Immunochemical characterization of six monoclonal antibodies to human apolipoprotein A-I: epitope mapping and expression

Santica Marcovina,[1] Simona Fantappie,* Adele Zoppo, Guido Franceschini,* and Alberico L. Catapano*

Laboratory of Immunochemistry and Lipoproteins, Scientific Institute S. Raffaele, Milan, Italy, and Institute of Pharmacological Sciences,* University of Milan, Milan, Italy

Abstract We have produced and characterized six murine monoclonal antibodies to human apolipoprotein A-I named A-I-9, A-I-12, A-I-15, A-I-16, A-I-19, and A-I-57. All monoclonal antibodies were specific for apolipoprotein A-I and bound between 55% and 100% of ^{125}I-labeled high density lipoproteins (HDL) in a fluid phase radioimmunoassay. All antibodies possessed a higher affinity to apoA-I in HDL than to free, delipidated apoA-I. Two of them, particularly A-I-12 and A-I-15, which were directed to the same or very close epitopes on the molecule, recognized very poorly the delipidated protein. Binding of apoA-I to phospholipid restored the immunoreactivity of the monoclonal antibodies to the protein suggesting that lipids play an important role in determining the immunochemical structure of apoA-I. Using CNBr fragments and synthetic peptides, the epitopes for the antibodies were mapped as follows: A-I-19, CNBr fragment 1; A-I-12 and 15, CNBr fragment 2; A-I-9 and A-I-16, CNBr fragment 3; A-I-57, CNBr fragment 4. Antibody A-I-57 failed to recognized a mutant form of apoA-I, A-I_{Milano} (Arg_{173}→Cys) by immunoblotting and by competitive radioimmunoassay demonstrating that substitution of a single amino acid in human apoA-I may cause the loss of an antigenic determinant. —**Marcovina, S., S. Fantappie, A. Zoppo, G. Franceschini, and A. L. Catapano.** Immunochemical characterization of six monoclonal antibodies to human apolipoprotein A-I: epitope mapping and expression. *J. Lipid Res.* 1990. **31:** 375–384.

Supplementary key words high density lipoprotein • delipated apolipoprotein A-I • conformational changes • apolipoprotein A-I_{Milano}

Human plasma high density lipoprotein (HDL) contains approximately 45% protein and 55% lipid by weight (1); cholesteryl esters and phospholipid are major lipid constituents.

Apolipoprotein A-I and apolipoprotein A-II (apo) account for approximately 60% and 30%, respectively, of the HDL protein mass (2, 3). Human apoA-I is synthesized mainly by the liver and small intestine (4) as preproapoA-I; it undergoes co-translational cleavage to proapoA-I, and is eventually converted to mature apoA-I (243 amino acids) in plasma (5, 6). This apolipoprotein is a cofactor of the enzyme lecithin:cholesterol acyltransferase (7). ApoA-I is also the ligand responsible for the binding of the HDL to a putative receptor present on the plasma membrane of peripheral cells (8). The immunochemical properties of apoA-I have been the object of several investigations. Monoclonal antibodies have been used to study the expression of antigenic sites and the immunological heterogeneity of apoA-I in HDL (9–11). In this study we report on the isolation and characterization of six monoclonal antibodies to apolipoprotein A-I, their application in mapping distinct epitopes of the mature apolipoprotein, and the study of their reaction with apoA-I_{Milano}, a mutant form of apolipoprotein A-I (12).

MATERIALS AND METHODS

Isolation of lipoproteins and apolipoproteins

Human plasma lipoproteins were isolated from plasma obtained from fasting (14 h) normal subjects by sequential ultracentrifugation in a 50.2 Ti rotor (Beckman, Palo Alto, CA) as described by Havel, Eder, and Bragdon (13) at the following densities: very low density lipoprotein (VLDL) 1.006 g/ml; low density lipoprotein (LDL) between densities 1.019 to 1.063 g/ml, and high density lipoproteins 2 and 3 (HDL_2, HDL_3) between densities 1.063 to 1.125 g/ml and 1.125 to 1.210 g/ml, respectively. Lipo-

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; egg PC, egg phosphatidylcholine; RIA, radioimmunoassay.

[1]Send correspondence and reprint requests to: Santica Marcovina, Northwest Lipid Research Center, ZA-36, Harborview Medical Center, 326 Ninth Avenue, Seattle, WA 98104-2499. A limited amount of the monoclonal antibodies can be obtained upon request.

proteins were extensively dialyzed at 4°C against phosphate-buffer saline (PBS), pH 7.4, containing 0.01% EDTA, sterilized through a 0.45-μm filter, stored at 4°C, and used within 2 weeks from isolation. HDL from an apolipoprotein A-I_{Milano} heterozygote subject (V. D. in the kindred) (12) were isolated as described above and stored at −20°C for 2 months before use. Apolipoprotein A-I was isolated from HDL_3 by column chromatography over a Sephadex G-200 column (2.5 × 130 cm) in a buffer containing 8 M urea and 0.1 M Tris-HCl (pH 7.4). The peak containing apoA-I was collected and further purified by a second passage on the column. ApoA-II was isolated using a similar procedure followed by an ion-exchange chromatography step on the DE52 column. ApoA-I and apoA-II purity was determined by SDS gel electrophoresis; a single band was detected by loading 20 μg of purified protein. Apolipoproteins C-II and C-III were purified by preparative isoelectric focusing (14). The purity of the fractions was assessed by analytical isoelectric focusing as described (15). ApoC-II showed only a slight contamination with apoC-III (less than 4% as determined by densitometric scanning of the gels); apoC-III showed a single band (12 μg load). Apo-A-I_{Milano} was isolated by affinity chromatography on thiopropyl-Sepharose. The purity of the preparation was greater than 85%.

Apolipoprotein A-I–egg phosphatidylcholine complexes

Egg PC–cholesterol–apoA-I complexes were prepared as described (16). Egg PC and cholesterol, dissolved in chloroform–methanol 2:1, in a molar ratio 100:2 were added to a glass tube and dried on the wall of the tube under a stream of N_2. ApoA-I, (1 M:100 M of egg PC) on 0.15 M NaCl, 2 mM EDTA, pH 7.4, was added and the mixture was vortexed for 30 sec. Sodium cholate was then added under continuous stirring to a final concentration of 4% (w/v). Complexes were separated from the detergent by column chromatography. Chemical analysis showed that the chemical composition of the vesicles was as expected. Complexes with apoA-I_{Milano} were prepared by using the same procedure and were similar in size and composition to those prepared with apoA-I.

Production of monoclonal antibodies

Male Balb/c mice were immunized with purified apoA-I by receiving intraperitoneal injections of 100 μg of apoA-I in Freund's complete adjuvant (Behringwerke AG, Marburg, F.R.G.).

The same dose was repeated 2 weeks later in Freund's incomplete adjuvant. The mouse with the highest specific antibody titre, as determined by enzyme-linked immunoassay, was boosted with intravenous injections of 50 μg of apoA-I, without any adjuvant, for 3 consecutive days before the fusion. Splenocytes from the immunized mouse were fused with mouse hybridoma cell line SP2/0-Ag14. As cell proliferation approached confluency, supernatants were screened for the presence of specific antibodies by an enzyme-linked immunoassay as previously described (17); the wells of the microtitre plates were coated overnight at 4°C with 100 μl of 0.05 M carbonate buffer, pH 9.6, containing 300 ng of purified apoA-I or 500 ng of HDL. The cells from the most positive wells were cloned by limiting dilutions in 96-well microculture plates, yielding 26 clones that were recloned twice. Six stable clones showing the highest antibody titre to both purified apoA-I and apoA-I in HDL were selected to be characterized after being further subcloned three times. The immunoglobulin class and subclass were determined by enzyme immunoassay (Mouse Hybridoma Sub-Iso Typing Kit, Calbiochem-Behring, La Jolla, CA). To obtain a large amount of antibodies, the hybridoma cells were injected into the peritoneal cavity of "pristane-primed" Balb/c mice. The ascitic fluids were collected 7–14 days later and centrifuged. The monoclonal antibodies were purified from ascitic fluids by adsorption to Protein A-Sepharose (Affi-Gel Protein A, Bio-Rad, Richmond, CA) and stored at −80°C in aliquots of a 1 mg/ml solution.

Iodination of lipoproteins, apolipoproteins, and monoclonal antibodies

HDL_2 and HDL_3 subfractions were labeled with ^{125}I using the procedure described by Bilheimer, Eisenberg, and Levy (18). Free ^{125}I was removed by gel filtration on a Sephadex PD25 column followed by extensive dialysis at 4°C against PBS containing 0.01% Na EDTA.

The ^{125}I precipitable with 10% trichloroacetic acid (TCA) was always greater than 98%. The lipid-associated radioactivity ranged from 3 to 8% of TCA-precipitable radioactivity; the specific activity was 300–400 cpm/ng of protein. ApoA-I was labeled with the Iodogen procedure (19) to a specific activity of 400–500 cpm/ng. TCA-precipitable radioactivity was always greater than 98%. Both labeled HDL and apoA-I were stored at 4°C under sterile conditions, and used within a week. Monoclonal antibodies were labeled with ^{125}I with the Iodogen procedure to a specific activity of 2,000–3,000 cpm/ng (19). Free iodine was removed as described for lipoproteins. Trichloroacetic acid-precipitable radioactivity was always more than 98%.

Direct binding assay of ^{125}I-labeled HDL to monoclonal antibodies and Scatchard plot analysis

Fluid phase radioimmunoassay was performed in duplicate as previously reported (20, 21). Briefly, 100 μl of serial dilution of monoclonal antibodies was incubated with 100 μl of ^{125}I-labeled HDL (30,000 cpm, containing about

80 ng of protein). After overnight incubation at 4°C, 100 μl of rabbit anti-mouse IgG was added (diluted to give a slight antibody excess), and the tubes were incubated for 3 h at room temperature. At the end of the incubation, 100 μl of Standardized Pansorbin Cells (Calbiochem-Behring), was added and incubated for 30 min. Alternatively, a one-step procedure was used in which the rabbit anti-mouse IgG (diluted 1:50) was preincubated for 24 h at 4°C with Pansorbin cells (1 ml of cell suspension per 10 ml of diluted antibody) prior the use in the RIA assay. Rabbit anti-mouse IgG/Pansorbin cells (100 μl) were added to the tubes and incubated for 6 h at room temperature. The assay tubes were then washed with 2 ml of cold assay buffer and centrifuged (2000 *g*, 30 min). Supernatants were removed and the radioactivity of the pellet was determined in a gamma counter (Behring Gamma Counter 1612). Maximum precipitable radioactivity was determined by replacing the rabbit anti-mouse IgG with trichloroacetic acid (220 g/l). The minimum precipitable activity or nonspecific binding was determined by replacing the specific monoclonal antibodies with supernatant fluid from an irrelevant hybridoma. For calculations, the percent of total ^{125}I-labeled HDL bound was expressed by B_0/T, where B_0 is the total ^{125}I-labeled HDL bound to antibodies, minus nonspecific binding, and T was the maximum TCA-precipitable radioactivity. A similar technique was used for determining the affinity constants of monoclonal antibodies by Scatchard plot analysis (22). Bound HDL was determined by incubating increasing doses of competing antigen (HDL or purified apoA-I) in the presence of ^{125}I-labeled HDL. Each monoclonal antibody was used at the dilution required for approximately 50% maximum binding. For determination of the affinity constants, the molar concentration of the competing antigen was calculated from the molecular weight of apoA-I (28,000 g/mol).

Competitive antibody binding assay

Solid phase radioimmunoassay was performed in duplicate as follow. HDL were diluted in coating buffer containing 0.015 M Na_2CO_3, 0.35 M $NaHCO_2$, 0.01% NaN_3 (pH 9.5), to a final concentration of 4 μg/ml. Aliquots (100 μl) were distributed in 75 × 11 mm tubes (Maxisorp, A/S Nunc, Denmark) and left to incubate overnight at 4°C to allow the binding of HDL to the tubes. The tubes were then emptied, washed three times with saline, and incubated with 300 μl of 10% bovine serum albumin in assay buffer to saturate nonspecific binding sites. After 1 h incubation at room temperature, the tubes were washed as above. Serial two-fold dilutions of monoclonal antibodies were prepared in assay buffer and 100-μl aliquots were transferred into the tubes and incubated for 3 h at room temperature, emptied, and washed as above. Aliquots of 100 μl of ^{125}I-labeled monoclonal antibody were then added to each tube. After 3 h incubation at room temperature, the tubes were washed three times and then counted in a gamma counter. The results were expressed as $B/B_0 \times 100$, where B is the counts per minute (cpm) bound, minus nonspecific binding, and B_0 is the cpm bound in the absence of competing antibody minus nonspecific binding.

Gel electrophoresis and immunoblotting

SDS gel electrophoresis was performed in a 10% polyacrylamide gel according to Weber and Osborne (23) using a Bio-Rad minicell (Bio-Rad, Richmond, CA). Isolated lipoproteins were delipidated by the method of Scanu and Edelstein (24) and solubilized in buffer containing 2% sodium dodecyl sulfate, 1% β-mercaptoethanol (w/v). After fractionation by SDS-PAGE, the apolipoproteins were electrophoretically transferred to nitrocellulose as described by Towbin, Staehelin, and Gordon (25). The nitrocellulose paper was then cut into strips and each strip was placed into a 500-fold dilution of each monoclonal antibody in PBS containing 0.5% BSA and incubated for 1 h. After washing with PBS containing 0.5% BSA, the strips were incubated for 1 h in a 1000-fold dilution of goat anti-mouse IgG conjugated with peroxidase (Bio-Rad) and washed extensively. The antibody binding was localized by staining the strips with peroxidase or, alternatively, by using ^{125}I-labeled protein A. Protein A, labeled with Iodogen to a specific activity of 4000–5000 cpm/ng, was incubated ($2–3 \times 10^5$ cpm) with the nitrocellulose strips in PBS containing 1% BSA for 2 h. The strips were washed, dried, and processed for autoradiography using an intensifier screen. For bidimensional gel electrophoresis, apolipoproteins were first focused on a 6% acrylamide gel (0.2 × 6 cm) containing 2% ampholines (pH 4 to 6). When focusing was completed, the gel was removed and processed for the second dimension on a 10% polyacrylamide gel containing 1% SDS (26). Electrophoresis transfer and immunological detection were performed as described above.

Synthetic fragments of apoA-I

Synthetic fragments of apolipoprotein A-I were kindly provided by Dr. J. T. Sparrow (Houston, TX). Fragments spanning regions 87–124, 142–182, and 224–243 were used. Peptides were synthesized using protected amino acids as previously described, based upon the published sequence of apoA-I (4). Peptides were homogeneous by analytical isoelectric focusing (15) and gave the expected amino acid analysis.

Dot-blot assay

The interaction between monoclonal antibodies and the apoA-I synthetic peptides was studied by spotting in-

creasing aliquots (from 0.05 to 5 μg) of peptide protein onto nitrocellulose and processing as described above for the immunoblotting.

CNBr fragments of apolipoprotein A-I

Purified apoA-I was cleaved with CNBr at a molar ratio protein:CNBr, 1:500 in PBS at room temperature in a sealed vial for 24 h. The excess reagent was removed by overnight dialysis at 4°C against water using a membrane with a cut-off of 3,500 daltons. The peptides were then lyophilized and stored in sealed vials at −20°C until use. CNBr fragments were separated by a bidimensional gel electrophoresis system essentially as described above with the only difference that the first dimension was in 8 M urea-acrylamide gel and the SDS-acrylamide gel in the second dimension contained 12% polyacrylamide (27). Blotting and immunological detection were as described above. The CNBr fragments were identified according to their molecular weight and electrophoretic mobility.

Interaction of monoclonal antibodies with apolipoprotein A-I_{Milano}

The interaction of monoclonal antibodies with apoA-I_{Milano} was studied by immunoblotting as described above with the difference that the isoform separation was performed by isoelectric focusing (pH range 4–6) after dissociation of apolipoprotein complexes with 2% β-mercaptoethanol. In a second set of experiments, apoA-I_{Milano} was reconstituted to form vesicles and the interaction with monoclonal antibodies was studied by fluid phase radioimmunoassay.

RESULTS

Specificity of monoclonal antibodies

From the fusion between the cell line SP2/0-Ag14 and the splenocytes of a Balb/c mouse immunized with human apoA-I, six mouse hybridomas that secreted antibodies specific for human plasma apoA-I were selected to be characterized. The details of the six monoclonal antibodies used in this study are shown in **Table 1**. After SDS electrophoresis of the plasma apolipoproteins, all monoclonal antibodies recognized a single band of mol wt 28,000. The same protein was identified by a polyclonal anti-human apoA-I raised in rabbit against purified apoA-I. Bidimensional gel electrophoresis also showed that these antibodies recognized all isoforms of apoA-I including proapoA-I (data not shown). Altogether these data suggest monospecificity of the antibodies without any stringent specificity for apoA-I isoforms. Studies with purified apoA-II, C-II, and C-III in an RIA assay showed no competition of these apolipoproteins with apoA-I for all the six monoclonal antibodies (data not shown).

TABLE 1. Monoclonal antibodies to human apolipoprotein A-I

Immunizing Antigen	Hybridoma Antibody	Monoclonal Antibody	Antibody Chain Type	K_a 10^9 l/mol
ApoA-I	9-B2-D2	A-I-9	IgG1 k	1.3
ApoA-I	12-C4-B1	A-I-12	IgG2a k	1.9
ApoA-I	15-C4-H1	A-I-15	IgG2a k	2.0
ApoA-I	16-C6-C4	A-I-16	IgG1 k	0.3
ApoA-I	19-D4-B6	A-I-19	IgG2b k	1.5
ApoA-I	57-H1-H9	A-I-57	IgG1 k	2.1

Immunoprecipitation of ^{125}I-labeled HDL

Preliminary experiments conducted on the immunoprecipitation of ^{125}I-labeled HDL in liquid phase RIA showed that the binding of the six monoclonal antibodies was 55% for the clone A-I-16 and about 90% for all the other clones. To verify whether these data reflected the true availability of the epitopes on HDL surface, or whether the HDL binding could be improved by optimization of the assay conditions, we used rabbit anti-mouse IgG preincubated with Pansorbin cells instead of using the two-step procedure. This approach did not modify the maximal binding of monoclonal antibodies A-I-16 and A-I-9 which remained 55 and 90%, respectively. Monoclonal antibodies A-I-12, A-I-15, and A-I-57, however, precipitated all of the ^{125}I-labeled HDL, while the binding of clone A-I-19 increased from 88 to 96% of the total HDL (**Fig. 1**). The data reported are the mean of seven different experiments; furthermore, the results were independent of the amount of antigen added in the range from 40 to 100 ng of ^{125}I-labeled HDL. By using the same assay we investigated the binding of monoclonal antibodies to ^{125}I-labeled apoA-I. The binding of monoclonal antibodies A-I-16 and A-I-57 was only slightly decreased, that of antibodies A-I-9 and A-I-19 was decreased about 15%, while monoclonal antibodies A-I-12 and A-I-15 bound less than 25% of the total ^{125}I-labeled apoA-I (data not shown).

Displacement curves of ^{125}I-labeled HDL

To measure the antibody affinities for apolipoprotein A-I in HDL and soluble apoA-I, a fluid phase radioimmunoassay was performed in which the ability of HDL and purified apoA-I to compete for binding of ^{125}I-labeled HDL was analyzed (**Fig. 2**). Full displacement of ^{125}I-labeled HDL by HDL was obtained with each monoclonal antibody except A-I-16. The affinity constants, calculated by Scatchard plot analysis are reported in Table 1.

All monoclonal antibodies showed a lower affinity to free apoA-I than to apoA-I in HDL (Fig. 2). Monoclonal antibodies A-I-12 and A-I-15, particularly, seem to be directed to epitope(s) very sensitive to the changes that apoA-I undergoes in the lipid-free form. To further investigate the role of lipids in maintaining the antigenic struc-

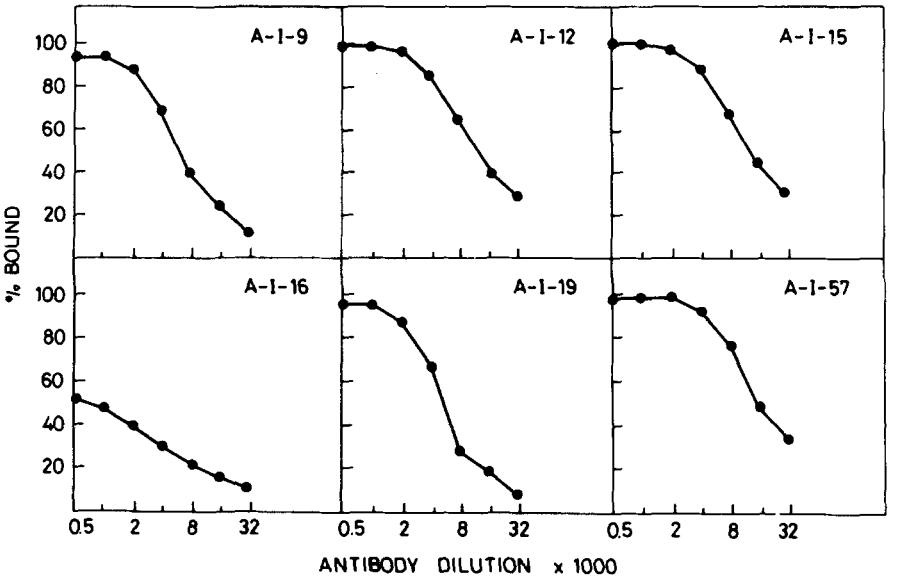

Fig. 1. Immunoprecipitation of ^{125}I-labeled HDL by monoclonal antibodies. The percentage of trichloroacetic acid-precipitable radioactivity bound by each antibody is shown as a function of antibody dilution. The data reported are the mean of seven different experiments. The results were independent of the amount of antigen added in the range from 40 to 100 ng of ^{125}I-labeled HDL.

ture of apoA-I, the ability of apoA-I vesicles to compete for binding of ^{125}I-labeled HDL was analyzed in a fluid phase radioimmunoassay. We compared the displacement curves obtained by monoclonal antibodies A-I-12 and A-I-15, which showed almost no displacement with purified apoA-I, to that of monoclonal antibody A-I-57, which is the antibody that more effectively recognizes purified apoA-I. As shown in **Fig. 3**, the displacement curves indicate that the binding of purified apoA-I to lipids restores the interaction of monoclonal antibodies to the protein, and the binding affinity, as judged by the slopes of the displacement curves, is almost the same for HDL and apoA-I vesicles, thus suggesting that reversible conformational changes occurred in purified apoA-I.

Epitope mapping

To further characterize our monoclonal antibodies we attempted epitope mapping on apolipoprotein A-I. First, we studied by immunoblotting the interaction of the antibodies with apoA-I CNBr fragments. The results (**Fig. 4**) are consistent with A-I-19 recognizing an epitope in CNBr fragment 1, A-I-15 and A-I-12 an epitope in CNBr 2, A-I-16 and A-I-9 an epitope in CNBr fragment 3, and A-I-57 an epitope in CNBr fragment 4. To further verify these assignments we used synthetic peptides reproducing amino acid sequences 87–124, 142–182, and 224–243 of mature human apoA-I. In a dot-blot analysis, A-I-19, A-I-16, and A-I-9 failed to recognized any synthetic peptide;

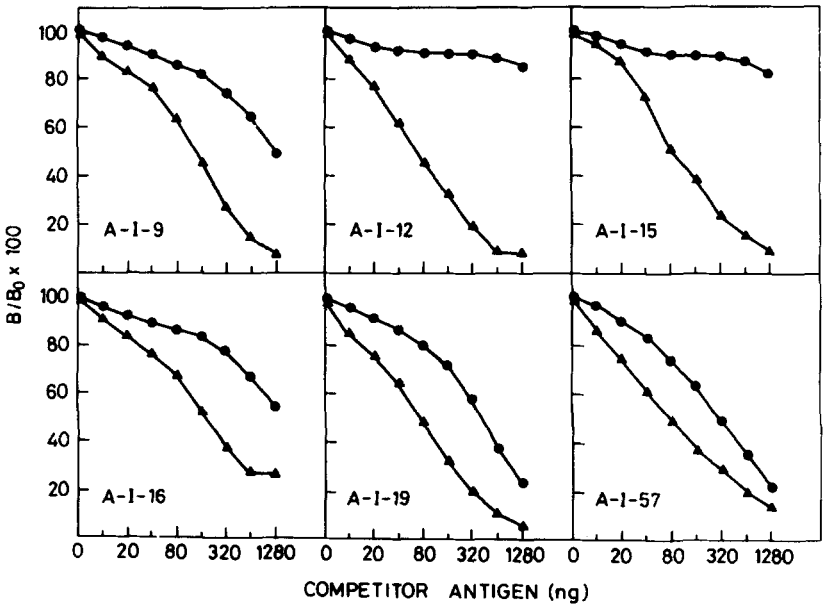

Fig. 2. Displacement curves of ^{125}I-labeled HDL by HDL (△) and purified apoA-I (●). Bound HDL was determined by incubating 100 μl of ^{125}I-labeled HDL (80 ng of protein) with 100 μl of competing antigen (HDL or apoA-I) ranging from 10 to 1280 ng, and 100 μl of monoclonal antibody at the dilution required for about 50% maximum binding. For determination of affinity constants by Scatchard plot analysis, the molar concentration of the competing antigen was calculated from the molecular weight of apoA-I (28,000 g/mol).

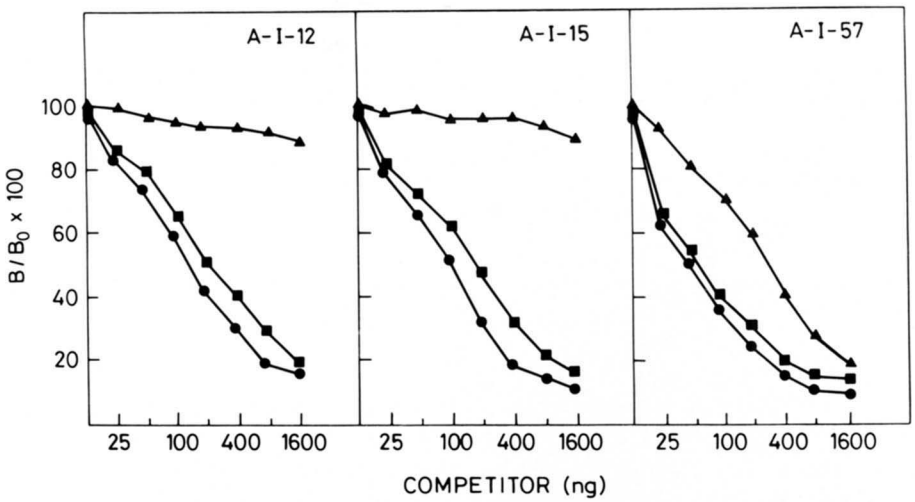

Fig. 3. Displacement curves of ^{125}I-labeled HDL by HDL (●), purified apoA-I (▲), and apoA-I-egg-PC-vesicles (■). ApoA-I vesicles were prepared as described under Methods.

A-I-15 and A-I-12 recognized peptide 87–124; and A-I-57 recognized peptide 142–182 (data not shown). By combining these data with the antibodies' specificity for CNBr fragments, a map of the epitopes was constructed (**Fig. 5**). An alternative way to map epitopes for antibodies is to take advantage of naturally occurring mutations of apolipoproteins. We studied, therefore, the interaction of our antibodies with apoA-I_{Milano}, a mutant protein that carries a Cys instead of an Arg at amino acid position 173. ApoA-I_{Milano}, in the reduced form, presents with a typical pattern in isoelectric focusing characterized by the presence of more negatively charged isoforms. **Fig. 6**

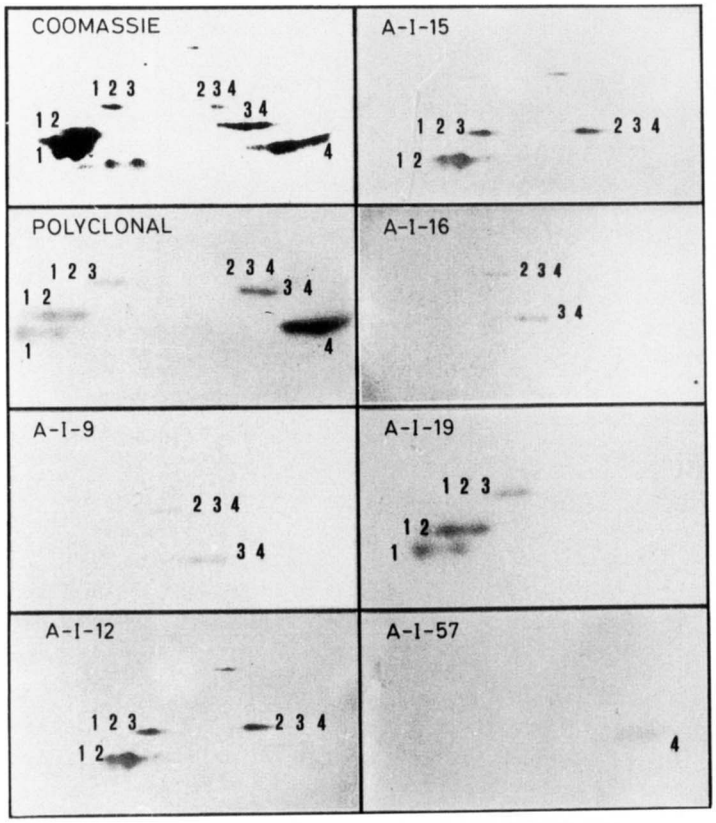

Fig. 4. Interaction of monoclonal antibodies with CNBr fragments of apoA-I. Purified apoA-I was cleaved with CNBr and the fragments were separated as described under Methods. The bound antibody was detected with ^{125}I-labeled Protein A. Numbers identify CNBr fragments: 1 = A-I (1–86); 1-2 = A-I (1–112); 1-2-3 = A-I (147); 2-3-4 = A-I (87–243); 3-4 = A-I (113–243); 4 = A-I (148–243).

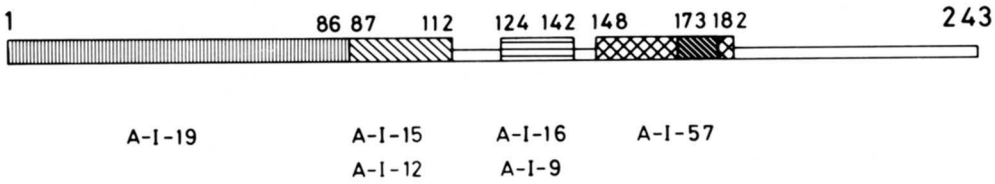

Fig. 5. Linear map of the epitopes for six monoclonal antibodies to human apoA-I. The epitope localization is indicated by the matched boxes.

shows the interaction of monoclonal antibodies A-I-15 and A-I-57 with apoA-I from a normal subject and from a heterozygote carrier of apoA-I_{Milano} after immunoblotting. Antibody A-I-15 recognized apoA-I and the typical isoforms of apoA-I_{Milano}, while antibody A-I-57 failed to detect the a pattern of apoA-I_{Milano}, only recognizing the normal apoA-I isoforms. As tested by a fluid phase radioimmunoassay, apoA-I_{Milano} reconstituted into vesicles failed to compete for the binding of monoclonal antibody A-I-57 to ^{125}I-labeled HDL (**Fig. 7**). Same displacement curves between apoA-I_{Milano} and HDL were obtained with the other monoclonal antibodies (data not shown). This finding indicates that substitution of a single amino acid at position 173 completely abolishes the expression of the epitope for antibody A-I-57, further confirming the mapping of the epitope for this antibody in this region.

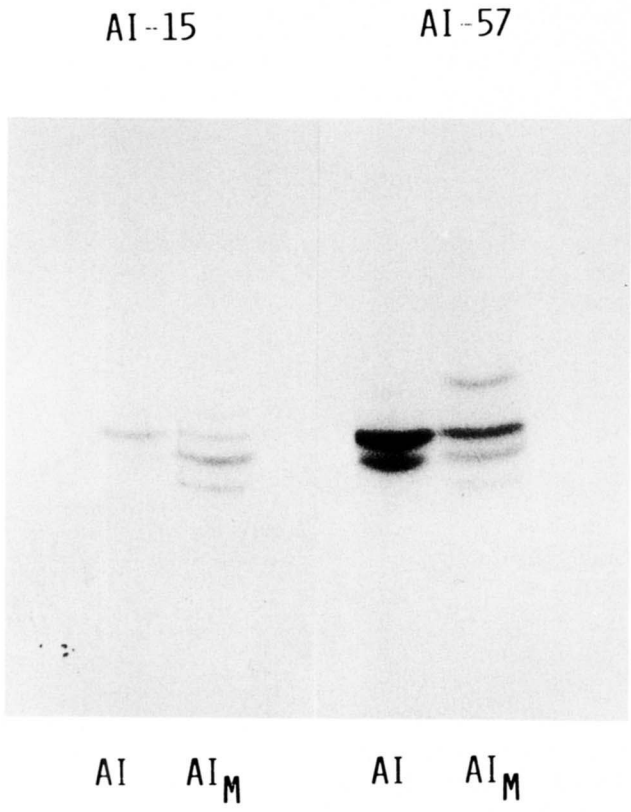

Fig. 6. Interaction of monoclonal antibody A-I-15 and A-I-57 with apoA-I and apoA-I_{Milano} after isoelectric focusing, as determined by immunoblotting. HDL from a control and from a heterozygote carrier of apoA-I_{Milano} were isolated, delipidated, and apoA-I was separated as described under Methods. Antibody A-I-15 recognizes apoA-I and apoA-I_{Milano} as shown by the typical pattern of isoelectric focusing that is shifted towards more acidic forms in apoA-I_{Milano} carrier. Antibody A-I-57 fails to detect the typical A-I_{Milano} pattern and this reactivity is consistent with the recognition of only the normal A-I isoforms.

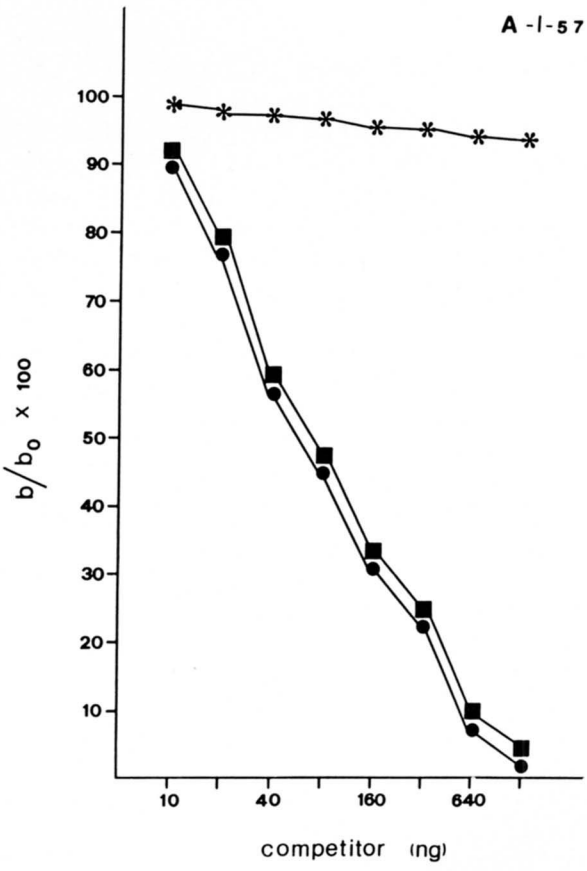

Fig. 7. Displacement curves of ^{125}I-labeled HDL by HDL (●), apoA-I-egg-PC-vesicles (■), and apoA-I_{Milano}-egg-PC-vesicles (*).

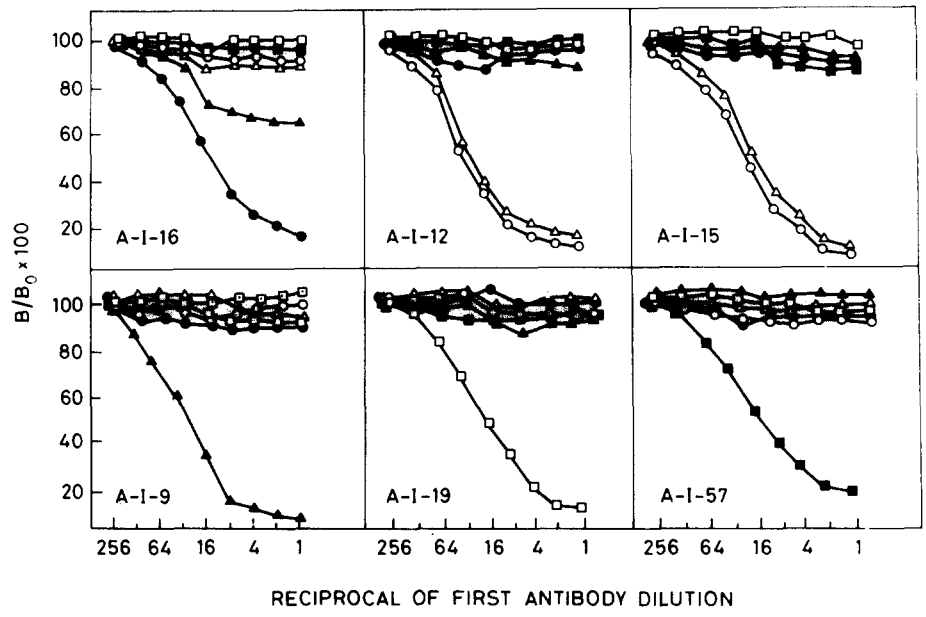

Fig. 8. Competition of monoclonal antibodies performed sequentially by a solid phase radioimmunoassay. The monoclonal antibodies were radioiodinated and the capacity of increasing amounts of one unlabeled antibody to interfere with the binding of a limited amount of a second ^{125}I-labeled antibody was assessed. The initial dilution of the unlabeled antibodies was 1:1000. Monoclonal antibody A-I-9, ▲; A-I-12, △; A-I-15, ○; A-I-16, ●; A-I-19, □; A-I-57, ■. The radioiodinated second antibody used in each experiment is marked at the bottom.

Competition experiments

Because the epitopes for monoclonal antibodies A-I-12 and A-I-15 and those for A-I-16 and A-I-9 were located respectively in regions 87–112 and 124–142 of the apoA-I sequence, we wished to study whether they did or did not recognize the same epitope. To this end we performed a solid phase radioimmunoassay. Each monoclonal antibody was labeled with ^{125}I and the ability of increasing amounts of one unlabeled antibody to interfere with the binding of a limited amount of a second radioiodinated antibody was assessed. As shown in **Fig. 8**, each of the unlabeled antibodies was able to specifically block its ^{125}I-labeled homologue, indicating the specificity of the binding. Monoclonal antibodies A-I-12 and A-I-15 appeared to sterically hinder the binding of one another, showing the same concentration-dependent inhibition that was obtained by the respective homologue and indicating that they are directed to the same or spatially very close epitopes on apoA-I. Antibodies A-I-9 and A-I-16 showed a peculiar behavior. Antibody A-I-16 did not compete at all with ^{125}I-labeled antibody A-I-9 for HDL, while antibody A-I-9 partially inhibited the binding of ^{125}I-labeled A-I-16. However, the competition was not fully effective since a plateau was reached at 30% inhibition. No competition by other antibodies was detected with antibodies A-I-19 and A-I-57.

DISCUSSION

Two of the monoclonal antibodies used in this study have been used previously in a radial immunodiffusion assay (28). Here we report their detailed characterization and the mapping of epitopes on the apoA-I molecule. Each of the six reported monoclonal antibodies was monospecific for apoA-I. Furthermore, no preferential binding of the antibodies to apoA-I isoforms isolated by isoelectric focusing or bidimensional gel electrophoresis was detected; this is consistent with an evenly distributed epitope expression among isoforms, including proapoA-I. Similar data were previously reported by different authors (9–11). All our antibodies, except A-I-16, recognized the vast majority of ^{125}I-labeled HDL in a fluid phase RIA, thus suggesting little heterogeneity of epitope expression on HDL for these antibodies. These data are not in agreement with those of Curtiss and Edgington (9) who suggested the existence of a high degree of heterogeneity for the epitopes recognized of apoA-I by several monoclonal antibodies. A possible explanation for their finding could be the use of an "aged" antigen for immunization and for the screening procedure (29). From our data, however, we cannot exclude that heterogeneity of the expression of apoA-I epitopes exists. Furthermore, antibody A-I-16 recognized only 55% of ^{125}I-labeled HDL. Additional experiments are required to elucidate the heterogeneity of

epitope expression detected with this antibody which, in solid phase radioimmunoassay or in enzyme immunoassay, binds apoA-I or HDL to the same extent as the other monoclonal antibodies. A difference in the affinity constant (monoclonal antibody A-I-16 has a $K_a = 3 \times 10^8$ l/mol, while that of the other antibodies is in the range of 10^9) does not provide a satisfactory explanation.

The properties of the monoclonal antibodies were further investigated by competitive RIA with HDL, purified apoA-I and apoA-I–egg-PC vesicles. The different reactivity of monoclonal antibodies to apoA-I in the purified, lipid-free form as compared to apoA-I in HDL or reconstituted into phospholipid particles suggests that the epitopes for all the monoclonal antibodies are partially inaccessible or totally unavailable in the lipid-free form of the apoA-I molecule. Chemical modifications that can occur during purification steps as well as self-association of apoA-I might have contributed to this finding. However, association of apoA-I with phospholipids fully restores epitope expression, suggesting that lipids play an important role in determining the expression of epitopes for apoA-I. However, the monoclonal antibodies showed nearly the same immunoreactivity to isolated HDL and purified apoA-I in solid phase assays. We speculate that binding of apoA-I to plastic wells mimics the binding of apoA-I to lipids, at least with respect to the expression of the epitopes for our antibodies. A very similar finding was reported by Silberman et al. (30) for monoclonal antibody M30. Recently, Curtiss and Smith (31) described two antibodies, AI-16 and AI-18 that, like ours, reacted poorly with purified apoA-I but effectively recognized A-I in HDL.

Our finding that the epitopes for A-I-57 and A-I-19 are slightly affected by the delipidation of apoA-I, while those of A-I-12 and A-I-15 are almost completely lost, is consistent with the hypothesis that some areas of apoA-I undergo conformational arrangements upon lipid binding while some other areas undergo little change. Alternatively, the epitopes for A-I-57 and A-I-19 are determined by a linear sequence, while those of the other antibodies involve amino acids that are relatively distant in the unfolded protein and become close upon protein folding.

To further characterize our monoclonal antibodies, we mapped their epitopes on apoA-I. Several authors reported monoclonal antibodies against the NH_2 terminal or the middle portion of apoA-I (10, 11, 32). Antibody A-I-57 is directed to CNBr fragment 4. Only one other monoclonal antibody to this portion of apoA-I has been reported (11). The epitope of antibody 57 was further narrowed down to the 148–182 amino acid sequence, and the finding that the antibody does not recognize the apoA-I_{Milano} indicates that the area around Arg_{173} may be involved. We do not know, however, whether this amino acid is part of the epitope since the $Arg_{173} \rightarrow$Cys substitution perturbs the alpha helix structure in this region of apoA-I_{Milano} (12), thus also affecting conformation of distant areas. Ehnholm et al. (11) reported analogous results with an antibody which failed to recognize an apoA-I mutant form that bears a $Gly_{136} \rightarrow$Lys substitution. Antibodies 15 and 12 recognized the same CNBr fragment (87–112) and the competition data suggest that these antibodies interact with the same epitope. This conclusion is further stressed by the fact that the antibodies have the same chain type, affinity constant, and immunochemical behavior. Antibodies A-I-16 and A-I-9 both recognize CNBr fragment 3. However, as determined by competition assay, they bind to distinct, but spatially related, epitopes. Antibody A-I-19 reacts with CNBr fragment 1. Several monoclonal antibodies to apoA-I have been reported that recognize this region, which apparently seems to be one of the most immunogenic in mice (10, 11, 32).

In summary, we have reported the characterization of six monoclonal antibodies specific for apoA-I that are directed to five different epitopes on the apoA-I molecule. One of them, monoclonal antibody A-I-57, failed to recognize a genetic variant of apoA-I, apoA-I_{Milano}, showing that a single amino acid substitution may cause the loss of the antigenic determinant. All antibodies, except one, recognize apoA-I in HDL better than in purified apoA-I and may prove useful in the definition of functionally relevant areas of intact apoA-I. All monoclonal antibodies, except A-I-16, bound from 90 to 100% of ^{125}I-labeled HDL, thus indicating that despite the heterogeneity of apoA-I-containing lipoproteins, several regions of apoA-I are uniformly expressed. This finding is important for the development of accurate immunochemical methods for determining apoA-I in human plasma. ■

This work was supported in part by grants no. 104320.56.8706658 and no. 02241.56115.11485 from the Italian Council of Research, Preventive Medicine Project. We wish to thank Dr. J. T. Sparrow for the generous gift of the synthetic fragments of apoA-I.

Manuscript received 24 June 1988 and in revised form 19 September 1989.

REFERENCES

1. Eisenberg, S. 1984. High density lipoprotein metabolism. *J. Lipid Res.* **25:** 1017–1058.
2. Schaefer, E. J. 1984. Clinical, biochemical, and genetic features in familial disorders of high density lipoprotein deficiency. *Arteriosclerosis.* **4:** 303–322.
3. Cheung, M. C., and J. J. Albers. 1977. The measurement of apolipoprotein A-I and A-II levels in men and women by immunoassay. *J. Clin. Invest.* **60:** 43–50.
4. Brewer, H. B., Jr., T. Fairwell, A. Larue, R. Ronan, A. Houser, and T. J. Bronzert. 1978. The amino acid sequence of human apoA-I, an apolipoprotein isolated from high density lipoproteins. *Biochem. Biophys. Res. Commun.* **80:** 623–630.
5. Law, S. W., G. Gray, and H. B. Brewer, Jr. 1983. DNA cloning of human apoA-I: amino acid sequences of prepro apoA-I. *Biochem. Biophys. Res. Commun.* **112:** 257–264.

6. Bojanovski, D., R. E. Gregg, G. Ghiselli, E. J. Schaefer, J. A. Light, and H. B. Brewer, Jr. 1985. Human apoliprotein A-I isoprotein metabolism:proapoA-I conversion to mature apoA-I. *J. Lipid Res.* **26:** 185–193.
7. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46:** 1493–1498.
8. Schmitz, G., H. Robenek, U. Lohmann, and G. Assmann. 1985. Interaction of high density lipoproteins with cholesteryl ester-laden macrophages: biochemical and morphological characterization of cell surface receptor binding, endocytosis and resecretion of high density lipoproteins by macrophages. *EMBO J.* **4:** 613–622.
9. Curtiss, L. K., and T. S. Edgington. 1985. Immunochemical heterogeneity of human plasma high density lipoproteins. Identification with apolipoprotein A-I and A-II specific monoclonal antibodies. *J. Biol. Chem.* **260:** 2982–2993.
10. Weech, P. K., R. W. Milne, P. Milthorp, and Y. L. Marcel. 1985. Apolipoprotein A-I from normal human plasma: definition of three distinct antigenic determinants. *Biochim. Biophys. Acta.* **835:** 390–401.
11. Ehnholm, C., M. Lukka, I. Rostedt, and K. Harper. 1986. Monoclonal antibodies specific for different regions of human apolipoprotein A-I. Characterization of an antibody that does not bind to a genetic variant of apoA-I (Glu_{136}→Lys). *J. Lipid Res.* **27:** 1259–1264.
12. Weisgraber, K. H., T. P. Bersot, and R. W. Mahley, G. Franceschini, and C. R. Sirtori. 1980. $A\text{-}I_{Milano}$ apoprotein. Isolation and characterization of a cysteine-containing variant of the A-I apoprotein from human high density lipoprotein. *J. Clin. Invest.* **66:** 901–907.
13. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1340–1352.
14. Kane, J. T., T. Sata, R. L. Hamilton, and R. J. Havel. 1975. Apoprotein composition of very low density lipoproteins of human serum. *J. Clin. Invest.* **56:** 1622–1630.
15. Catapano, A. L., R. L. Jackson, E. B. Gilliam, A. M. Gotto, Jr., and L. C. Smith. 1978. Quantification of apoC-II and apoC-III of human very low density lipoproteins by analytical isoelectric focusing. *J. Lipid Res.* **19:** 1047–1052.
16. Matz, C. E., and A. Jonas. 1982. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem.* **257:** 4535–4540.
17. Marcovina, S., P. J. Price, J. S. McDougal, and L. Cremonesi. 1984. Monoclonal antibodies directed against human low density lipoprotein and their characterization by the immunodot technique. *In* Protides of the Biological Fluids. Vol. 31. H. Peeters, editor. Pergamon Press Ltd., Oxford, England. 985–989.
18. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. Metabolism of very low density lipoproteins. Part 1 (Preliminary in vitro and in vivo observations). *Biochim. Biophys. Acta.* **260:** 212–221.
19. Fraker, D. J., and J. C. Speck. 1978. Protein and cell membrane iodination with a sparingly soluble chloramide 1,3,4, 6-tetra-chloro-3,6-diphenyl-glycoluril. *Biochem. Biophys. Res. Commun.* **80:** 849–851.
20. Marcovina, S., D. France, R. A. Phillips, and S. J. T. Mao. 1985. Monoclonal antibodies can precipitate low-density lipoprotein. I. Characterization and use in determining apolipoprotein B. *Clin. Chem.* **31:** 1654–1658.
21. Marcovina, S., B. A. Kottke, and S. J. T. Mao. 1985. Monoclonal antibodies can precipitate low-density lipoprotein. II. Radioimmunoassays with single and combined monoclonal antibodies for determining apolipoprotein B in serum of patients with coronary artery disease. *Clin. Chem.* **31:** 1659–1663.
22. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:** 660–672.
23. Weber, K., and M. Osbourne. 1969. The reliability of molecular weight determination by dodecyl sulphate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
24. Scanu, A. M., and C. Edelstein. 1971. Solubility in aqueous solutions of ethanol of the small molecular weight peptides of serum very low density and high density lipoproteins. *Anal. Biochem.* **44:** 576–588.
25. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
26. Hames, B. D., and D. Rikwood. 1981. Gel Electrophoresis of Proteins. IRL Press, Oxford.
27. Pownall, H. J., Q. Pao, D. Hickson, J. T. Sparrow, S. K. Kosserow, and J. B. Massey. 1981. Kinetics and mechanism of association of human plasma apolipoproteins with dimyristoyl phosphatidylcholine. Effect of protein structure and lipid clusters on reaction rates. *Biochemistry.* **20:** 6630–6635.
28. Marcovina, S., G. Di Cola, and A. L. Catapano. 1986. Radial immunodiffusion assay of human apolipoprotein A-I with use of two monoclonal antibodies combined. *Clin. Chem.* **32:** 2155–2159.
29. Curtiss, L. K., and R. S. Smith. 1987. Immunochemical heterogeneity of high density lipoproteins. *In* Proceedings of the Workshop on Lipoprotein Heterogeneity. NIH Publication 87-2646:366–377.
30. Silberman, S. R., F. Bernini, J. T. Sparrow, A. M. Gotto, Jr., and L. C. Smith. 1987. Monoclonal antibodies as probes of high density lipoprotein structure: identification and localization of a lipid-dependent epitope. *Biochemistry.* **26:** 5833–5843.
31. Curtiss, L. K., and R. S. Smith. 1988. Localization of two epitopes of apolipoprotein A-I that are exposed on human high density lipoproteins using monoclonal antibodies and synthetic peptides. *J. Biol. Chem.* **263:** 13779–13785.
32. Marcel, Y. L., D. Jewer, C. Vézina, P. Milthorp, and P. K. Weech. 1987. Expression of human apolipoprotein A-I epitopes in high density lipoproteins and in serum. *J. Lipid Res.* **28:** 768–777.