Enzyme-linked immunoassay for Lp[a]

Gunther M. Fless,* Margaret L. Snyder,* and Angelo M. Scanu*,†

Departments of Medicine* and Biochemistry and Molecular Biology,† The Pritzker School of Medicine, The University of Chicago, Chicago, IL 60637

Abstract Based on our findings that rabbit antisera raised against human Lp[a] or apo[a] have the potential to cross-react with plasminogen, and in some cases have nearly equal affinities for plasminogen and Lp[a], we have developed an assay for plasma Lp[a] based on a "sandwich" ELISA that is insensitive to the presence of plasminogen. This was accomplished through the use of anti-apo[a] as a capture antibody and quantitation of the bound Lp[a], i.e., the apoB-100–apo[a] complex, with an anti-apoB antibody. Although apo[a] is heterogeneous in size, all Lp[a] particles tested, either in pure form or contained in whole plasma, gave parallel dose–response curves and were immunologically equivalent. However, when purified Lp[a] particles with different apo[a] isoforms were studied, those having larger isoforms were, on a weight basis, less reactive than those having a smaller size. Nearly equivalent reactivity was observed when protein concentration was expressed on a molar basis. The distribution of Lp[a] in a population of 84 subjects was skewed with one-third of the individuals having less than 1 mg/dl Lp[a] protein. All subjects tested had measurable concentrations of Lp[a] with a lower limit of detection of 0.030 mg/dl Lp[a] protein. The mean level was 3.2 mg/dl with a range of 0.045 to 13.3 mg/dl. These studies demonstrate the successful development of an ELISA for Lp[a] protein that is insensitive to the presence of plasminogen; that heterogeneity of Lp[a] and apo[a] are an important source of variation in the assay; and the need for an appropriate Lp[a] standard in order to minimize this variation.—**Fless, G. M., M. L. Snyder, and A. M. Scanu.** Enzyme-linked immunoassay for Lp[a]. *J. Lipid Res.* 1989. **30:** 651–662.

Supplementary key words lipoprotein[a] • plasminogen • apolipoprotein[a] • ELISA • heterogeneity • cross-reactivity • population study

Lipoprotein[a] (Lp[a]) resembles low density lipoprotein (LDL) in that both contain apolipoprotein B-100 (apoB), have a similar lipid composition, and have a size and density intermediate to that of VLDL and HDL. However, it differs from LDL because the protein component of Lp[a] includes a second protein designated apolipoprotein[a] (apo[a]) that is disulfide-linked to apoB (1–6). The molecular weight of apo[a] is not fixed and has been reported to vary from 280,000 to 700,000 (1, 2, 5, 7). Recent experiments have revealed that apo[a] has a striking homology to plasminogen (8–10). Both have a serine protease, a kringle 5, and a kringle 4 domain; however, in contrast to plasminogen, apo[a] has multiple kringle 4 domains. The sharing of common domains or epitopes by plasminogen and apo[a] is also demonstrated by their cross-reactivity against either anti-apo[a] or anti-plasminogen polyclonal antibodies (8–11). The homology between these proteins is so close that a monoclonal antibody directed to kringle 4 of plasminogen reacted with Lp[a] (8, 11), and, conversely, a monoclonal apo[a] antibody reacted with kringle 4 of plasminogen (12). In light of this cross-reactivity, we developed an enzyme-linked immunoassay for the determination of Lp[a] that is insensitive to the presence of plasminogen. The method involves contacting plasma with anti-apo[a] antibody (attached to the microtiter plate) to form an immobilized Lp[a]–anti-apo[a] complex. Upon the addition of anti-apo B antibody a ternary complex is formed which allows the enzymatic assay of Lp[a] based on the amount of anti-apoB in the complex. Furthermore, we examined the immunoreactivity of Lp[a] particles with different apo[a] isoforms in order to assess their effect on the quantitation of plasma Lp[a].

METHODS

Blood samples

For the population study, samples were obtained from healthy adult male and female volunteers after an overnight (8–10 h) fast. All subjects gave informed consent. Venous blood was drawn into Vacutainer tubes (5 ml) containing 0.05 ml 15% EDTA. The plasma was promptly separated by low speed centrifugation at 4°C, and within 60 min 0.2-ml aliquots were frozen in liquid nitrogen and stored at −70°C until immunochemical analysis. Blood was also obtained from six subjects to study the effect of hypertriglyceridemia on the Lp[a] assay. Two individuals gave blood before and 3 h after the ingestion of a breakfast containing 100 g fat. All of the subjects were studied prior to drug treatment and gave informed consent. They were patients of the Lipid Clinic of the University of Chicago.

Abbreviations: Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; LDL, low density lipoprotein; apoB, apolipoprotein B-100; ELISA, enzyme-linked immunoassay; M_r, molecular weight; K_d, dissociation constant; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

Isolation of Lp[a] and LDL

Blood from healthy male or female donors (500 ml) was drawn into sterile bottles that were immersed in wet ice and that contained, at a final concentration, 0.15% Na_2EDTA and 0.4 mM soybean trypsin inhibitor. The plasma was separated immediately by a low speed centrifugation at 4°C and was made 1 mM with respect to diisopropyl fluorophosphate to minimize proteolysis. Total lipoproteins were obtained by adjusting plasma to d 1.21 g/ml with solid NaBr and centrifugation in a 60 Ti rotor at 59,000 rpm for 20 h at 15°C. LDL and Lp[a] were isolated from the total lipoproteins using a combination of rate zonal and density gradient ultracentrifugations in the 60 Ti rotor as previously described (7). Lp[a] or LDL preparations were checked for purity by SDS-gradient gel electrophoresis (2 to 16% acrylamide gels, Pharmacia, Uppsala, Sweden). When necessary, purification was conducted by FPLC-ion exchange chromatography using a Mono-Q column (Pharmacia, Uppsala, Sweden) (4). Sample load varied from 1 to 10 mg; Lp[a] or LDL were eluted with a 20-min gradient from 0 to 0.5 M NaCl superimposed on 0.01 M Tris buffer, pH 7.4, at a flow rate of 1 ml/min at 8°C. Lp[a] eluted at 0.41 M NaCl and LDL at 0.29 M NaCl. These fractions were checked again for purity by SDS-polyacrylamide gradient gel electrophoresis. The Lp[a] standard had the following chemical composition: 23.6% protein, 21.8% phospholipid, 8% free cholesterol, 37.6% cholesteryl ester, and 9.0% triglyceride. The factor for converting Lp[a] protein to lipoprotein was 4.2.

Fractionation of plasma by single-spin centrifugation

One ml plasma was fractionated by single-spin centrifugation on a step-density gradient as previously described (7). After centrifugation the gradient was separated into 26 tubes. The top 2 tubes were pooled and contained VLDL; tubes 3 through 20 were pooled and contained besides LDL and HDL all of the Lp[a]; the bottom fraction, e.g., tubes 21 through 26, contained lipid-free apoB-100–apo[a] and "free" apo[a].

Isolation of apo[a] from Lp[a]

Apo[a] was isolated from Lp[a] after reduction and carboxymethylation by rate zonal ultracentrifugation as previously described (3).

Isolation of plasminogen

Plasminogen was isolated from the d > 1.21 g/ml bottom fraction that had been dialyzed extensively against 0.1 M phosphate, 0.01% Na_2 EDTA, 0.02% NaN_3, and 1 mM benzamidine, pH 7.4, by lysine-Sepharose chromatography (13, 14). The bottom fraction from 250 ml plasma was diluted to 500 ml with phosphate buffer and applied to a 2.5 × 20 cm column of Sepharose-lysine equilibrated with the same buffer at a flow rate of 30 ml/h at 4°C. The column was washed with the same solvent until the absorbance of the eluate at 280 nm was essentially zero. To elute nonspecifically bound proteins the column was washed with 0.3 M phosphate, 1 mM benzamidine, pH 7.4, at room temperature. This was followed with a 200 ml gradient of 6- amino hexanoic acid (0 to 20 mM) superimposed on 0.1 M phosphate, 0.01% Na_2 EDTA, 0.02% NaN_3, 1 mM benzamidine, pH 7.4. Plasminogen was eluted at 4°C at a flow rate of 30 ml/h and examined for purity by SDS gel electrophoresis before forms 1 and 2 were pooled.

Preparation of antisera to Lp[a], LDL and to apo[a]

Antisera to FPLC-purified preparations of Lp[a] and LDL, and reduced and carboxymethylated apo[a] that were shown to be pure by Western blotting were prepared in the rabbit (Lp[a], apo[a]), and goat (LDL). The animals were injected intramuscularly with an emulsion consisting of 2 parts complete Freund's adjuvant and 1 part antigen. In the case of Lp[a] and LDL, 1 mg protein was injected per animal; in the case of apo[a], 350 μg/animal was injected in the form of a polyacrylamide slurry. Apo[a] was purified before injection by 2–16% gradient gel electrophoresis and the apo[a] band was excised from the gel. After 6 weeks, the animals were injected with a second dose (equivalent to the first dose) which was emulsified with incomplete Freund's adjuvant. The animals were bled 2 weeks later. The anti-Lp[a] serum was purified by passage over LDL-Sepharose, HDL-Sepharose, and albumin-Sepharose. The goat anti-human LDL was precipitated with saturated ammonium sulfate (33% final) to prepare a crude IgG fraction. All antisera and IgG fractions were stored at −70°C.

Affinity-purified rabbit anti-human apo[a] IgG was prepared by passing the antiserum over LDL- and Lp[a]-Sepharose columns attached in tandem. The LDL-sepharose column was included in order to remove any contaminating antibodies to apoB. Both columns were equilibrated with phosphate (10 mM)-buffered saline, pH 7.4, containing 0.01% Na_2EDTA and 0.02% NaN_3. To remove nonspecifically bound proteins, the Lp[a]–Sepharose column was first washed with 0.5 M NaCl, 0.1 M $NaHCO_3$, followed by 0.15 M NaCl, pH 7.4, before the anti-human apo[a] IgG was eluted from the Lp[a]–Sepharose column with 0.01 M glycine/HCl, pH 2.5. The eluted IgG was then titrated immediately to pH 7.4 with a predetermined amount of 1 M Tris and dialyzed against 0.15 M NaCl, pH 7.4, containing 0.01% Na_2EDTA and NaN_3, and frozen in small aliquots at −70°C.

Comparison of reactivity of Lp[a] and plasminogen to anti-Lp[a] and anti-apo[a] sera

To quantitate plasminogen cross-reactivity to anti-Lp[a] and anti-apo[a] sera, plastic microtiter plates (Costar, #3590 flat-bottom 96-well, Cambridge, MA) were coated with equivalent concentrations (4 μg/100 μl) of Lp[a] protein and plasminogen for 1.5 h at 37°C. Unbound antigen

was then removed and the wells were washed 3 times and then blocked for 1 h at 37°C with 10 mM Tris, 0.15 M NaCl, 1% BSA, pH 7.4. Dilutions of antiserum (100 μl) ranging from 100- to 100,000-fold were added to the wells and allowed to incubate 1 h at 37°C. This was followed with 100 μl of goat anti-rabbit IgG labeled with horseradish peroxidase (diluted 1:1000 with blocking buffer containing 0.1% Tween-20). The plate was washed again and color was developed with 1 mg/ml *o*-phenylene diamine for 10 min in the dark. The reaction was stopped with 75 μl 2 M H_2SO_4 and the plate was read at 490 nm.

To obtain some measure of the actual amounts of Lp[a] and plasminogen that were bound to the plates under these conditions, the two particles were iodinated according to the iodine monochloride procedure (15), and coated to the plate as shown above. After washing and counting we calculated that 12% more ^{125}I-labeled plasminogen than ^{125}I-labeled Lp[a] was bound to the plate.

Double immunodiffusion was carried out in 1% agarose (Sigma, Type VII) in 0.06 M barbital buffer, pH 8.6. Antigens were loaded at a concentration of 0.2 mg/ml protein into outside wells (4 μl per well). The antisera were applied twice, 4 μl each, into the central well. The plates were incubated in a humid chamber at room temperature for 24 h. The gels were then washed extensively with saline, dried, and stained with 0.1% Coomassie Blue.

When grading plasma samples for Lp[a] positivity, 4 μl plasma was added to the outside wells, and 4 μl antisera #6 was applied twice to the central well. The plates were developed and stained as outlined above. Depending on the intensity of the precipitin band, they were graded visually as negative, slightly positive, positive, double positive, and triple positive.

ELISA procedure for Lp[a]

Polystyrene microtiter plates (flat-bottom 96-well EIA plates, catalog no. 3590 Costar, Cambridge, MA) were coated with 100 μl of affinity-purified rabbit anti-human apo[a]-antibody, (2 μg/ml) in 0.01 M Tris, 0.15 M NaCl, pH 7.6. The plates were sealed with a thin adhesive-coated plastic sheet and incubated overnight at room temperature. The next day the unbound antibodies were removed by washing the plates 3 times, 5 min/wash, with Tris–saline containing 1% BSA. The remaining binding sites in the wells were blocked by incubating the plates for 2 h with 200 μl/well Tris–saline–1% BSA. After blocking, the plates were dried by blotting, sealed, and stored at 4°C until use (within 12 days). After equilibrating plates to room temperature (30 min), 100 μl of each dilution of the standard and samples, made in 0.1 M $NaHCO_3$, 0.5 M NaCl containing 1% BSA and 0.1% Tween-20, pH 8.1, were added to the wells. The Lp[a] standard usually ranged from 1 ng to 1 μg per well; the plasma samples were diluted from 1:10 to 1:25000. The microtiter plates were incubated for 2 h at 37°C, sitting on 6-mm-thick preheated aluminum plates, cut to the exact size of the bottom of the microtiter plates. The aluminum plates serve to conduct heat uniformly to all 96 wells of the microtiter plate. The microtiter plates were washed 3 times, 5 min/wash, with sodium bicarbonate buffer then incubated for 1 h at 37°C (on the aluminum plates) with 100 μl per well of goat anti-human apoB antibody. After washing, 100 μl per well of rabbit anti-goat alkaline phosphatase antibody conjugate (Pel-Freez Biologicals, Rogers, AR) (diluted 1:3500 in sodium bicarbonate buffer) was added and the plates were incubated at 37°C for 1 h on the aluminum plates. After washing, 100 μl of the substrate (1 mg/ml *p*-nitrophenyl phosphate, (Sigma, St. Louis, MO) in diethanolamine buffer containing 0.01% $MgCl_2$) was added to each well. The plate was put into a dark chamber and the enzyme reaction was allowed to proceed for 30 min at room temperature. After completion, the reaction was stopped by adding 100 μl of 1 N NaOH to each well, and the absorbance was read at 410 nm using a Dynatech MR 600 microplate reader.

Each plate contained five dilutions of the Lp[a] standard in triplicate; one dilution (1 μg/well) to obtain the absorbance maximum and four dilutions (31 to 3.9 ng/well) to establish the straight line portion of the sigmoidal dose–response curve. A high Lp[a] and a low Lp[a] plasma sample (one dilution each) in addition to an Lp[a] free dilution were included with each plate as controls. Each plasma sample was added to the plate in three different dilutions in triplicate. To establish the proper dilution, each plasma was graded for Lp[a] positivity by double-diffusion before analysis by ELISA. Those grading negative or slightly positive were diluted 10- to 40-fold; positive samples were diluted 25- to 100-fold; double positive samples were diluted 200- to 800-fold; and triple positive samples were diluted 800- to 3200-fold. Because of edge effects due to uneven heating and exposure to light, the outside wells of the plate were not used. They were, however, filled with water to aid in even heat distribution.

Electrophoretic methods

Gradient gel electrophoresis in the presence of SDS on 2–16% polyacrylamide gels (Pharmacia) was carried out according to the methods outlined by Pharmacia and described by us previously (7). Sample load when gels were blotted was equivalent to 1 μg Lp[a] protein per lane. Electrophoretic transfer of proteins to nitrocellulose (0.45 μm membrane, Millipore) was done at 50 v overnight, with cooling. Antigens were identified with a double antibody technique involving IgG coupled to horseradish peroxidase using 4-chloronaphthol as the chromogen. Gels of samples containing multiple apo[a] isoforms and stained with Coomassie Blue were scanned directly with an LKB ultroscan XL laser densitometer (LKB, Bromma, Sweden). In the case of transfers to nitrocellulose, the patterns were photographed and the negative was scanned with the densitometer.

Chemical analyses

Protein content was determined by the method of Lowry et al. (16) as modified by Markwell et al. (17) using bovine serum albumin as standard. Phospholipid (18), cholesterol, both free and esterified (19, 20), and triglyceride (21) analyses were also performed as previously described (7).

Calculations

The sigmoidal dose-response curves were transformed using the logit function as outlined by Tijssen (22) and analyzed by least square regression using the Statview program (Brain Power, Inc., Calbasa, CA). Affinity of anti-apo[a] towards Lp[a] standards with different isoforms was calculated as outlined by Khalil et al. (23). For comparative purposes, the absorbance maxima of the different response curves were normalized to 100%. The dose-response curves were then analyzed for their dissociation constants using a nonlinear least square fit program and employing the damping Gauss-Newton method (24). Within and between assay variance was calculated as described by Rodbard (25). Determination of statistical significance was carried out by Student's t test.

RESULTS

Immunological cross-reactivity of Lp[a] and plasminogen

To quantitate plasminogen cross-reactivity with anti-Lp[a] and anti-apo[a] sera, plastic microtiter plates were coated with equivalent concentrations of plasminogen and Lp[a] protein and the reactivity against seven different antisera was determined in an enzyme-linked immunoassay (**Table 1**). The seven different antisera used were all raised in the rabbit. Three each were raised against human Lp[a] and apo[a]; one was a commercial Lp[a] antiserum (Calbiochem, San Diego, CA; Cat. No. 425827, lot no. 607681). All four antisera directed against Lp[a] and one specific to apo[a] had a significant affinity for plasminogen which was only 0.7- to 8-fold less than that for Lp[a] (**Fig. 1**). Two antisera specific to apo[a] (#7 and #8) had essentially no or very weak affinity for plasminogen (Fig. 1). The affinities were determined by assuming that the plates coated at equivalent protein bound the same quantity of plasminogen or Lp[a] protein. However, the binding of iodinated plasminogen was 12% higher than that of Lp[a]. If the binding of the native particles is similar to that of the iodinated proteins, then the relative affinities of Lp[a] to plasminogen may actually be somewhat higher.

The affinities of the different antisera for Lp[a] or plasminogen were compared at equivalent protein concentrations. Yet the molecular weight of plasminogen is 92,000 and the M_r of the protein moiety of the Lp[a] used in this study was 930,000 not including carbohydrate (7). Therefore, on a molar basis, the affinities of the antisera for plasminogen are considerably less than for Lp[a] (Table 1). The concentration of the two proteins can also be expressed in terms of kringle 4 domains. The Lp[a] standard used in this study has two identical apo[a] subunits, each with a M_r of 280,000 of which 200,000 is due to protein. Based on the structural information encoded in the cDNA sequence of apo[a] (9), we may infer that each unit contains one protease and kringle 5 domain in addition to 13 kringle 4 domains resulting in 26 kringle 4 domains per Lp[a] particle. Thus on an equal protein basis, Lp[a] would have 2.6 times more kringle 4 domains than plasminogen. Expressed as the numbers of kringle 4 domain, the affinities

TABLE 1. Properties of apo[a]-specific antisera

Antiserum	Antigen	Apo[a] Isoform[a]	Host	Relative Affinity of Lp[a]/Plasminogen			
				Double Diffusion	ELISA[b]		
					Molar	Weight	Kringle-4
1	Lp[a]	F	rabbit	–	7.1	0.71	0.27
2	Lp[a]	F	rabbit	–	26	2.6	1.0
3	Lp[a]	ND	rabbit	+	29	4.9	1.9
4	Lp[a][c]	ND	rabbit	+	82	8.2	3.2
5	Lp[a]	B	goat	+ + +	ND	ND	ND
6	Apo[a]	F	rabbit	–	24	2.4	0.92
7	Apo[a]	F	rabbit	–	NC[d]	NC	NC
8	Apo[a]	F	rabbit	–	NC	NC	NC

[a]F and B stand for apo[a] isoforms with a mobility faster than or equal to apoB; ND, not determined.
[b]Affinity of antisera for Lp[a] as compared to plasminogen. These relative affinities were determined with Lp[a] and plasminogen concentration expressed either on a molar, weight, or kringle 4 concentration.
[c]Commercial anti-Lp[a] (Calbiochem, San Diego, CA; Cat. no. 425827, Lot no. 607681).
[d]NC, no cross-reactivity.

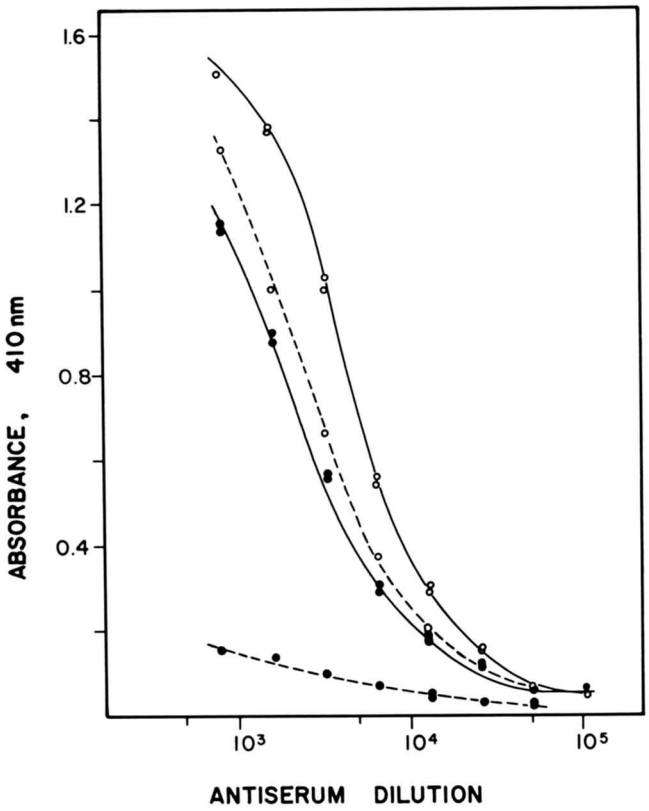

Fig. 1. Differential affinity of Lp[a] (○) and plasminogen (●) to anti-Lp[a] antibodies (solid line) and anti-apo[a] antibodies (dotted line). Lp[a] and plasminogen were coated at equivalent protein concentrations on plastic microtiter plates. After blocking, the plates were exposed to various dilutions of anti-Lp[a] No. 4 or anti-apo[a] No. 7 (see Table 1). The bound antibodies were then determined with the aid of a horseradish peroxidase-coupled antibody specific to rabbit IgG colorimetrically at 490 nm.

of the antisera for plasminogen are therefore only 0.27-3-fold less than for Lp[a] (Table 1).

The same rabbit antisera, in addition to an antiserum specific to Lp[a] but raised in the goat, were also tested for cross-reactivity with plasminogen by double diffusion (Table 1). None of the antisera specific to apo[a] gave visible precipitin lines. Two anti-Lp[a] sera were negative but two other anti-Lp[a] sera rasied in the rabbit (including the Calbiochem anti-Lp[a]) gave weak precipitin lines. The anti-Lp[a] serum raised in the goat gave a strong reaction with plasminogen (**Fig. 2**). These results obtained with different immunological methods demonstrate that antisera to Lp[a] or apo[a] have the potential to cross-react with plasminogen. The use of such antisera could therefore potentially compromise immunoassays for Lp[a] in plasma.

Assay for Lp[a]

The assay was designed in such a way as to be insensitive to the presence of plasminogen. The method employs immobilized rabbit anti-human apo[a] antibody (antiserum #6, Table 1) as the capture antibody and goat anti-human apoB antibody as the second antibody. A third antibody-alkaline phosphatase conjugate is used in the final step to quantitate the bound anti-apoB antibody. A standard curve obtained with purified Lp[a] is shown in **Fig. 3A**. Significant displacement from baseline was obtained with 30 ng/ml Lp[a] protein (open circles). The working range of the assay was between 50 and 500 ng/ml Lp[a] protein. This was also the range in which the assay was linear.

During the development phase of the assay, LDL consistently gave positive results. This reactivity was not due to small amounts of Lp[a] contaminating the LDL preparation. It was subsequently shown that LDL obtained from an Lp[a]-negative subject and then purified by FPLC on a Mono Q (Pharmacia) column still reacted in the assay. When microtiter plates were coated with 100 ng/well non-immune rabbit IgG, both LDL and Lp[a] gave positive results above 0.5 μg/well lipoprotein protein, with LDL reacting 2.5 times stronger than Lp[a]. This nonspecific interaction between rabbit IgG and human LDL or Lp[a] was finally eliminated with the inclusion of 0.5 M NaCl and 0.1 M $NaHCO_3$ in the wash buffer.

Specificity of assay

To test whether plasma components other than Lp[a] might react in the assay, we removed Lp[a] from the plasma of an individual (apoB = 28 mg/dl, apoA-I = 110 mg/dl) by two passages over a column of anti-human apo[a]-IgG-Sepharose 4B. The anti-apo[a] used did not react with plasminogen. During this process, the plasma was diluted 3-fold and was then diluted serially and tested in the assay. As seen in Fig. 3B, this plasma did not react in the assay when diluted 10-fold or more. The slight reactivity with 3-fold diluted Lp[a]-free plasma may have been due to residual amounts of Lp[a] not removed by the affinity column.

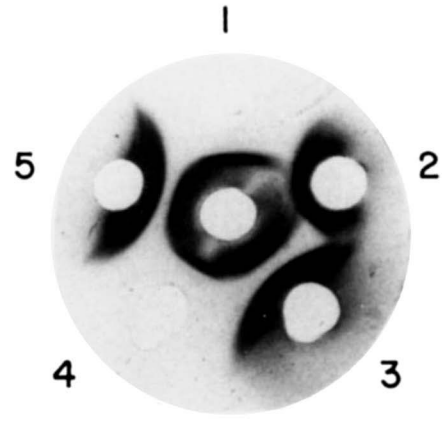

Fig. 2. Cross-reactivity of Lp[a] and plasminogen as demonstrated by double immunodiffusion. Lp[a] and plasminogen were loaded at a concentration of 0.2 mg/ml. Wells 1 and 4, plasminogen; well 2, Lp[a]; wells 3 and 5, Lp[a]-positive plasma; center well, goat anti-human Lp[a] (Antiserum 5, Table 1).

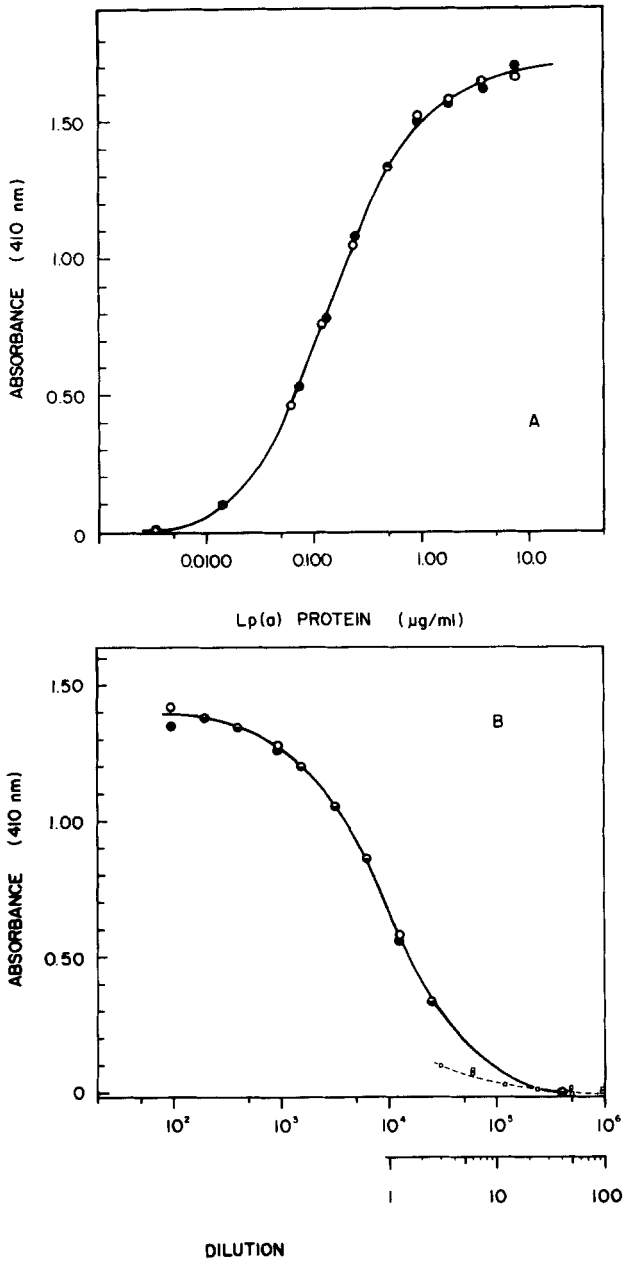

Fig. 3. Validation of enzyme linked immunoassay for Lp[a]. A: Dose-response curve obtained with Lp[a] standard alone (○) and in the presence of an Lp[a]-positive plasma diluted 100-fold (●). B: Dose response curve obtained with Lp[a] standard alone (○) and in the presence of an Lp[a]-free plasma diluted 10-fold (●). The plasma was freed of Lp[a] by passage over an anti-apo[a]-Sepharose affinity column. The anti-apo[a] did not react with plasminogen. The Lp[a] concentration of the 100-fold dilution was 10 μg protein/ml. The dotted curve was obtained with the Lp[a]-free plasma in the absence of added standard Lp[a]. The lower scale on the abcissa refers to the dilution of the Lp[a]-free plasma.

To further verify the assay, purified LDL at final concentrations up to 30 μg protein per well was added to the above and two other low Lp[a] plasma samples. Although the LDL in these 10-fold diluted plasma specimens was approximately three times the normal plasma level, all of them had the same absorbance response as the no-LDL controls. Purified plasminogen at a concentration of 2 μg per well, which represents roughly a 10-fold dilution of plasma, did not react in the assay. As expected, apo[a] was found not to react in the assay at a concentration of 1 μg/well.

Free apo[a] was not expected to contribute significantly to total plasma Lp[a], because previously we were unable to detect Lp[a] by Western blotting in VLDL and bottom fractions (7) that were obtained upon density gradient centrifugation of plasma. Since the ELISA technique is more sensitive than immunoblotting, we determined Lp[a] in the VLDL fraction, the d 1.01 to d 1.21 g/ml fraction, and the bottom fraction of plasma from three normolipidemic individuals by single-spin density gradient ultracentrifugation. The intermediate fraction contained 98.2 ± 0.2 percent of plasma Lp[a] whereas 1.8 ± 0.2 percent of Lp[a] was present in the bottom fraction as apparently lipid-free apoB-100-apo[a] complex. The amount in VLDL was too small to contribute significantly to the total Lp[a]. By Western blotting apparently free apo[a] could be demonstrated in the bottom fraction of one high Lp[a] plasma. If we assume that apo[a] and apoB-100-apo[a] stain similarly after blotting, then laser densitometry of the two bands indicated that apo[a] is only 37% of the apoB-100-apo[a] complex or approximately 0.6% of the total plasma Lp[a] in this sample.

Assay variability

The variability of the assay was assessed over a 3-month period by determining the Lp[a] concentration of a high Lp[a] (9.5 mg/dl) and a low Lp[a] plasma (0.96 mg/ml). The plasma samples were stored at −70°C and were included in triplicate on every plate. During this period, different lots of the substrate and secondary and tertiary antibodies were used; the primary antibody was the same throughout. The intra- and inter-assay coefficient of variation for means of triplicates (n = 33) of the high Lp[a] plasma were 2.9 and 8.1%, respectively; those for the low Lp[a] plasma were 2.7 and 7.6%, respectively.

Validation of assay

The validity of the assay in the presence of plasma was tested by adding different concentrations of exogenous Lp[a] to a 100-fold diluted plasma that contained 1.42 μg/dl endogenous Lp[a] protein. The curve generated from the sum of endogenous and exogenous Lp[a] (closed circles) in the presence of 100-fold diluted plasma superimposed upon that obtained with the Lp[a] standard alone (open circles) (Fig. 3A). A second assay was conducted in which exogenous Lp[a] was added to a 10-fold diluted Lp[a]-free plasma. Again, the curve obtained in the presence of plasma coincided with the one obtained with the Lp[a] standard

(Fig. 3B). These experiments demonstrate that the determination of Lp[a] is unaffected by the plasma constituents and that the assay is valid.

Validation of assay with high triglyceride plasma samples

Two subjects were given a 100 g-fat breakfast and plasma samples were obtained in the fasting state and 3 h postprandially when chylomicrons peaked. At this time, plasma triglycerides had risen from 69 to 272 mg/dl in subject 1 and from 301 to 640 mg/dl in subject 2. Aliquots of plasma from both time points were serially diluted and tested in the ELISA. The data was linearized using a log-logit transformation and the resulting slopes and intercepts of the fasted and postprandial samples were compared. The slopes obtained with the fasting plasma samples (0.355 ± 0.070 and 0.401 ± 0.018) did not differ significantly from that of the post-prandial plasma samples (0.363 ± 0.031 and 0.409 ± 0.024). Similarly, the intercepts that represent the log of the dilution at half-maximal absorbance did not differ. The values for the fasting plasma samples were (−2.911 ± 0.017 and −2.573 ± 0.024) whereas the postprandial samples were (−2.944 ± 0.031 and −2.556 ± 0.030).

In four separate experiments, plasma from subjects with elevated triglyceride levels was used to further test the validity of the assay (see **Table 2**). Different concentrations of exogenous Lp[a] were added to 100-fold diluted plasma samples and the slopes and intercepts of the transformed data were compared to the standard. In every case examined, the slope of the high triglyceride plasma did not differ significantly from that of the standard. Similarly, three of the four plasma samples had intercepts that did not differ significantly from that of the standard. However one sample had a significantly different ($P < 0.001$) intercept indicating that, in the presence of this high triglyceride plasma, 9.4% less Lp[a] protein was needed to get the same response obtained with the standard.

Reactivity of Lp[a] having different apo[a] isoforms

It is now established that apo[a] of different Lp[a] particles can vary in molecular weight from 280,000 to 700,000 as determined by SDS-gel electrophoresis (1, 2, 5, 7). To assess the effect of heterogeneity on the assay, five preparations of Lp[a] having different apo[a] isoforms were prepared and tested in the assay (**Fig. 4**). Two Lp[a] fractions having different apo[a] isoforms were isolated from one donor (KB); one had an isoform with a mobility faster than apoB and was used as the standard. The other preparation was a mixture of two Lp[a] species that could not be resolved and consisted of 75% of an Lp[a] with a slow moving apo[a] isoform, and 25% of an Lp[a] identical to the standard. This was determined by laser densitometry of a 2–16% gradient gel stained with Coomassie Blue and of an immunoblot. Densitometry gave the same ratio with either method. The other three preparations came from different individuals and had apo[a] isoforms with mobilities slightly slower or faster than that of apoB (Fig. 4, **Table 3**).

A logit transformation of the results obtained with each Lp[a] preparation indicated that the slopes did not differ significantly from that of the standard and were parallel (Table 3). However, an examination of their dissociation constants revealed that the K_d of the standard was significantly different from the other Lp[a] species. When their dose–response was compared to the standard Lp[a] at equivalent protein concentrations, the reactivity of the three Lp[a] species with a mobility similar to apoB was 11–12% lower. The reactivity of the Lp[a] species with the slow moving apo[a] isoform was 16% lower than the standard after correction for the contribution caused by the presence of 25% of the fast apo[a] isoform.

In addition to isolated Lp[a], five different plasma samples whose apo[a] isoforms had previously been established by Western blotting were examined for parallelism with respect to the standard Lp[a]. The mobilities of their isoforms were 83–92% slower than that of apoB; however their slopes

TABLE 2. Validation of assay with high triglyceride plasma samples

Plasma	Triglyceride	Total Cholesterol	Lp[a]	Slope	Intercept
		mg/dl			
1	440	203	0.26	0.356 ± 0.038[a]	−4.057 ± 0.055[a]
Standard				0.364 ± 0.025	−4.061 ± 0.029
2	841	278	1.35	0.376 ± 0.038[a]	−3.996 ± 0.047[a]
Standard				0.377 ± 0.035	−3.999 ± 0.046
3	940	439	0.27	0.351 ± 0.035[a]	−3.957 ± 0.028[b]
Standard				0.359 ± 0.021	−3.914 ± 0.023
4	2900	469	2.40	0.330 ± 0.073[a]	−3.846 ± 0.111[a]
Standard				0.358 ± 0.042	−3.886 ± 0.055

[a]Not statistically different from standard.
[b]Statistically different from standard ($P < 0.001$).

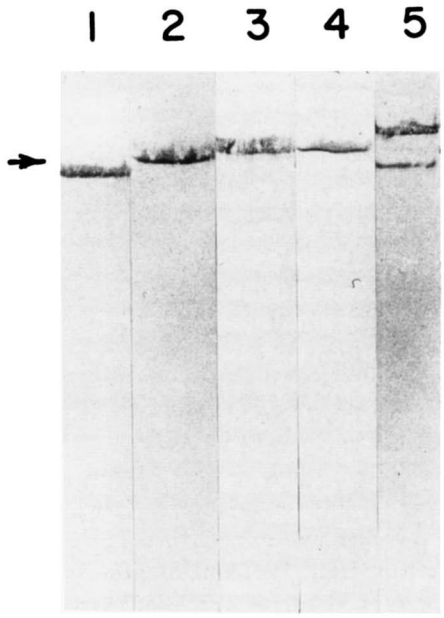

Fig. 4. Western blot of five different Lp[a] preparations showing several different apo[a] isoforms. One μg of Lp[a] was submitted to SDS-PAGE on 2 to 16% gradient gels under reducing conditions. Lp[a] in lane 1 was used as the standard. A second Lp[a] fraction was obtained from the same donor (KB) that contributed the standard Lp[a] and is shown in lane 5. This Lp[a] preparation consisted of a mixture of Lp[a] particles with different isoforms. From laser-densitometry it was estimated to contain 25% Lp[a] with the fast isoform and 75% Lp[a] with the slow isoform (see Methods and Results). Lp[a] preparations from lanes 2, 3, and 4 were obtained from three different individuals: SK, LP, and TT, respectively (see Table 2). The arrow refers to the position of apoB-100.

did not differ statistically from the standard (mean difference = 1.0%; range −7.4% - +3.6%).

Stability of standard and plasma samples at 4°C

The standard Lp[a] was isolated from the same subject at 2-month intervals. The preparation was filter-sterilized and stored in 0.5-ml aliquots in sterile tubes (with minimal air space) at 4°C. Each preparation of new Lp[a] standard was compared to the previous standard to assess the stability of the Lp[a]. A logit transformation of the dilution curves of the old and new standard gave slopes (2.9%) and intercepts (1.6%) that were not significantly different from each other. We also compared a standard that was kept 4 months at 4°C with a fresh standard. In this case there was also no difference in the slopes (0.007%) and intercepts (0.003%).

The effect of storage at 4°C was assessed on two Lp[a]-positive plasmas. The concentration of Lp[a] was determined on the day plasma was obtained and then after 1 week of storage at 4°C. The concentration of the Lp[a] in the fresh samples was 1.66 ± 0.067 and 6.11 ± 0.33 mg/dl, whereas after 1 week the corresponding values were 1.63 ± 0.048 and 6.02 ± 0.33 mg/dl. Thus storage of plasma for 1 week at 4°C did not affect Lp[a] concentration.

We also investigated the effect of refrigeration time on the Lp[a] concentration of two plasma samples that had been freshly frozen and thawed at room temperature and stored at 4°C. Both samples were assayed six times over a 2-week period. One exhibited no change over this time interval, while the second sample was constant for only 4 days before exhibiting a linear decrease to 50% of its original Lp[a] concentration.

Effect of freezing and storage

Initially, freshly obtained plasma samples were frozen in freezers kept at −20°C or −70°C. However, subsequent analyses of these frozen samples gave variable results that were generally higher when compared with the unfrozen specimens. Better results were obtained by freezing plasma more rapidly in liquid nitrogen. In **Table 4** are shown data from an experiment in which two plasma samples were put through both one and three freeze–thaw cycles. The analysis was done on the same day the blood was collected. Values represent the mean of four different dilutions each done

TABLE 3. Dose-response of Lp[a] preparations having different isoforms

	Lp[a] Preparation				
	KB LD-Lp[a] (Standard)	SK	LP	TT	KB[a] HD-Lp[a]
Mobility of apo[a] isoform relative to apoB	1.19	1.05	0.95	0.95	0.64
Slope of logit-log curve	0.313 ± 0.036	0.329 ± 0.033[b]	0.324 ± 0.028[b]	0.302 ± 0.016[b]	0.321 ± 0.029[b]
K_d (μg/ml)	0.117 ± 0.0087	0.133 ± 0.0093[c]	0.133 ± 0.0116[c]	0.131 ± 0.0047[c]	0.134 ± 0.001[d]
Relative dose-response at equivalent protein	100%	88.0%	88.0	89.3	83.8[e]

[a]This Lp[a] preparation was a mixture containing 25% LD-Lp[a] and 75% HD-Lp[a]. LD-Lp[a] stands for Lp[a] with relatively low density and was used as standard. HD-Lp[a] stands for Lp[a] of relatively high density.
[b]Not statistically different when compared to standard Lp[a].
[c]Statistically different ($P < 0.001$) when compared to standard Lp[a].
[d]Statistically different ($P < 0.005$) when compared to standard Lp[a].
[e]Dose-response is for the slow isoform only. This value was calculated by taking into account the contribution to the K_d by the LD-Lp[a] with the fast isoform which encompassed 25% of the total Lp[a].

TABLE 4. Effect of freezing plasma in liquid nitrogen on quantification of Lp[a]

Plasma	Unfrozen	Same Day Frozen Once	Frozen 3 Times	1 Week at −70°C	1 Month at −70°C
1	9.69 ± 0.46	9.88 ± 0.30	9.57 ± 0.17		9.63 ± 0.07
2	4.18 ± 0.02	4.22 ± 0.23	3.99 ± 0.33	4.12 ± 0.22	
3	1.68 ± 0.07			1.76 ± 0.07	
4	0.714 ± 0.016			0.706 ± 0.059	
5	3.05 ± 0.04				3.12 ± 0.14

in triplicate. No difference was found between the fresh and frozen samples.

To examine the effect of storage time and freezing in liquid nitrogen, several plasma samples were analyzed before and after either 1 week or 1 month at −70°C. Again, no statistical differences were found between fresh and frozen samples.

Population study

The plasma of 84 subjects (50 males, 34 females) was analyzed for the Lp[a] content. The population consisted of 78 caucasians, 4 blacks, and 2 orientals. The mean age was 31.5 ± 9.5 years (range 18–62); mean total plasma cholesterol was 191 ± 38 mg/dl (range 130–301); and mean plasma triglyceride was 101 ± 46 mg/dl (range 39–260). A bar graph showing the distribution of Lp[a] protein in the population is shown in **Fig. 5A**. The distribution is skewed with one-third of the individuals having Lp[a] protein levels falling into the smallest interval (0.045 mg/dl to 0.925 mg/dl). The mean Lp[a] protein value was 3.16 ± 3.43 mg/dl; the median was 1.61 mg/dl; and the highest value was 13.25 mg/dl. To better view the distribution of Lp[a] in the population, especially with respect to values below 1 mg/dl, Lp[a] protein was also plotted on a logarithmic scale (Fig. 5B). Presented this way, the distribution is spread out more and no interval holds more than 13% of the population. When Lp[a] protein is converted to Lp[a] lipoprotein by applying the factor 4.2, 15.5% of the population had Lp[a] lipoprotein values greater than 30 mg/dl.

DISCUSSION

These studies were initiated by our original observations that apo[a] is strikingly homologous to plasminogen and that antisera specific to Lp[a] cross-react with plasminogen (8, 11). However the Western blotting technique used gave only a qualitative assessment of cross-reactivity. In the current study this reactivity was quantitated by an ELISA that indicated that four anti-Lp[a] and one of three anti-apo[a] sera had an affinity for plasminogen that was only 0.7- to 8-fold less than that of Lp[a] when expressed on a protein basis. Since the greater part of apo[a] consists of kringle 4 domains (approximately 80% of the protein for the small isoform), it is possible that these structures may be responsible for the cross-reactivity. When the data are expressed in moles of kringle 4 domains, the affinities of the antisera to Lp[a] and plasminogen are nearly equal, suggesting that kringle 4 domains of either particle may contain the antigenic determinants recognized by these antisera. Thus, plasminogen may potentially interfere with the immunological assays of Lp[a].

Interestingly, two out of three anti-apo[a] sera reacted very poorly with plasminogen by ELISA and none by dou-

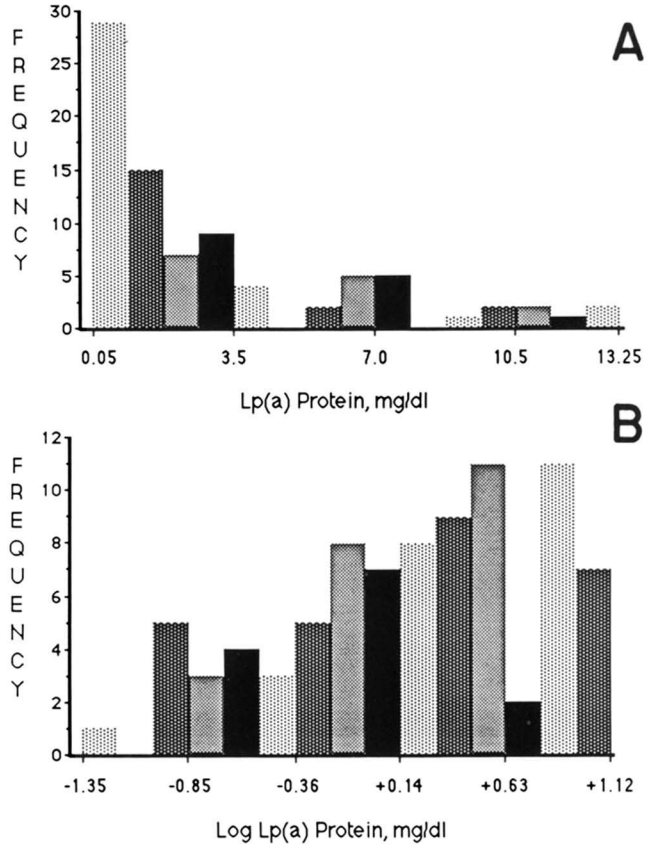

Fig. 5. Bar graph showing the distribution of Lp[a] protein in a population of 84 subjects (50 males, 34 females). A: Number of observations plotted as a function of Lp[a] protein. B: Number of observations plotted against log Lp[a] protein.

ble diffusion. Since the apo[a] used to inject the rabbits was reduced and carboxymethylated, its kringles, which are held together by three disulfide bonds each, were presumably destroyed. This may explain why antibodies produced against this denaturated apoprotein reacted poorly to plasminogen by not recognizing the native structure of kringle 4. The short (36 amino acids) linking regions connecting the many kringle 4 domains of apo[a] are devoid of disulfide bonds and were probably less affected by reduction and carboxymethylation. In contrast to plasminogen, these linkage regions are highly glycosylated (six O-linked sites) in apo[a] (10). Since most of the sequence divergence between apo[a] and plasminogen occurs in these linkage regions (9) this may explain why, in some cases, antibodies produced to reduced and carboxymethylated apo[a] can still recognize Lp[a] but not plasminogen.

Cross-reactive antisera are an important problem in the assay of Lp[a], particularly when one considers that the plasminogen content of plasma ranges from 12 to 25 mg/dl (26), and that the mean plasma levels of the Lp[a] protein as determined in this study are 3.2 mg/dl (range 0.045–13.3 mg/dl). Yet by taking advantage of the fact that the protein moiety of Lp[a] consists of two distinct proteins, apo[a] and apoB, we designed an ELISA for Lp[a] that can make use of anti-Lp[a] antisera that also have affinity for plasminogen. The capture antibody, anti-apo[a], immobilizes any apo[a]-containing molecule and potentially also plasminogen; the inclusion of a second antibody (anti-apoB) in the assay permits the bound Lp[a] to be quantitated based on the amount of apoB in the ternary complex. Because of this design, the assay will detect apo[a] complexed to apoB but not free apo[a]. However, this does not compromise the assay for Lp[a] because the amount of free apo[a] in the bottom fraction of plasma appears to be small. Whether these small amounts represent native or large proteolytically derived fragments of apo[a] remains to be established. Moreover, when different forms of Lp[a] were examined, either purified from or present in different plasma samples, all of them had parallel dose–response curves, indicating that the expression of apo[a] antigenic determinants in these diverse Lp[a] particles is the same. Our results also indicate that Lp[a] may be assayed in plasma samples having as much as 300 mg/dl apoB, and that high triglyceride levels do not affect the slope of the dose–response curves. However, in one out of six cases, somewhat higher Lp[a] values were obtained for reasons that are not understood at the present time.

The selection of an Lp[a] standard having an apo[a] isoform with a mobility faster than apoB was based on physiochemical considerations since this lipoprotein, unlike Lp[a] particles with larger isoforms, is stable and does not undergo cryoprecipitation (7, 27). The validity of this decision was borne out by the fact that the Lp[a] standard did not undergo detectable immunological change when stored under sterile conditions for up to 4 months at 4°C. We also found that there was no loss of immunodetectable Lp[a] when plasma samples were frozen in small aliquots in liquid nitrogen and then stored at −70°C for prolonged time.

Size heterogeneity of apo[a] (1, 2, 5, 7, 28) was anticipated to present problems in the development of the assay. This concern was justified when dose–response curves of purified Lp[a] preparations containing high M_r apo[a] isoforms were displaced from the Lp[a] standard that contained the low M_r isoform. This indicated that, on a protein basis, the affinity of the antibody for the Lp[a] with the larger apo[a] isoforms was about 20% less than for Lp[a] with the smaller apo[a] isoform. However, we found that this difference largely disappears when the data are expressed on a molar basis. This is verified by the results presented in Table 3 relating to subject KB in whom the molecular weights of Lp[a] and Lp[a] protein were determined previously (7). This subject had two types of Lp[a], one of relatively low density with a protein M_r of 930,000 which we have used as the standard and one with a high density with a protein M_r of 1,100,000 which represents a mixture of two Lp[a] particles containing two apo[a] isoforms (25% small and 75% large). From these data, the molecular weight of Lp[a] protein of the high M_r isoform species is calculated to be 1.19×10^6. At equivalent protein concentrations its molarity is therefore only 80% of the standard. This number compares favorably with the experimentally determined value of 83.8% (Table 2) which indicates that by plotting Lp[a] protein on a molar basis, these differences in avidity would largely disappear. However, in practice, the M_r of the apo[a] isoforms of the test plasma is unknown and data are expressed on a weight basis. Since subjects with high Lp[a] concentrations and thus presumably at a high risk for cardiovascular disease tend to have Lp[a] particles with low M_r apo[a] isoforms (28, 29), choosing a standard Lp[a] with a similar isoform as the test sample would increase the accuracy of the assay, particularly when evaluating clinically relevant samples.

The distribution of Lp[a] in the population was highly skewed which confirms similar qualitative observations made by others (30–34). However, a comparison of quantitative parameters describing this and other distributions is difficult because of the use of different standards, subject populations, and methods of Lp[a] determination. Mean Lp[a] values as determined by other investigators range from 10 to 20 mg/dl and 90th percentile values range from 25 to 50 mg/dl (30–34). Upon conversion to Lp[a] lipoprotein, our mean and 90th percentile values of 13.3 and 34.7 mg/dl fall within the range reported by others. Less attention has been paid to the analysis of Lp[a] values in the lower percentiles, possibly due to limits of detection that vary from 1 to 5 mg/dl Lp[a] lipoprotein for electro- and radial immunodiffusion, respectively. The only assay sensitive enough to determine Lp[a] in all plasma samples was the radioimmunoassay (RIA) developed by Albers, Adolphson, and Hazzard (31). Initially, these authors

screened a population of 1000 individuals using radial immunodiffusion and found that 8.9% were Lp[a]-negative. These 89 samples were subsequently tested for Lp[a] using RIA in which they found values ranging from 0.5 to 8 mg/dl. When we convert our data to Lp[a] lipoprotein, we find that 7% of our population had Lp[a] values lower than the lowest value reported by Albers et al. (31) and 21% with values lower than 8 mg/dl. Whether these differences are due to potential cross-reactivity with plasminogen or to other factors pointed out above is unclear.

Based on current information about the structure of Lp[a], it may be pertinent to comment on the most appropriate way to report quantitative data obtained by Lp[a] assays. Commonly, data are expressed in terms of whole lipoprotein mass (sum of protein plus lipid). However, this may not be the best or most convenient way to represent the results, because this requires the determination of Lp[a] mass either gravimetrically or from the Lp[a] lipid composition. For example, a previous study has shown that in different Lp[a] particles the protein to Lp[a] mass ratio can vary by more than 30% (7). Thus we recommend that results of Lp[a] immunoassays be expressed in terms of Lp[a] protein of defined apo[a] isoform.

The authors thank Sherra McIntyre for help in the preparation of this manuscript. This research was supported by Program Project Grant USPHS HL-18577.

Manuscript received 18 July 1988 and in revised form 21 October 1988.

REFERENCES

1. Gaubatz, J. W., C. Heideman, A. M. Gotto, Jr., J. D. Morrisett, and G. H. Dahlen. 1983. Human plasma lipoprotein[a]. *J. Biol. Chem.* **258:** 4582–4589.
2. Utermann, G., and W. Weber. 1983. Protein composition of Lp[a] lipoprotein from human plasma. *FEBS Lett.* **154:** 357–361.
3. Fless, G. M., M. E. ZumMallen, and A. M. Scanu. 1985. Isolation of apolipoprotein[a] from lipoprotein[a]. *J. Lipid Res.* **26:** 1224–1229.
4. Armstrong, V. W., A. K. Walli, and D. Seidel. 1985. Isolation, characterization, and uptake in human fibroblasts of an apo[a]-free lipoprotein obtained on reduction of lipoprotein[a]. *J. Lipid Res.* **26:** 1314–1323.
5. Fless, G. M., M. E. ZumMallen, and A. M. Scanu. 1986. Physiochemical properties of apolipoprotein[a] and lipoprotein[a-] derived from the dissociation of human plasma Lp[a]. *J. Biol. Chem.* **261:** 8712–8718.
6. Seman, L. J., and W. C. Breckenridge. 1986. Isolation and partial characterization of apolipoprotein[a] from human lipoprotein[a]. *Biochem. Cell Biol.* **64:** 999–1009.
7. Fless, G. M., C. A. Rolih, and A. M. Scanu. 1984. Heterogeneity of human plasma lipoprotein[a]. *J. Biol. Chem.* **259:** 11470–11478.
8. Eaton, D. L., G. M. Fless, W. J. Kohr, J. W. McLean, Q-T. Xu, C. G. Miller, R. M. Lawn, and A. M. Scanu. 1987. Partial amino acid sequence of apolipoprotein[a] shows that it is homologous to plasminogen. *Proc. Natl. Acad. Sci. USA.* **84:** 3224–3228.
9. McLean, J. W., J. E. Tomlinson, W-J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein[a] is homologous to plasminogen. *Nature.* **330:** 132–137.
10. Kratzin, H., V. W. Armstrong, M. Niehaus, N. Hilschmann, and D. Seidel. 1987. Structural relationship of an apolipoprotein[a] phenotype (570kDa) to plasminogen: homologous kringle domains are linked by carbohydrate-rich regions. *Biol. Chem. Hoppe-Seyler.* **368:** 1533–1544.
11. Fless, G. M., and A. M. Scanu. 1987. Immunological cross-reactivity of human Lp[a] with plasminogen. *Arteriosclerosis.* **7:** 505a.
12. Karadi, I., G. M. Kostner, A. Gries, J. Nimpf, L. Romics, and E. Malle. 1988. Lipoprotein[a] and plasminogen are immunochemically related. *Biochem. Biophys. Acta.* **960:** 91–97.
13. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen purification from human plasma by affinity chromatography. *Science.* **170:** 1095–1096.
14. Brockway, W. J., and F. J. Castellino. 1972. Measurement of the binding of antifibrinolytic amino acids to various plasminogens. *Arch. Biochem. Biophys.* **151:** 194–199.
15. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
17. Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.
18. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
19. Allain, C. C., L. S. Poon, C. S. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
20. Gallo, L. L., R. Atasoy, G. V. Vahouny, and C. R. Treadwell. 1978. Enzymatic assay for cholesterol ester hydrolase activity. *J. Lipid Res.* **19:** 913–916.
21. Wahlefeld, A. W. 1974. Triglycerides: determination after enzymatic hydrolysis. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. 2nd English edition. Academic Press, New York. 1831–1835.
22. Tijssen, P. 1985. Practice and Theory of Enzyme Immunoassays. Elsevier, Amsterdam.
23. Khalil, A., N. Bramson, F. J. Kezdy, E. T. Kaiser, and A. M. Scanu. 1986. Affinity of nonhomologous amphiphilic peptides towards a monoclonal antibody raised against apolipoprotein A-I. *Proteins.* **1:** 280–286.
24. Yamaoka, K., Y. Tanigawara, T. Nakagawa, and T. Una. 1981. A pharmacokinetic analysis program (multi) for microcomputers. *J. Pharmacobiodyn.* **4:** 879–885.
25. Rodbard, D. 1974. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20:** 1255–1270.
26. McKee, P. A. 1982. Disorders of blood coagulation. *In* Cecil Textbook of Medicine. J. B. Wyngaarden and L. H. Smith, editors. 16th ed. W. B. Saunders Co., Philadelphia. 922–1009.
27. Jurgens, G. 1983. Cryoscopic behavior of isolated lipoprotein[a], and possible consequences for its use as a standard. *Clin. Chem.* **29:** 1856.
28. Utermann, G., H. J. Menzel, H. G. Kraft, H. C. Duba, H. G. Kemmler, and C. Seitz. 1987. Lp[a] glycoprotein phenotypes: inheritance and relation to Lp[a]-lipoprotein concentrations in plasma. *J. Clin. Invest.* **80:** 458–465.

29. Utermann, G., H. G. Kraft, H. J. Menzel, T. Hopferwieser, and C. Seitz. 1988. Genetics of the quantitative Lp[a] lipoprotein trait. I. Relation of Lp[a] glycoprotein phenotypes to Lp[a] lipoprotein concentrations in plasma. *Hum. Genet.* **78:** 41–46.
30. Albers, J. J., and W. R. Hazzard. 1974. Immunochemical quantification of human plasma Lp[a] lipoprotein. *Lipids.* **9:** 15–26.
31. Albers, J. J., J. L. Adolphson, and W. R. Hazzard. 1977. Radioimmunoassay of human plasma Lp[a] lipoprotein. *J. Lipid Res.* **18:** 331–338.
32. Kostner, G. M., P. Avogaro, G. Cazzolato, E. Marth, G. Bittolo-Bon, and G. B. Qunici. 1981. Lipoprotein Lp[a] and the risk for myocardial infarction. *Atherosclerosis.* **38:** 51–61.
33. Schriewer, H., G. Assmann, and M. Sandkamp. 1984. The relationship of lipoprotein[a] (Lp[a]) to risk factors of coronary heart disease. *J. Clin. Chem. Clin. Biochem.* **22:** 591–596.
34. Guyton, J. R., G. H. Dahlen, W. Patsch, J. A. Kautz, and A. M. Gotto, Jr. 1985. Relationship of plasma lipoprotein Lp[a] levels to race and apolipoprotein B. *Arteriosclerosis.* **5:** 265–271.