papers and notes on methodology

Quantitation of plasma apolipoprotein A-I using two monoclonal antibodies in an enzyme-linked immunosorbent assay

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Abstract A rapid sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitation of human apolipoprotein (apo) A-I was developed. The assay uses a pair of noncompeting purified monoclonal antibodies to detect apoA-I in plasma. The antibodies used in this assay were selected because they bind >90% of radioiodinated high density lipoprotein (HDL), they identify "fresh" nondeamidated epitopes on apoA-I, and they have comparable binding affinities for isolated HDL and HDL in plasma. The assay was standardized with a plasma secondary standard composed of lyophilized human serum. The assay was used to measure the apoA-I levels in normal subjects, patients with coronary artery disease, and patients with familial hypercholesterolemia. The results indicate that certain monoclonal antibodies can be used to reliably measure plasma levels of apoA-I in diverse groups of subjects. — **Hogle, D. M., R. S. Smith, and L. K. Curtiss.** Quantitation of plasma apolipoprotein A-I using two monoclonal antibodies in an enzyme-linked immunosorbent assay. *J. Lipid Res.* 1988. **29:** 1221–1229.

Supplementary key words apoA-I • HDL

Recent studies have established the association between elevated cholesterol levels and increased risk for premature atherosclerosis (1–5). Epidemiologic studies have implied that elevated levels of plasma cholesterol specifically associated with low density lipoproteins (LDL) are a risk factor for atherosclerosis. Furthermore, the prevalence of coronary artery disease (CAD) was shown to be inversely correlated with elevated levels of plasma high density lipoprotein (HDL) (3, 6). More recently, measurements of the predominant protein constituents of HDL and LDL, apoprotein (apo) A-I and apoB, respectively, were shown to provide additional information for discriminating those at risk for ischemic heart disease (7–11).

A number of immunoassay methods are used for the determination of apoA-I in plasma. These include radioimmunoassay (RIA) (12, 13), radial immunodiffusion (RID) (14), enzyme immunosorbent assay (ELISA) (15, 16), and nephelometric procedures (17). However, problems associated with reproducibility, assay matrix, and antibody specificity have hindered widespread acceptance of these procedures (18). Moreover, quantitation of apoA-I using monoclonal antibodies has been complicated by reports of heterogeneity of HDL and apoA-I species (19), and by the observation that the immunoreactivity of apoA-I can be altered by chemical modifications or by storage (20–22). Previously, we characterized the binding of three apoA-I-specific monoclonal antibodies (19). These antibodies bind both native HDL and isolated apoA-I, but no single monoclonal antibody can bind and identify 100% of HDL. These antibodies were generated with isolated and stored HDL as the immunogen, and in subsequent studies were shown to have enhanced binding for alkaline-treated and/or deamidated HDL. Therefore, a second set of antibodies was generated with fresh HDL as the immunogen (22). Some of these antibodies bound fresh HDL well and alkaline-treated HDL poorly, indicating that some epitopes on apoA-I are sensitive to the protein deamidation that can occur with alkaline treatment.

Abbreviations: CAD, coronary artery disease; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; apo, apolipoprotein; RIA, radio immunoassay; RID, radial immunodiffusion; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; d, density in g/ml relative to water.

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Two of these second generation antibodies, AI-10 and AI-11, bound greater than 90% of radioiodinated HDL in a fluid phase RIA (22).

Here we report the development and characterization of a solid phase enzyme-linked immunoassay for the measurement of apoA-I in human plasma utilizing these monoclonal antibodies. The ELISA utilizes the apoA-I-specific monoclonal antibodies, AI-10 and AI-11 (22), in a "sandwich" format. The plasma samples do not require pretreatment with lipid solubilizing agents to expose apoA-I antigenic sites, and the total reaction time is 1 hr at room temperature. Panels of plasma samples from normal subjects, patients with CAD, and patients with heterozygous familial hypercholesterolemia were quantitated and compared to the amounts of apoA-I measured with commercially available RID and RIA assays. The results indicate that the ELISA accurately measures apoA-I levels in these specimens.

MATERIALS AND METHODS

Isolation and characterization of lipoproteins and apolipoproteins

The lipoprotein fractions: VLDL (d < 1.006 g/ml), IDL (d 1.006–1.019 g/ml), LDL (d 1.025–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated by standard ultracentrifugation techniques from pooled, fresh normal plasma that contained protease inhibitors and antioxidants as described (23). After separation from plasma, the HDL fraction was subjected to a clean-up spin at d 1.063 g/ml to remove residual "heavy" LDL. All lipoproteins were dialyzed extensively against a lipoprotein buffer containing 150 mmol NaCl, 300 mmol EDTA, and 0.005 ml of α-tocopherol per liter, pH 7.4. The lipoproteins were filter-sterilized and stored at 4°C. "Fresh" HDL refers to HDL that was used within a week of isolation.

The apoprotein composition of the isolated lipoproteins was analyzed by polyacrylamide (75–200 g/l acrylamide gradient) slab gel electrophoresis in the presence of 10 g/l of SDS as described (19). The apoA-I isoforms were separated by isoelectric focusing in polyacrylamide (60 g/l acrylamide) slab gels containing 8 mol urea and 20 ml ampholine (pH 4 to 6) per liter as described (19, 22). Lipoproteins were delipidated by boiling for 3 min in 10 g/l SDS before electrophoresis, and the gels were stained after electrophoresis with either Coomassie Brilliant Blue (1 g/l) R250 or silver nitrate. Gels containing radioiodinated lipoproteins were visualized by autoradiography as described (19).

ApoA-I was isolated from ether/ethanol-delipidated HDL by high performance liquid chromatography with G300SW TSK gel permeation columns as described by Kinoshita et al. (24). Fractions containing apoA-I were pooled and dialyzed against 1 mM ammonium bicarbonate and stored at −70°C. Purity of this apoA-I was verified by SDS-polyacrylamide gel electrophoresis and silver staining. Purified apoA-I and apoA-II also were purchased from Chemicon International, Inc. (El Segundo, CA). Protein content of all lipoprotein and apolipoprotein fractions was determined by a modified Lowry procedure (25) with bovine serum albumin (BSA) as a standard.

Preparation and characterization of the monoclonal antibodies

Monoclonal antibodies AI-10 and AI-11 were obtained from two separate fusions of spleen cells from BALB/c mice immunized as described (19, 22) with either fresh glutaraldehyde-fixed HDL (AI-10) or fresh native HDL (AI-11) using previously published protocols (19, 23). The glutaraldehyde-cross-linked HDL was prepared by exposing fresh HDL in phosphate-buffered saline at 20°C for 18 hr to glutaraldehyde at a final concentration of 0.04% (22). Culture supernatants were screened for their capacity to bind both immobilized HDL in a solid-phase RIA and radiolabeled HDL in a fluid-phase RIA as previously described (19). Positive hybridomas were cloned by limiting dilution. Ascites fluids containing the antibodies were obtained from BALB/c mice, which had been primed with 0.3 ml of mineral oil and injected intraperitoneally with 5×10^5 cloned hybridoma cells.

IgG subclasses of the ascites fluids were determined by a commercial RID kit (Meloy Laboratories, Inc., Springfield, VA). Antibody AI-10 is an $IgG2_a$ and AI-11 is an IgG_1 subclass. The monoclonal antibodies were purified from ascites fluid by passage through a Mono-Q column (Pharmacia, Piscataway, NJ) on a Fast, High Performance Liquid Chromatography (FPLC) System (Pharmacia) with a 0–0.5 mol/l gradient of NaCl in 10 mmol/l Tris buffer, pH 8.0. Purified AI-10 and AI-11 were conjugated to horseradish peroxidase (HRPO) as described (26).

Immunoblot analysis of each of the apoA-I antibodies was performed as described previously (19, 22). Electrophoretically separated lipoproteins were transferred to nitrocellulose for reaction with dilutions of ascites fluids specific for apoA-I. Monoclonal antibody binding was detected by a second incubation with radioiodinated goat anti-mouse IgG followed by autoradiography. Antibody competition experiments (27) were performed to determine whether antibodies AI-10 and AI-11 recognized identical or unrelated epitopes on HDL and apoA-I. Twenty ng of purified AI-10 or AI-11 conjugated to HRPO were incubated with 0–400 ng of unlabeled AI-10 or AI-11 for 30 min at 25°C in microtiter wells coated with 5 μg/ml of purified HDL or apoA-I. After washing the plates, *o*-phenylenediamine substrate was added and the assay was completed as described below.

The affinities of the antibodies for isolated HDL and for plasma HDL were compared in competitive solid-phase radioimmunoassays as described (19). The slopes of the logit transformed lines were obtained by linear regression analysis and were subjected to tests of equality as described (28).

Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter plates (Nunc-Immuno Plate I; Irvine Scientific, Santa Ana, CA) were coated for 16 hr at 4°C with 0.15 ml of a 0.1 mol/l sodium bicarbonate buffer, pH 9.0, containing 5.0 μg of purified AI-10 antibody per ml. The plates were washed three times with 0.2-ml volumes of phosphate-buffered saline (PBS), pH 7.3, containing 0.136 mol of NaCl, 1.46 mmol of KH_2PO_4, 8.17 mmol of Na_2HPO_4, and 2.68 mmol of KCl per liter, that also contained 10 g/l of BSA and 5 ml/l of Tween 20. Residual binding sites on the 96-well plates were blocked by a 1-hr incubation at 37°C with 0.2 ml of PBS that contained 100 g/l of BSA and 0.02 ml/l of aprotinin. The wells were washed three times with washing buffer, and the plates were stored at 4°C in an airtight moist chamber for up to 30 days.

A standard curve was prepared for each plate using either HDL or purified apoA-I as a primary standard and a lyophilized plasma or serum pool as a secondary standard. The secondary standard was prepared as pooled plasma from 20 normal subjects. The pool contained 0.1 g/l sodium azide and was sterile-filtered. Aliquots of 0.5 ml were lyophilized in vials, sealed, and stored at 4°C for up to 12 months. The apoA-I value of the secondary standard was determined by 20 repetitive assays in the sandwich ELISA using purified apoA-I as a primary standard. The primary standards were diluted in PBS to concentrations ranging from 1.0 to 0.031 μg/ml.

In each assay, five commercially available controls were run in triplicate: *a*) Calbiochem Apolipoprotein A, Human, Standard Serum (no. 178447; Behring Diagnostics, La Jolla, Ca); *b*) Omega Lipid Fraction Control Serum (no. 46106001A; Cooper Biomedical Diagnostic Division, Freehold, NJ); *c*) Kontroll-Plasma (human) for Partigen Immunodiffusion Plates (no. 512804; Behring Diagnostics, La Jolla, CA), *d*) Isolab Lipotype Control (no. 407020; Isolab Inc., Akron, OH), *e*) Lipotrol (no. 5069; Helena Laboratories, Beaumont, TX). Each control was prepared according to the manufacturer's instructions.

Plasma or serum samples were diluted 5000-fold in PBS. A volume of 0.05 ml of standards, controls, and unknowns was added to the wells in triplicate, immediately followed by 0.05 ml of PBS containing a fixed concentration of antibody AI-11-HRPO conjugate. All plates were set up within 4–5 min. The plates were incubated for exactly 30 min at 25°C, then washed three times. A volume (0.1 ml) of freshly prepared substrate (distilled water containing 30 ml of 30% H_2O_2 and 0.67 g of *o*-phenylenediamine per liter) was added to each well and color was allowed to develop at 25°C for 30 min. The reaction was stopped by the addition of 0.05 ml of 4 mmol/l H_2SO_4 and the absorbance of each well was determined at 490 nm using a 96-well plate reader (MR600; Dynatech, Alexandria, VA).

Plasma collection and characterization

Matched plasma or serum samples were obtained from 20 patients (39–73 years of age) with coronary artery disease (CAD) as diagnosed in the cardiac catheterization laboratory at the San Diego VA Hospital and from 20 patients with heterozygous familial hypercholesterolemia (FH) from a clinic of the University of California, San Diego. Protocols to obtain these samples were approved by the Human Use Committee of the University of California, San Diego. Blood was collected as well from 20 fasting subjects with normal cholesterol and triglyceride levels. Samples were collected into tubes containing EDTA (1.5 mg/ml of blood) and promptly separated by centrifugation at 1500 *g* at 4°C. Total cholesterol and triglycerides were measured in fresh plasma samples on an ABA-200 bichromatic analyzer (Abbott Laboratories, Irving, TX), with use of High Performance Cholesterol Reagent (no. 236691; Boehringer-Mannheim Diagnostics, Indianapolis, IN) and Triglycerides A-gent (no 6097; Abbott Laboratories). LDL and HDL cholesterol were measured using the Lipid Research Clinic procedures (29). All plasmas were aliquoted and stored frozen at −70°C. Plasma apoA-I concentration was determined by use of two commercially available RID kits: "M-Partigen" (Calbiochem-Behring, La Jolla, CA) RID #1 and "Diffugen" (Tago, Ins., Burlingame, CA) RID #2. A radioimmunoassay kit available from Iso-Tex Diagnostics (Friendswood, TX) was also used. Each of the test kits was performed according to the enclosed instructions using the standards supplied by the manufacturer.

To test the effects of plasma storage on the measurements of apoA-I, 0.5-ml aliquots of individual plasmas from five normolipidemic subjects were obtained as described above, placed into airtight polypropylene tubes, and stored at 4°C, −20°C, and −70°C. At approximately weekly intervals the apoA-I in the samples was quantitated.

RESULTS

Characterization of the monoclonal antibodies

In this assay, immobilized monoclonal antibody AI-10 was used as the capture antibody and soluble antibody AI-11 conjugated with HRPO was used as the probe. These antibodies were selected from a panel of 31 hybri-

domas for their ability to bind to immobilized HDL and apoA-I in a solid-phase RIA. Specificity of AI-10 and AI-11 for apoA-I was demonstrated by protein staining and immunoblotting of the electrophoretically separated lipoproteins (22). In a fluid-phase RIA, these antibodies bound 92.6% and 100% of freshly iodinated HDL, respectively (22). Furthermore, it was shown previously that

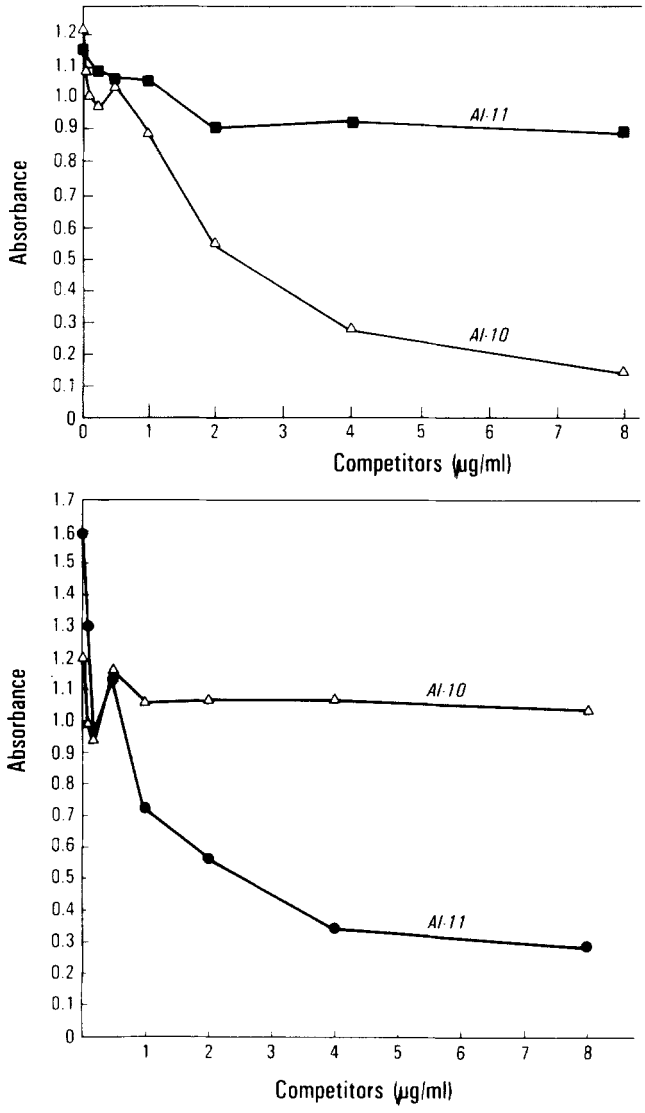

Fig. 1. Antibodies AI-10 and AI-11 bind separate epitopes on apoA-I. Polyvinyl chloride, microtiter plates were coated with purified HDL (5 μg/ml in PBS) for 18 hr at 4°C. The plates were washed three times and blocked with BSA in PBS (30 g/l) for 1 hr at 25°C. The plates were again washed and 0.05 ml containing 20 ng of HRPO-labeled AI-10 (top) or 35 ng of HRPO-labeled AI-11 (bottom) was added to each well in the presence of 0.05 ml of unlabeled purified AI-10 or AI-11 antibody. The plates were incubated for 30 min at 25°C, washed three times, and 0.05 ml of substrate was added to all wells. Plates were incubated for an additional 30 min at 25°C, and the color development was stopped by the addition of 0.05 ml of 4 N H_2SO_4. The absorbance was measured at 490 nm.

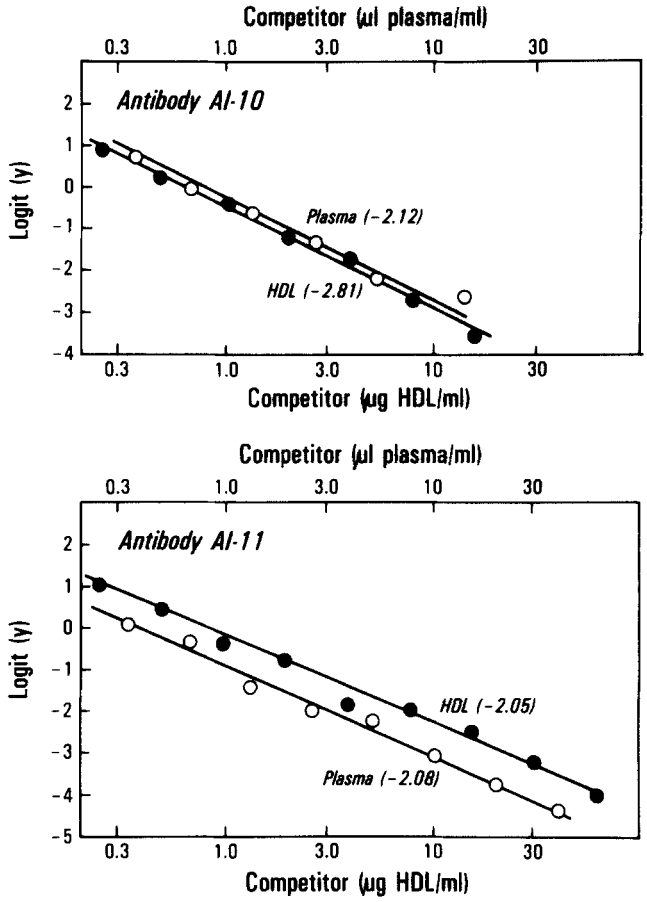

Fig. 2. Antibodies AI-10 and AI-11 bind isolated HDL and plasma HDL with the same affinity. The affinities of the antibodies for isolated HDL and HDL in plasma were compared in a solid-phase RIA employing HDL-coated plates. The capacity of plasma and HDL to compete for a limiting amount of monoclonal antibody was measured as described in Methods. The slopes of the logit transformed lines (shown in parentheses) were obtained by linear regression analysis and subjected to tests of equality. No differences between the slopes for HDL or plasma were observed for either antibody at $P > 0.1$ using the two-tailed Student's t test.

antibodies AI-10 and AI-11 bound separate and distinct CNBr fragments of apoA-I (22).

Verification that these antibodies identified separate and distinct epitopes on HDL was obtained in antibody competition assays. A 20-fold excess of purified antibody AI-11 did not compete with a limiting amount of HRPO-labeled AI-10 for binding to immobilized HDL-coated wells (**Fig. 1,** top). Similarly, a 12-fold excess of unlabeled antibody AI-10 did not compete with peroxidase-labeled AI-11 for binding to HDL (Fig. 1, bottom). Similar results were obtained with apoA-I-coated plates (data not shown).

To establish that both antibodies identified isolated HDL and HDL in plasma with the same apparent affinity, HDL and plasma were added as competitors to a solid-phase RIA. Plates were coated with HDL and increasing amounts of homologous HDL and plasma were added in

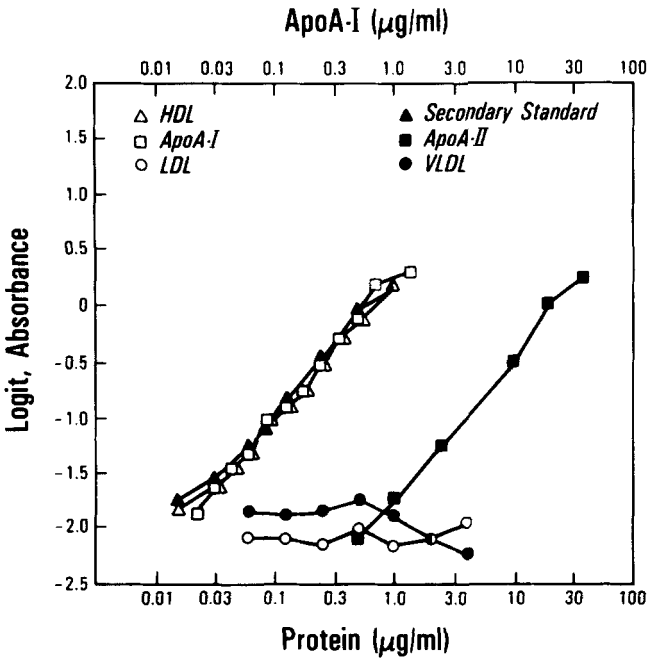

Fig. 3. Detection with the ELISA of apoA-I in apoproteins, lipoproteins, and plasma. AI-10-coated polystyrene microtiter plates were prepared as described in Methods. Increasing amounts (0.05 ml) of HDL, apoA-I, apoA-II, LDL, VLDL, and the lyophilized secondary plasma standard were assayed as described with HRPO-conjugated AI-11 antibody. The apoA-I and apoA-II used in this assay were purchased from Chemicon. The concentration of apoA-I in the secondary standard (μg/ml) and in HDL was determined by separate immunoassay. The protein concentration of the other competitors was determined by a modification of the Lowry procedure (25). Isolated HDL, apoA-I, and the secondary standard exhibited essentially the same slope as assessed by tests for equality of the slopes (28).

the presence of limiting amounts of antibody. No differences in affinity of either antibody for plasma or isolated HDL were observed, as evidenced by the fact that there were no differences in the slopes of the logit transformed regression lines of either competitor (**Fig. 2**). Similar analyses performed on four different occasions with four different sources of HDL and plasma gave similar results.

Validation of the ELISA

First, the abilities of antibodies AI-10 and AI-11 to react with isolated lipoproteins, apoproteins, and human plasma in the sandwich ELISA were compared. Increasing concentrations of isolated VLDL, LDL, HDL, apoA-I, apoA-II, and human plasma (secondary standard) were added to the capture assay (**Fig. 3**). Results of binding studies observed with the secondary standard and with HDL were plotted on the basis of their apparent content of apoA-I assessed by separate immunoassay. All others were plotted on the basis of total protein added. As determined by slope analysis, HDL, apoA-I, and the secondary standard were recognized with similar affinities in this sandwich ELISA format. In this assay the apoA-II was also recognized. However, a 40-fold increase in apoA-II protein was required to achieve binding similar to that of the apoA-I. This observation could be accounted for by the presence of small amounts of apoA-I in this commercially prepared apoA-II fraction, because no binding of apoA-II was observed by either of these antibodies with Western blot analysis (22). ApoA-I was not detected in the ELISA in isolated VLDL or LDL at protein concentrations of ≤ 3 μg/ml (Fig. 3).

The intra-assay coefficient of variation (cv), which was determined by assaying three different plasma samples at least 23 times in a single assay, ranged from 5.2% to 8.6% (**Table 1**). The interassay cv, which was determined by testing three samples in numerous assays over a 3-month period, ranged from 9.8% to 11.7% (Table 1). To assess the accuracy of the assay, we performed spike and recovery experiments in which known quantities of isolated HDL or apoA-I were added to five separate plasma samples that were then assayed as unknowns. In these experiments the observed recovery of HDL ranged from 97% to 109%, and the observed recovery of apoA-I was 106% to 112% (**Table 2**). The sensitivity of the apoA-I ELISA, which was defined as the smallest concentration that could be distinguished from zero, was 0.13 mg/dl of apoA-I. An assessment of the linearity of the measurements showed that apoA-I concentrations in three separate plasmas could be measured accurately over an eightfold range of plasma dilutions (**Table 3**).

Finally, to assess the effects of sample storage on measurements of apoA-I with the ELISA, aliquots of five normolipidemic samples were stored at 4°, −20°, and −70°C and assayed over a period of 32 weeks. The results demonstrate that samples stored at 4°C had dramatically lower apoA-I values after 8 weeks of storage (**Fig. 4, A**). However, the apoA-I values for the five plasma samples

TABLE 1. Intra- and interassay variability of the apoA-I ELISA

	Intra-assay			Interassay		
	Plasma 1	Plasma 2	Plasma 3	Plasma 4	Plasma 5	Plasma 6
No. of determinations	24	24	23	102	26	38
Mean apoA-I ± SD (mg/dl)	60 ± 5	238 ± 14	274 ± 14	183 ± 10	130 ± 13	236 ± 28
cv (%)	8.6	5.9	5.2	11.6	9.8	11.7

TABLE 2. Accuracy of the apoA-I ELISA. Recovery of HDL or apoA-I added to normal human plasma

	HDL			ApoA-I	
	Plasma 1	Plasma 2	Plasma 3	Plasma 4	Plasma 5
Sample (mg/dl)	82	82	78	78	43
Spike (mg/dl)	50	100	200	10	50
Theoretical (mg/dl)	132	182	278	88	93
Observed (mg/dl)	144	180	270	94	104
Recovery (%)	109	98.9	97.3	106	112

stored at −20°C or −70°C appeared to be stable for up to 32 weeks of storage (Fig. 4, B and C).

Application of the ELISA

To assess the capacity of the ELISA to accurately measure apoA-I over a broad range of plasma apoA-I concentrations, we measured the levels of apoA-I in 23 normal subjects, 24 patients diagnosed as having coronary artery disease, and 22 patients with untreated heterozygous familial hypercholesterolemia. Among the 69 subjects a significant correlation was observed between apoA-I values obtained by the ELISA and the plasma levels of HDL cholesterol (r = 0.7123, P = 0.001) (**Fig. 5**). To compare the accuracy of the ELISA with other estimates of plasma apoA-I, we measured apoA-I in the same samples with three other immunoassays including two separate commercial RID assays and a commercial RIA. Each assay was performed as described by the manufacturer using the standards supplied with each assay. For each separate assay a significant correlation was observed between apoA-I and HDL cholesterol. The correlations were 0.42, 0.64, and 0.54 for RID #1, RID #2, and the RIA, respectively. Thus, the correlation between HDL cholesterol and apoA-I was the greatest with the ELISA. Furthermore, although the correlations of the apoA-I measurements obtained with the ELISA and the other three assays were each significant (P = 0.001), the r values were lower (**Fig. 6**). In fact, systematic biases were observed with each of the assays that could be attributed to differences in assay methodologies, standardization,

TABLE 3. Linearity of apoA-I measurement

	ApoA-I		
Dilution	Plasma 1	Plasma 2	Plasma 3
		mg/dl	
1:2,000	167	171	146
1:4,000	181	206	148
1:8,000	141	173	126
1:16,000	162	206	147
Mean ± SD	163 ± 14	189 ± 17	142 ± 9
cv	8.8%	8.9%	6.4%

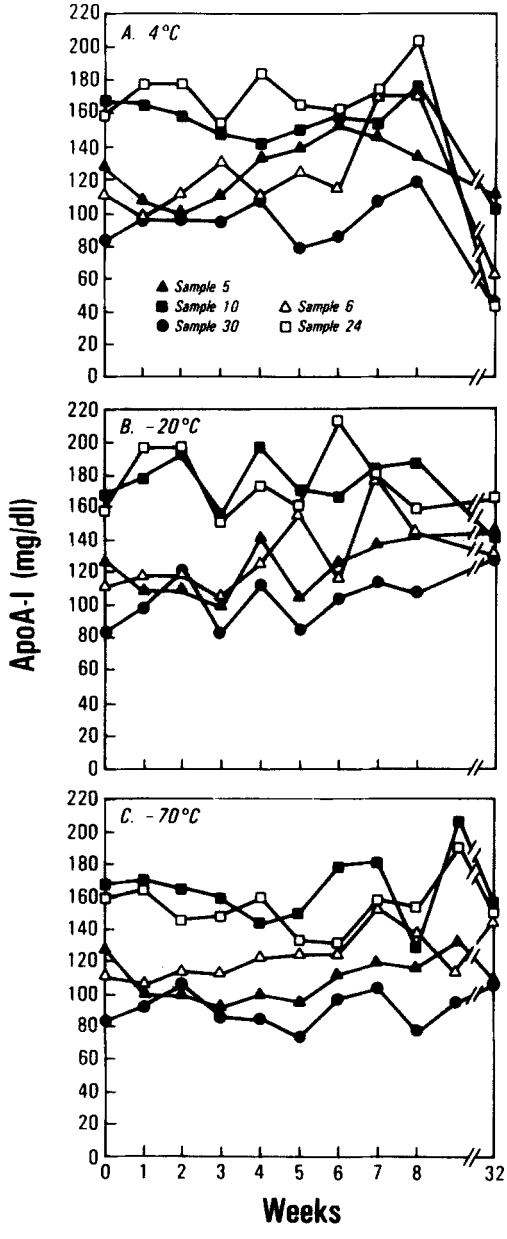

Fig. 4. Effects of sample storage on apoA-I levels in plasma. Five normolipidemic plasmas were filter-sterilized and stored in airtight polypropylene tubes at 4°, −20°, and −70°C. The aliquots were assayed at the time intervals noted on the ordinate in the apoA-I ELISA. The interassay coefficient of variation for these assays ranged from 9.8% to 11.7%.

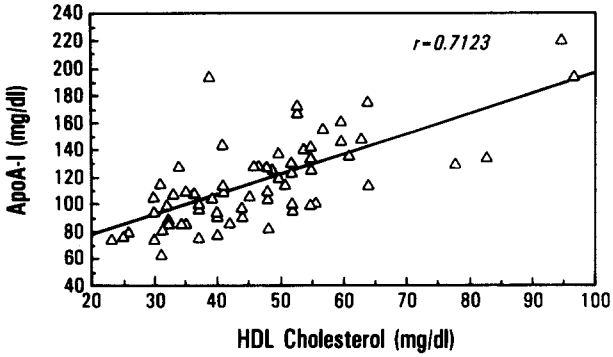

Fig. 5. Correlation between apoA-I of plasma measured with the ELISA and HDL cholesterol. The plasmas of 69 subjects, including 23 normals, 24 patients with CAD, and 22 patients with heterozygous type II FH, were assayed with the apoA-I ELISA. All plasmas were assayed after storage at −70°C.

and the characteristics of the antibodies used. Listed in **Table 4** are apoA-I values determined by each of the assays in each subject group. In normal subjects, the mean apoA-I value measured in the ELISA was 125 ± 28 mg/dl. This apoA-I value is in agreement with published values for apoA-I (18). No significant differences were observed in plasma apoA-I levels from patients with heterozygous familial hypercholesterolemia and normal subjects. However, apoA-I levels in patients with coronary artery disease were significantly lower than those of the normal control group.

DISCUSSION

In this report, we have described a new ELISA to quantitate apoA-I in plasma. This assay is in a format that is identical to our apoB ELISA including the use of a common secondary standard (27). Dilutions of untreated human plasma or serum along with peroxidase-labeled antibody AI-11 are incubated in microtiter wells coated with antibody AI-10. The assay uses phosphate-buffered saline as a diluent and a total incubation time of 1 hr at room temperature. The common methodologies and secondary standards should permit simultaneous measurements of both apoA-I and apoB.

Our earlier attempts to quantitate apolipoprotein A-I by immunochemical means using monoclonal antibodies were hindered by the lack of immunologic reagents that would bind greater than 60% of apoA-I or HDL. This heterogeneity of apoA-I epitopes on purified HDL was documented with our first generation of antibodies specific for apoA-I (19). In these studies, none of the antibodies, separately or in combination, could bind 100% of HDL. Measurement of apoA-I has also been complicated by problems due to protein/protein or protein/lipid interactions that could mask antigenic sites (12). ApoA-I often had to be exposed by detergent- or heat-treatment before it could be accurately quantitated. Recently, it was reported that apoA-I could be deamidated in vitro by storage or by exposure to alkaline solutions (20–22). This deamidation of apoA-I was shown to affect the immunoreactivity of apoA-I when some monoclonal reagents were used (20–22). Therefore, careful choices of the monoclonal antibodies, which are to be used for plasma assays of total apoA-I, must be made. In this report, we

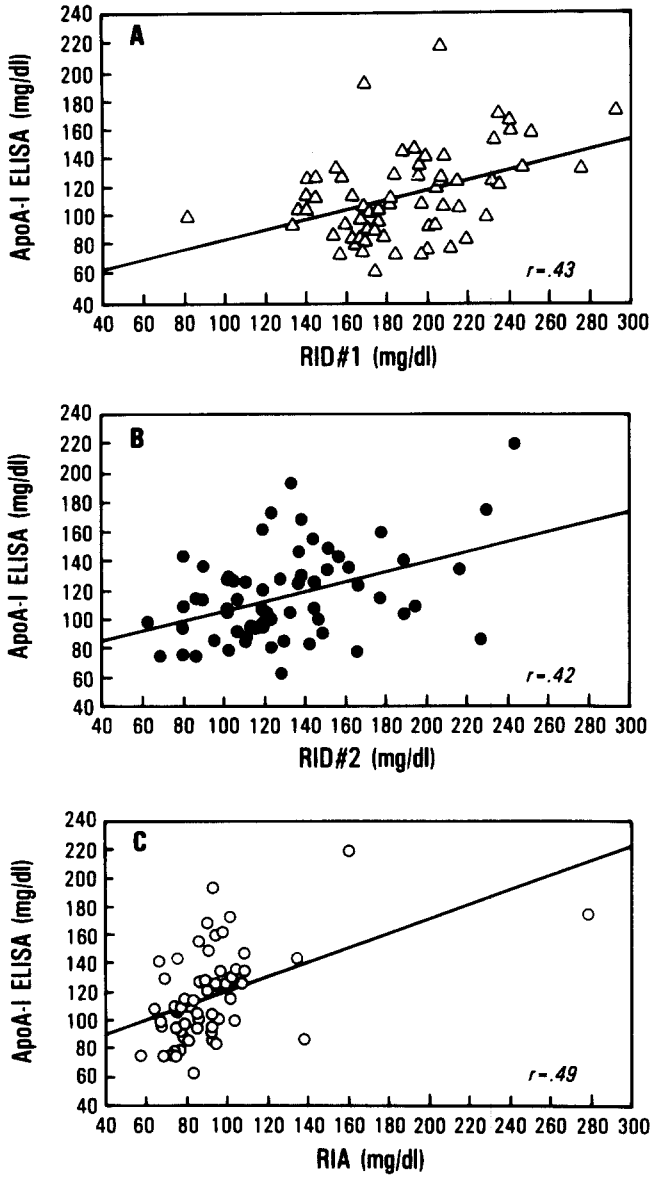

Fig. 6. Correlation of apoA-I measured with the ELISA to apoA-I values measured by three other commercial immunoassays. RID #1 (A) was the Calbiochem-Behring "M-Partigen" assay; RID #2 (B) was the Tago "Diffugen" assay, and the RIA (C) was from Iso-Tex Diagnostics. Each assay was performed exactly as described by the manufacturers using the standards supplied with the kit.

TABLE 4. Summary of apoA-I values for normal, coronary artery disease and familial hypercholesterolemia subjects[a]

	ApoA-I			
	RID #1[b]	RID #2[c]	RIA[d]	ELISA[e]
Normal subjects				
n	21	21	20	23
Mean ± SD (mg/dl)	214 ± 34	144 ± 41	107 ± 42	125 ± 28
Coronary artery disease patients				
n	23	24	24	24
Mean ± SD (mg/dl)	186 ± 26	107 ± 46	75 ± 12	99 ± 23
Familial hypercholesterolemia patients				
n	22	22	22	22
Mean ± SD (mg/dl)	166 ± 33	130 ± 33	94 ± 18	131 ± 36

[a]Differences observed between the normal and CAD patients were significant at $P = 0.01$. No significant differences were observed between the normal subjects and the FH patients.
[b]Behring-Calbiochem Radial Immunodiffusion Assay.
[c]Tago Radial Immunodiffusion Assay.
[d]Isotex Radioimmunoassay.
[e]ApoA-I ELISA.

described the use of a new generation of monoclonal antibodies to apoA-I in a sandwich type ELISA for the quantitation of apoA-I.

The binding characteristics of both antibodies have been reported previously (22). The epitope on apoA-I bound by antibody AI-10, the immobilized capture antibody, is not labile to alkaline treatment. The antibody binds greater than 92% of total HDL in a fluid-phase RIA and recognizes a spectrum of CNBr fragments that includes CNBr fragments 2 and/or 3. The epitope on apoA-I bouind by antibody AI-11, the antibody used as the enzyme-conjugated detecting antibody, is expressed preferentially on fresh HDL. The antibody binds 100% of radiolabeled HDL and recognizes an epitope on CNBr fragment 1 (22). Competition results reported here confirm that these two antibodies recognize distinct epitopes on apoA-I, confirming that they would be ideally suited for use in a sandwich enzyme immunoassay. More importantly it was shown that these antibodies bind both isolated HDL and HDL in plasma with the same apparent affinity. The assay framework used here with AI-10 as the immobilized capture antibody and AI-11 as the labeled probe, consistently yielded optimal assay sensitivity, assay linearity, and accurate and reliable quantitation of apoA-I in plasma.

The need for a stable apoA-I standard, exhibiting reliable reactivity upon long-term storage, is obvious. Purified apoA-I or HDL standards do not fulfill the criteria (18). Therefore, the choice was made to incorporate a pooled plasma secondary standard into the assay. Purified apoA-I was then used to assign an apoA-I value to this secondary standard. In fact, patient sample values obtained in assays calibrated with a secondary standard correlated very well with values obtained in assays calibrated with a primary standard ($r = 0.933$).

This assay procedure is simple and rapid, and offers numerous advantages over previously described apoA-I assays. Either plasma or serum can be used for analysis with no sample pretreatment to expose masked antigenic sites. Also, samples can be assayed after short-term (days) storage at 4°C or long-term storage (weeks) at −20° or −70°C. Total incubation time is only 1 hr at room temperature allowing completion of the assay within 2 hr. Finally, the use of antibodies AI-10 and AI-11, which recognize greater than 90% of iodinated HDL, as the capture and conjugated antibodies, insures the accuracy of apoA-I quantitation.

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