Selection of monoclonal antibodies for linear epitopes of an apolipoprotein yields antibodies with comparable affinity for lipid-free and lipid-associated apolipoprotein

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Abstract A direct approach is described for generating human apolipoprotein (apo) A-I-specific monoclonal antibodies with defined linear epitopes. The antibodies have comparable binding affinity for delipidated apoA-I and apoA-I on high density lipoproteins (HDL). The antibodies were obtained by immunizing mice with HDL, screening the fusions first for antibodies that bind native HDL and second, for antibodies that bind defined apoA-I synthetic peptides. ApoA-I antibody epitopes assigned on the basis of synthetic peptide binding were confirmed by solid phase and fluid phase antibody competition assays. These antibodies, which bind epitopes that encompass greater than 60% of apoA-I, should prove useful for identifying discrete functional domains of apoA-I on HDL.—**Curtiss, L. K., and C. L. Banka.** Selection of monoclonal antibodies for linear epitopes of an apolipoprotein yields antibodies with comparable affinity for lipid-free and lipid-associated apolipoprotein. *J. Lipid Res.* 1996. **37:** 884–892.

Supplementary key words apolipoprotein A-I • monoclonal antibodies • linear epitopes

Plasma lipoproteins play key roles in cholesterol metabolism and are important factors in identifying those individuals at risk for atherosclerosis, stroke, and cardiovascular disease. Individuals with high levels of circulating high density lipoproteins (HDL) are at a lower risk for the complications of atherosclerosis (1, 2). Apolipoprotein (apo) A-I is the major apoprotein of HDL. Numerous functions of apoA-I contribute to the apparent beneficial properties of HDL in humans. These include the capacity of apoA-I to: *a)* activate the enzyme that esterifies cholesterol in plasma, lecithin:cholesterol acyltransferase (LCAT) (3); *b)* facilitate cholesterol efflux from certain nonhepatic peripheral cells such as cholesteryl ester-loaded foam cells of the atherosclerotic lesion (4); and *c)* mediate cholesterol delivery to steroidogenic tissues (5). Thus, apoA-I plays a pivotal role in cholesterol metabolism and vascular biology.

Knowledge of the structure of apoA-I could provide for the rational design of therapeutic drugs that mimic the beneficial effects of apoA-I and potentially alter the course of lesion development in atherosclerosis. The primary structure of apoA-I is known (6). Also, circular dichroism, tryptophan fluorescence, NMR spectroscopy, quanidine denaturation, polarized attenuated total reflection infrared spectroscopy, and computer models have revealed certain properties of the secondary structure of apoA-I (7–11). ApoA-I, like other related exchangeable apolipoproteins, contains multiple 11 amino acid residue sequences that share a high degree of homology. These 11 residue repeats make up 69% of the protein of apoA-I and are assembled into eight, 22-residue tandem repeats (12). The 22-residue tandem repeats are alpha helices interrupted by beta turns imposed by proline or glycine residues. Each alpha helix is amphipathic; the hydrophobic face interacts with the lipids of HDL and the hydrophilic face interacts with the aqueous environment. The definitive secondary structure of a protein is elucidated by X-ray crystallography. However, apoA-I, like other proteins that interact with lipids, has proven refractory to this analysis. Even though isolated apoA-I or fragments of apoA-I potentially can be crystallized and studied, information gained from this approach may not provide an accurate representation of the tertiary structure of apoA-I as it is assembled on spherical HDL. Because of this level of

Abbreviations: apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline; HDL, high density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ig, immunoglobulin.

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complexity, alternate approaches to delineating the secondary and tertiary structure of apoA-I are necessary. Recent studies of protease-resistant regions and deletion mutants have enabled researchers to assign specific functions or properties to discrete domains within the molecule (8, 13, 14). We have identified specific functional domains using an immunochemical approach (15, 16).

Monoclonal antibodies with defined linear epitopes can be used to probe the conformation of specific regions on apoA-I as it is assembled on HDL. Previously, we have identified the epitopes of five monoclonal antibodies that bind human apoA-I on HDL (15–17). These antibodies were obtained by immunizing mice with human HDL, screening for antibodies that bind native HDL, and then defining the epitopes of key antibodies by testing their binding to fragments or synthetic peptides of apoA-I. In the work described here we used a more direct approach to obtain 12 new antibodies. These antibodies were generated by immunizing mice with native HDL or isolated apoA-I and screening for antibodies that bound only native HDL. All HDL-binding antibodies were subsequently selected for their ability to bind apoA-I synthetic peptides in competitive fluid phase immunoassays. The epitopes identified by these antibodies span approximately 60% of the 243 amino acid residues of apoA-I. More importantly, these antibodies all have comparable affinity for lipid-free and lipid-associated apoA-I so they can be used to precisely identify apoA-I irrespective of its state.[2]

MATERIALS AND METHODS

Isolation of lipoproteins and apoproteins

Human HDL (d 1.063–1.21 gm/ml) was isolated from normal human plasma by standard ultracentrifugation techniques in the presence of protease inhibitors and antioxidants as described (16). After isolation, HDL was dialyzed extensively against 0.15 M NaCl containing 0.3 mM EDTA and 1 μM probucol, filter-sterilized, and stored at 4°C for up to 30 days. The apolipoprotein composition of the HDL fraction was defined by electrophoresis on 4–15% polyacrylamide gels in the presence of SDS (SDS-PAGE) as described (18).

ApoA-I was isolated from 40 mg of ether/ethanol-delipidated HDL proteins by preparative SDS-PAGE in a Bio-Rad Prep Cell 491 (Richmond, CA). After delipida-

[2]Qualified investigators can obtain antibody by contacting L. K. Curtiss at The Scripps Research Institute; fax: (619) 554-6146.

TABLE 1. Human apoA-I-specific monoclonal antibodies

Antibody Number[a]	Immunogen	Ig Heavy Chain	ApoA-I Epitope[b]	A-I/HDL Affinity[c]
AI-16	PAGE HDL[d]	IgG_2	1–15	different
AI-1.2	ApoA-I	IgG_2	1–19	same
AI-19.2	ApoA-I	IgG_1	19–31	same
AI-19.4	ApoA-I	IgG_{2a}	19–31	same
AI-18	PAGE HDL[d]	IgG_1	95–105	different
AI-11	HDL	IgG_1	96–111	different
AI-4	Apo VLDL[e]	IgG_1	99–121	different
AI-115.1	ApoA-I	IgG_1	115–126	same
AI-115.3	ApoA-I	IgG_{2a}	115–126	same
AI-119.1	ApoA-I	IgG_1	119–144	same
AI-119.3	ApoA-I	IgG_1	119–144	same
AI-119.8	ApoA-I	IgG_1	119–144	same
AI-137.1	HDL	IgG_1	137–147	same
AI-141.1	HDL	IgG_1	141–164	same
AI-178.1	HDL	IgG_1	178–200	same
AI-187.1	HDL	IgG_1	187–210	same

[a]Antibodies AI-16, AI-18, AI-11 and AI-4 have been described previously (see references 16, 17, and 20).
[b]Inclusive amino acid residues of the synthetic peptide that was bound by antibody with the highest affinity as illustrated in Fig. 1 for each new antibody.
[c]A qualitative comparison of the slope analysis for antibody binding to lipid-free apoA-I and HDL as represented in Fig. 3 for six of the antibodies.
[d]Polyacrylamide immobilized HDL.
[e]The soluble apoproteins recovered after ether/ethanol delipidation of normal VLDL.

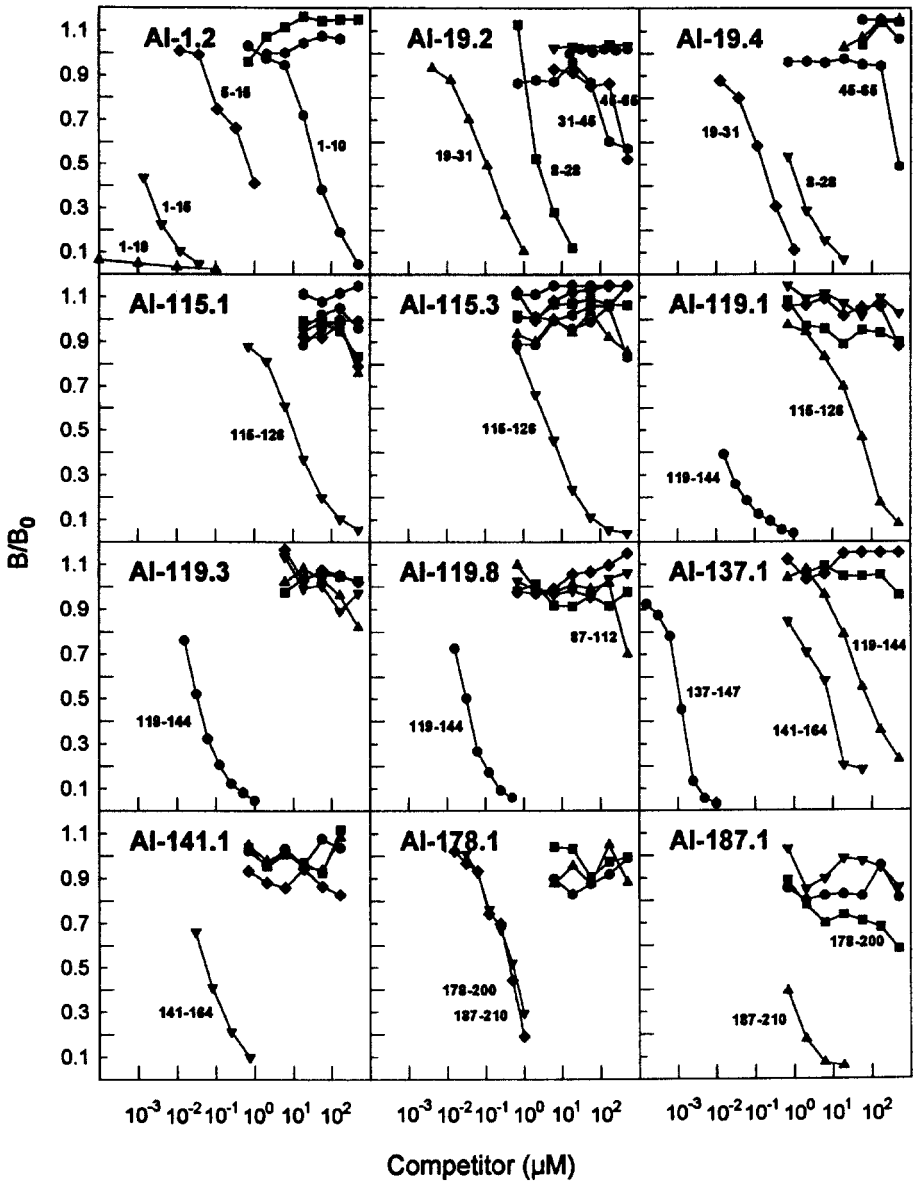

Fig. 1. Epitope assignment. Microtiter plates were coated with 5 μg/ml of HDL and incubated with a limiting amount of each monoclonal antibody in the absence or presence of increasing concentrations of an apoA-I synthetic peptide as described in Methods. Antibodies are designated in top left of each panel. Informative synthetic peptides are identified by their inclusive amino acid residue numbers. Non-competing peptides for antibody AI-1.2 included amino acid residues 8–28 and 19–31. Peptide 1–19 was a potent competitor and inhibited binding by 50% at 10^{-5} μM. Non-competing flanking peptides for each of the other antibodies are as follows: peptides 1–15 and 26–40 for antibody AI-19.2; peptides 1–15, 26–40, and 31–45 for antibody AI-19.4; peptides 94–125, 99–114, 99–121, 108–121, and 141–164 for antibody AI-115.1; peptides 94–125, 99–114, 99–121, 108–121, 119–144, and 141–164 for antibody AI-115.3; peptides 108–121, 137–147, and 141–164 for antibody AI-119.1; peptides 95–108, 108–121, 115–126, 137–147, and 141–164 for antibody AI-119.3; peptides 87–112, 115–126, 119–144, and 137–147 for antibody AI-119.8; peptides 156–174 and 115–126 for AI-137.1; peptides 87–112, 119–144, 137–147, and 156–174 for antibody AI-141.1; peptides 164–187, 156–174, and 203–227 for antibody AI-178.1; and peptides 203–227 and 164–187 for antibody AI-187.1. Antibody epitopes were assigned on the basis of the ability of a given synthetic peptide to inhibit the binding of the antibody to immobilized HDL. The epitope assignments shown in Table 1 were based on the data shown in this figure for the 12 new antibodies.

tion, the proteins were dissolved in electrophoresis sample buffer that contained 4% sodium dodecyl sulfate, 50 mM Tris, pH 7.0, 10% glycerol, 0.1% Bromphenol Blue and electrophoresed through 0.5 cm of a 4% acrylamide stacking gel and 10 cm of a 12% acrylamide resolving gel. Two-ml fractions were collected and the fractions containing apoA-I were pooled, dialyzed against 25 mM ammonium bicarbonate, and stored at -20°C. Purity of the isolated apoA-I was monitored by SDS-PAGE and the apoA-I was quantitated with a modified Lowry assay (19).

ApoA-I peptides were synthesized on an Applied Biosystems ABI 430-A automated peptide synthesizer using HOBt/DCC activation as described (16). Each peptide was purified by high pressure liquid chromatography as described (16), and analyzed by amino acid composition analysis. All peptides were lyophilized and stored under vacuum at room temperature.

Generation and characterization of monoclonal antibodies

Four of the monoclonal antibodies, including AI-16, AI-18, AI-11, and AI-4, have been described previously (16, 17). The remaining 12 antibodies were obtained from five separate fusions of spleens from immunized Balb/c mice with P3Ag8.653.1 myeloma cells using standard fusion protocols (17). The antibodies and the immunogens are listed in **Table 1.** All animals received 20–30 μg of protein in the presence of Hunter's TiterMax (CytRx; Norcross, GA) for a minimum of four injections. After cloning by limiting cell dilution, ascites fluids containing antibodies were generated in 10-week-old Balb/c mice that had been primed with 98% 2,6,10,14-tetramethylpentadecane (Aldrich Chemical Co.) and injected intraperitoneally with 3–50 × 10^5 hybridoma cells. Ig heavy chain subclasses were identified using one of two commercial kits, Isostrip Mouse Monoclonal Antibody Isotyping Kit (Boehringer Mannheim Corporation) and Mouse Monoclonal Sub-Isotyping Kit EK-5150 (HyClone Laboratories, Inc.). Antibodies were purified from ascites fluid by FPLC on a Mono-Q HR 15/10 (100 mm × 16 mm ID) anion exchange column (Pharmacia) (16). Ig was eluted with a 0–0.5 M NaCl gradient in 10 mM Tris buffer, pH 8.0, dialyzed against phosphate-buffered saline (PBS) and stored frozen at -20°C. Isolated antibodies were iodinated using the Iodobead method as described (16). Radioiodinated antibody was separated from free iodine by dialysis. Trichloroacetic acid (TCA) precipitability of the labeled antibodies was in all cases > 95%.

Western blots

To perform Western blots, 20–40 μg of isolated lipoproteins, VLDL, IDL, LDL, and HDL, were electrophoresed on 3–20% gradient SDS polyacrylamide gels under reducing conditions (18). Separated proteins were electrophoretically transferred to Immun-lite membranes (Bio-Rad, Richmond, CA), and the Western blots were visualized using an Immun-lite chemiluminescence assay kit for detection of primary mouse antibody.

Solid phase immunoassays

Hybridoma supernatants were screened for antibodies that bound HDL and synthetic peptides in solid phase immunoassays performed in Falcon 3911 Microtest III flexible assay plates using the volumes and buffers described previously (18). The plates were coated with 5 μg/ml of HDL, pools of synthetic peptides, or

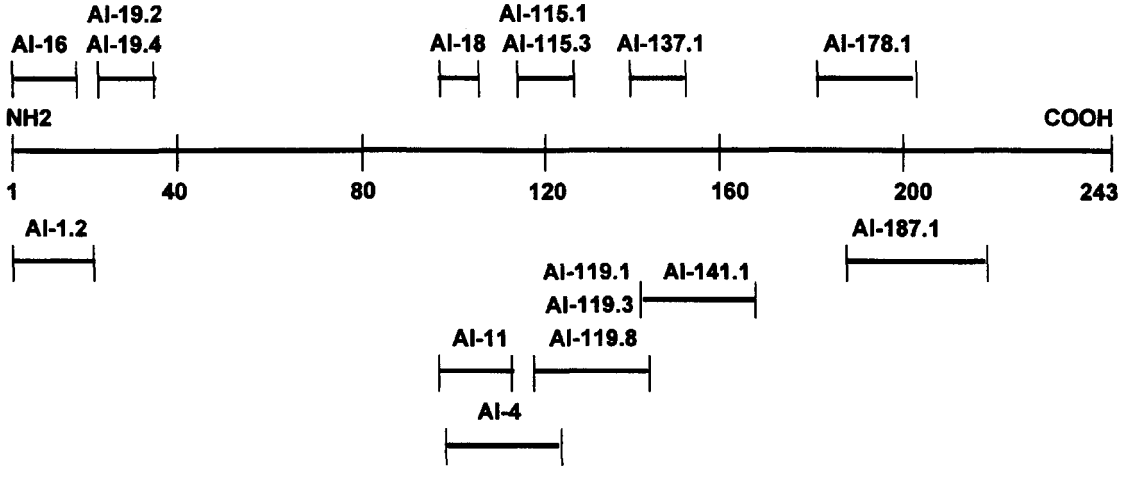

Fig. 2. Linear model of apoA-I primary structure. The continuous epitopes for all 16 antibodies used in this study are indicated on this linear representation of apoA-I. The antibodies define continuous epitopes on apoA-I from residues 1–31, 95–164, and 178–210. No antibodies were selected from the five fusions that bound residues between 32 and 94, 165 and 177, or 211 and 243.

individual synthetic peptides. After post-coating, the plates were incubated with dilutions of hybridoma culture supernatants for 18 h at 4°C. After washing, antibody binding was detected by a second 1-h incubation at 37°C with 10 ng/well of ^{125}I-labeled goat anti-mouse Ig (3–4 μCi/μg).

Competitive solid phase immunoassays were used to compare the binding of each of the antibodies to HDL and isolated apoA-I, and to identify binding to apoA-I synthetic peptides. Native HDL (5 μg/ml) was immobilized on the assay plates. Solubilization of all the peptides was achieved at 1 mM in PBS and all peptide

^{125}I LABELED ANTIBODY

COMPETITOR

	AI-16	AI-1.2	AI-19.2	AI-19.4	AI-18	AI-11	AI-4	AI-115.1	AI-115.3	AI-119.1	AI-119.3	AI-119.8	AI-137.1	AI-141.1	AI-178.1	AI-187.1	
AI-16	<0.5	0.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-16
AI-1.2	<0.5	<0.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-1.2
AI-19.2	>12.5	>12.5	1	2.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-19.2
AI-19.4	>12.5	>12.5	>12.5	0.5	>12.5	>12.5	>12.5	>12.5	>12.5	7	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-19.4
AI-18	>12.5	>12.5	>12.5	>12.5	<0.5	1	<0.5	>12.5	>12.5	>12.5	2	>12.5	>12.5	>12.5	>12.5	>12.5	AI-18
AI-11	>12.5	>12.5	>12.5	>12.5	2	1	1	>12.5	10	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-11
AI-4	>12.5	>12.5	<0.5	>12.5	0.6	>12.5	<0.5	8	<0.5	>12.5	>12.5	>12.5	>12.5	>12.5	9	>12.5	AI-4
AI-115.1	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	<0.5	<0.5	<0.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-115.1
AI-115.3	>12.5	>12.5	>12.5	>12.5	>12.5	<0.5	8	>12.5	<0.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	AI-115.3
AI-119.1	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	10	10	<0.5	10	10	>12.5	>12.5	>12.5	>12.5	AI-119.1
AI-119.3	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	<0.5	<0.5	<0.5	>12.5	>12.5	>12.5	AI-119.3
AI-119.8	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	<0.5	<0.5	0.5	>12.5	>12.5	>12.5	AI-119.8
AI-137.1	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	2	<0.5	1	1	2.5	>12.5	>12.5	AI-137.1
AI-141.1	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	2	<0.5	>12.5	>12.5	AI-141.1
AI-178.1	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	<0.5	<0.5	AI-178.1
AI-187.1	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	2	<0.5	AI-187.1
	AI-16	AI-1.2	AI-19.2	AI-19.4	AI-18	AI-11	AI-4	AI-115.1	AI-115.3	AI-119.1	AI-119.3	AI-119.8	AI-137.1	AI-141.1	AI-178.1	AI-187.1	

Fig. 3. Identification of pairs of antibodies that compete with each other for binding to immobilized HDL in solid phase assays. Radioiodinated antibodies are listed horizontally and the unlabeled competing antibodies are listed vertically. Antibodies are ordered from left to right and top to bottom according to their defined epitopes beginning with the amino terminal. Numbers represent μg/ml of purified competing antibody required for 50% inhibition of ^{125}I-labeled antibody binding to HDL and were obtained from a minimum of two assays per antibody pair. Numbers > 12.5 μg/ml indicate no competition or interference. Numbers < 0.5 μg/ml indicate that 50% inhibition of ^{125}I-labeled antibody binding was observed at antibody concentrations of < 0.5 μg/ml. Homologous competitors are illustrated by the diagonal line of vertically stripped boxes running from top left to bottom right. In each case competition by homologous antibodies was observed at concentrations of ≤ 1 μg/ml. Dark stippled boxes indicate competition between antibodies that bound similar or overlapping epitopes on the linear sequence of apoA-I as illustrated in Fig. 2. Horizontally stripped boxes were described in the text and represent competing pairs of antibodies that did not bind overlapping regions based upon synthetic peptide binding assays.

concentrations were confirmed by amino acid composition analysis. HDL, purified apoA-I, and peptides were diluted in PBS containing 3% normal goat serum and added at the same time as the antibodies. The plates were incubated overnight at 4°C. After washing, mouse antibody binding to the immobilized HDL was detected by a second 1-h incubation at 37°C with ^{125}I-labeled goat anti-mouse Ig. All data were expressed as B/B_o where B is the cpm bound in the presence of competitor, and B_o is the cpm bound in the absence of competitor. To compare affinity of the antibodies for HDL and apoA-I, the slopes of the straight lines drawn through logit-transformed B/B_o ratios were obtained by linear regression for each competitor and subjected to tests of equality as described (17).

Antibody competition assays

Both solid phase and fluid phase antibody competition assays were used to assess the degree to which one antibody interfered with the binding of another. A solid phase assay was used to assess the competition of all possible pairs of antibodies for HDL immobilized at 5 μg/ml onto a 96-well Microtest plate. Increasing amounts of a purified antibody (0.5–12.5 μg/ml) were incubated for 18 h at 4°C with a limiting amount (0.1 or 0.5 μg/ml) of a second radioiodinated antibody (16). Data were expressed as the concentration (μg/ml) of competing unlabeled antibody required to inhibit the binding of radioiodinated antibody by 50%.

For selected antibody combinations, a fluid phase assay also was used to measure the degree to which one antibody interfered with the binding of another antibody on HDL. The assay mixture contained five compartments in a total volume of 0.45 ml per 10 × 75 mm siliconized glass tube. All incubations were performed at 4°C. The first compartment contained 25 ng of HDL in 0.05 ml of PBS with 4% human serum albumin (RIA grade). The second was 0.014–100 μg of purified unlabeled competing monoclonal antibody in 0.1 ml of PBS containing 5% dextran. This mixture was incubated for 18 h before the third compartment was added, which contained 4–20 ng of ^{125}I-labeled monoclonal antibody in 0.05 ml of PBS containing 5% dextran. After a 2-h incubation, antigen-bound ^{125}I-labeled antibody was separated from free ^{125}I-labeled antibody by precipitation of the HDL. This was accomplished with the addition of the fourth and fifth compartments, a saturating amount of affinity-purified rabbit-human apoA-I-specific polyclonal antibodies (15 μg) followed 75 min later by a second 1-h incubation with a 1:10 dilution of a goat anti-rabbit Ig antiserum. The tubes were centrifuged, the supernatants were aspirated, and the radioactivity of the pellets was counted as described above. Nonspecific precipitation was assessed with samples exposed only to goat anti-rabbit Ig and was, in all cases, less than 5%. In preliminary studies it was determined that 12.5 μg/ml of each monoclonal antibody did not directly precipitate HDL and did not interfere with the precipitation of HDL by the rabbit anti-HDL followed by the goat anti-rabbit Ig. Results were expressed as the ratio B/B_o.

RESULTS

Antibodies

The HDL binding properties of 16 human apoA-I-specific monoclonal antibodies were studied. Four of these antibodies, AI-16, AI-18, AI-11, and AI-4, have been described previously, and their epitopes on apoA-I are defined (16, 17). The 12 new antibodies were obtained from five separate fusions using the spleens of mice immunized with HDL or freshly isolated apoA-I. The immunogens used for the generation of each antibody are shown in Table 1, as are the Ig heavy chain subtypes.

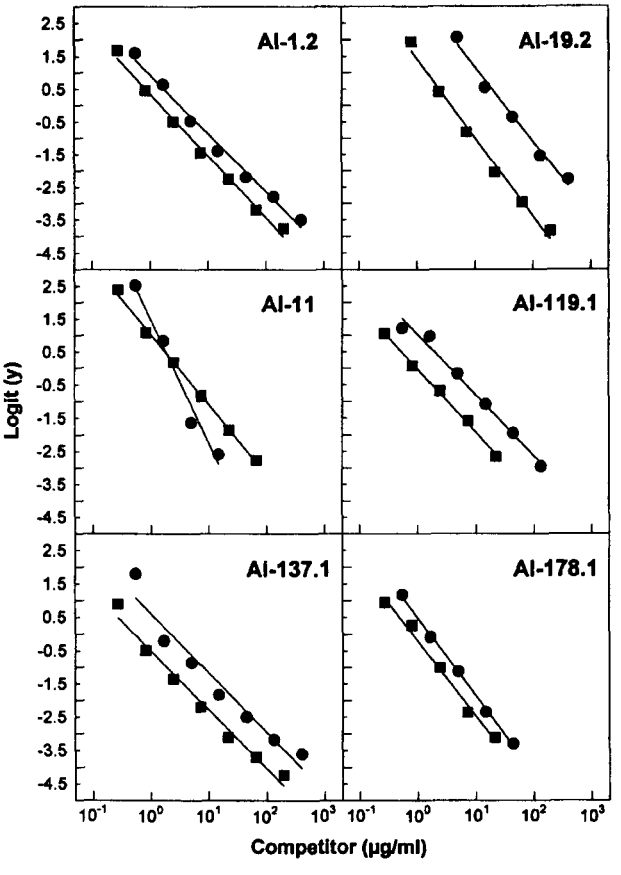

Fig. 4. Comparison of antibody affinities for isolated apoA-I and HDL. All antibodies were tested in multiple assays and the data for six antibodies is shown. Results of similar competitive solid phase assays with all antibodies are summarized in Table 1. The B/B_o binding ratios were subjected to logit transformation where logit (y) equaled the ln (y/1 –y) and $y = B/B_o$. The slopes were calculated by linear regression analysis of the logit-transformed data and were subjected to tests of equality as described (17). Competitors were HDL (solid circles) and lipid-free apoA-I (solid squares).

Each hybridoma supernatant was screened initially for an antibody that bound immobilized native HDL. To select the 12 new antibodies, each HDL-binding hybridoma supernatant was then screened for its capacity to bind six different pools of immobilized synthetic peptides. The pools represented 24 different regions of human apoA-I. Overlapping peptides representing the entire sequence of apoA-I were used. Hybridoma supernatants that bound a pool of immobilized synthetic peptides were examined subsequently for their ability to bind individual peptides within that pool. After ascites production, each antibody was shown to be specific for apoA-I by Western blotting of isolated VLDL, IDL, LDL, and HDL separated by SDS-PAGE (data not shown).

Epitope identification

To establish epitope specificity, the 12 new antibodies were tested in competitive immunoassays for their ability to bind individual apoA-I synthetic peptides in solution. Antibodies that bound peptides in solution were removed by washing and were therefore not available to bind immobilized HDL. The data shown in **Fig. 1** include only competitive binding data for the peptides that were the most informative for each antibody, including all positive peptide competitors as well as noncompeting peptides that flanked the putative epitope. Good binding to synthetic peptides was observed at low μM concentrations of peptides, whereas negative reactions were characterized by the absence of peptide competition at concentrations > 500 μM. The epitopes listed for each new antibody in Table 1 were defined on the basis of these peptide binding assays.

TABLE 2. Fluid phase antibody competition assays[a]

Radioiodinated Antibody	Competitor Antibody	Competitor Concentration Required for 50% Inhibition of Antibody Binding (μg/ml)
^{125}I-AI-19.2	AI-19.2	4
	AI-4	> 27
^{125}I-AI-11	AI-11	0.05
	AI-115.3	> 27
^{125}I-AI-115.3	AI-115.3	0.5
	AI-11	> 27
^{125}I-AI-119.1	AI-119.1	7.5
	AI-19.4	> 27
^{125}I-AI-119.3	AI-119.3	0.8
	AI-18	> 27
^{125}I-AI-178.1	AI-178.1	1.4
	AI-4	> 27

[a]Assays were performed with 25 ng/ml of HDL.

The apoA-I epitopes for each of the 12 new antibodies as well as four previously described antibodies are illustrated in **Fig. 2.** The epitopes were all considered continuous and were drawn onto a linear model of apoA-I. As represented in this model, the epitopes of 16 antibodies encompassed three large discrete regions of apoA-I. Antibodies completely defined the regions from residues 1 to 31, 95 to 164, and 178 to 210, and thus covered greater than 60% of the linear sequence of the apoA-I molecule.

Antibody competition assays

To verify the epitopes assigned on the basis of synthetic peptide binding, we measured the competition between all possible pairs of antibodies for binding to HDL. To perform these studies, each of the 16 antibodies was purified, radioiodinated, and added to solid phase immunoassays with immobilized HDL. Dose titrations were performed to confirm that all antibodies bound HDL after radioiodination and to determine an optimal limiting concentration of radioiodinated antibody for use in the competition assays. In subsequent competitive assays increasing amounts of homologous or heterologous unlabeled antibody were added to an HDL-coated plate to determine the capacity of unlabeled antibody to interfere with the binding of a limiting amount of a radioiodinated antibody. Results of these assays are summarized in **Fig. 3.** Antibodies are ordered in this figure according to their epitopes from the N-terminal to the C-terminal. For ease of analysis, an unlabeled antibody that successfully competed with a radioiodinated antibody is shown as the concentration of antibody (μg/ml) required to achieve 50% inhibition. Numbers > 12.5 μg/ml indicate no competition and numbers < 0.5 μg/ml indicate efficient competition.

As expected, homologous competition was observed for each antibody and is represented as a diagonal line of vertically striped boxes from top left to bottom right of Fig. 3. Dark stippled boxes represent competition by nonhomologous but cross reacting antibodies. The competition between these pairs of antibodies was in complete agreement with the defined linear epitopes that were assigned on the basis of the synthetic peptide binding. For example, antibodies AI-16 and AI-1.2 compete with each other for binding to HDL because their epitopes (residues 1–15 and 1–19, respectively) overlap. Similar expected pairs of competing antibodies include: antibodies AI-19.2 and AI-19.4; antibodies AI-18, AI-11, and AI-4; antibodies AI-4, AI-115.1, AI-115.3, and AI-119.1; antibodies AI-119.1, AI-119.3, AI-119.8, AI-137.1, and AI-141.1; antibodies AI-137.1 and AI-141.1; and antibodies AI-178.1 and AI-187.1. In some cases reciprocal competition between pairs of antibodies was not observed and these cases are designated in the boxes of **Fig. 4** by the bold outline with light stippling. For

example, antibody AI-19.4 did not interfere with the binding of ^{125}I-labeled AI-19.2, but antibody AI-19.2 competed for the binding of ^{125}I-labeled AI-19.4. The most likely explanation for non-reciprocal interference was a major difference in the affinities of the two antibodies (e.g., the affinity of antibody AI-19.4 is considerably less than the affinity of antibody AI-19.2) for immobilized HDL.

Six combinations of competing antibody pairs were found that were not predicted from the epitopes assigned on the basis of the synthetic peptide binding. These combinations are indicated in Fig. 3 by the horizontally striped boxes. To eliminate any artifacts imposed by using immobilized HDL, these competing pairs were studied further in fluid phase immunoassays. The assays were first optimized by determining the proper proportions of rabbit anti-human HDL and goat anti-rabbit Ig required to precipitate fixed amounts of HDL. The antibody competition assays were then performed by incubating increasing amounts of unlabeled monoclonal antibody with HDL for 18 h followed by a 2-h incubation with limiting amounts of homologous or heterologous radioiodinated monoclonal antibody. Bound radioiodinated antibody was separated from free antibody by direct precipitation of the HDL with the polyclonal antisera. Results are shown in **Table 2.**.

Direct evidence was obtained that HDL immobilized on a plastic immunoassay plate behaved differently than fluid-phase HDL. Competition for HDL between five of the original six antibody pairs was not observed in the fluid phase antibody competition assays, although good competition for 25 ng of ^{125}I-labeled antibody by 0.05–7.5 μg/ml of the homologous unlabeled competitor was observed always in these fluid phase assays. Reasons for the loss of interference between non-homologous antibodies in fluid phase assays were not explored further.

Binding to HDL and isolated apoA-I

Although all of these antibodies were specific for apoA-I as shown by Western blotting, it was necessary to verify that they reacted with apoA-I on HDL for these antibodies to be useful probes of the structure of apoA-I on HDL. This was accomplished by comparing the affinities of each antibody for apoA-I and HDL. Each antibody was assessed for its ability to bind HDL versus isolated apoA-I. Shown in Fig. 4 are representative results of these assays for six of the antibodies. Slope analysis of the straight lines drawn through the logit-transformed B/B_o ratios was used to compare the affinities of each antibody for HDL and apoA-I. Each of the 12 antibodies selected by peptide binding bound HDL and apoA-I with comparable affinities. In contrast, each of the previously described antibodies including AI-16, AI-4, AI-11, and AI-18 bound the two antigens with different affinities. The data for antibodies AI-16, AI-18, and AI-4 have been published (17, 20). The qualitative results for all 16 antibodies are listed in Table 1.

DISCUSSION

Monoclonal antibodies with defined epitopes on apoA-I have proven useful in delineating both molecular domains of functional importance and structural features. An epitope is a relational entity defined only by the binding of a specific antibody. Protein epitopes have been classified as continuous (linear) or discontinuous (conformational) (21). Continuous epitopes are composed of residues that are in close proximity in the linear peptide sequence. Discontinuous epitopes are made up of residues from different parts of the primary sequence that are brought together by folding of the protein into its native tertiary structure. Marcel et al. (22) has proposed that most epitopes on apoA-I are discontinuous and are assembled topographically from a series of residues that are not contiguous in the primary amino acid sequence but are brought together by folding of a single apoA-I molecule or by the interaction of multiple molecules of apoA-I. Nonetheless, we demonstrated here that antibodies that bind continuous linear epitopes on a protein can be selected by altering the antibody screening strategy. We used a strategy to identify HDL-specific antibodies that also bound small, defined synthetic peptide fragments representing linear sequences of apoA-I. A caveat of this strategy is that the precision of each epitope assignment is a direct reflection of the size and sequence of the peptides used. More explicit definitions of the epitopes will require use of either additional peptides or a precise identification of the apoA-I residues that physically contact the antibody binding domain and truly contribute to the binding energy.

Each new antibody that bound a continuous linear epitope, as shown in Fig. 1, bound HDL and apoA-I with comparable affinity (Fig. 3). This outcome probably resulted from our screening strategy. Only antibodies with continuous linear epitopes were obtained, because only those that bound both HDL and a defined apoA-I synthetic peptide were selected. Thus, the screening strategy used to select antibodies for probing protein conformation on complex structures is extremely critical. It is interesting to note that the antibodies we selected previously, which were not selected on the basis of their ability to bind a continuous epitope (including antibodies AI-16, AI-18, AI-11, and AI-4), all have different affinities for lipid-free apoA-I and apoA-I on spherical HDL (17, 20), and thus support the notion that many antibodies identify conformation-dependent epitopes.

In summary, our approach for selecting monoclonal antibodies with discreet, continuous linear epitopes has proven successful. Furthermore, such a screening strategy has given rise to a panel of antibodies with defined epitopes that have comparable affinity for lipid-free and lipid-associated apoA-I. Such antibodies should prove useful for further defining domains of apoA-I that are responsible for its many functions.

The authors wish to acknowledge the technical contributions of Kathi Richards, D. J. Bonnet, Yili Guo, Ian J. Dawson, Jill Patch, and Audrey S. Black. Dr. Richard Smith helped with preparation of the peptides, and we thank Anna Meyers for preparation of the manuscript. These studies were supported by NIH Grant HL43815. This is manuscript #8736-IMM from The Scripps Research Institute.

Manuscript received 18 December 1995.

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