDL

Table I: Cotitration of Anti-HDL Mabs[a]

	anti-apoA-I mabs					anti-apoA-II mabs		
mab	M-25	M-28	M-29	M-30	M-31	M-7	M-26	M-32
M-25	1.53	1.74	1.80	2.13	2.18	1.58	1.50	1.85
M-28		1.46	1.32	1.49	1.42	1.50	1.54	1.53
M-29			1.43	1.37	1.34	1.70	1.76	1.47
M-30				1.37	1.37	1.64	1.75	1.51
M-31					1.41	1.46	2.08	1.54
M-7						0.96	1.16	1.40
M-26							1.17	1.73
M-32								1.27

[a] The relationship of antigenic epitopes bound by each mab was determined by cotitration of a concentration of each antibody in the plateau region of maximum binding. Mabs at twice the predetermined concentration for maximal response were mixed at a 1:1 ratio (v/v) and incubated overnight at 4 °C on plates coated with 3 $\mu g\ mL^{-1}$ HDL. The values shown are the A_{414} values after incubation with a second antibody anti-murine IgG HRP conjugate, and the chromogenic ABTS solution, as described under Materials and Methods.

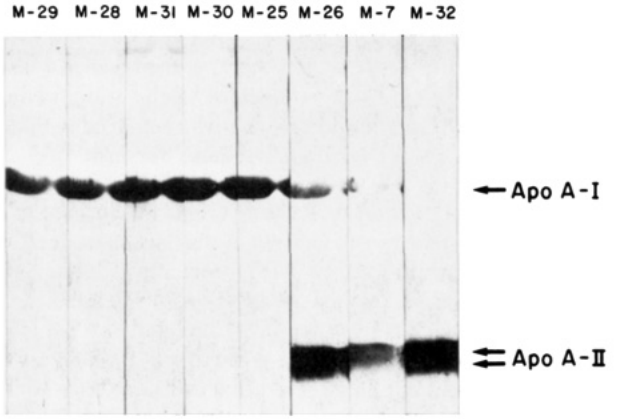

FIGURE 1: Immunoblot of affinity-purified anti-HDL mabs. Transfer of apoprotein to nitrocellulose from 12.5% SDS–polyacrylamide gels and successive incubations of strips in mab and anti-mouse IgG HRP-conjugated anti-mouse IgG followed by color development in a solution of *o*-dianisidine were done as detailed under Materials and Methods. Lanes a–h (from left to right) are strips of nitrocellulose which were incubated with mab M-29, mab M-28, mab M-31, mab M-30, mab m-25, mab M-26, mab M-7, and mab M-32, respectively. The position of apoA-I and apoA-II as determined by amido black staining of lanes containing the isolated apoproteins and polyclonal anti-apoA-I and anti-apoA-II antibodies are shown by a single arrow or a double arrow, respectively.

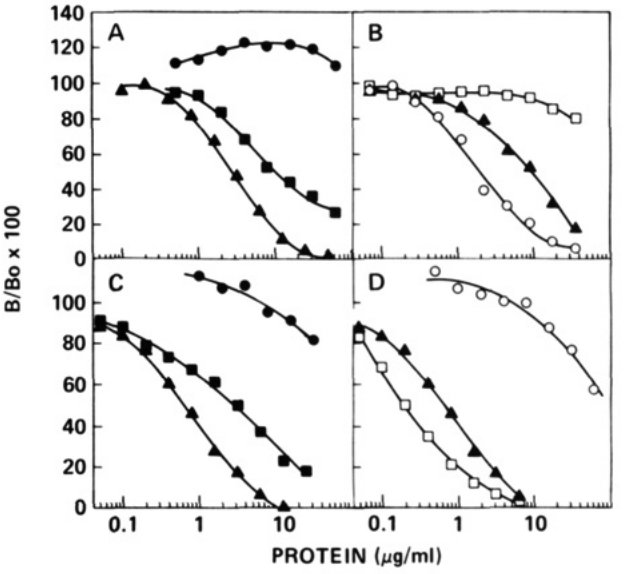

FIGURE 2: Solid phase competitive RIAs with mab M-30 and mab M-32. Typical displacement curves of competitive radioimmunoassay with lipoproteins and apoproteins of human plasma are shown. Plates coated with 25 $\mu g\ mL^{-1}$ mab M-30 (A and B) and mab M-32 (C and D) were incubated overnight at 4 °C with (1:2) serial dilutions of each competing antigen and 50 ng of ^{125}I-HDL. Each antigen was diluted in a 1% BSA/PBS solution and is designated as follows: HDL (▲), apoA-I (●), apoA-II (■), POPC–apoA-I (○), and POPC–apoA-II (□). Similar assays utilizing ^{125}I-HDL from at least three different preparations of HDL gave essentially the same displacement patterns (slopes) as shown here, but there were significant differences between the absolute protein concentrations needed for similar displacement. HDL used in panel B was obtained from a separate preparation. Results shown are the average of duplicate determinations.

apoproteins were separated by electrophoresis on 12.5% SDS–polyacrylamide gels and transferred to nitrocellulose for immunoblotting. The bands stained by *o*-dianisidine had the mobility of apoA-I and/or apoA-II. The identity of these bands was confirmed with monospecific polyclonal anti-apoA-I and anti-apoA-II antibodies. By this criterion, five of the mabs, mab M-25, mab M-28, mab M-29, mab M-30, and mab M-31, were specific for apoA-I. Only one, mab M-32, was specific for apoA-II. The final two mabs, mab M-26 and mab M-7, show a high reactivity with apoA-II and slight cross-reactivity with apoA-I. Reactivity toward human apolipoprotein E, human apolipoprotein C, and human apolipoprotein B was not detected (data not shown).

HDL–Sepharose-purified mabs were assessed by ELISA to determine the relative differences between the affinities of individual anti-HDL mabs. With immobilized HDL on microtiter plates, the apparent K_a values (Materials and Methods) of mabs M-7, M-25, M-26, M-28, M-29, M-30, M-31, and M-32 were (3.6, 1.5, 6.3, 9.9, 6.6, 8.0, and 4.9) × 10^{10} M^{-1}, respectively. Virtually no reactivity above background was observed with LDL- and VLDL-coated plates.

Cotitration Analysis of Anti-HDL Mabs. To investigate the relationship of the antigenic determinants on HDL, cotitration at antibody excess was performed using HDL-coated microtiter plates. All the apoA-I-specific mabs, with the exception of mab M-25, showed no increase in detectable bound IgG when mixed in a 1:1 (v/v) ratio at a final concentration (of each) equal to the predetermined plateau concentration and incubated overnight at 4 °C on coated plates (Table I). These results indicate that four anti-apoA-I mabs, mab M-28, mab M-29, mab M-30, and mab M-31, have very similar or overlapping epitopes on HDL, while mab M-25 binds to a spatially distant epitope on apoA-I. It is apparent that anti-apoA-II mabs M-26 and M-7 appear to bind to different domains on apoA-II, compared with anti-apoA-II mab M-32. However, mab M-26 and mab M-7 have similar or overlapping domains. All anti-apoA-I mabs did not compete with any anti-apoA-II mabs for similar sites on HDL.

Solid Phase Competition RIA. To establish the immunoreactivity of these mabs toward soluble lipid-free human apolipoproteins, affinity-purified mabs were bound to microtiter plates. Isolated apoproteins and lipoproteins were used as competitors for the binding of ^{125}I-HDL to the immobilized

mabs. Typical competitive displacement curves with the representative anti-apoA-I and anti-apoA-II mabs, mab M-30 and mab M-32, are shown in Figure 2. HDL was an effective competitor with all mabs (Figures 2A,C), averaging at least 80% displacement of ^{125}I-HDL in a range of competitor protein concentrations between 50.0 and 0.1 μg mL^{-1}. ApoA-II was almost equally effective as HDL, while apoA-I gave virtually no displacement of labeled antigen. Only at high concentrations of competitor, greater than 1.5 μg mL^{-1}, did apoA-I show some displacement of labeled antigen with mab M-32 coated plates. A solution containing greater than 50-fold more LDL and 100-fold more VLDL compared to HDL (based on protein concentration) was required to obtain similar 50% displacement of ^{125}I-HDL with all mabs. The levels of ^{125}I-HDL binding ($B/B_0 \times 100$) greater than 100 observed both with mab M-30 and with mab M-32 are likely due to transfer of free apoprotein to ^{125}I-HDL under the assay conditions employed. Displacement of ^{125}I-labeled apoprotein from HDL by addition of unlabeled apoprotein at 23 °C has been described by other investigators (Edelstein et al., 1982; Van Tornout, 1981) and may account for the competition of apoA-II with mab M-30, which is an anti-apoA-I antibody by the criteria of immunoblotting. Alternatively, apoA-II may transfer to the HDL without a concomitant loss of protein, thereby decreasing the lipid to protein ratio, and/or transfer may alter the conformation of apoA-I and diminish the expression of the apoA-I epitope for mab M-30. Size-exclusion chromatography, performed in PBS containing 1% BSA at both 23 and 4 °C, of ^{125}I-HDL mixed with ^{131}I-labeled apoA-II (at the same concentrations employed in RIAs) showed that ^{131}I-apoA-II elutes with the HDL fraction and displaced ^{125}I-apoprotein. However, which apoprotein is displaced has not been determined because of the low protein concentrations and high BSA content. Therefore, which of these two possible explanations is the cause of the observed phenomenon has not been established. Self-association of apoA-I does not explain the lack of apoA-I competition for ^{125}I-HDL binding to mab M-30, since, at the concentrations employed, less than 10% self-association of apoA-I occurs (Formisano, 1978).

The reactivities of these mabs were characterized further by competitive RIA with the sHDL, POPC–apoA-I, and POPC–apoA-II. As illustrated in Figure 2B these competition assays showed a pattern of reactivity different from the profiles of Figure 2A. The sHDL containing apoA-I was a very effective competitor of ^{125}I-HDL binding to mab M-30, competing over a 700-fold range of concentrations, from 35 to 0.05 μg mL^{-1}. POPC–apoA-I was a more effective competitor than HDL on the basis of total protein because the displacement curves shift to the left. When these data are plotted by using the molar apoA-I content of HDL, the plot of HDL is shifted to the left about 33%, but the pattern remains essentially the same as in Figure 2B. Representative displacement curves with mab M-32 are shown in Figure 2D. The sHDL containing apoA-II was similarly effective and required less protein than HDL to obtain the same displacement. When plotted on the basis of molar apoA-II content, the HDL curve shifts to the left by 88%, indicating that 31% less molar concentration of HDL is needed for the same displacement as POPC–apoA-II. These results indicate that both POPC–A-II and HDL are effective competitors of ^{125}I-HDL, with the latter slightly more effective in competing for mab M-32 binding.

The relative affinities of mab M-30 and mab M-32 toward HDL, lipid-bound apoprotein, and lipid-free apoprotein were obtained from several competition RIA experiments by analysis of the slopes of logit transformation of the data. The affinity with mab M-30 for apoA-I, as a component of the POPC–apoA-I complex (slope = -3.11 ± 0.52, $n = 5$), is similar (two-sided paired Student's t test, $p > 0.1$) to that of HDL (slope = -2.34 ± 0.78, $n = 5$). With four to five different preparations of HDL and POPC–apoA-I as competitors, large standard deviations were obtained. However, with a single HDL preparation as competitor, interassay and intraassay coefficients of variation were less than 9%, indicating the variability is due to differences in the HDL apoprotein composition of different preparations. Mab M-32 showed equal affinity, $p > 0.1$, with apoA-II under all conditions: in the lipid-free state (slope = -1.57 ± 0.21, $n = 4$) or as a component of either HDL (slope = -1.98 ± 0.10, $n = 3$) or a POPC–apoA-II complex (slope = -1.87 ± 0.22, $n = 3$).

Expression of HDL Epitopes. Polyclonal antisera have detected an expression of sterically masked HDL antigenic domains after partial delipidation of HDL in the nonionic detergent Tween-20 (Mao, 1980). Our mabs were tested for enhancement of binding of ^{125}I-HDL, ^{125}I-apoA-I, and ^{125}I-apoA-II in the presence of Tween-20. Using concentrations from 0.1% to 5.0×10^{-5}% (v/v), we noted no increase in ^{125}I-HDL binding, but rather a decrease in binding was observed with mab M-30 and mab M-32 at concentrations of Tween-20 higher than 0.01%. Within a Tween-20 concentration range of 2.5×10^{-2}% to 6.3×10^{-3}%, the binding of ^{125}I-apoA-I to mab M-30 increased dramatically from essentially zero to maximal binding of 36% at 12.5×10^{-3}% Tween-20 followed by a return to background levels. By contrast, ^{125}I-apoA-II binding showed virtually no increases with Tween-20 concentration. These results may be due to a micellar formation of Tween-20 and apoA-I into a "lipid" conformation, which increases binding to mab M-30. The critical micellar concentration of Tween-20 is 0.006% (Helenius, 1975) which is about half the concentration at which ^{125}I-apoA-I binding was maximal. The decreased ^{125}I-HDL binding indicates that Tween-20 does not expose additional "masked" mab M-30 or mab M-32 sites and appears to denature HDL or to alter the conformation of HDL apoproteins.

Mab Binding of ^{125}I-Labeled Apolipoprotein and Lipoprotein. Further investigation of the specificity of anti-HDL mabs were undertaken to test our hypothesis that the expression of the mab M-30 epitope is lipid dependent and to exclude transfer of apoproteins between HDL in competitive assays. Direct binding of ^{125}I-antigen was performed by using the mabs coated on microtiter plates as solid phase antigen receptors. The binding curves obtained with mab M-30 and ^{125}I-HDL and ^{125}I-POPC–apoA-I complexes (Figure 3A) are typical of curvilinear high-affinity binding and consistent with a high degree of immunoreactivity for apoA-I associated with phospholipid. Although some degree of binding of POPC–apoA-II occurred at higher concentrations of antigen, the linearity of these binding curves suggests nonspecific interactions. Not more than 6–3% binding of ^{125}I-apoA-I and ^{125}I-apoA-II, respectively, to mab M-30 was observed. Therefore, apoA-I and apoA-II associated very poorly with mab M-30 in the absence of lipid.

Direct binding studies with mab M-32 (Figure 3B) confirmed the antigen specificity previously determined by solid phase RIA. Binding of apoA-II, POPC–apoA-II complex, and HDL to mab M-32 coated plates showed curvilinear asymptotic binding, characteristic of high-affinity antigen binding. By contrast, low-affinity linear binding, characteristic of nonspecific interactions, was observed with mab M-32 and both free apoA-I and POPC–apoA-I complexes. Maximal binding of apoA-II was more than 2-fold higher than that of HDL or

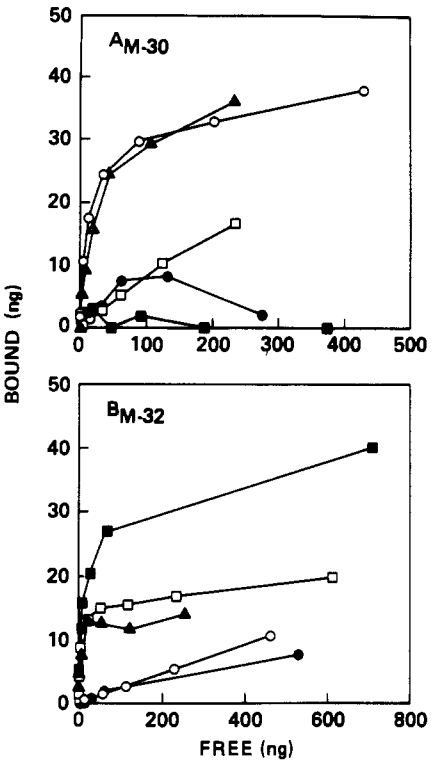

FIGURE 3: Direct binding of ^{125}I-labeled apoproteins and lipoproteins to mab M-30 and mab M-32. Microtiter plates coated with anti-apoA-I mab M-30 and anti-apoA-II mab M-32 were used to determine the level of reactivity of these mabs to ^{125}I-labeled apoproteins and lipoproteins. Individual samples were incubated overnight at 4 °C in 1% BSA/PBS. After the plates were washed, the wells were counted, and the amount of bound sample was determined. The results of binding to mab M-30 (A) and mab M-32 (B) are expressed as nanograms of protein bound, calculated from the specific radioactivity of the initial sample. Synthetic lipid vesicles, POPC–^{125}I-apoA-I complexes and POPC–^{125}I-apoA-II complexes, were prepared by the cholate dialysis method as described under Materials and Methods, using a 100:1 ratio of lipid to ^{125}I-apoprotein. Antigens were diluted in a 1% BSA/PBS solution and are designated by the following: ^{125}I-HDL (▲), ^{125}I-apoA-I (●), ^{125}I-apoA-II (■), POPC–^{125}I-apoA-I (○), and POPC–^{125}I-apoA-II (□). At 25 μg mL^{-1}, 727 and 279 ng of ^{125}I-mab M-30 and ^{125}I-mab M-32 were bound to the plates, respectively.

POPC–apoA-II complex. Under these experimental conditions, at low concentrations of added antigen, immobilized mab M-30 and mab M-32 bound all of the added POPC–apoA-I and POPC–apoA-II complexes.

Levels of HDL Epitope Expression. The percentage of HDL detected under conditions of maximal binding was measured to determine if the epitopes recognized by these mabs are homogeneously expressed on all HDL particles. Optimal conditions for binding of mab to microtiter wells and HDL to the immobilized mab were determined by solid phase RIA using ^{125}I-mab and ^{125}I-HDL, respectively. A single incubation of ^{125}I-HDL with plates coated with mabs M-25, M-28, M-29, M-30, M-7, M-26, and M-32 gave a range of binding from 65% to 86% of the added ^{125}I-labeled HDL (data not shown).

Successive incubations of three concentrations of ^{125}I-HDL on plates coated with mab M-25, mab M-30, or mab M-32 were performed by removing unbound ^{125}I-HDL from each well and placing this supernatant solution in a second well coated with the same mab. These incubations showed that two incubations were sufficient to achieve maximal binding. Maximal binding with mab M-30 and mab M-32 was 77% and 86% of the added ^{125}I-HDL, respectively. The higher maximal binding could not be explained by differences in the relative affinities of the mabs. A 2-fold difference in the concentrations of HDL showed no significant differences in maximal binding after the second incubation. The different levels of HDL binding obtained with our anti-apoA-I mabs suggested that this difference may be due to HDL subfraction heterogeneity similar to that found by others (Curtiss & Edgington, 1985; Cheung & Albers, 1984).

Heterogeneous HDL Epitope Expression. To further investigate this possible heterogeneity of HDL, three experiments were performed. First, ^{125}I-HDL were incubated on anti-apoA-II mab coated plates, and then the "unbound" fraction was transferred to plates coated with either anti-apoA-I or anti-apoA-II mabs. Mab M-32 or mab M-26 was the initial anti-HDL antibody. Between 19% and 27% of the ^{125}I-HDL fraction was subsequently bound by anti-apoA-I mab M-30, compared with only 9% bound by the anti-apoA-II mab M-32. Initial incubations with anti-apoA-II mabs appeared to remove a subfraction of HDL. The HDL remaining in the unbound fraction bound predominantly to the anti-apoA-I mabs in the second incubation.

Second, several of the unbound and bound ^{125}I-HDL fractions from single incubations with mab M-30 and mab M-32 coated plates were subjected to SDS–polyacrylamide gel electrophoresis, followed by autoradiography and laser densitometry scanning. The apoA-I and apoA-II patterns of bound and unbound fractions are shown in Figure 4. Mab M-30 and mab M-32 bound particles containing apoA-I and apoA-II in proportions different from those found in the HDL. Comparisons of the profiles of the unbound fraction after exposure to mab M-30 revealed a much lower apoA-I:apoA-II ratio, 0.64:1, than in native HDL. This selective removal of ^{125}I-HDL with an apoA-I:apoA-II ratio different from the HDL solution added to each well indicated that this apoA-I epitope is heterogeneously expressed. The apoA-I:apoA-II profile of unbound HDL after exposure to mab M-32 was essentially free of apoA-II. Therefore, mab M-32 removed most of the apoA-II-containing particles, leaving mainly apoA-I-containing particles unbound.

Third, we studied the expression of mab M-30 epitope(s) on HDL_2 and HDL_3 with zonally isolated HDL from postprandial (pp) and postabsorptive (pa) states. Solid phase competitive RIAs of the HDL_2 and HDL_3 from four different plasma sources revealed an apparent difference in the affinity of mab M-30 and in the relative expression of this epitope on HDL_2 and HDL_3. As shown in Table II, the mean value of the logit slope with HDL_2 samples was significantly higher ($p < 0.005$, unpaired Student's t test) than with HDL_3 samples. Expression of the mab M-30 epitope on HDL_2 was higher than HDL_3, shown by about a 6-fold difference in the amount of HDL_2 needed for 50% displacement, compared with the amount of HDL_3 for the same effect. The high molar ratios of the apoA-I–apoA-II content of the HDL_2 samples show a possible correlation to a more negative slope and the lower protein concentration necessary for 50% displacement. No correlation between affinity or expression and the ratio of triglyceride to cholesterol in each HDL sample was observed. Assuming all other protein levels were similar except apoA-I and apoA-II, the higher apoA-I content of HDL_2 would account for 67% of its higher mab M-30 apoA-I epitope expression but would not affect the affinity. Therefore, these results support the view that the apoA-I:apoA-II ratio of HDL regulates the affinity and expression of this apoA-I epitope.

Cooperativity with ApoA-II Mabs. The relationship of apoA-I and apoA-II epitopes on HDL was examined by an overnight incubation of HDL with a 28-fold molar excess of anti-apoA-II antibody or nonimmune mouse IgG (Table III).

Table II: Differential Affinity and Expression of the Mab M-30 Epitope on HDL_2 and HDL_3[a]

source	sample	slope	concn needed for 50% displacement (ng mL^{-1})	apoA-I:apoA-II molar ratio[b]	[TG/(TG + CE)] × 100[c]
1	HDL_3 (pp)	−2.42	529	7.0	21.1
2	HDL_2 (pp)	−2.85	672	2.8	56.9
3	HDL_2 (pa)	−2.87	511	5.8	20.2
4	HDL_2 (pa)	−3.02	647	6.7	10.6
	mean (n = 4)	−2.79	590	5.6	27.2
	SD	0.26	82	1.9	20.4
1	HDL_3 (pp)	−2.13	2666	1.6	19.6
2	HDL_3 (pp)	−2.19	5315	1.3	67.5
3	HDL_3 (pa)	−2.30	2205	1.6	9.7
4	HDL_3 (pa)	−2.00	3706	1.2	29.9
	mean (n = 4)	−2.15	3473	1.4	31.7
	SD	0.13	1379	0.2	25.3
	POPC–A-I				
	mean (n = 5)	−3.11	630		
	SD	0.52	298		

[a] HDL_2 and HDL_3 samples were diluted in 1% BSA/PBS for use as competitors of ^{125}I-labeled HDL binding to immobilized mab M-30 as described for the solid phase RIA. Logit transformation of the RIA data are performed to obtain relative affinities as defined by the slope of the displacement curve. All regression lines had correlation coefficients greater than 0.97. HDL were isolated from subjects in a postprandial state (pp) or postabsorptive (pa) state (Patsch et al., 1984) and were a generous gift from Dr. Josef R. Patsch, Department of Medicine, Baylor College of Medicine and The Methodist Hospital, Houston, TX. [b] Molar ratios were determined from laser densitometric scanning of SDS–polyacrylamide gels containing HDL samples and standards. [c] Triglyceride (TG) and cholesterol (CE) levels were determined enzymatically with Boehringer Mannheim kits 701912 and 692905, respectively.

Table III: Cooperativity Effect between ApoA-I and ApoA-II Mabs[a]

	immobilized mab in second incubation	% of ^{125}I-HDL-bound anti-apoA-I antibody in first incubation			
		mab M-7	mab M-26	mab M-32	murine IgG
anti-apoA-II mabs	M-7	7.2	4.4	3.9	19.5
	M-26	20.7	18.0	14.8	30.1
	M-32	7.4	4.2	1.8	35.2
anti-apoA-I mabs	M-25	100	100	100	43.0
	M-28	100	100	100	41.8
	M-29	100	100	95.1	39.5
	M-30	100	100	100	37.5
	M-31	100	100	100	58.7

[a] A 28-fold molar excess of either mab M-7, mab M-26, mab M-32, or nonimmune normal murine IgG was incubated overnight at 4 °C with 40 ng of ^{125}I-HDL. Samples containing antibody and ^{125}I-HDL complexes were then incubated overnight at 4 °C on duplicate wells coated with 15 μg mL^{-1} each mab, washed, and counted as described under Materials and Methods. The values are the percentage of added ^{125}I-HDL bound by each immobilized mab. With immobilized normal mouse IgG in the second incubation, low levels of nonspecific ^{125}I-HDL were bound (<1% of added ^{125}I-HDL) using all of the antibodies from the first incubation.

As expected, all immobilized anti-apoA-II mabs bound less ^{125}I-HDL after incubation with anti-apoA-II mabs than with nonimmune mouse IgG. By contrast, all anti-apoA-I mabs bound more ^{125}I-HDL after incubation with anti-apoA-II mabs than with normal mouse IgG. The binding percentages were 100% compared to 44%. The complete binding of HDL after exposure to anti-apoA-II mabs suggested either that mab M-32 cross-linked two or more HDL particles or that an unusual form of cooperative binding was elicited.

To determine if mab M-32 can cause two or more HDL particles to cross-link through an M-32 bridge and to study the effect of mab concentration on this increased binding, the following experiments were performed. Increasing concentrations of mab M-32 were preincubated with a fixed amount (20 ng) of ^{125}I-HDL overnight, followed by a second overnight incubation on mab M-30 coated plates. A dose-dependent cooperative increase in HDL binding to mab M-30 (Figure 5) was elicited as determined from the percentage of ^{125}I-HDL bound. This increased binding, from 63% to 100%, was obtained by using mab M-32:HDL molar ratios from 0.05:1 to 12.5:1. Whether a dose-dependent cooperative increase of ^{125}I-HDL binding can be produced by each anti-apoA-II mab and each anti-apoA-I mab has not been established. However, since incubations of ^{125}I-HDL with each anti-apoA-II mab (IgG) had the effect of increasing the binding of HDL to all anti-apoA-I mabs tested, similar dose-dependent results with any anti-apoA-II and anti-apoA-I IgG pair would be expected.

Incubation of ^{125}I-HDL with Fab fragments of mab M-32 gave the same percentage of binding to mab M-30 as did ^{125}I-HDL alone. With POPC–^{125}I-apoA-II or ^{125}I-apoA-II, there was no effect of mab M-32 addition. This indicates that this cooperativity effect is specific for HDL and excludes any possible conversion of apoA-II to an apoA-I-like conformation by mab binding. In addition, ^{125}I-mab M-32 did not bind to mab M-30 coated plates using the same or higher concentrations of mab M-32 as in dose-dependent assays (data not shown).

The molar ratio comprising the mab M-32–HDL immune complex bound by immobilized anti-apoA-I mab M-30 was calculated from the amount of radioactivity bound by mab M-30 in concurrent incubations with either component, HDL or mab M-32, prelabeled with ^{125}I. Shown in Figure 5 as the amount of mab M-32 increased, the HDL:mab M-32 ratio bound by mab M-30 simultaneously decreased from 6.0 to 0.02. As the cooperative effect of mab M-32 binding to HDL is elicited, mab M-30 binds immune complexes containing less HDL per mole of mab M-32. If these data were the function of cross-linking of HDL through an mab M-32 bridge, it would

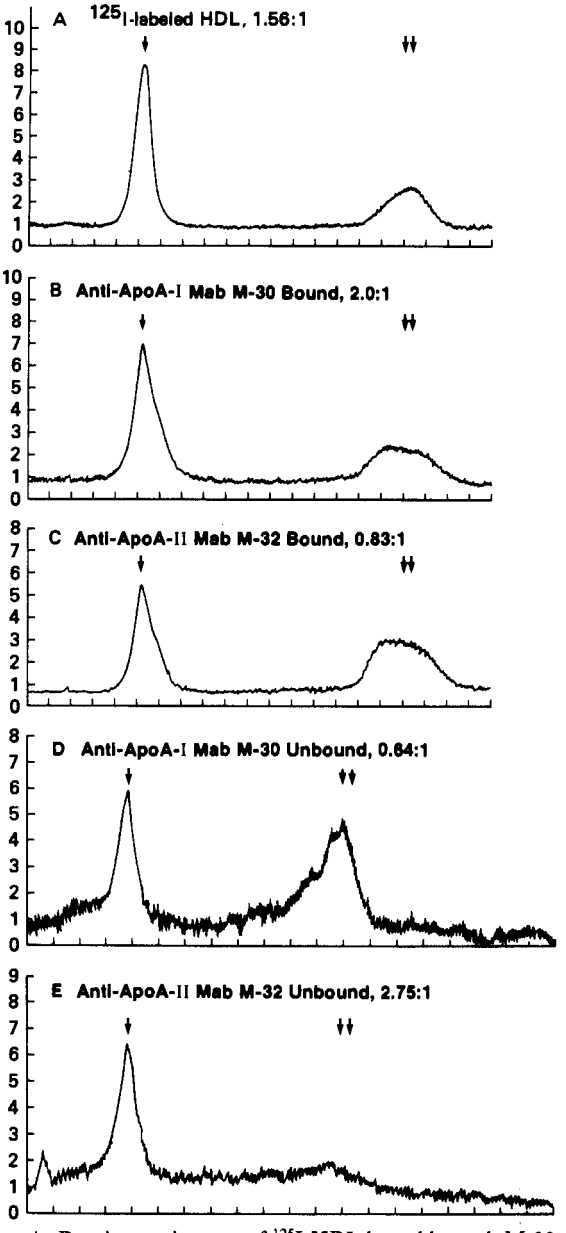

FIGURE 4: Densitometric scans of ^{125}I-HDL bound by mab M-30 and mab M-32. Bound ^{125}I-HDL from incubations in 12 wells were released from the wells by an incubation in 50 μL of a buffer containing 2% SDS and 5% 2-mercaptoethanol for 1 h at 23 °C. Electrophoresis was performed on 12.5% SDS–polyacrylamide gels. Autoradiography of the gels was performed at 23 °C with Kodak X-Omax R film for 3–4 days. The profiles of the autoradiographs were obtained with an LKB automatic laser densitometry scanning system. The positions of apoA-II and apoA-II are indicated by a single arrow and a double arrow, respectively. Both abscissa and ordinate scales are in arbitrary units. The apoA-I:apoA-II ratios shown are based on integration of peak areas for apoA-I and apoA-II. Figure 5A is the profile of untreated ^{125}I-HDL; Figure 5B, HDL bound to mab M-30; and Figure 5C, HDL bound by mab M-32. The ^{125}I-HDL that was not bound by either mab M-30 or mab M-32 were boiled with SDS gel sample buffer similarly applied to a 12.5% SDS–polyacrylamide gel. The profile of the unbound HDL fraction shown in Figure 5D was obtained with mab M-30 and the profile shown in Figure 5E with mab M-32. The differences in salt concentration due to PBS in these unbound samples changed the electrophoretic migration of apoA-I and apoA-II, compared to those of the bound samples.

have resulted in a higher HDL:mab M-32 ratio. In contrast, we obtained an inverse relationship between HDL binding and the HDL:mab M-32 ratio bound by mab M-30.

With several concentrations of HDL, and up to an 80-fold molar excess of mab M-32, no significant increase, over moderate background levels with HDL alone, was detected

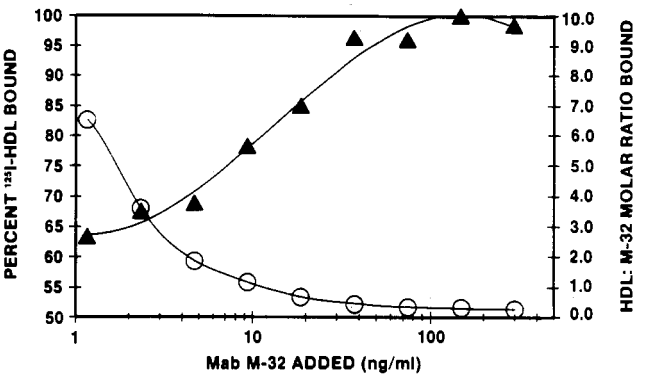

FIGURE 5: Dose-dependent cooperativity effect of mab M-32 binding to HDL. Increasing amounts of either purified anti-apoA-II mab M-32 and 20 ng of ^{125}I-HDL or ^{125}I-labeled mab M-32 and 20 ng of unlabeled HDL were preincubated overnight at 4 °C. Samples were then added to anti-apoA-I mab M-30 coated wells, incubated overnight at 4 °C, and washed and counted as described (see Materials and Methods). The percentage of ^{125}I-HDL (▲) bound by mab M-30 was determined by subtracting counts of nonspecific binding (wells without mab M-30) from the counts of bound wells (wells with mab M-30), divided by the total cpm originally added to each well, multiplied by 100. The HDL:mab M-32 molar ratio (○) was calculated from the two separate incubations by determining the amount of labeled component specifically bound to the mab M-30 coated wells. Radioactivity (cpm) of nonspecific wells was subtracted from the counts of bound wells (as above) divided by the specific radioactivity of either ^{125}I-HDL or ^{125}I-mab M-32 (451 and 757 cpm/ng, respectively), and then divided by their relative molecular weight of 2.5×10^5 and 1.5×10^5, respectively. The titer of mab M-32 was not changed by ^{125}I-labeling as determined by ELISA. All values are the average of duplicate determinations and were within 3% of the mean.

by right-angle light scattering (data not shown).[2] Therefore, all our evidence indicates that cross-linking of HDL is not a factor in the cooperativity we observe with mab M-32 and HDL. This cooperativity is most likely due to either an increased affinity of apoA-I or an increased availability and expression of the apoA-I epitope on a subfraction of HDL which contains a higher proportion of apoA-II.

Localization of ApoA-I Epitope. Synthetic apoA-I peptides corresponding to amino acid residues 87–124, 142–182, and 224–243 were initially tested by dot blotting each peptide on nitrocellulose, followed by incubation with mab M-30 and a peroxidase second antibody detection. Only one synthetic peptide fragment, apoA-I_{87-124}, showed any reactivity with mab M-30. Solid phase competitive RIAs with two of the three synthetic peptides described below, apoA-I_{87-124} and apoA-I_{99-124}, did not compete but gave a dose-dependent increase in the level of ^{125}I-HDL bound by mab M-30. The $B/B_0 \times 100$ value increased from 100 to 200 over a range from 1.0 to 100 μg mL^{-1} (data not shown). These results suggest that peptide, free in solution, is incorporated in HDL and increases HDL binding to mab M-30. To determine the relative expression of the domain of mab M-30 on smaller peptides, it was necessary to incorporate the peptides to a phospholipid matrix.

Using the same procedure which enabled the classification of this lipid-dependent mab M-30 epitope, synthetic HDL composed of POPC–apoA-I peptides were tested as competitors of ^{125}I-HDL binding (Figure 6). Logit transformation of the data shows a large difference in the slopes obtained with POPC–apoA-I peptides, compared with HDL. The slopes were −8.0, −8.4, −7.0, and −2.2 for POPC–apoA-I_{87-124},

[2] Right-angle light scatter was measured with an SLM 8000S fluorometer (excitation, 340 nm; emission, 340 nm).

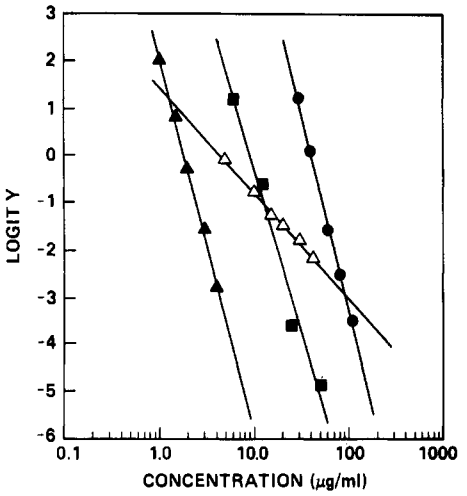

FIGURE 6: Mab M-30 RIAs with sHDL containing apoA-I peptides. The effects of sHDL containing apoA-I peptides on ^{125}I-HDL binding to mab M-30 are shown by using logit transformation of the data for slope comparisons. The conditions used for these RIAs with ^{125}I-HDL and mab M-30 coated plates were the same as in Figure 2. sHDL were prepared, as described under Materials and Methods, containing the synthetic peptides apoA-I_{87-124} (▲), apoA-I_{99-124} (●), and apoA-$I_{107-124}$ (■). Competition by these sHDL for ^{125}I-HDL binding to mab M-30 is compared with unlabeled HDL (△).

POPC–apoA-I_{99-124}, POPC–apoA-$I_{107-124}$, and HDL, respectively. Even on the basis of the molarity of incorporated peptide, POPC–apoA-I_{87-124} requires 25-fold less than POPC–apoA-I_{99-124} and 10-fold less than POPC–apoA-$I_{107-124}$ for equal competition. This pattern of epitope expression by synthetic apoA-I peptides suggests that the higher level of expression on POPC–apoA-I_{87-124} is due to some aspect of its larger molecular size. The reason why POPC–apoA-$I_{107-124}$ is a better competitor than POPC–apoA-I_{99-124} is unclear. These data indicate that the 18 apoA-I amino acid residues of apoA-$I_{107-124}$ are sufficient for high-affinity lipid-dependent binding to mab M-30.

Discussion

Eight monoclonal antibodies were generated with HDL as the immunogen and as the primary screening antigen. Five clones, mab M-25, mab M-28, mab M-29, mab M-30, and mab M-31, show absolute reactivity to apoA-I by immunoblotting analysis of each affinity-purified mab. An additional antibody, mab M-32, showed absolute specificity for apoA-II bound to nitrocellulose. By contrast, mab M-7 and mab M-26 are highly reactive with apoA-II and are slightly cross-reactive with apoA-I by the criteria of immunoblotting. The reason for this cross-reactivity is not precisely known but is attributed to the homologous regions shared by apoA-I and apoA-II (Barker & Dayhoff, 1977).

Cotitration analysis indicates that, with the exception of mab M-25, all anti-apoA-I mabs bind to a similar epitope or region of apoA-I within HDL. By the same method, mab M-7 and mab M-26 appear to recognize a site on apoA-II different from that bound by mab M-32. The small molecular volume of apoA-I and apoA-II (28 000 and 17 400 daltons, respectively), in contrast to an IgG or its Fab fragment, limits the interpretation of the cotitration analysis. Steric hindrances by the antibody could cause large differences in accessibility to epitopes that may actually be relatively far apart on the surface of HDL. Thus, any mabs thought to bind to similar or proximal sites may in fact bind to distinct epitopes. However, in spite of these considerations, these studies show that distinct mabs can be identified. The classification of competing antibodies into a noncompeting group specific for a single epitope is therefore only provisional; however, mabs placed in different groups are free of these considerations. Additional studies with peptides of apoA-I and apoA-II are necessary to map unequivocally the binding sites of each group of mabs. Cotitrations together with the different immunoblotting reactivities of two (mab 7 and mab 26) of the three apoA-II mabs are consistent with the view that these mabs can identify two discrete epitopes on apoA-II. There is only one other report of a monoclonal antibody specific for apoA-II (Curtiss & Edgington, 1985).

Quantitative competitive RIAs of apoproteins have routinely used lipid-free ^{125}I-apoprotein as the antigen and either whole plasma or delipidated plasma as competitor. Several problems with this method have been noted. They include self-association (Formisano et al., 1978; Vittelo et al., 1976), limited or variable detection of all apoA-I in HDL or plasma, and the absence of the appropriate standards. In this report, we describe a sensitive solid phase competitive RIA that utilizes the specificity of anti-apoA-I mab M-30 for HDL and sHDL. The difficulties inherent in interpretation of competitive RIAs are very much apparent with mab M-30 and lipid-free apoA-II as competitor (Figure 2A). It apoA-II is a component of the synthetic POPC vesicle, it no longer inhibits the binding of ^{125}I-HDL to mab M-30 (Figure 2B). The shift in the position of the displacement curves with POPC–apoA-I to the left (Figure 2) of HDL indicates that the expression of apoA-I on HDL is modulated by either the lipids (cholesterol, triglycerides, and phospholipids), the ratio of lipids to apoprotein, the presence of other apoproteins, or the accessibility of the antibody to apoA-I due to more apoA-I per particle. The change from highly competitive to essentially nonreactive upon addition of apoA-II to synthetic POPC complexes shows that these assays (which utilize ^{125}I-HDL as antigen) are sensitive to conformational changes in the apoprotein. Moreover, the presence of HDL lipids other than phosphatidylcholine is not necessary for this effect to be observed. The increased binding seen upon addition of lipid-free apoA-I peptides to RIAs with HDL, and the reversal of apoA-II competition in our RIA, indicates that protein transfer can be detected by mab M-30.

The similarity in the slopes of RIAs with POPC–apoA-I and HDL as competitor implies that apoA-I is structurally organized in POPC complexes in a manner similar to that of HDL. However, in HDL, the mab M-30 epitope is not completely expressed. There also was essentially no significant difference between the affinity of mab M-32 for apoA-II, as a component either of an POPC–apoA-II complex or of HDL.

Direct binding studies and competitive RIA results provide independent evidence that establishes the preferential reactivity of mab M-30 for apoA-I associated with lipids. Mab M-30 binds to apoA-I as a component of either POPC–apoA-I or HDL, but not to free apoA-I. More free apoA-II was bound to mab M-32 when the apoprotein was in the lipid-free state, as compared to apoA-II in POPC–apoA-II or HDL. This suggests either that all the apoA-II within POPC–apoA-II may not be expressed or that the restriction imposed by solid phase assays may give lower maximal binding with the POPC–apoA-II vesicles or HDL.

Without the data from direct binding studies, the cross-reactivity seen by competitive inhibition assays at only high protein concentrations (Figure 2) may be explained by component contamination. However, the direct binding experiments show that POPC–apoA-I binds nonspecifically to anti-apoA-II mab M-32, as does POPC–apoA-II with anti-apoA-I mab M-30. The competitive RIAs show that there are at least 2 orders of magnitude difference in the concen-

tration required for similar levels of competitive inhibition between apoA-I– or apoA-II–POPC complexes. Therefore, we feel that all our evidence indicates that mab M-30 and mab M-32 are in fact very specific antibodies which have distinct non-cross-reacting specificity.

Our studies with POPC–^{125}I-apoA-I and POPC–^{125}I-apoA-II argue against any intrinsic immunochemical heterogeneity of the apolipoprotein epitopes, since *complete* binding of both synthetic complexes is obtained with mab M-30 and mab M-32, respectively. Immunoblotting of apoA-I isoforms separated by isoelectric focusing in polyacrylamide gels did not give any apparent differences in the reactivity with all our anti-apoA-I mabs (data not shown).

The heterogeneity of HDL has been described in several studies on the basis of immunochemical and classical separation techniques. HDL contains various subfractions which have different molar ratios of apolipoproteins. All species of HDL described to date contain apoA-I. Our anti-apoA-I mab M-30 could bind HDL which contain a higher apoA-I:apoA-II ratio. Therefore, we conclude that in those HDL which contain a high proportion of apoA-II, the binding to mab M-30 is reduced either because the presence of apoA-II or because of other conformational constraints imposed by an intrinsic heterogeneity in the composition of HDL subfractions. The absence of apoA-II in the unbound fraction from HDL incubated on immobilzed mab M-32 supports previous studies (Curtiss & Edgington, 1985; Cheung & Albers, 1984) in which anti-apoA-II antibodies remove all the apoA-II-containing particles, leaving HDL particles containing only apoA-I. Complete HDL binding in the presence of excess mab M-32 (Figure 5) supports the views that mab M-30 can bind to HDL containing only apoA-I, in addition to HDL containing both apoA-I and apoA-II.

With our mabs, the apparent immunochemical heterogeneity of HDL can be eliminated by addition of any one of the anti-apoA-II mabs. Removal of ^{125}I-labeled apoA-II from HDL would not produce the complete binding of HDL that is observed. Complete HDL binding also suggests that the mab M-30 epitope is not "altered" as described by Milthorp et al. (1986), although the different levels of reactivity with various preparations of HDL and sHDL, containing apoA-I, may be due to similar phenomena. Any possible transformation of apoA-II into an apoA-I conformational state upon mab binding was excluded. No direct binding of ^{125}I-mab M-32 to mab M-30 coated plates eliminated the possibility of nonspecific interaction between mabs and an HDL–mab complex. By enhancing the binding of ^{125}I-HDL to one solid phase apoA-I "receptor", mab M-30, after preincubation with one anti-apoA-II mab, mab M-32, our monoclonal pair elicited a cooperativity independent of any cross-linking or precipitation. Since the cooperative effect shown by Curtiss and Edgington (1985) required three monoclonals, two anti-apoA-I and one anti-apoA-II monoclonals, for complete immunoprecipitation of all ^{125}I-HDL, a direct comparison to our findings is not possible. Their results may have reflected a removal of apoA-II from HDL prior to complete immunoprecipitation. However, it is possible that the complete precipitation reported by Curtiss and Edgington was due to a similar but unrecognized phenomenon such as the cooperativity reported here. Therefore, all the evidence suggests that the sites identified by our mabs are homogeneous but are expressed heterogeneously on HDL.

This unusual form of cooperativity with only two monoclonals may be due to a conformational change in apoA-I induced by binding of an anti-apoA-II mab to the apoA-II in HDL containing both apoA-I and apoA-II. There appear to be two possible explanations for this conformational change. First, there is a conformational change in apoA-I which increases the affinity of apoA-I in complexes which contain both apoA-I and apoA-II. Second, mab M-32 binds to apoA-II on HDL in such a way that accessibility of the antibody to apoA-I is changed in some HDL subfractions, particularly those containing greater amounts of apoA-II. This change in affinity or accessibility could be due to a change in the following on particles containing both apoA-I and apoA-II: the interaction of apoA-I with apoA-II caused by binding of mab M-32 to apoA-II; a modified microenvironment, i.e., a different lipid to protein ratio which may affect distribution of apoA-I on the surface of HDL; or an electrostatic interaction of apoA-I and the Fc portion of the IgG bound to apoA-II. The lack of cooperativity found when using the Fab fragment of M-32 indicates that the Fc region of the monoclonal participates in the interaction with apoA-I. If there is a direct interaction of apoA-I with apoA-II, our results are in contrast to those of Swaney and Palmieri (1984), who found no strong apoA-I and apoA-II protein–protein interactions by chemical cross-linking either as delipidated species or in lipid complexes.

The activation of LCAT by apoA-I is well established (Fielding et al., 1972; Chung et al., 1979). Recent evidence by Chen and Albers (1986) indicates that the level of apoA-II in apoA-I liposomes can modulate the amount of LCAT activation. From these findings, we propose that the ratio of apoA-I to apoA-II may regulate the expression and conformation of the mab M-30 lipid-dependent epitope and thereby regulate the level of LCAT activity. Quantitation of this sensitive mab M-30 epitope from plasma may provide a simple method of establishing the metabolic state of HDL. In support of this possibility, we have shown that mab M-30 and mab M-32 exhibit differences in binding affinity and detect differences in the expression of epitopes on HDL_2 and HDL_3 isolated by zonal centrifugation (Table III). Further studies are in progress to characterize the effects of these antibodies on LCAT activation by apoA-I and its synthetic peptides and to define the nature of the postulated involvement of apoA-II on the mab M-30 antigenic site.

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