

# Localization of apolipoprotein A-I epitopes involved in the activation of lecithin:cholesterol acyltransferase

Patrizia Uboldi,\* Monica Spoladore,\* Simona Fantappiè,\* Santica Marcovina,<sup>§</sup> and Alberico L. Catapano<sup>1,\*</sup>,<sup>†</sup>

Institute of Pharmacological Sciences,\* Centro per lo Studio dell' Ateroaterosclerosi,<sup>†</sup> University of Milano, 20133 Milano, Italy, and Department of Medicine,<sup>§</sup> University of Washington, Seattle, WA 98195

**Abstract** Eight murine monoclonal antibodies (Mab) to apolipoprotein A-I were characterized for their epitopes and for their ability to interfere with lecithin:cholesterol acyltransferase (LCAT) activation mediated by apoA-I using a synthetic substrate. Using overlapping synthetic peptides we have identified six continuous epitopes that span amino acids 1–10 (Mab A-I-19), 96–101 (Mab A-I-15), 133–141 (Mab A-I-5), 140–145 (Mab A-I-9), 144–148 (Mab A-I-8), and 167–174 (Mab A-I-57). Furthermore, antibodies A-I-11 and A-I-16 recognized discontinuous epitopes, namely amino acids 124–128 and 144–148. When antibodies were tested for their ability to inhibit LCAT activation, an inhibitory effect was observed with those whose epitopes covered the area of apoA-I encompassing amino acids 96–174. From these data we conclude that several areas of apoA-I spanning the middle region of the apolipoprotein act in concert to stimulate LCAT activity, possibly by cooperative interaction with the enzyme.—**Uboldi, P., M. Spoladore, S. Fantappiè, S. Marcovina, and A. L. Catapano.** Localization of apolipoprotein A-I epitopes involved in the activation of lecithin:cholesterol acyltransferase. *J. Lipid Res.* 1996. **37**: 2557–2568.

**Supplementary key words** monoclonal antibodies • epitopes • HDL • Fab fragments

Human plasma high density lipoprotein (HDL) is an heterogeneous class of lipoproteins that contain approximately 45% protein and 55% lipid by weight; cholesteryl esters and phospholipid are major lipid constituents (1). ApoA-I and apolipoprotein A-II account for about 60% and 30% of HDL protein mass (2, 3), respectively. Human apoA-I is synthesized mainly by the liver and small intestine as preproapoA-I (1–4); it undergoes co-translational cleavage to proapoA-I, and is eventually converted to mature apoA-I (243 amino acids) in plasma (5, 6). ApoA-I serves as a cofactor for the enzyme lecithin:cholesterol acyltransferase (LCAT) (7, 8), and is also believed to be responsible for the

binding of HDL to a putative receptor present on the plasma membrane of peripheral cells (9–11). Both these properties appear to be of relevance in the early clearance of excess cholesterol from peripheral cells to the liver and may offer a biological explanation for the known inverse relation between plasma levels of HDL cholesterol and the risk of atherosclerosis (12).

Monoclonal antibodies (Mabs) have been used to study the exposure of antigenic sites and the immunological heterogeneity of apoA-I in HDL (13–20). Furthermore, Mabs provide a tool to study the areas involved in determining the physiological functions of apoA-I. Allan, Tetaz, and Fidge (20) used antibodies directed towards different regions of apoA-I to identify the areas of the protein involved in the interaction with HDL binding sites on liver cell membranes. The authors concluded that the COOH-terminal region of apoA-I is involved in the interaction. Recently, however, Banka, Black, and Curtiss (21) showed that antibody A-I 11, whose epitope lies between amino acids 96 and 111, modulates the efflux of cholesterol from THP-1 monocytic cells and Dalton and Swaney (22) suggested that the area encompassing amino acids 148–219 possesses the capacity to bind to “specific” proteins on hepatic membranes. Others failed to obtain clear-cut evidence that Mabs to apoA-I may interfere with HDL binding to specific receptors (23).

Monoclonal antibodies have also been used to ad-

Abbreviations: apoA-I, apolipoprotein A-I; Mab, monoclonal antibody; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoproteins; HDL<sub>2</sub>, high density lipoprotein<sub>2</sub>; HDL<sub>3</sub>, high density lipoprotein<sub>3</sub>; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TLC, thin-layer chromatography; MoBt, 1-hydroxybenzotriazole.

<sup>†</sup>To whom correspondence should be addressed.

dress the question whether specific areas of apoA-I are involved in LCAT activation (24, 25). Only antibodies directed towards the middle portion of apoA-I (aa 95–121) inhibited apoA-I-mediated LCAT activation (24). However, Fab fragments were not used and it could be argued that steric hindrance or formation of multimeric complexes could have been responsible for the inhibition. More recently, Meng et al. (25) addressed the same question by using a different panel of monoclonals and concluded that the area between aa 96 and 186 spans the apoA-I domain involved in LCAT activation.

The aim of our work was to further address this question by using a panel of eight well-characterized murine monoclonal antibodies to human apolipoprotein A-I; six of these antibodies have been previously reported (26). In this paper we characterize the epitopes of all antibodies (down to 5–8 aa), and report data on their ability to interfere with apoA-I-mediated LCAT activation.

## MATERIALS AND METHODS

### Isolation of lipoproteins and apolipoproteins

Human plasma lipoproteins were isolated from plasma of fasting (14 h) normolipidemic donors by sequential ultracentrifugation in a 50.2 Ti rotor (Beckman, Palo Alto, CA) as described by Havel, Eder, and Bragdon (27), at the following densities: very low density lipoprotein (VLDL) 1.006 g/ml; low density lipoprotein (LDL) 1.019–1.063 g/ml, and high density lipoproteins 2 and 3 (HDL<sub>2</sub>, HDL<sub>3</sub>) 1.063–1.125 g/ml and 1.125–1.210 g/ml, respectively. Lipoproteins were extensively dialyzed at 4°C against phosphate-buffered saline (PBS), pH 7.4, containing 0.01% EDTA, sterilized through a 0.45- $\mu$ m filter, stored at 4°C, and used within 2 weeks from isolation. Apolipoprotein A-I was isolated from HDL<sub>3</sub> by gel filtration on a Sephadex G-200 column (2.5  $\times$  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 through the column. ApoA-II was isolated by ion exchange chromatography on a DE52 column. ApoA-I and apoA-II purity was determined by SDS and polyacrylamide gel electrophoresis (28); a single band was detected by loading 20  $\mu$ g of purified protein. Apolipoproteins C-II and C-III were purified by preparative isoelectric focusing (29). The purity of the fractions was assessed by analytical isoelectric focusing and PAGE as described (28–30).

### Production of monoclonal antibodies

Male Balb/c mice were immunized with purified apoA-I by receiving two intraperitoneal injections (2 weeks apart) of 100  $\mu$ g of apoA-I in Freund's complete adjuvant (Boehringer AB, Marburg, Germany). The mouse with the highest specific antibody titer, as determined by enzyme-linked immunoassay, was boosted with intravenous injections of 50  $\mu$ g of apoA-I, without any adjuvant, for 3 consecutive days before the fusion. Splenocytes from the immunized mouse were fused with the mouse hybridoma cell line SP2/0-Ag14. As cells approached confluency, supernatants from different wells were screened for the presence of specific antibodies by an enzyme-linked immunoassay, as previously described (30); the wells of the microtiter plates were coated overnight at 4°C with 100  $\mu$ l of 0.05 M carbonate buffer, pH 9.6, containing 300 ng of purified apoA-I or 500 ng of HDL. The cells from positive wells were cloned by limiting dilutions in 96-well microculture plates, and the clones obtained were recloned twice. Eleven stable clones showing the highest antibody titer to purified apoA-I and apoA-I in HDL were selected to be characterized after three more subclonings. 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 in aliquots at –80°C at the concentration of about 1 mg/ml.

### Iodination of lipoproteins, apolipoproteins, and monoclonal antibodies

HDL<sub>2</sub> and HDL<sub>3</sub> subfractions were labeled with <sup>125</sup>I using the procedure described by Bilheimer, Eisenberg, and Levy (31). Free <sup>125</sup>I was removed by gel filtration on a Sephadex G-25 column, followed by extensive dialysis at 4°C against PBS containing 0.01% Na EDTA. The <sup>125</sup>I precipitable with 10% trichloroacetic acid (TCA) was always greater than 98% of the total. Lipid-associated radioactivity ranged from 2 to 8% of TCA-precipitable radioactivity. Specific activity was 300–400 cpm/ng of protein. Purified apoA-I was labeled with the Iodogen procedure (32) to a specific activity of 400–500 cpm/ng. TCA-precipitable radioactivity was always greater than 99% of total. Both labeled HDL and apoA-I were stored at 4°C under sterile conditions, and used within a week. Monoclonal antibodies were labeled with <sup>125</sup>I with the Iodogen procedure (32) to a specific activity

of 2,000–3,000 cpm/ng. Free iodine was removed as described for lipoproteins. TCA-precipitable radioactivity was greater than 98% of total.  $^{125}\text{I}$ -labeled monoclonal antibodies were used within a week.

#### Direct binding assay of $^{125}\text{I}$ -labeled HDL to monoclonal antibodies

Fluid-phase radioimmunoassay was performed in duplicate as previously reported (26, 33, 34). Briefly, 100  $\mu\text{l}$  of serial dilutions of monoclonal antibodies was incubated with 100  $\mu\text{l}$  of  $^{125}\text{I}$ -labeled HDL (30,000 cpm, containing approx. 80 ng of protein). After an overnight incubation at 4°C, 100  $\mu\text{g}$  of rabbit anti-mouse IgG was added to give a slight antibody excess and the tubes were incubated for 3 h at room temperature. At the end of the incubation, 100  $\mu\text{l}$  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 to the use in the RIA assay. Rabbit anti-mouse IgG/pansorbin cells (100  $\mu\text{l}$ ) were added to the tubes and incubated for 6 h at room temperature after centrifugation (2000 g, 30 min) and the assay tubes were washed with 2 ml of cold assay buffer and centrifuged again. Supernatants were removed and the radioactivity of the pellet was determined in a gamma counter (Packard Multi-Prias). Maximum precipitable radioactivity was determined by replacing the rabbit anti-mouse IgG with trichloroacetic acid (220 g/l). Minimum precipitable radioactivity, or nonspecific binding, was determined by replacing the specific monoclonal antibodies with supernatant fluid from an irrelevant hybridoma. For calculations, the percent of total  $^{125}\text{I}$ -labeled HDL bound was expressed as  $B_0/T$ , where  $B_0$  is the total  $^{125}\text{I}$ -labeled HDL bound to antibodies, minus nonspecific binding, and T is the maximum TCA-precipitable radioactivity. A similar technique was used for determining the affinity constants of monoclonal antibodies by Scatchard plot analysis (35). Bound HDL was determined by incubating increasing doses of competing antigen (HDL or purified apoA-I) in the presence of  $^{125}\text{I}$ -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).

#### Binding of proteoliposomes to monoclonal antibodies

Proteoliposomes were incubated with affinity-purified antibodies (molar ratios apoA-I: antibody, 1:0.12, 1:0.25, 1:1, 1:2) for 1 h at 37°C. Equal amounts of protein from the different samples were loaded on agarose

gel (0.8%) and electrophoresed at 60 V for 1.30 h. After electrophoretic separation the proteins were blotted onto a nitrocellulose membrane (36) and incubated with a 1:50,000 dilution of rabbit antiserum to human apolipoprotein A-I (Behring), then with a 1:25,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad). Immunocomplexes were detected by an enhanced chemiluminescence method (ECL, Amersham) followed by autoradiography.

#### Competitive antibody binding assay

Solid-phase radioimmunoassay was performed in duplicate as follows. Affinity-purified antibodies were diluted in coating buffer containing 0.015 M  $\text{Na}_2\text{CO}_3$ , 0.35 M  $\text{NaHCO}_2$ , pH 9.6, to a final concentration of 10  $\mu\text{g}/\text{ml}$ . Aliquots (100  $\mu\text{l}$ ) were distributed in Immuno Module Break apart plates (Maxisorp Nunc, Denmark) and incubated overnight at 4°C to allow the binding of the antibody to the wells. The plates were then emptied, washed three times with PBS containing 0.05% Tween-20 and once with PBS, then incubated with 200  $\mu\text{l}$  of 3% BSA to saturate nonspecific binding sites. After 2 h incubation at room temperature, the plates were washed as above. Serial 2-fold dilutions of monoclonal antibodies were prepared in PBS and 50- $\mu\text{l}$  aliquots were transferred into the wells with 50  $\mu\text{l}$  of  $^{125}\text{I}$ -labeled HDL. After an overnight incubation at room temperature, the plates were washed as above and the single wells were counted in a gamma-counter. The results were expressed as  $B/B_0$ , where B is the counts per minute (cpm) bound, and  $B_0$  is the cpm in the absence of competing antibody.

#### Gel electrophoresis and immunoblotting

SDS gel electrophoresis was performed in a 10% polyacrylamide gel according to Weber and Osborn (37) using a Bio-Rad minicell (Bio-Rad, Richmond, CA). Isolated lipoproteins were delipidated by the method of Scanu and Edelstein (38) and solubilized in PBS containing 2% SDS (w/v), 1%  $\beta$ -mercaptoethanol (v/v). After separation by SDS-PAGE, the apolipoproteins were electrophoretically transferred onto nitrocellulose, as described (38). The nitrocellulose paper was then cut into strips and each strip was incubated for 1 h in a 500- to 1000-fold dilution of each monoclonal antibody in PBS containing 0.5% BSA. 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. Antibody binding was localized by peroxidase staining or, alternatively, by using  $^{125}\text{I}$ -labeled protein A. Protein A, labeled with Iodogen to a specific activity of 4000–5000 cpm/ng, was incubated ( $2\text{--}3 \times 10^5$  cpm) with the nitrocellulose strips in PBS containing

1% BSA for 2 h. The strips were washed, dried, and processed for autoradiography. 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 (39). Transfer and immunological detection were performed as described above.

### 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 (39). Blotting and immunological detection were as described above. CNBr fragments were identified according to their molecular weight and electrophoretic mobility.

### Peptide synthesis

Overlapping hexapeptides and octapeptides of apoA-I were synthesized using a kit purchased from Chiron Mimotopes (Epitope Scanning kit, Clayton, Australia). The solid polyethylene pins used for the Multipin Peptide Synthesis strategy developed by Geysen (40), are radiation-grafted with acrylic acid. This provides a reactive functionality predominantly on the surface of the otherwise inert polyethylene upon which synthesis may be initiated. All amino acids used bore  $\alpha$ -amino groups protected by the S-fluorenylmethyloxycarbonyl (Fmoc) group (Fluka). Highly purified dimethyl formamide (DMF) was purchased from Sigma. Synthesis of peptides was accomplished by repetitive cycling of Fmoc-deprotection, washing and coupling at the rate of addition of one amino acid per day.

The pins were first incubated for 30 min with 20% piperidine in DMF at room temperature, and then washed 1 × 5 min in DMF, 3 × 2 min in methanol, air-dried for a minimum of 30 min, and finally washed 1 × 5 min in DMF. Solutions of Fmoc amino acids (60 mM) were prepared in MoBt/DMF and dispensed in polypropylene microtiter plates according to the sequence schedule. The blocks were placed in the trays and incubated at room temperature for 18 h. The pins were then washed 1 × 2 min in DMF, 3 × 2 min in methanol, and air dried for at least 30 min. Deprotection, washing, coupling, and washing steps were re-

peated until all required amino acids were coupled. The  $\gamma$ -terminus of each peptide was acetylated by incubation of the pins with DMF-acetic anhydride-triethylamine 50:5:1 for 90 min at room temperature. The pins were then washed 1 × 15 min in a methanol bath and air-dried for at least 15 min. Some amino acids had a side-chain protecting group that was removed by incubating the pins in polypropylene baths containing one of the following cleavage mixtures: mixture 1: trifluoroacetic acid-ethanedithiol-anisole 38:1:1 (v/v/v); mixture 2: trifluoroacetic acid-anisole 18:1 (v/v) for 4 h at room temperature. The pins were air-dried for 10 min and sonicated in 0.1% HCl in methanol-distilled water 1:1 (v/v) for 15 min then the pins were air-dried or stored in a sealed plastic bag containing silica gel unless the blocks were used immediately.

### ELISA assay for the interaction of Mabs with synthetic peptides

Monoclonal antibodies were tested for their reactivity with synthetic hexa- and octapeptides of apoA-I using an ELISA assay. Each block of pins was incubated in a microtiter plate containing 200  $\mu$ l/well 2% BSA in PBS, 0.1% Tween 20, pH 7.2 (pre-coat buffer) for 1 h at room temperature. The pins were then incubated overnight at 4°C in microtiter plates containing 175  $\mu$ l/well of the antibody at an appropriate dilution (e.g., 1:500) in pre-coat buffer containing 0.1% NaN<sub>3</sub>. The pins were washed 4 times for 10 min in PBS, pH 7.2, then incubated in a microtiter plate containing 175  $\mu$ l/well of peroxidase-conjugated affinity-purified rabbit anti-mouse IgG (M + L), diluted 1:1000 in conjugate diluent containing 0.1% BSA in PBS and 0.1% Tween 20, for 2 h at room temperature. The pins were washed 4 times for 10 min in PBS, pH 7.2. To detect the presence of peroxidation, the pins were incubated in the dark in microtiter plates containing 150  $\mu$ g/well of 0.5 mg/ml diammonium 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonate] in 1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.8 M citric acid, pH 4. The reaction was stopped by removing the pins when enough color had developed (usually 10 min). Absorbance at 405 nm was read immediately in a plate reader. In order to test the reaction with other antibodies, the blocks of pins were sonicated for 20 min at 65°C in 1% SDS in PBS, pH 7.2, containing 0.1%  $\beta$ -mercaptoethanol. The pins were then rinsed twice in distilled water at 60°C for 30 sec, then with distilled water at 60°C for at least 30 min, and finally soaked in hot methanol for at least 15 sec and air dried. In our experience, pins could be used for a maximum of 6-8 times, then the background became too high to give reliable results.

### LCAT partial purification and LCAT assay

LCAT was partially purified according to the procedure described by Chen and Albers (41) without ultra-

centrifugation. Briefly, to 250 ml of plasma 25 ml of a dextran sulfate (MW 50,000 10 g/liter, 500 mM MgCl<sub>2</sub>) was added under continuous stirring at 4°C. The mixture was incubated for 10 min and then centrifuged at 2500 rpm at 4°C for 30 min. To the supernatant 14.5 g of solid NaCl was added and dissolved. The mixture was then applied to a phenyl-Sepharose column. LCAT activity was eluted with distilled water. The phenyl-Sepharose-bound fractions containing LCAT activity were pooled, dialyzed against 20 mM sodium phosphate buffer, pH 7.1, and passed through an Affi-Gel Blue column. LCAT activity was eluted with phosphate buffer (20 mM, pH 7.1). The partially purified enzyme (purified about 400-fold as compared to the activity in whole plasma) was stored at -80°C until use.

#### Determination of LCAT activity

The activity of LCAT was determined by using a synthetic substrate preparation of egg yolk lecithin (42, 43). For this, 0.154 ml of a 50 mg/ml solution of synthetic substrate in ethanol, 0.116 ml of 1 mg/ml solution of cholesterol, and 10.8 µl of radioactive cholesterol were dried under a stream of nitrogen at room temperature; 3.3 ml of the assay buffer (10 mM Tris, 140 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.88 mg of purified apoA-I was added together with 0.3 ml of 7.25 mM sodium cholate solution. The solution was incubated with continuous shaking at 24°C for 30 min and then dialyzed extensively (at least 72 h) against the assay buffer at 4°C. At the end of the dialysis the substrate volume was adjusted to 4 ml with assay buffer. The assay mixture contained 250 nmol lecithin, 7.5 nmol unlabeled cholesterol,  $6 \cdot 10^{-3}$  nmol [<sup>3</sup>H]cholesterol, 0.8 nmol apoA-I, 0.5% FAF-BSA, 5 mM β-mercaptoethanol and 5–15 µl of partially purified enzyme in a final volume of 500 µl. The assay mixture was incubated at 37°C for 30 min. Appropriate controls were run simultaneously. The reaction was stopped by the addition of 2 ml of ethanol and the bottom phase was extracted twice with 4 ml of hexane. The extracts were collected, dried under nitrogen, and dissolved in 0.5 ml chloroform. Aliquots were subjected to TLC and the radioactivity in each region of the TLC plate was determined by liquid scintillation counting. Activity was expressed as nanomoles cholesteryl esters per hour per ml of partially purified enzyme. In a subset of experiments we studied the size heterogeneity of the substrate by gel filtration on a Sepharose 2B column and by nondenaturing gel electrophoresis. Column chromatography indicated that the substrate was monodisperse, with a size in the range of HDL. Upon gel electrophoresis two particles could be detected, one with a size slightly larger than HDL<sub>3</sub>, the other slightly larger than HDL<sub>2</sub> (data not shown). When particles purified by column chromatography were used to study the inhibition of LCAT activa-

tion by Mabs A-I-19 and A-I-9, the results were essentially identical to those obtained with the nonpurified particles. Therefore, the results obtained with the latter are presented.

## RESULTS

### Monoclonal antibodies

Murine monoclonal antibodies used in this study were obtained as described under Materials and Methods and their main characteristics are reported in Table 1.

### Specificity of monoclonal antibodies

The details of the monoclonal antibodies used are reported in Table 2. The data for antibodies A-I-9, 15, 16, 19, and 57 have been reported in part previously (26). Monospecificity of all the antibodies used in the present work was demonstrated by immunoblotting after SDS gel electrophoresis of plasma apolipoproteins. Bidimensional gel electrophoresis also showed that these antibodies recognized all isoforms of apoA-I, including proapoA-I (data not shown, and ref. 26).

### Epitope mapping

To further characterize our Mabs we attempted epitope mapping. First, we studied the interaction of the antibodies with apoA-I CNBr fragments by immunoblotting. Data obtained were consistent with the localization of the epitopes for antibodies A-I-5, A-I-8, and A-I-11 within CNBr fragment 3 (amino acids 113–148, data not shown), and with the epitope assignment for previously characterized antibodies (26, Table 2). Taking into account these data, as well as those for the previously characterized antibodies, we designed synthetic peptides to be used in the mimotope assay. The results are reported in Fig. 1 and Table 2. In most cases a single predominant antigenic site was detected; moreover, some antibodies recognized other areas along the linear sequence. This finding may reflect the presence of "discontinuous epitopes" (16).

To gain further insight into the spatial organization of the epitopes we studied the ability of each antibody to compete with all others for the binding to <sup>125</sup>I-labeled HDL<sub>3</sub>. Representative data are reported in Fig. 2. Binding was inhibited only by the homologous antibody for all Mabs, with the exception of antibody A-I-11, whose binding was also inhibited by antibody A-I-5. These data are in general agreement with the data reported in Fig. 1 and Table 2.

TABLE 1. Monoclonal antibodies to human apolipoprotein A-I

Immunizing Confirm	Hybridoma	Monoclonal Antibody	Antibody Type	Chain	K <sub>d</sub> , 10 <sup>3</sup> l/mol
ApoA-I	9-B2-D2	A-I-9	IgG1	K	1.3
ApoA-I	15-C4-B1	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
ApoA-I	11-C5-E11	A-I-11	IgG1	K	0.9
ApoA-I	8-B11-C8	A-I-8	IgG1	K	1.1
ApoA-I	5-F6-C5	A-I-5	IgG2a	K	1.5

TABLE 2. Immunoreactivity of monoclonal antibodies

	HDL	ApoA-I	CNBr Fragment	Epitope from Synthetic Peptides
A-I-9	+	+	3	140-145
A-I-15	+	+	2	96-101
A-I-16	+	+	3	144-148
A-I-19	+	+	1	124-128 (minor) 1-10
A-I-57	+	+	4	167-174
A-I-11	+	+	3	124-128 144-148 (minor)
A-I-8	+	+	3	144-148
A-I-5	+	+	3	133-141

**Effect of the Mabs on LCAT activation by apoA-I**

The capacity of the eight antibodies studied to interfere with LCAT activation was assessed by measuring the degree of esterification of cholesterol incorporated into proteoliposomes containing phosphatidylcholine, cholesterol, and apoA-I in molar ratios of 250:7.5:0.8. Before the addition of LCAT, affinity-purified antibodies were preincubated with the proteoliposomes for 1 h at 37°C, to allow interaction with apoA-I. The rate of reaction was linear for at least 90 min; therefore, the percentage of total cholesterol esterified/hour (fractional esterification rate, FER) was calculated after 1 h incubation. In control samples the FER ranged between 24 and 33% (n = 10); esterification in the absence of partially purified LCAT was less than 0.1%. The antibody-mediated effects on LCAT activity are depicted in Fig.

3. In these experiments the apoA-I to Mab molar ratio ranged from 0.5 to 4. Out of eight antibodies tested, six inhibited LCAT activation up to 80% in a dose-dependent manner, while antibodies A-I-11 and A-I-19 were ineffective.

To address the question whether steric hindrance could have played a major role in determining LCAT inhibition by the antibodies, we used monovalent Fab fragments. The general trend observed with intact antibodies was preserved, although the curves moved slightly to the right; this could reflect a reduced affinity of the monovalent Fab for the antigen (Fig. 3).

**Fluid-phase radioimmunoassays**

It has been previously reported that some Mabs (14) fail to bind all HDL particles present in plasma. This has been ascribed to different apoA-I conformations that may, at least in part, depend upon particle size (13, 14). In this study we used a synthetic substrate to study the effects of anti-apoA-I Mabs on LCAT activation. We therefore studied the ability of Mabs to interact with apoA-I present on the proteoliposomes used in the LCAT assay. Experiments were performed under conditions identical to those of the LCAT assay as described under Materials and Methods. The binding of antibodies to proteoliposomes is depicted in Fig. 4. The binding of the proteoliposomes by a 4-fold molar excess of antibody under the conditions of the LCAT assay is compared with the inhibition of the LCAT activation in-

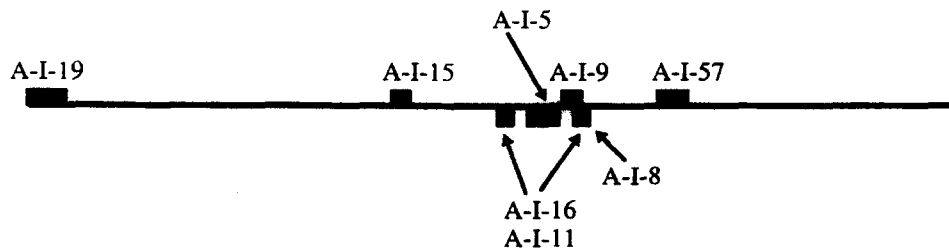


Fig. 1. Linear map of the epitopes for monoclonal antibodies to human apoA-I. The epitope localization is indicated by the boxes.

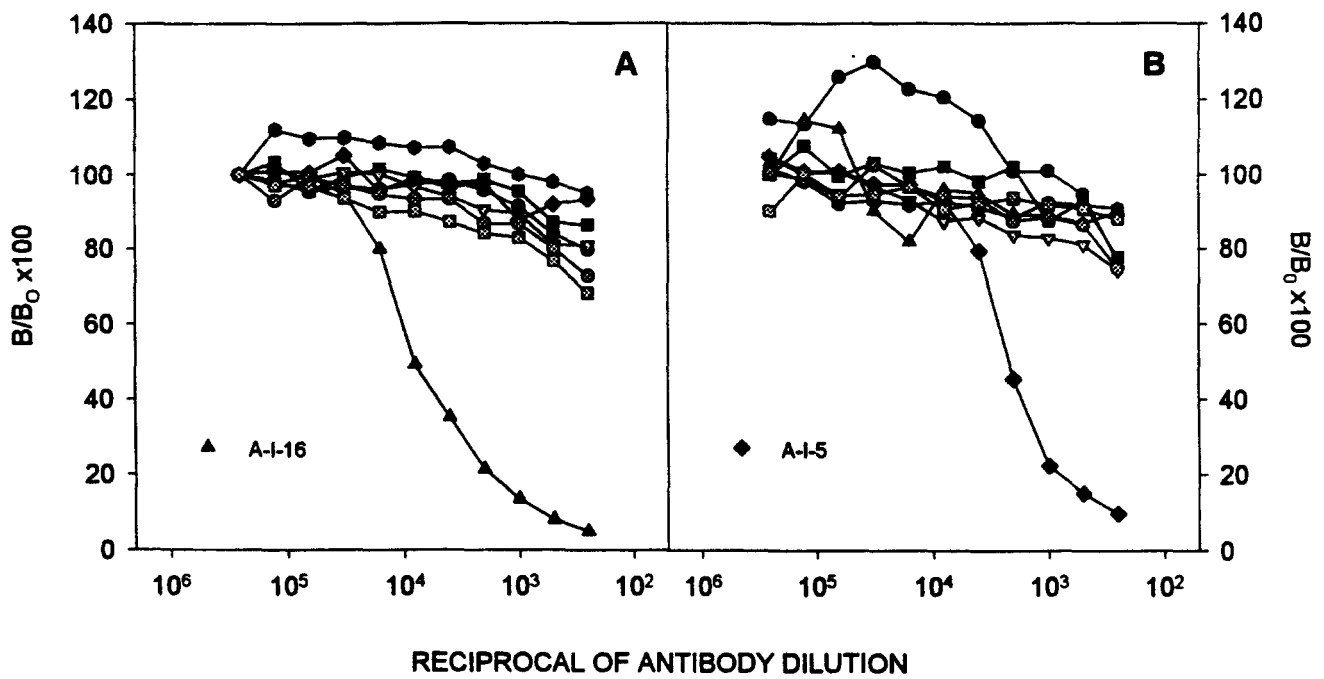


Fig. 2. Representative experiments of the competitive antibody binding assay: competition with monoclonal antibodies A-I-16 (panel A) and A-I-5 (panel B) was performed by a solid phase radioimmunoassay as described under Materials and Methods. Data are means of quadruplicates that did not differ by more than 5%.

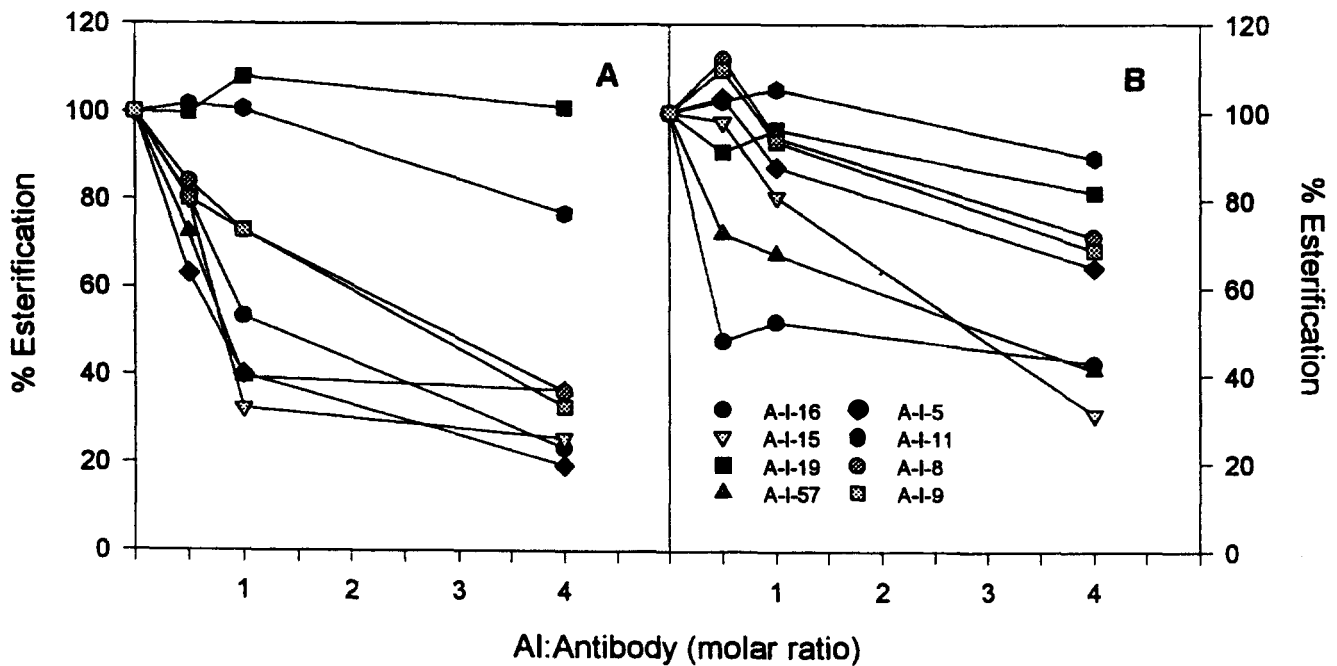
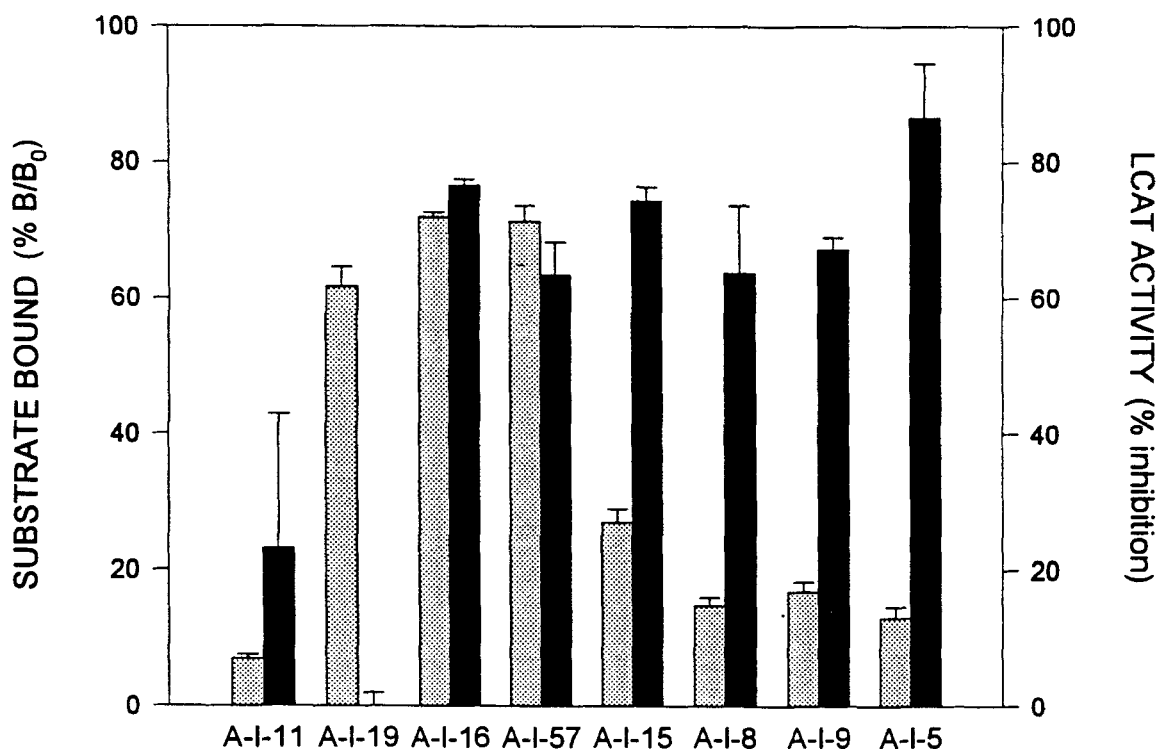


Fig. 3. Effect of increasing amounts of apoA-I monoclonal antibodies and Fab fragments on LCAT-mediated cholesterol esterification. Data represent LCAT-mediated cholesterol esterification in apoA-I proteoliposomes in the presence of increasing concentrations of antibodies (panel A) or Fab fragments (panel B). Esterification in the absence of antibodies or Fab fragments was taken as 100%. Data are means of triplicates that did not differ by more than 5%.



**Fig. 4.** Effect of anti-A-I monoclonal antibodies on LCAT activity and their binding to the synthetic substrate. Inhibition of LCAT activity (■) was determined at a 1:4 molar ratio (substrate: antibody) as described in Material and Methods. Binding to the substrate (▨) was determined at the same molar ratio. Data are the mean  $\pm$  SD of triplicate experiments.

duced by a 4-fold molar excess of the antibody. As assessed by this procedure, not all antibodies bound completely to the substrate.

The difference in the level of immunoprecipitation of the substrate and the LCAT inhibition by certain antibodies may appear contradictory. It should be considered, however, that immunoprecipitation may reflect not only the affinity of the antibody for the antigen but also the stability of the antibody-antigen complex upon centrifugation in the presence of Tachisorb N IgG Immunoabsorbent (Calbiochem). It was suggested that the immunoprecipitation with pansorbin may disrupt the immunocomplex (25). We therefore used a more gentle procedure to assess interaction of the antibody with the substrate by nondenaturing gel electrophoresis and immunoblotting. According to these experiments at least one antibody was bound per particle (**Fig. 5**).

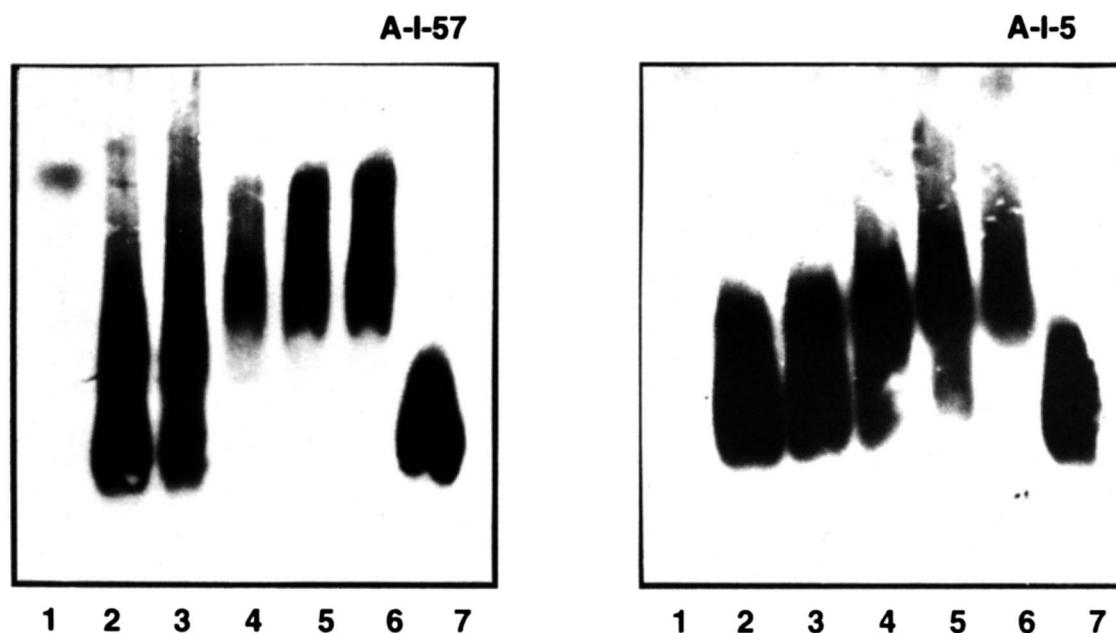
## DISCUSSION

A variety of physiological functions have been attributed to HDL; perhaps the most significant are those associated with "reverse cholesterol transport" (8, 44). A

key component in determining reverse cholesterol transport is the LCAT-mediated cholesterol esterification (7, 8, 44). This enzyme preferentially acts on cholesterol present in HDL and its full activity is dependent upon the presence of the cofactor apoA-I. To study the areas of apoA-I responsible for LCAT activation we have elected to use an immunochemical approach, by using a panel of well-characterized monoclonal antibodies. To do this a number of conditions should be met: 1) a number of antibodies spanning the entire apoA-I sequence should be available; 2) their affinity must be high, so that they can compete with LCAT for binding apoA-I; 3) their epitopes must be known; and 4) the monovalent Fab fragment should be used to avoid oligomer formation.

Five of the eight monoclonal antibodies used in this study have been previously described and their epitopes were partially defined by using purified apoA-I, CNBr fragments, and synthetic peptides (26). In this paper we report a more detailed characterization of their epitopes as well as the characterization of new Mabs produced in our laboratory. All antibodies have an apparent  $K_d$  in the range of  $10^9$  l/mol (25, and data not shown) and in a fluid-phase RIA recognize more than 90% of labeled HDL, with the exception of Mab A-I-16





**Fig. 5.** Representative experiments for the study of the interaction of monoclonal antibodies with synthetic substrate. Data are representative immunoblots of agarose gel of the proteoliposomes incubated at 37°C for 1 h in the presence or absence of monoclonal antibodies. The blots were incubated with a polyclonal anti apoA-I antibody. For experimental details refer to the Materials and Methods. Lane 1: monoclonal antibody; lane 2: 1:0.12 molar ratio (substrate:antibody); lane 3: 1:0.25 molar ratio; lane 4: 1:0.5 molar ratio; lane 5: 1:1 molar ratio; lane 6: 1:2 molar ratio; lane 7: substrate.

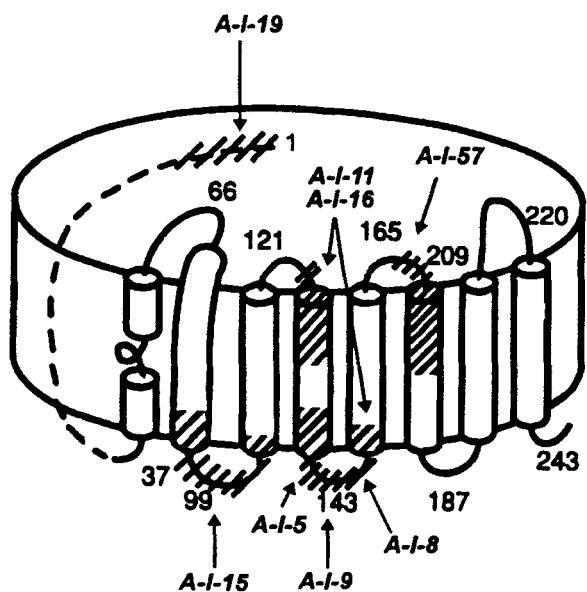
(26). The properties of the monoclonal antibodies were further investigated by competitive RIA with HDL. We previously reported that antibodies A-I-15, A-I-16, A-I-19, and A-I-57 failed to recognize purified apoA-I in the free form, but recognized the protein well when it was associated with lipids or bound to nitrocellulose (26). To overcome this problem we elected to coat the wells of a microtiter plate with the antibody and compete for the binding with HDL with other antibodies. The results of these experiments confirm previous findings for antibodies A-I-9, A-I-57, A-I-16, A-I-15, and A-I-19 and indicate that antibodies A-I-5, A-I-11 and A-I-8 recognize epitopes that differ among each other and from those of the antibodies previously described. The characterization of the epitopes for these antibodies was obtained by using several approaches, including the “mimotope” technique. While the first series of antibodies we produced spanned most of the apoA-I sequence, the second series was directed towards epitopes in the CNBr-3 fragment, confirming the experience of several authors that antibodies to the NH<sub>2</sub>-terminal or the central portion of apoA-I are frequently obtained (14, 16, 21, 25).

Some of our antibodies appear to have epitopes overlapping those of antibodies described by others (24, 25). However, some of them are unique and recognize epitopes immunologically distinct from each other as demonstrated by competitive binding of <sup>125</sup>I-labeled

HDL. The use of CNBr fragment only allows determination of relatively extended epitopes. The use of the mimotope technique results in a better definition of the areas of apoA-I involved in the interaction with Mabs.

This approach has been previously used by Marcel et al. (16) to characterize the epitopes for a series of Mabs to apoA-I. They reported a large variability in the immunoreactivity of the peptides. To overcome this problem we synthesized a series of overlapping peptides and tested their reactivity with Mabs in at least four independent experiments. The data obtained were in agreement with those obtained with CNBr fragments (Table 1) and with previous assignments (Fig. 1). As opposed to Marcel et al. (16) we found relatively few discontinuous epitopes (2 out of 8 Mabs vs. 11 out of 17). The reason for this discrepancy may relate either to the procedure for selecting antibodies or to their affinity. Alternatively, one may consider the fact that our monoclonals were produced by using purified apoA-I for immunization, while Marcel et al. used HDL (16).

The presence of “discontinuous” epitopes for monoclonals on apoA-I indicates either that these sequences are present on one face of an  $\alpha$ -helix or are relatively distant and brought together by the folding of the peptide chain. This might be the case for antibodies A-I-11 and A-I-16. The two areas recognized are relatively distant on the linear sequence of apoA-I, but, according to the current view of A-I structure on HDL, they are on



**Fig. 6.** Artistic view of the conformation of A-I in HDL. The different  $\alpha$ -helices are represented by cylinders. The epitopes for the antibodies are presented. The model is from reference 25 with minor modifications (with permission of ASBMB. *J. Biol. Chem.* 1993. 268:16966–16973).

antiparallel helices and therefore relatively close when apoA-I is bound to lipids (Fig. 6). Marcel et al. (16) suggested that as all discontinuous epitopes lie in the  $\text{NH}_2$  terminal half of the molecule; this area is relatively structured in HDL. We only have one antibody that recognizes a  $\text{NH}_2$ -terminal epitope, A-I-19, and this antibody apparently does not recognize a discontinuous epitope, although interacting with the same area of apoA-I antibody 4H1 described by Marcel et al. (16).

A relatively large number of our Mabs interact with the middle region of apoA-I (see Fig. 1). This is not surprising, as many groups have experienced the fact that this portion of human apoA-I appears to be most immunogenic in mice (24, 25).

Essentially all areas of the middle region of apoA-I (amino acids 70–180) appear to be available to form epitopes for Mabs. The high frequency of continuous epitopes in this areas has been taken as an indication of a relatively “unstable” structure of this portion of the molecules, with high mobility and accessibility (16, 24). Perhaps the observation that the only two antibodies with discontinuous epitopes in our series lies in this area (A-I-16 and A-I-11) may suggest that within an “unstable” structure there are areas of conformational constraint. Finally, we have characterized the epitope for antibody A-I-57 as lying in the area 167–174, a region that also appears to be immunoreactive with other antibodies (13, 16).

Overall, our data are in agreement with the concept that the central portion of human apoA-I is highly im-

munogenic in mice and that the major antigenic sites are represented by  $\beta$ -turns and adjacent portion of  $\alpha$ -helices, and further stress the concept that this domain of apoA-I is mobile.


Having characterized these antibodies, we aimed at identifying the areas of apoA-I involved in LCAT activation. Our data indicate that the area between amino acids 96 and 101 and the one between amino acids 124 and 174 are important for LCAT activation. Several laboratories have identified peptide sequences of apoA-I that associate with phospholipids and activate LCAT, including amino acids 121–164 and 142–185 (24). However, these peptides were only 30 and 24% as active as apoA-I. Activation was also obtained with the amino (1–85) and carboxy (199–243) terminal regions of apoA-I (7). Data obtained with naturally occurring point mutations or point mutations introduced by site-directed mutagenesis suggest that modifications at the COOH- and  $\text{NH}_2$ -terminals of apoA-I have no effect on LCAT activation, while mutations in the 107–165 region reduce LCAT activation (45–50). Deletion studies (51, 52) further confirmed these findings and indicate the 143–203 region of apoA-I as the most important determinant in LCAT activation, while COOH-terminal deletion mutants were activators of LCAT.

By using monoclonal antibodies, Banka et al. (24) indicated the 95–116 region as the most important in LCAT activation, while antibody A-I-16, that binds an epitope in the amino terminal amino acids 1–15, did not inhibit the activation of LCAT by apoA-I. In this study, however, Fab fragments were not used and therefore steric hindrance, as well as formation of large complexes, could have played a role. Meng et al. (25) recently addressed the same question by using proteoliposomes of different size. In general, antibodies had the same behavior with different substrates. Three antibodies were the most efficient inhibitors of the apoA-I-induced LCAT activation: 3G10 (aa 96–122) A 03 (aa 135–148) and A 44 (aa 149–186). Our results are in agreement with these data, including the fact that antibodies directed towards the  $\text{NH}_2$ -terminal do not affect activation, and further stress the concept that the area between aa 96 and 174 of the apoA-I sequence is essential to LCAT activation.

The identification of at least three different areas of apoA-I that potentially play a role in LCAT activation can be interpreted in different ways. 1) Upon binding, the antibodies induce a change in conformation that results in a reduced ability to activate LCAT. This explanation is unlikely, as antibodies binding in the 116–148 region failed to inhibit (21). 2) Steric hindrance: this possibility is also unlikely, as data with purified antibodies were similar to those with monovalent Fabs. 3) Because the vast majority of the antibodies that inhibit LCAT activation interact with areas including  $\beta$ -turns in

the structural region of apoA-I, one could speculate that antibody binding to these regions results in a dramatic reduction of the "mobility" of these areas that are "frozen" in a particular conformation. This conformation might not favor the interaction with LCAT. In line with this hypothesis is the fact that all the antibodies reported so far to inhibit LCAT activation encompass areas of  $\beta$  turns or adjacent regions. In HDL these areas may be those regulating dissociation of the "mobile domains" from the surface of the lipoprotein. If this hypothesis is correct, apoA-I mutations that reduce the chances of  $\alpha$  helix formation in these areas should be less active than native A-I in enhancing LCAT activity. Further studies are required to address this issue.

Meng et al. (25) have also reported that some antibodies may actually enhance LCAT activity on synthetic substrates resembling HDL of various sizes. We have not experienced this effect with any of our antibodies. The reasons for these discrepancies are unclear, but may relate to the different overall structure of apoA-I in different substrates.

In summary, our results demonstrate that unrestricted access of the antibodies to apoA-I between amino acids 96 and 174 is essential for inhibition of LCAT reaction. This finding confirms and extends previous data on the areas of apoA-I required for LCAT activation and suggests that several areas spanning the middle region of the apolipoprotein may act in concert to stimulate LCAT activity, possibly by cooperative interaction with the enzyme. 

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