methods

A new enzyme-linked immunosorbent assay with two monoclonal antibodies to specific epitopes measures human lecithin-cholesterol acyltransferase

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Abstract **We established five monoclonal antibodies that reacted with human LCAT and recognized different epitopes on LCAT. These are mouse anti-human LCAT monoclonal antibodies designated 36487, 36454, 36442, 36405, and 36486, which react with the peptides corresponding to human LCAT amino acid residues R159-E179, M258-S273, S274-S294, D352-S376, and N415-E440, respectively. We also successfully used two of these antibodies to develop an ELISA, which uses a solid phase monoclonal antibody, 36486, that reacts with the C-terminus of LCAT, and a detection monoclonal antibody, 36487, that reacts with an epitope located in the center of the LCAT primary structure. We observed a significant positive correlation between the values of LCAT protein determined with ELISA and LCAT activity determined with liposome substrate (r = 0.871, $P < 0.001$) or the endogenous self-substrate method (r = 0.864, $P < 0.001$), and we obtained inter- and intra-assay coefficients of variation less than 6.1%, minimum detection limit of 0.1 μg/ml. Highly specific monoclonal antibodies will be useful in the study of the molecular pathology of LCAT. Therefore, this precise and sensitive LCAT assay will help clarify the role of this enzyme in the metabolism of HDLs, and can be used for diagnostic purposes in investigating liver function.** We obtained five monoclonal antibodies that recognized different epitopes on LCAT and developed a sandwich-type ELISA. Highly specific monoclonal antibodies provide a sensitive and specific analytical system for measurements of LCAT protein.—Kobori, K., K. Saito, S. Ito, K. Kotani, M. Manabe, and T. Kanno. **A new enzyme-linked immunosorbent assay with two monoclonal antibodies to specific epitopes measures human lecithin-cholesterol acyltransferase.** ***J. Lipid Res.*** **2002.** 43: **325–334.**

Supplementary key words ELISA • LCAT • epitope

HDL plays an important role in reverse cholesterol transport, a process by which excess cholesterol from peripheral tissues is returned to the liver for use or excretion. There is a strong inverse correlation between plasma HDL cholesterol concentration and the incidence of atherosclerosis. LCAT performs a central role in HDL metabolism by catalyzing the formation of cholesteryl esters on HDL through the transfer of fatty acids from the *sn*-2 positions of phosphatidylcholine (PC) to cholesterol (1–3). Plasma and serum LCAT activity can be measured by a variety of chemical and radiochemical methods, which measure either enzyme activity on a common substrate or the cholesterol esterification rate. However, abnormality of plasma lipids and lipoprotein profiles adversely affects these assays. Although such assays may reflect the physiological esterification rate, they do not readily distinguish influences of the enzyme from those of the substrate, cofactors, and products (4). Genetic deficiencies of human LCAT have been recently reviewed by Kuivenhoven et al. (5). Briefly, two classes of genetic deficiencies are known: familial LCAT deficiency (FLD) and fish-eye disease (FED). FLD is caused by either null or missense mutations; in Class 1 defects, null mutations cause total loss of catalytic activity and virtual absence of LCAT mass, whereas in Class 2, missense mutations are characterized by loss of activity and either normal, reduced, or absent LCAT mass. FED is caused by missense mutations only; these mutations affect either LDL or HDL activity in Class 3 defects, and LCAT mass is reduced. In Class 4 defects, the missense mutations are associated with partial loss of activity against HDL only, and reduced LCAT mass. Direct measurement of the enzyme mass and activity may contribute to the differentiation of LCAT defects. We describe

Abbreviations: CV, coefficient of variation; DMPC, dimyristoylphosphatidylcholine; FCA, Freund's complete adjuvant; FED, fish-eye disease; FIA, Freund's incomplete adjuvant; FLD, familial LCAT deficiency; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; PAF-AH, platelet activating factor-acetylhydrolase; PBS, 10 mM phosphate buffer at pH 7.2 containing 150 mM NaCl; PBS-T, PBS containing 0.05% Tween 20; PC, phosphatidylcholine; PVDF, polyvinylidene difluoride.

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the establishment of five monoclonal antibodies that recognize different epitopes, the development of a specific and sensitive enzyme immunoassay for human plasma and serum LCAT, and the application of this assay to the measurement of LCAT in normal subjects.

MATERIALS AND METHODS

Serum and plasma samples

Serum and plasma samples from apparently healthy volunteers (eight women and 32 men) who had fasted overnight were collected at the Daiichi Pure Chemicals Diagnostics laboratories (Ibaraki, Japan). Serum samples from patients with liver disease were collected at the Department of Laboratory Medicine (Hamamatsu University School of Medicine, Hamamatsu, Japan). The study was approved by the Institutional Review Board.

Serum lipids and apolipoproteins were determined by enzymatic analysis and immuno-turbidimetric assay according to the manufacturer's instructions for commercially available kits (Daiichi Pure Chemicals, Tokyo, Japan). LCAT activity was determined by assay either with dimyristoyl-phosphatidylcholine (DMPC)-cholesterol liposomes as the substrate (liposome substrate method, Daiichi Pure Chemicals) (6) or by measurement of the decrease in unesterified cholesterol (endogenous self-substrate method; Azwell, Osaka, Japan) (7).

Purification and preparation of LCAT antigen

LCAT was purified 100-fold to homogeneity from normolipidemic human plasma (6). Plasma was chromatographed sequentially on Butyl-Toyopearl (Tosoh, Tokyo, Japan), hydroxylapatite (Bio-Rad, Hercules, CA), and DEAE-Toyopearl (Tosoh) columns. Two liters of pooled human plasma was diluted in the same volume of 1 M NaCl, 20 mM Tris-HCl, pH 7.4, and mixed with 500 ml of Butyl-Toyopearl resin equilibrated with 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4. After being stirred at 4°C for 2 h, the resin was washed with 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4, and packed into a column. A protein peak containing LCAT was eluted with 5% ethanol in water. The eluted protein was adsorbed to a hydroxylapatite column and eluted with a linear gradient of sodium phosphate. Protein containing LCAT activity was eluted at a phosphate concentration of 20 to 25 mM, pooled, and passed through a DEAE-Toyopearl column. The enzyme was eluted with a linear gradient of NaCl in 20 mM Tris-HCl, pH 7.4. A single protein peak, containing enzyme activity, was eluted at a NaCl concentration of 150 to 220 mM. Each fraction was assayed for LCAT activity with the liposome substrate method (6). Samples were prepared for assay by mixing 50 μl of eluate with 50 μl of plasma that was heated at 65°C for 30 min. When the activity was measured in the presence of heat-inactivated original plasma, purification was approximately 100-fold. SDS-PAGE and silver staining of the purified LCAT preparation revealed a single protein band with a relative mobility of 65 ± 2 kDa. The molecular mass of LCAT ranges from 65 to 69 kDa when determined with SDS-PAGE (8) and 59 or 60 kDa when determined with sedimentation equilibrium ultracentrifugation (9–10). Protein concentration was measured either with a modified Lowry method (11) or by determining optical density with use of a molar extinction coefficient of 2.0 $mg^{-1}cm^2$ at 280 nm (12).

Preparation of monoclonal antibodies to human LCAT

For our monoclonal antibody trial, immunization of BALB/c mice with purified LCAT emulsified in Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) produced IgMs reactive with a dominant epitope consisting of human LCAT amino acids 352–376. No other Igs were produced, including the IgG class, which is more useful than the IgM class (data not shown). We circumvented this problem by emulsifying purified human LCAT protein with the Ribi adjuvant system (Ribi ImmunoChem Research, Inc, Hamilton, MT), which contains detergents and generates almost no nonspecific Igs. Three times at 14 day intervals, purified human LCAT (25 μg of protein) emulsified in Ribi adjuvant system was subcutaneously injected into either the back or the abdomen of 4-week-old female BALB/c mice (SLC, Shizuoka, Japan).

We used two criteria related to hydrophobicity and similarity of amino acid sequence in various mammal species to design eight peptide antigens that correspond to parts of the human LCAT protein (13). We selected eight human LCAT sequences that corresponded to hydrophilic regions with little homology across species and to good potential antigens. We identified several peptides in the LCAT amino acid sequence that were likely to serve as good antigens (**Table 1**). Eight peptides corresponding to potential immunogenic epitopes of human LCAT were synthesized chemically and conjugated with keyhole limpet hemocyanin (KLH) (14). FCA (Sigma, St. Louis, MO) was added to 50 μg of each KLH-peptide conjugate in PBS and emulsified. Emulsions were injected subcutaneously into the backs of BALB/c mice. Two weeks later, the mice were immunized again with the same peptides emulsified in FIA (Sigma) followed by two more immunizations at 14 day intervals.

Antibody titers were determined 3 days after the final immunization, at which time blood was drawn from the tail vein of each mouse, allowed to stand at 4°C for 24 h, and then centrifuged at 3,000 × *g* for 30 min to harvest antiserum. Each antiserum was diluted serially, added in 50 μl aliquots to a 96-well microtiter plate coated with purified LCAT, and incubated at room temperature for 2 h. After three washes with PBS containing 0.05% Tween 20 (PBS-T), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgGs (Biosource International, Camarillo, CA) were diluted in PBS-T to the working concentrations recommended by the manufacturer, and 50 μl of the appropriate reagent was added to each well. The plates were incubated for 1 h at room temperature and washed again. The wells were finally incubated with 50 μl of the substrate solution containing 0.3% *o*-phenylenediamine-HCl and 0.003% H_2O_2 at room temperature for exactly 15 min. The reaction was terminated by the addition of 50 μl/well of 1.5 N H_2SO_4, and absorbance was measured at 492 nm with an MPR4A*i* microplate reader (Tosoh).

Three days after the final immunization, spleen cells (2.2×10^8) from each mouse were prepared and fused to myeloma cells (P3-63-AG8-658, 7×10^7) in the presence of 50% (w/w) polyethylene glycol. After 2 min, the cell suspension was diluted with 20 ml of serum-free RPMI-1640 medium, and the cells were collected via centrifugation at 1,000 × *g* for 5 min. The cell pellet was suspended in an RPMI-1640 medium containing 15% FBS, and the cell suspension was incubated for 2 h at 37°C in a

TABLE 1. Synthetic LCAT peptides used in this study

Peptide No.	Amino Acid Residue	Peptide Sequence	Length
1	27–48	LLNVLFPPHTTPKAELSNHTRP	22
2	56–74	LGNQLEAKLDKPDVVNWMC	19
3	159–179	RDETVRAAPYDWRLEPGQQEE	21
4	258–273	MSSIKLKEEQRITTTS	16
5	273–294	SPWMFPSQMAWPEDHVFISTPS	22
6	352–376	DHGFPYTDPVGVLYEDGDDTVATRS	25
7	384–407	QGRQPQPVHLLPLHGIQHLNMVFS	24
8	415–440	NAILLGAYRQGPPASPTASPEPPPPE	26

5% CO_2 atmosphere. The cells were collected by centrifugation, suspended in hypoxanthine, aminopterine, and thymidine (HAT) medium, and the resulting cell suspension seeded into a 96-well microtiter plate (1×10^5 cells/well). Plates were incubated at 37°C in a 5% CO_2 atmosphere for approximately 1 week. Hybridomas producing anti-human LCAT antibody were selected by testing the hybridoma culture medium with an ELISA that used pure LCAT fixed to a 96-well microtiter plate. The antibody-antigen complexes were visualized with the use of HRP-conjugated goat anti-mouse IgGs by the same colorimetric technique as that mentioned above. Positive hybridomas were subcloned with the limiting dilution method. Antibodies produced by some hybridomas displayed strong immunoreactivity against fixed LCAT antigen. These five hybridoma have been deposited with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan, under the deposition numbers FERM P-17611, P-17609, P-17608, P-17607, and P-17610 for LCAT monoclonal antibodies designated 36405, 36422, 36454, 36486, and 36487, respectively. Ascites fluid containing concentrated monoclonal antibodies was produced by intraperitoneally injecting hybridomas (2×10^6) into BALB/c mice treated with pristane. IgGs were purified from ascites fluid by ammonium sulfate fractionation and protein A affinity chromatography. Subclasses of these monoclonal antibodies were determined with a mouse monoclonal typing kit (Zymed Laboratories, South San Francisco, CA).

Electrophoresis and Western blot analysis

SDS-PAGE was carried out according to the method of Laemmli (15). Samples were processed with 1% SDS and 5% 2-mercaptoethanol and electrophoresis in a polyacrylamide gradient (4–20%) gel (Daiichi Pure Chemicals). Proteins in gels were visualized with Coomassie brilliant blue R-250 and silver staining. For Western analysis, proteins were transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane at 100 mA for 1 h. The membrane was incubated with Block Ace™ (Dainihon Pharmacy, Osaka, Japan) for 1 h at room temperature. Then the membrane was incubated for 1 h at room temperature with 5 ml of 10 μg/ml anti-human LCAT monoclonal antibodies diluted in distilled water containing 25% Block Ace. After a wash with 25 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20 buffer, pH 7.5 (TBS-T), the membrane was incubated for 1 h at room temperature with 5 ml of HRP-conjugated goat anti-mouse IgGs in 25% Block Ace. After a wash with TBS-T, the membrane was stained with 3,3′-diaminobenzidine tetrahydrochlroride. Part of the membrane was stained with Coomassie brilliant blue R-250 and destained in 10% acetic acid and 50% methanol.

Localization of the epitope reactive with monoclonal antibodies

For initial mapping of epitopes, the reactivity of monoclonal antibodies was tested against synthetic peptides passively coated onto the wells of microtiter plates (16). For passive-coating, peptides were dissolved in PBS at a concentration of 10 μg/ml, and 50 μl of this solution was added to the wells of 96-well microtiter plates. The plates were sealed, incubated at room temperature for 2 h, and washed twice with PBS-T. The wells were filled with Block Ace and incubated for 1 h to block nonspecific protein binding. Test monoclonal antibodies were diluted in PBS-T to a concentration of 10 μg/ml, and 50 μl/well of these dilutions was added to the peptide-coated wells and incubated for 1 h at room temperature. Then the antibody-antigen complexes were visualized with the use of HRP-conjugated goat anti-mouse IgGs by the same colorimetric technique described above.

Alternatively, the reactivity of the monoclonal antibody to peptide was examined by a competitive binding assay against the purified LCAT. One microgram of human purified LCAT was incubated in the wells for 2 h. After being blocked with Block Ace, the monoclonal antibody, 1 μg/ml, and 4 to 400 μg/ml of peptide, both in the T-PBS, were mixed at 1:1 (v/v) and added to the LCAT wells. The bound antibody was measured with the use of HRP-conjugated goat anti-mouse IgGs by the colorimetric technique described above. The values are expressed as the percentage of the control without peptide (100% binding). All assays were performed in duplicate.

ELISA for LCAT in human sera

The ELISA was based on a sandwich method involving two monoclonal antibodies against human LCAT. The monoclonal antibodies 36486 and 36487 were selected via a grouping test of the monoclonal antibodies reactive with human LCAT, which was performed by identifying two monoclonal antibodies that sandwich purified human LCAT antigen and human LCAT in sera. Fab′ fragments of monoclonal antibody 36487 were conjugated with HRP according to a previously described method (17). The microtiter plate was coated with 50 μl/well of monoclonal antibody 36486 (10 μg/ml in PBS) stored at 4°C overnight. After three washes with PBS-T, the wells were incubated with 300 μl of PBS-T containing 1% BSA at room temperature for 1 h to block non-specific binding. The wells were then incubated at room temperature for 2 h with 50 μl of the LCAT standard solution (1 μg/ml to 35.0 μg/ml of LCAT) or with samples diluted with PBS-T containing 0.2% BSA. After three washes with PBS-T, the wells were incubated at room temperature for 1 h with 50 μl of HRP-conjugated Fab′ 36487 diluted with PBS-T containing 0.2% BSA. Finally, after three washes with PBS-T, the wells were measured by the calorimetric technique described above. All assays were performed in duplicate. The quantity of LCAT in the sample was determined by comparing the sample absorbance with absorbance obtained from the standards.

Localization of LCAT protein in human serum lipoprotein fractions

The lipoprotein fractions in human serum were isolated by FPLC on a Superose™ 6HR column (Amersham Pharmacia Biotech, Buckinghamshire, England) according to a previously described method (18). Two hundred microliters of human serum was fractionated with a Superose™ 6HR column equilibrated with PBS, and 2 ml fractions were collected. Lipoprotein patterns were determined by measuring cholesterol content enzymatically with a commercial kit described above (Daiichi Pure Chemicals). Each fraction was subjected to the LCAT sandwich described above.

Statistical analysis

Pearson's correlation coefficient r was used to show the degree of linear association between the different variables. The significance of r was found from the t distribution. $P < 0.05$ was considered significant.

RESULTS

Properties of monoclonal antibodies against human LCAT

Hybridomas producing anti-human LCAT antibody were established by fusing mouse spleen cells that had high antibody titers to the immunized purified LCAT or peptide-KLH with myeloma cells. Antibodies produced by five of the hybridomas displayed strong immunoreactivity against purified LCAT antigen. These are the mouse anti-

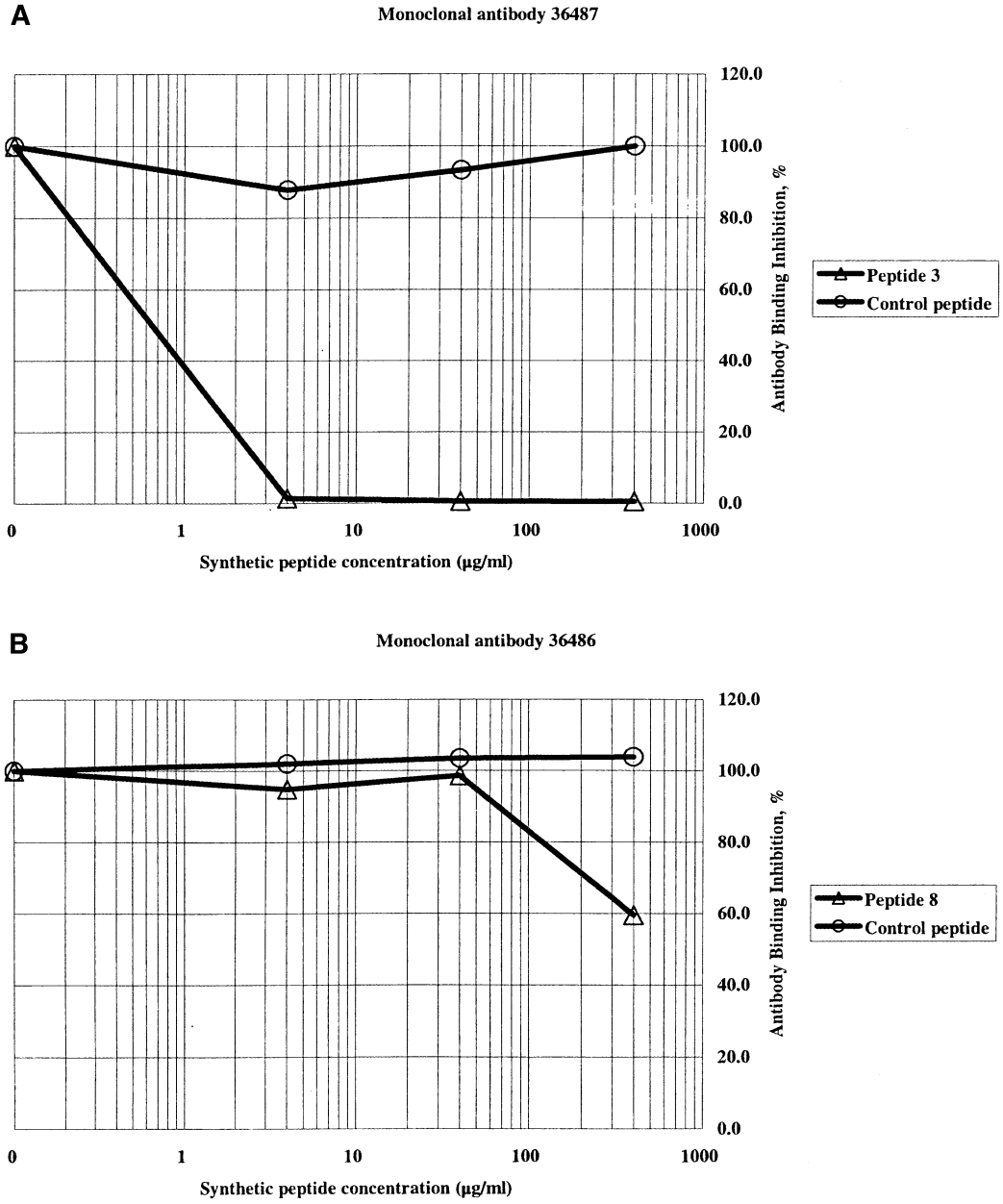

Fig. 1. Inhibition by peptide of the binding of each monoclonal antibody against the purified LCAT. Binding is expressed as the absorbance at 492 nm minus that at 620 nm as the percentage of the control peptide. Experimental details are described in Materials and Methods. Control peptide is Peptide No. 2 in Table 1.

human LCAT monoclonal antibodies designated 36487, 36454, 36442, 36405, and 36486. Three monoclonal antibodies, 36487, 36442, and 36486, were established by immunization of mice with the purified LCAT emulsified by the Ribi adjuvant system. The epitopes of these three monoclonal antibodies were determined by testing for immunoreactivity against the synthetic peptide antigen. Alternatively, the reactivity of each monoclonal antibody to peptide was examined by a competitive binding assay against the purified LCAT. Where the start methionine of the amino acid sequence predicted by the human LCAT mRNA is residue number 1, monoclonal antibody 36487 reacts with the peptide corresponding to human LCAT amino acid residues 159–179 (RDETVRAAPYDWRLEPGQQEE), 36442 reacts with the peptide corresponding to human LCAT amino acid residues 258–273 (MSSIKLKEEQRITTTS), and 36486 reacts with the peptide corresponding to human LCAT amino acid residues 415–440 (NAILLGAYRQGP PASPTASPEPPPPE). As shown in **Fig. 1**, each peptide corresponding to the epitope of monoclonal antibody 36486 or 36487 was able to compete with the coated LCAT. Monoclonal antibodies 36486 and 36487 were progressively displaced in the presence of increasing concentrations of each peptide corresponding to their respective epitopes.

Immunization of mice with KLH-peptide conjugate in FIA and FCA yielded monoclonal antibody 36454, which is established as the peptide corresponding to human LCAT amino acid residues 273–294 (SPWMFPSQMAW

TABLE 2. Summary of five monoclonal antibodies

Properties of Monoclonal Antibodies		Localization of the Epitope of Monoclonal Antibodies Synthetic Peptides[a]								Purified LCAT	Control[b]
Clone No.	Subclass	1	2	3	4	5	6	7	8		
36487	IgG2b	−	−	+++	−	−	−	−	−	++	−
36442	IgG1	−	−	−	+	−	−	−	−	+	−
36454	IgG1	−	−	−	−	+++	+	−	−	++	−
36405	IgG2a	−	−	−	−	−	+++	−	−	+++	−
36486	IgG1	−	−	−	−	−	−	−	+++	+++	−
Control[c]		−	−	−	−	−	−	−	−	−	−

[a] Binding is expressed as the absorbance at 492 nm minus that at 620 nm as a background for solid phase assay. +++: O.D. > 1.2. ++: O.D. 1.2–0.6. +: O.D. 0.6–0.1. −: O.D. < 0.1.
[b] Negative control, PBS.
[c] Negative control, Mouse Myeloma IgG.

PEDHVFISTPS), and monoclonal antibody 36405, which is established as the peptide corresponding to human LCAT amino acid residues 352–376 (SPWMFPSQMAW PEDHVFISTPS). Each mouse immunized with the other 6 peptide antigens showed immunoreactivity with purified LCAT antigen too. But hybridomas producing anti-human LCAT antibody were not established. The properties of the five monoclonal antibodies are summarized in **Table 2**.

The specificity of anti-human LCAT monoclonal antibodies was further ascertained with Western blot analysis (**Fig. 2**). These five monoclonal antibodies reacted with the LCAT protein, revealed as a single band with a relative mobility of 65 kDa.

Khalil et al. (19) developed monoclonal antibody (B10) that is reactive with the purified LCAT cross-reacted with pancreatic and snake venom phospholipase from many different A2 species. Therefore, we assayed the five new monoclonal human LCAT antibodies via ELISA with microtiter plates prepared by incubation of the wells with phospholipase A2 from either bee venom, bovine pancreas, or

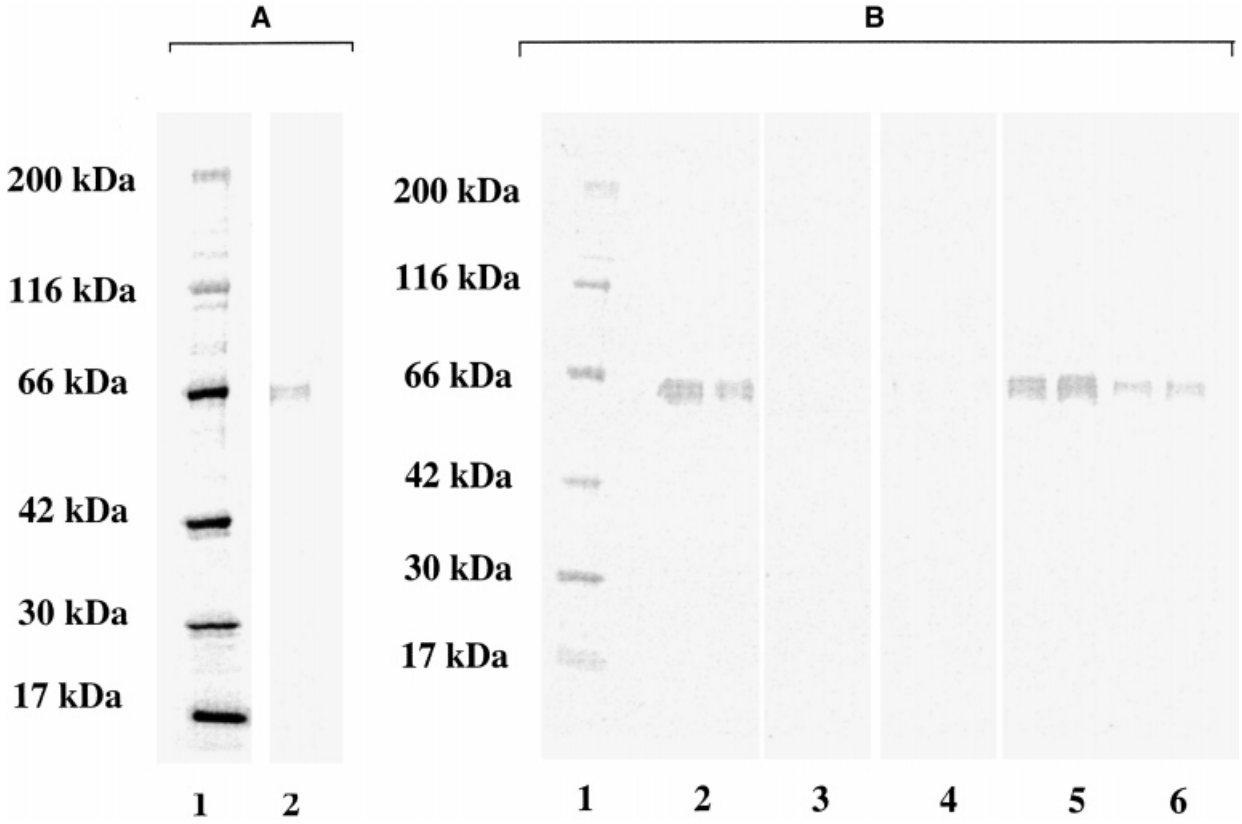

Fig. 2. Electrophoresis and Western blot analysis of purified LCAT. Purified LCAT and molecular weight standard (lane 1) were subjected to electrophoresis under reducing conditions on 4–20% polyacrylamide gradient gels. A: Each gel was fixed and silver stained as recommended by the manufacturer (Daiichi Pure Chemicals). Lane 2 corresponds to purified LCAT. B: A gel was analyzed by immunoblotting with monoclonal antibodies 36487 (lane 2), 36442 (lane 3), 36454 (lane 4), 36405 (lane 5), and 36486 (lane 6). Each lane shows results of two samples. The molecular mass marker kit (Daiichi Pure Chemicals) consists of rabbit muscle myosin (200 kDa), *Escherichia coli* β-galactosidase (116 kDa), BSA (66 kDa), rabbit muscle aldolase (42 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), and horse muscle myoglobin (17 kDa).

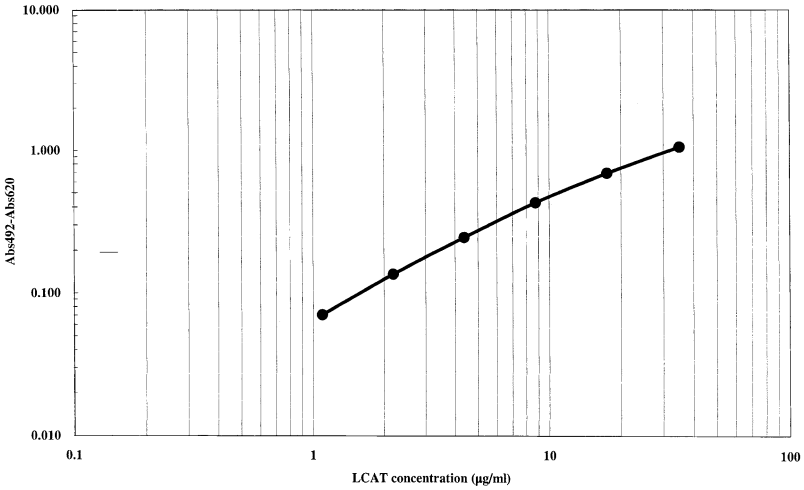

Fig. 3. Calibration curve for LCAT detection with 36486 and 36487 antibodies in standard plasma. Means of triplicate samples at each concentration are shown.

porcine pancreas at concentrations greater than 10 μg/ml. No cross-reactivity was detected (data not shown).

A sandwich ELISA for human LCAT was developed by screening the five monoclonal human LCAT antibodies for a combination of two IgGs that would capture and detect both purified antigen and LCAT in serum. Antigen was captured onto a solid phase coated with monoclonal antibody 36486. Antigen was detected with an HRP-conjugated Fab′ fragment of monoclonal antibody 36487. This system showed a dose-dependent response to purified LCAT and serum.

Standardization of ELISA for LCAT concentration

For calibration of the ELISA, the primary standard was frozen plasma [kindly provided by Dr. Marcovina, Northwest Lipid Research Laboratories (NWLRL), University of Washington, Seattle, WA] with LCAT concentrations assigned by the RIA (20). The calibration curve, constructed from a set of six dilutions of the primary standard, was used in our laboratory to determine the LCAT concentration in a pool of normolipidemic sera, which constituted the secondary standard. With use of the secondary standard, the recovery rates of LCAT assessed in three QC (quality control) sera of NWLRL with LCAT concentrations assigned by the RIA (20) were 96.1%, 92.2%, and 102.8% with LCAT levels of 10, 8.8, and 7.2 μg/ml, respectively. A calibration curve for each assay was constructed with six duplicate dilutions of the secondary standard in LCAT concentrations from 0.0 to 35.0 μg/ml (**Fig. 3**). A curve was fitted to the data points, and LCAT concentrations were calculated with data analysis software. Two dilutions of each sample were assayed, and LCAT concentration was calculated by averaging the two values. There was a good linear relation between the dilution ratio and concentration of LCAT detected by the ELISA (**Fig. 4**) although the minimum detection limit for LCAT in serum was 0.1 μg/ml.

In the intra-assay (n = 10), the means of three serum sample sets measured were 12.2, 7.9, and 5.25 μg/ml, and their coefficient variations were 5.2%, 2.7%, and 5.2%, respectively. In the inter-assay (n = 6), the means of three sample sets were 9.6, 8.1, and 7.2 μg/ml, and their coefficient variations were 5.3%, 6.1%, and 4.5%, respectively (**Table 3**).

The validity of the sandwich ELISA for LCAT was further examined in the presence of a high concentration of triglyceride and other substances including hemoglobin, conjugated bilirubin, and unconjugated bilirubin. Samples with hemoglobin at less than 500 mg/dl, unconjugated bilirubin at less than 20 mg/dl, conjugated bilirubin at less than 20 mg/dl, and Intralipos® at less than 5% had no effect on the LCAT assay.

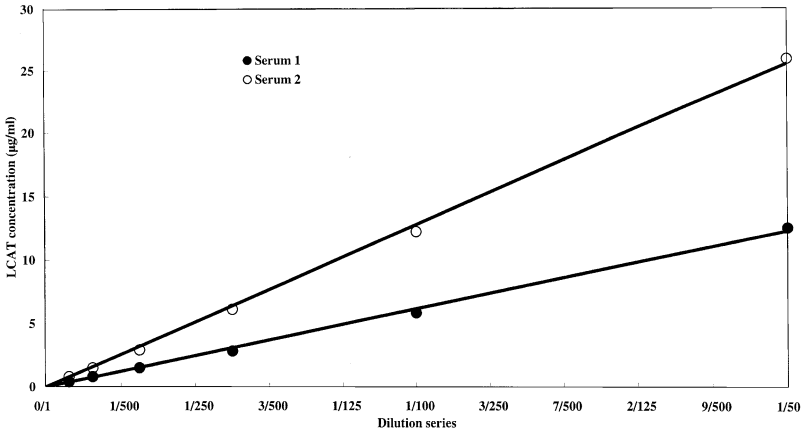

Fig. 4. Dilution test of serum samples. Two samples were serially diluted from 50- to 1,600-fold. Means of triplicate samples at each concentration are shown.

TABLE 3. Sandwich ELISA precision test for the determination of LCAT protein

Sample	Mean	SD	C.V.
	μg/ml	*μg/ml*	%
Intra-assay validation (n = 10)			
Serum 1	12.2	0.6	5.2
Serum 2	7.9	0.2	2.7
Serum 3	5.3	0.3	5.2
Inter-assay validation (n = 6)			
Serum 4	9.6	0.5	5.3
Serum 5	8.1	0.5	6.1
Serum 6	7.2	0.3	4.5

In the results of 40 adult healthy volunteers and five patients with liver disease, sera for LCAT mass and activity showed good correlation. Correlation was detected between LCAT levels determined by ELISA and LCAT activity obtained with the liposome substrate method (n = 45, y = 0.019x + 0.849, $r = 0.871$, $P < 0.001$) or LCAT activity obtained with the endogenous self-substrate method (n = 45, y = 0.079x + 1.534, $r = 0.864$, $P < 0.001$) (**Figs. 5** and **6**).

Distribution of LCAT protein in lipoprotein fractions of human serum

To determine the distribution of LCAT in lipoproteins, we used an FPLC chromatography system for isolation of lipoprotein. A representative elution profile of total cholesterol and LCAT protein is shown in **Fig. 7**. LCAT protein was detected in the HDL fraction and not in other fractions such as VLDL and LDL fractions in normolipidemic serum (Fig. 7A). A major peak of LCAT protein was detected in fraction 25 in the HDL region. Our data indicate that LCAT may be associated with large HDLs but not with small HDLs. Compared to normolipidemic serum, LCAT protein in hyperlipidemic serum was bimodally distributed (Fig. 7B). The major peak of LCAT protein detected in fractions 19–24 corresponded to HDL particles, and the minor peak of LCAT protein detected in fractions 15–18 corresponded to LDL particles. In some normolipidemic serum samples, a minor peak of LCAT protein was detected in the LDL region (fraction 17), and this peak corresponds to a cholesterol peak, suggesting that under the appropriate conditions, LCAT can associate with lipoproteins of lower density.

DISCUSSION

The antioxidant activity of HDL is apparently associated with several HDL-bound proteins such as paraoxonase (21, 22), platelet activating factor-acetylhydrolase (PAF-AH) (23), and LCAT (24). Purified LCAT is not only capable of esterifying cholesterol in the plasma but also can prevent the accumulation of oxidized lipids in LDL (25). Whereas the physiologic importance of this novel activity remains unclear, clinical studies suggest that a deficiency of this enzyme can result in oxidative tissue damage (26). Mutations in the LCAT gene underline either FLD or FED that are both inherited in an apparent autosomal recessive manner (5). Patients with complete LCAT deficiency have premature coronary artery disease or severe renal disease, both of which are associated with the accumulation of oxidized lipid-enriched foam cells in glomerular tufts (5, 26). Since the mid-1990s, studies of transgenic animals that overexpress human LCAT have confirmed the importance of this enzyme in cholesterol homeostasis and have suggested the LCAT gene as a target of therapeutic intervention for preventing or reversing arteriosclerosis (27). A variety of chemical and radiochemical techniques, which measure either enzyme activity on a common substrate or the cholesterol esterification rate, have been used to quantify this enzyme. Only one assay is currently available for measurement of LCAT protein. It requires radioisotopes, highly purified LCAT, and polyclonal antibodies that inhibit LCAT activity (20).

Attempts to prepare specific, polyclonal LCAT antibodies have had limited success (19, 28–32). A previously reported study showed that attempts to produce anti-LCAT sera from a sheep and five different types of rabbits were

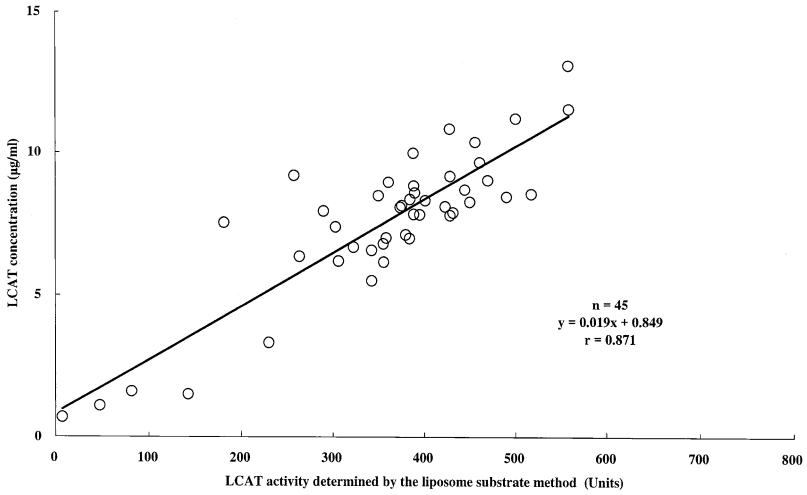

Fig. 5. Relation between LCAT concentration and LCAT activity measured with the liposome substrate method in 40 normolipidemic subjects and five liver disease patients (n = 45, y = 0.019x + 0.849, $r = 0.871$, $P < 0.001$).

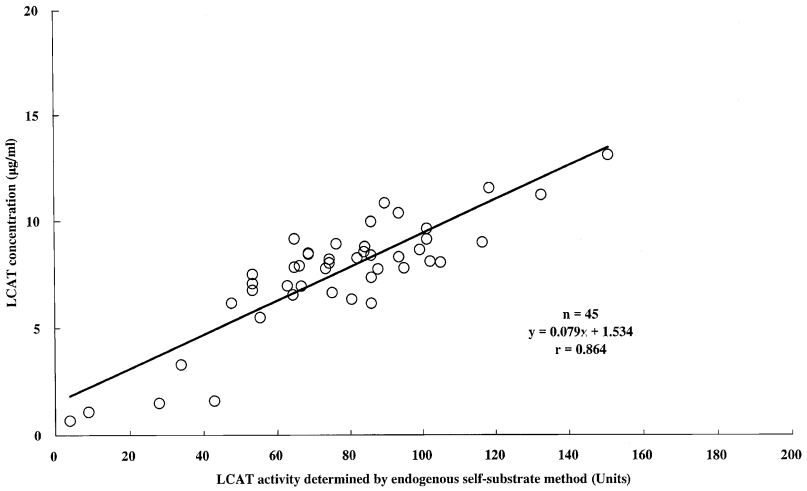

Fig. 6. Relation between LCAT concentration and LCAT activity determined with endogenous self-substrate method in 40 normolipidemic subjects and five liver disease patients (n = 45, y = 0.079x + 1.534, $r = 0.864$, $P < 0.001$).

unsuccessful (20). Researchers have experienced great difficulty in developing antibodies with purified LCAT protein, Freund's adjuvant, or standard immunization protocols. In our immunologic studies of BALB/c mice, the characteristics of the primary and of the memory IgM- and IgG-type antibody responses showed that LCAT, which contains an immunodominant epitope from amino acids 352 to 376, was capable of inducing a strong IgM-type antibody response but was barely able to induce an IgG-type antibody response. We were unable to produce any Igs against any region of the LCAT molecule other than the single immunodominant epitope. However, we were able to avoid this problem by immunizing mice with antigen emulsified in a detergent adjuvant. Antibody production against bacterial cell wall components, such as those found in Freund's adjuvant, does not occur, thus lowering non-specific immunoreactivity. We used two criteria based on hydrophobicity and similarity of amino acid sequence to design eight peptides corresponding to human LCAT protein for antigen production (13).

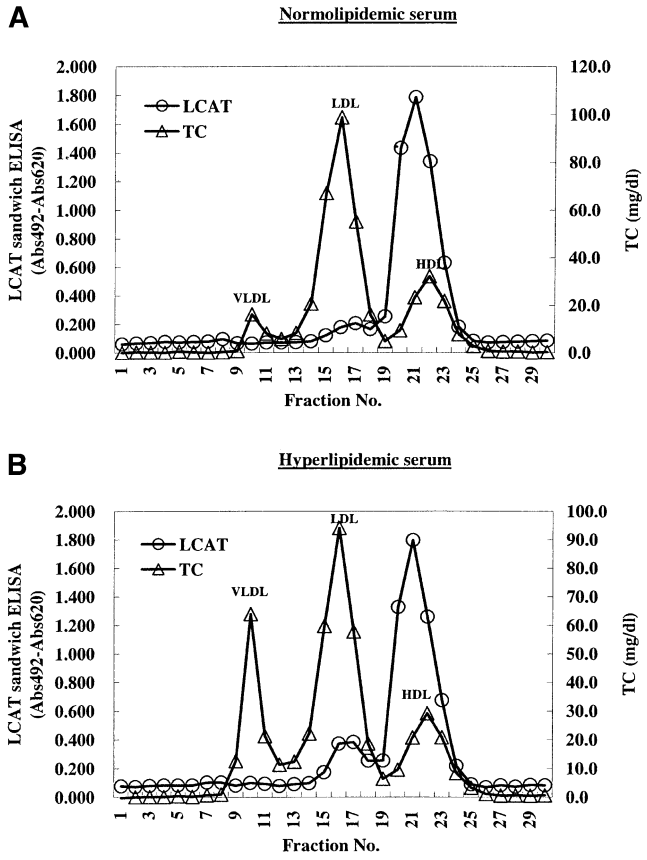

Fig. 7. Distribution of LCAT protein in a normolipidemic serum sample and a hyperlipidemic serum sample. Two hundred microliters of either normolipidemic (A) or hyperlipidemic (B) serum samples was fractionated with a Superose 6HR column equilibrated with PBS, and 2 ml fractions were collected. Lipoprotein patterns were determined via enzymatic assays to measure cholesterol content. Each fraction was subjected to the LCAT sandwich ELISA described in Materials and Methods.

Our accomplishment in producing the five mouse monoclonal antibodies and in producing LCAT antibodies with negligible cross-reactivity with phospholipase A2 (19) from a variety of animal species shows that we have developed procedures for designing and producing monoclonal antibodies that react with multiple epitopes of a protein with a single, immunodominant epitope. Our five monoclonal antibodies that recognized different epitopes on LCAT enabled us to estimate the length of truncated LCAT.

For measurements of LCAT protein, highly specific monoclonal antibodies provide a sensitive and specific analytical system to diagnosis the LCAT deficiency syndromes. We successfully utilized a sandwich-type enzyme immunoassay, which uses a solid phase monoclonal antibody (36486) that reacts with the C-terminus of LCAT and a detection monoclonal antibody (36487) that reacts with an epitope located in the center of the LCAT primary structure. Since 36486 reacts with the C-terminus of the protein to capture the LCAT, our immunoassay easily differentiates full length of normal protein from truncated protein.

Although the minimum detection limit for purified LCAT is 0.1 μg/ml, the practical measurement range of this assay for clinical application is from 0.5 to 35.0 μg/ml.

We observed a significant positive correlation between the values of LCAT protein determined with ELISA and LCAT activity determined with the liposome substrate (6) or endogenous self-substrate method (7). This suggests that immunodetectable LCAT is available in our new ELISA and that the epitopes recognized by the monoclonal antibodies are expressed on LCAT-containing particles in serum. The validity of the present sandwich ELISA for LCAT was verified with precision (inter- and intra-assay) and dilution tests. Using the procedures described in this study, we obtained inter- and intra-assay coefficients of variation of less than 6.1% for quantitation of immunoreactive LCAT protein in human serum. Linear dose-response curves were derived with the use of either purified human LCAT or human serum standards. The present sandwich ELISA is not affected by high concentrations of any substance such as Introlipos®, hemoglobin, conjugated bilirubin, and unconjugated bilirubin. Thus, this method is suitable for quantitation of LCAT in plasma and serum from hyperlipidemic individuals and normolipidemic individuals.

Because of its high sensitivity, our LCAT immunoassay is useful for screening of lipoprotein-associated LCAT. The association of LCAT with LDL was previously reported (33), and a previously reported distribution of LCAT in plasma and plasma fractions (34) was similar to the distribution we observed in serum. In our study, LCAT protein was detected in the HDL. A major LCAT protein peak in the region of the chromatogram was associated with elution of large HDL, and this suggests that LCAT may be associated with large HDL. The bimodal distribution of LCAT in serum suggests that some forms of LCAT may be associated with lipoproteins that are lower in density than HDL is. Our two-dimensional electrophoresis and Western blotting studies showed that anti-LCAT monoclonal antibody 36486 showed strong immunostaining associated with HDL_2 migration, which was confirmed by anti-apolipoprotein A-I monoclonal antibody immunostaining. Further, weak immunostaining was detected associated with LDL migration (data not shown). Our LCAT immunoassay will hopefully enable screening for LCAT in lipoprotein subfractions, thus clarifying the role of these LCATs in cholesterol transport.

In conclusion, our highly specific monoclonal antibody and the enzyme immunoassay for LCAT reported here may be a useful tool to clarify the physiological role of LCAT and to clinically investigate LCAT deficiency syndrome, and it can be used for diagnostic purposes related to liver function.

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