Characterization of five mouse monoclonal antibodies to apolipoprotein[a] from human Lp[a]: evidence for weak plasminogen reactivity

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Abstract We describe the development of five murine monoclonal antibodies (14A12, 39A1, 53A9, 73A7, and 128A6) specific to human apolipoprotein[a] (M_r ~ 570,000), and their characterization by a number of procedures including cotitration, competition and inhibition enzyme-linked immunosorbent assays (ELISA), immunoblotting of native lipoproteins and of SDS-solubilized apolipoproteins electrophoresed in polyacrylamide gels, and dot immunobinding assays. The patterns of immunoreactivity of these antibodies were similar. Each reacted in ELISA assays and upon electroimmunoblotting with purified apo[a], with apo[a] liberated by reduction of Lp[a], and with delipidated Lp[a] solubilized in SDS, but by contrast, they reacted with native Lp[a] to a significant degree only upon electroimmunoblotting. No reactivity was seen with LDL-apoB-100 or with other apolipoproteins. The cross-reactivity of these antibodies with the homologous protein, plasminogen, was examined by comparison of the amount of plasminogen or apo[a] required for 50% inhibition of antibody binding to apo[a], and by an ELISA assay. The inhibition assay showed reactivity with plasminogen to be 37- to 50-fold lower than with apo[a], while dot immunobinding showed the lower limit of detection of plasminogen and of apo[a] to be ~320 and 31 μg, respectively. In an ELISA sandwich assay based on monoclonal antibodies LHLP-1, 14A12, and 53A9, the lower limit of Lp[a] detection (~1 ng/ml protein) was about 100-fold less than that of plasminogen. Chemical modification of apo[a] revealed a significant contribution of arginine residues to the epitopes of 14A12, 39A1, and 53A9. Modification of cysteine residues with iodoacetamide was without effect, thereby distinguishing these antibodies from LHLP-1. Each antibody reacted with the six major size forms of apo[a] (M_r ~ 450,000–750,000) in immunoblots of human sera electrophoresed in SDS-polyacrylamide gels. Marked heterogeneity in apo[a] phenotype was detected and both single and double band phenotypes were observed in a randomized study. Cotitration and competition binding studies showed varying degrees of interaction between all five epitopes, with the exception of 128A6 which appeared to be independent of 39A1 and 53A9 (and vice versa). These data suggest that our five monoclonal antibodies recognize epitopes on apolipoprotein[a] that are exposed and accessible on the native Lp[a] particle. We conclude that our monoclonal antibodies recognize a specific region of apo[a], and that this region undergoes a conformational change upon adsorption of Lp[a] to plastic thereby diminishing epitope recognition. Furthermore, these antibodies display weak reactivity with plasminogen, and have provided the basis for a specific assay for human Lp[a]. —**Guo, H-C., V. W. Armstrong, G. Luc, C. Billardon, S. Goulinet, R. Nustede, D. Seidel, and M. J. Chapman.** Characterization of five mouse monoclonal antibodies to apolipoprotein[a] from human Lp[a]: evidence for weak plasminogen reactivity. *J. Lipid Res.* 1989. **30:** 23–37.

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The Lp[a] lipoprotein was first detected in human plasma by Berg et al. (1, 2) and was originally thought to be a genetic variant of low density lipoprotein (LDL). Subsequently, extensive evidence has suggested that Lp[a] may be an independent risk factor for coronary heart disease and cerebral infarction (2–6). Furthermore, immunofluorescent studies have documented the localization of the Lp[a] antigen in human atherosclerotic plaques (7).

The Lp[a] lipoprotein and LDL share certain features of their lipid and protein composition, notably their high content of cholesteryl esters and the presence of apoB-100 (8–13). In contrast to LDL, however, Lp[a] is distinguished by its larger size (mol wt 3–5 × 10^6, diam. ~ 250 Å), its pre-β electrophoretic mobility on agarose or paper, and its higher content of protein and carbohydrate (8, 10–15). Most significantly, the Lp[a] particle is unique

Abbreviations: Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; Lp[a–], Lp[a] from which apo[a] has been removed by disulfide bond reduction; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apoB, apolipoprotein B; SDS, sodium dodedcyl sulfate; EDTA, ethylenediamine tetraacetate; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; DTT, dithiothreitol; MAb, monoclonal antibody; FPLC, fast protein liquid chromatography; NCS, newborn calf serum; TBS, Tris-buffered saline.

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in that it contains the carbohydrate-rich, [a] protein, which is covalently attached to the B-100 protein by one or more disulfide bonds (9, 11–17). In consequence of this additional protein moiety, the predominant form of Lp[a] is found in the density interval 1.05–1.12 g/ml in man (18–21). Moreover, recent investigations have shown Lp[a] to exhibit heterogeneity in its density distribution and physicochemical properties, not only in normolipidemic subjects (11, 13, 22, 23) but also in patients with homozygous familial hypercholesterolemia (24).

Apo[a] is distinct in both its amino acid and carbohydrate composition from apoB-100 and other known apolipoproteins (13, 14, 16, 17, 25). It exhibits a marked polymorphism, presenting several different molecular weight forms in the range of 280,000 to 710,000 (9, 13, 16, 17, 25, 26). In a large scale population study (26), six apo[a] phenotypes with apparent molecular weights ranging from 400,000 to 700,000 were detected by immunoblot analysis; on the basis of these data, it was concluded that the various apo[a] phenotypes are controlled by a series of autosomal alleles at a single locus.

Recently, determination of the partial amino acid sequence of an apo[a] with molecular weight 280,000 (27) and of the nucleotide sequence of the cloned complementary DNA (28) have revealed a striking homology to plasminogen, the [a] protein containing domains homologous to kringles 4 and 5 from plasminogen and an inactive protease domain (28). Similar studies on an apo[a] phenotype of molecular weight 570,000 have confirmed this structural similarity between apo[a] and human plasminogen (29).

The presence of apoB-100 and apo[a] in the same lipoprotein particle raises a number of important questions concerning the structure, formation, metabolism, and function of Lp[a]. At a structural level, information is lacking on the stoichiometric relationship of apoB-100 to apo[a] within Lp[a] particles differing in density and in apo[a] phenotype, on the precise nature of the interactions between apoB-100 and apo[a], and on the nature of chemical differences between the apo[a] phenotypes.

Monoclonal antibodies have proven powerful tools in studies of apolipoprotein structure and in the identification of functional domains. To date, immunological studies of Lp[a] have primarily concerned its quantitation with polyclonal, heterogeneous antibody populations (2–8, 30), and indeed, in view of the remarkable structural homology between apo[a] and plasminogen (27, 28), such antibodies would not be expected to be monospecific for the [a] protein. More recently, however, a monoclonal antibody (LHLP-1) was identified which is specific to a conformational epitope in Lp[a] and dependent on the presence of intact disulfide bonds (22).

As an initial step towards the epitopic mapping of apo[a], of the elucidation of its conformation in Lp[a] particles, and of the immunological relationship between apo[a] and plasminogen, we have developed a library of five mouse monoclonal antibodies to human apo[a]. We now describe the generation and characterization of these antibodies, their use in the investigation of the immunological reactivity of apo[a] in isolated form and in bound form in the Lp[a] particle, and the evaluation of their reactivity with the homologous protein, plasminogen.

METHODS

Isolation of plasma lipoproteins

Plasma was separated from the freshly drawn venous blood of normolipidemic and of hypercholesterolemic donors into sterile bottles containing EDTA and NaN_3 at final concentrations of 0.1% and 0.01%, respectively; all donors had fasted overnight for 12–14 hr. Plasma was immediately separated by low speed centrifugation at 4°C, and used on a single donor basis without pooling.

Lipoprotein fractions of d 1.024–1.050, 1.050–1.100, and 1.13–1.21 g/ml were isolated by sequential flotation in the 60Ti and 50Ti rotors of the Beckman L8.55 ultracentrifuge (Beckman, Palo Alto, CA) at 10°C (31). Each lipoprotein preparation was washed once at its upper limiting density. Exhaustive dialysis against a solution containing 0.01% NaN_3, 0.01% EDTA, and 0.1 M NaCl was then performed at 4°C. The final preparations were sterilized by ultrafiltration and stored at 4°C under a nitrogen atmosphere. The purity and integrity of these lipoprotein fractions were verified as described earlier (32).

Plasma lipoprotein subfractions were also prepared by density gradient centrifugation (32), in which case gradients were subfractionated into successive fractions of 0.5 ml.

Isolation and purification of apolipoprotein[a]

Lipoprotein Lp[a] was isolated from the plasma of a healthy donor with a plasma Lp[a] concentration of ~100 mg/dl. Briefly, the plasma was adjusted to a density of 1.15 g/ml with solid KBr and the d < 1.15 g/ml lipoproteins were separated by ultracentrifugation at 150,000 *g* for 20 hr (10°C). This fraction was then used as the dense solution in forming a linear gradient with a d 1.03 g/ml KBr solution. After centrifugation in a Beckman 75Ti rotor at 49,000 rpm for 20 hr (10°C), the gradient was fractionated and the fractions containing Lp[a] were pooled and dialyzed against 0.01 M Tris-Cl, 0.05 M NaCl, 0.001 M EDTA, and 0.02% NaN_3 at pH 8.0. Traces of HDL were removed by affinity chromatography over heparin-Sepharose 4B (Pharmacia) equilibrated with the same buffer. HDL were not retained by this column and after all A_{280} material had eluted, the NaCl concentration in the buffer was raised to 0.5 M to release bound Lp[a].

The purity of our Lp[a] preparations was assessed by anion exchange chromatography using a Mono QHR 5/5

resin on an FPLC system (Pharmacia, Uppsala, Sweden) as described by Armstrong, Walli, and Seidel (12); LDL were undetectable by this procedure.

To obtain pure apo[a], Lp[a] was reduced as described previously (12). DDT (final concentration 0.01 M) was added to a solution of Lp[a] containing apo[a] and centrifuged overnight (12); apo[a] sedimented as a pellet under these conditions. It was solubilized in a buffer containing 0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, 0.001 M DTT, and 1% sodium dodecyl sulfate at pH 8.0 and then extensively dialyzed against 0.001 M EDTA.

The apo[a] used in these studies migrated as a single band on 4–30% or 2–16% SDS-polyacrylamide gradient gels with an apparent molecular weight of ~570,000 unless indicated otherwise. Protein determinations were performed by the method of Lowry et al. (33), with bovine serum albumin as the working standard.

Production of monoclonal antibodies

Eight-week-old BALB/C mice were immunized by subcutaneous injection of 20 μg of apo[a] (M_r ~ 570,000) emulsified with an equal volume of Freund's complete adjuvant (Gibco BRL, Paisley, Scotland). The mice were boosted by subcutaneous injection of the same amount of antigen, emulsified in Freund's incomplete adjuvant, after 14 and 21 days. At 28 days and at 3 days before cell fusion was performed, 10 μg of apo[a] in phosphate-buffered saline was injected intravenously. The fusion procedure was conducted according to the method of Boumsell and Bernard (34) on the third day after intravenous challenge with the immunogen. For this purpose, the non-secretor mouse myeloma line P3-NS-1/Ag4-1(NS-1) with 8-azaguanine-resistance was cultured in RPMI-1640 (Gibco BRL) containing final concentrations of 20 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U penicillin–streptomycin, 0.2 U insulin, 0.132 mg oxaloacetic acid, and 2×15^{-5} M 2-mercaptoethanol, in addition to non-essential and essential amino acids. Myeloma cells (10^7) from a culture in log phase were first mixed with spleen cells (ca. 2×10^7) from immunized mice. The cell mixture was washed with serum-free medium, the cell pellet was resuspended in 1 ml of 33% polyethylene glycol 4000 (Merck, Darmstadt), and gently swirled for 3 min. The fusion mixture was then centrifuged for 2 min at 200 *g*, and 5 ml of myoclone fetal calf serum (Gibco BRL; heat-inactivated for 45 min at 56°C) was added for 10 min. The cells were subsequently resuspended in 19 ml of RPMI-1640-enriched medium and distributed into flat-bottom 96-well culture plates (0.1 ml per well) over a mouse thymocyte feeder layer (3×10^5 cells/well). The cultures were maintained in a humidified incubator containing 5% CO_2. The following day, 0.1 ml of a selective medium (HAT; 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 3×10^{-5} M thymidine) of double strength was added to each well. Hybrid clones began to appear macroscopically 7–15 days after fusion; supernatants were screened for the presence of antibodies to apo[a] by an ELISA method (see below). The antigen used for screening was apo[a] (M_r ~ 570,000) (see "Isolation and purification of apo[a]"). The cells corresponding to positive wells were subcloned at limiting dilution (0.5 cell/well) in 96-well microculture plates over a thymocyte feeder layer; antibody reactivity as determined by ELISA appeared identical to that of the respective parent clones. After identification, positive clones were transferred to larger culture vessels.

Of a number of clones that produced antibody to apo[a], five were studied in detail and are herein described. Both supernatant fluids and ascites fluid were used as sources of antibody. For ascites, Pristane-primed BALB/C mice were injected intraperitoneally with 5×10^6 cultured hybridoma cells (35). When sufficient ascitic fluid had accumulated, the ascites was collected by draining the peritoneal cavity with a large-gauge needle.

Immunoglobulin class and subclass

A sample of each hybridoma supernatant was analyzed by double immunodiffusion in 1% agarose in veronal buffer as described by Ouchterlony (36), using antisera to mouse IgG (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3), IgM, and IgA (Nordic Immunological Laboratories, Tilburg, Holland).

Monoclonal antibodies against Lp[a] and apoB-100

Supernatant and ascitic fluids containing monoclonal antibodies to human apoB-100 (1.8.C4) were produced from hybridoma cells that were kindly provided by Dr. T. L. Innerarity. The characterization of this antibody was described earlier (37). Monoclonal antibody LHLP-1 was produced as described by Duvic et al. (22) and the supernatant fluid was used in the present studies.

Polyclonal antibody against apo[a]

A polyclonal antibody to apo[a] was produced by subcutaneous injection of 100 μg apo[a] emulsified in Freund's complete adjuvant into sheep. The same amount, in Freund's incomplete adjuvant, was similarly injected at 3 and at 6 weeks after the initial immunization. After immunological testing of the antiserum, the sheep were bled out and serum was separated.

Enzyme-linked immunosorbent assay (ELISA)

This procedure is based on the assay of Engwall and Perlmann (38). Aliquots (100 μl) of the antigen (5 μg protein/ml) in coating buffer (0.05 M sodium carbonate, pH 9.6) were pipetted into the wells of 96-well microtiter plates (Titertek, Eflab, Helsinki, Finland) and incubated overnight at 4°C. The antigen solution was then shaken out, and each well was filled with 300 μl of a solution of 1% (w/v) bovine serum albumin in 0.9% NaCl. The plates were incubated at room temperature for 1 to 2 hr and subsequently washed three times with a solution of 0.05%

Tween in 0.9% NaCl. Each hybridoma supernatant was first diluted in this same solution (0.9% NaCl, 0.05% Tween 20) to which 1% (w/v) bovine serum albumin had been added, and aliquots were added to each well; the plates were then incubated for 2 hr at 37°C, and washed three times as above. Sheep anti-mouse immunoglobulin labeled with horseradish peroxidase (Dakopatts a/s; Glostrup, Denmark) was added to each well, and incubated for 2 hr at 37°C. After washing three times as before, 100-μl aliquots of substrate solution (peroxide-*o*-phenyldiamine) were added, and the plates were incubated for 15 min at room temperature. After color development, the reaction was terminated by addition of 1 M H_2SO_4 (50 μl) to each well, and the absorbances were read at 492 nm on an automated microtiter plate spectrophotometer (Titertek Multiskan Plus). All assays were performed in duplicate or triplicate. Antigens studied by this procedure included Lp[a] in both native and reduced forms, apo[a], LDL, and plasminogen (Boehringer Mannheim); reduction of these antigens was performed at room temperature for 1 hr in the presence of 7 mM DTT (final concentration).

Cotitration and competition of apo[a] antibodies

These assays were used to determine whether two monoclonal antibodies competed for a related epitope on the same antigen. In cotitration experiments, the method of Fisher and Brown (39) was modified for use with soluble antigens. Apo[a] (100 μl; 0.1 μg protein/ml) was first used to coat the microtiter plate. Serial dilutions of either individual antibodies (as culture supernates) or a 1:1 (v/v) combination of two different antibodies were prepared in a solution containing 1% BSA, 0.9% NaCl, and 0.05% Tween 20. Aliquots (100 μl) of each dilution were then added to the microtiter plate and the remainder of the assay was performed as above. For each antibody, activity was compared when presented as a mixture to that when presented alone.

In competition studies, the plastic wells were first coated with an affinity-purified monoclonal antibody (100 μl; 1 μg protein/ml); each antibody was purified from ascites fluid as described below. A second antibody, which had been separately preincubated with apo[a] antigen, was then added to the wells. After incubation for 1 hr at 37°C and washing as described for the ELISA assay above, aliquots of our sheep polyclonal antibody to apo[a] were added and incubated for 1 hr at 37°C. The amount of bound sheep IgG was then revealed by addition of a rabbit peroxidase-labeled antibody to sheep IgG (Dakopatts a/s). The remainder of the assay was carried out as before.

Determination of antibody affinity constants

The affinity constant of each monoclonal antibody was determined by ELISA according to the principle described by Friguet et al. (40). For this purpose, antibodies were purified by affinity chromatography on protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) (41). A constant amount of each antibody was mixed with apo[a] at various concentrations and incubated until equilibrium was attained. The concentration of free antibody was then determined by our ELISA assay described above, the antigen being adsorbed to the microtiter plate wells.

Chemical modification of apo[a]

After coating of microtiter plate wells with apo[a], this protein was chemically modified at specific amino acid residues in order to evaluate their potential role as structural elements of epitopes. Cyclohexanedione modification of arginine residues was carried out as described by Mahley et al. (42); lysine residues were modified by reductive methylation (43), and cysteine residues by reductive alkylation with iodoacetamide (43). Neuraminidase treatment of apo[a] was carried out to remove sialic acid from the carbohydrate side chains (44).

Electrophoresis of native lipproteins

Agarose electrophoresis and immunoblotting. Electrophoresis of native lipoproteins was carried out on agarose gel films (Corning, Palo Alto, CA) using the Corning ACI electrophoresis system. The procedure is essentially as described by Noble (45). Gels were either stained for lipid with Oil Red O, or immunoblotting was immediately performed. The agarose gel was first overlaid with nitrocellulose paper (Bio-Rad, Richmond, CA) in order to adsorb lipoprotein material by capillarity (46). After 1 hr, the nitrocellulose paper was removed and blocked by incubation in a solution of 10% newborn calf serum (NCS) in 10 mM Tris–150 mM NaCl (TBS) at pH 7.4 for 1 hr at room temperature. The nitrocellulose was then incubated overnight at room temperature with 10 μg/ml monoclonal antibody in TBS containing 10% NCS (v/v). After washing three times with TBS, the blots were incubated with peroxidase-conjugated sheep anti-mouse IgG (Dakopatts a/s, Denmark) at 20°C for 4 hr. The nitrocellulose was washed three times with TBS and developed with 4-chloro-1-naphthol (Fluka AG) (0.5 mg/ml) in a 1:5 mixture of methanol and 50 mM Tris–200 mM NaCl containing 0.01% H_2O_2, and at pH 7.4 (46).

Polyacrylamide gradient gels. Continuous gradient gel electrophoresis of native lipoproteins was performed in a Pharmacia electrophoresis apparatus GE-2/4 LS loaded with gels containing a 2–16% gradient (PAA 2/16; Pharmacia Fine Chemicals, Uppsala, Sweden). Approximately 15 μg of lipoprotein was applied to each well and electrophoresis was carried out at 125 V for 12 hr at 4°C in a Tris/borate buffer (0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA, pH 8.35) (47). Gels were subsequently stained with 0.7% Amido Black in 7% acetic acid and diffusion-destained in 7% acetic acid. A set of standard proteins with known hydrated diameters (thyroglobulin, 170 Å;

ferritin, 122 Å; catalase, 104 Å; lactic dehydrogenase, 81 Å; bovine serum albumin, 71 Å; HMW electrophoresis calibration kit, Pharmacia Fine Chemicals) was run in duplicate on each slab as a reference marker. From the migration distances of the different lipoprotein subfractions and those of the proteins contained in the calibration kit, it was possible to calculate the Stokes diameters of lipoprotein particles using the Stokes-Einstein equation (47).

For immunoblotting, lipoprotein bands were electrophoretically blotted onto nitrocellulose paper (see "Apolipoprotein electrophoresis" below). After transfer in this manner, the nitrocellulose paper was reacted with either polyclonal or monoclonal antibodies as described above under "Agarose electrophoresis and immunoblotting."

Apolipoprotein electrophoresis

Agarose-acrylamide gels and electroimmunoblotting. Serum (20 μl) and lipoprotein samples (2–20 μg protein) were first lyophilized and subsequently delipidated in ethanol–diethyl ether 3:1 (v/v) at 4°C overnight (32). After drying under nitrogen, apolipoprotein samples, as well as those of purified plasminogen (5–15 μg protein), were solubilized in running buffer (see below) containing 5% SDS and 20 mM DTT; they were then heated at 95°C for 10 min. Samples were loaded onto polyacrylamide slab gels made up from 2.75% acrylamide and 0.07% bisacrylamide and containing 0.5% agarose (Bio-Rad) and 0.2% sodium dodecyl sulfate (48, 49). A Tris-glycine buffer (pH 8.3) containing 0.2% SDS was used as a running buffer in both the upper and lower buffer chambers. Electrophoresis was performed at a constant voltage of 180 V and at 4°C for approximately 2 hr. On completion of electrophoresis, protein bands were electrophoretically blotted onto a nitrocellulose sheet. A transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at pH 8.3 was used in a Transblot cell (Bio-Rad); electrophoretic transfer was performed for 2.5 hr at a current of 500 mA (50). The nitrocellulose was then treated as described above (see "Electrophoresis of native lipoproteins—Agarose electrophoresis and immunoblotting").

In gels that were stained for apolipoprotein bands with Coomassie Brilliant Blue, molecular weights were calculated as outlined by Weber and Osborn (51). For calibration purposes, a series of polymerized protein standards (BDH Biochemicals, Poole, U.K.) was electrophoresed in parallel (52).

Polyacrylamide gradient gels. The molecular weights of apolipoproteins (apoLp[a], apo[a], and apoB-100) and plasminogen were also examined in 2–16% PAA polyacrylamide gradient gels (Pharmacia Fine Chemicals) in the presence of 0.2% SDS at pH 7.4. The running buffer (0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, and 0.2% SDS) and conditions of electrophoresis, staining with 0.02% Coomassie Brilliant Blue in 7% acetic acid and diffusion destaining were those recommended by Pharmacia (53).

In gels intended to be analyzed for apolipoprotein bands by immunoblotting, electrophoretic transfer to nitrocellulose paper and reaction with specific antibody were performed as indicated above.

Dot immunobinding assays. For comparison of the reactivities of our monoclonal and polyclonal antibodies to apo[a] and to Lp[a] with a series of antigens, including plasminogen (Boehringer Mannheim), we used a dot immunobinding assay. Our procedure corresponds essentially to that described earlier (52) and adapted from Hawkes, Niday, and Gordon (54).

Enzyme-linked immunoassay for Lp[a]. Serum samples were first diluted 1000-fold in 0.9% NaCl containing 1% (w/v) bovine serum albumin containing 0.05% Tween 20. Microtiter plate wells (Titertek) were coated with 100 μl affinity-purified monoclonal antibody LHLP-1 at 5μg/ml in coating buffer (0.05 M Na_2CO_3, pH 9.6) for 18 hr at 4°C.

Excess antibody was removed and the wells were saturated with 1% (w/v) bovine serum albumin in 0.9% NaCl at room temperature for 1 hr. After five washings with a solution of 0.05% Tween 20 in 0.9% NaCl, 100 μl of each serum sample was added to individual wells. After sample addition, the plates were incubated at 37°C for 2 hr. The plates were washed five times with 0.9% NaCl–Tween 20; 100 μl of a mixture of affinity-purified biotin-labeled monoclonal antibodies 14A12 and 53A9 (0.2 μg/ml in 0.9% NaCl–1% bovine serum albumin–Tween 20) was then added to each well. After incubation for 2 hr at 37°C, the plates were washed five times as above and incubated with 100 μl of a 1/1000 dilution of peroxidase-labeled Streptadivin (Amersham) in 0.9% NaCl–1% bovine serum albumin–Tween 20 for 1 hr at 37°C. The color reaction was developed in the wells after thorough washing and addition of 100-μl aliquots of substrate solution (peroxide-*o*-phenylenediamine); after color development, the reaction was terminated by addition of 1 M H_2SO_4 (50 μl) to each well and the absorbances were read at 490 nm as for the ELISA assay above.

Further details of this procedure will be described in a subsequent report from our laboratory (Guo et al., unpublished data).

For calibration purposes, either a serum standard (Immuno AG, Vienna, Austria) or Lp[a] purified as above (12) was used; the working concentration range was 1 to 500 ng/ml.

For comparison, Lp[a] concentrations were also determined in the same serum samples as those assayed by our ELISA procedure above, with the laser immunonephelometric method (Immuno-France SA, Rungis, France) as outlined elsewhere (52); the same working standards as above were used.

RESULTS

Cell fusion and monoclonal antibody production

Cell growth was observed in all 240 wells that were seeded; of these, 22 clones were initially shown to secrete antibodies to apo[a] by ELISA assay. Cells from 10 wells were recloned by limiting dilution. Five stable hybridomas, denoted 14A12, 39A1, 53A9, 73A7, and 128A6, were then selected for further study on the basis of their titer. Each hybridoma secreted antibody of the IgG1 subclass, the light chain being of the K type in each case.

Immunological specificity and binding characteristics of antibodies

Analysis of the specificity of our monoclonal antibodies by Western blot analysis following electrophoresis of the apolipoproteins of HDL_3 (d 1.13–1.21 g/ml) in SDS-polyacrylamide gels showed no reactivity with apolipoproteins A-I, A-II, C-I, C-II, C-III, D, and E.

The reactivity of our monoclonal and polyclonal antibodies with purified apo[a], purified native Lp[a], the reduced form of Lp[a], and native LDL (d 1.024–1.050 g/ml) was evaluated in ELISA assays (see above), in which aliquots of increasing dilutions of these antibodies were reacted with a fixed amount of each antigen coated onto microtiter plates (**Fig. 1**).

All of our monoclonal and polyclonal antibodies bound to apo[a], but bound to Lp[a] only after reduction in situ with dithiothreitol (Fig. 1A–F). By contrast, antibody LHLP-1 bound preferentially to intact Lp[a], and reacted only at low levels with the other antigens; such reactivity was markedly reduced (Fig. 1G) upon reduction of Lp[a], confirming the earlier findings of Duvic et al. (22).

These initial observations on the specificity of our monoclonal antibodies to apo[a] were then confirmed by a series of experiments involving localization of antibody reactivity to different antigens by dot blotting and by gel electrophoresis followed by electrophoretic transfer and immunoblotting.

By dot immunobinding (54), the reactivity of the various monoclonal antibodies with native Lp[a] was either low (39A1, 53A9, and 128A6) or at the limit of detection (14A12 and 73A7); by contrast, high reactivity was seen with LHLP-1. Upon reduction of Lp[a], the reactivities were modified in a manner comparable to that seen in the ELISA assay (data not shown).

In order to localize antibody reactivity to specific lipoprotein particles, we immunoblotted purified native Lp[a] and native LDL (d 1.024–1.050 g/ml) after electrophoresis in agarose gel films; one film was immunoblotted with our various monoclonal antibodies to apo[a] (**Fig. 2A**), the second with a monoclonal antibody (1.8.C4) to apoB-100 (data not shown). Lp[a] was seen to migrate as a pre-β-lipoprotein when stained for lipid with Sudan Black (not shown). Each monoclonal antibody to apo[a] reacted with Lp[a], but failed to react with LDL (Fig. 2A). By contrast, both Lp[a] and LDL were immunoreactive with 1.8.C4 attesting to their common content of apoB-100. Immunoblotting of native LDL and native Lp[a] was also performed in nondenaturing polyacrylamide gradient gels (PAA 2–16%). When immunoblotted with our monoclonal antibodies to apo[a], a single band was uniformly detected for Lp[a] with an average diameter of 24 nm (not shown). No reactivity was seen with LDL.

The protein moieties of the same fractions, in native and reduced forms, were also electrophoresed, after solubilization in SDS, in SDS-polyacrylamide-agarose gradient gels (Fig. 2B). All our monoclonal antibodies bound to both apoLp[a] (Fig. 2B); lanes 1, 3, 5, 7, and 9) and to the corresponding apo[a] obtained upon reduction of intact apoLp[a] (Fig. 2B, lanes 2, 4, 6, 8, and 10): these antibodies did not bind to apoB-100. In contrast, the anti-apoB-100 monoclonal antibody reacted with B-100 both when bound to apo[a] in native apoLp[a] and when in free form after dissociation of apo[a] from apoLp[a] by disulfide reduction.

Taken together, the above qualitative data indicate that monoclonal antibodies 14A12, 39A1, 53A9, 73A7, and 128A6 each react with delipidated Lp[a] (i.e., apoLp[a] and with isolated apolipoprotein [a]), but not with LDL. The antibodies also reacted with native Lp[a] upon blotting to nitrocellulose, but bound poorly when this antigen was adsorbed to plastic surfaces (Fig. 1); substantially higher reactivities were seen in sandwich ELISA assays in which antigen adsorption was avoided (see "Quantitation of Lp[a]" below). We then undertook further characterization of these antibodies.

Estimation of antibody affinity

After purification of each monoclonal antibody by protein A-Sepharose affinity chromatography, the affinity constant of each to apo[a] was estimated by ELISA assay (40). Our data (**Table 1**) showed that affinity constants for binding to apo[a] varied from $0.8 \times 10^{-8} \cdot M^{-1}$ for 53A9 to $1.5 \times 10^{-8} \cdot M^{-1}$ for 39A1.

Epitopic mapping

In an effort to determine the interrelationship of the various epitopes on the apo[a] protein, we performed competition and cotitration experiments and, in addition, evaluated the effect of the chemical modification of specific amino acid residues on the immunological reactivity of each monoclonal antibody.

As a preliminary to cotitration experiments, we first determined the concentration range of the antigen, apo[a], that resulted in a plateau in the amount of antibody bound; increasing dilutions of antibody were used for such studies, but the plateau was only seen at low antibody dilutions (<1,000-fold). Under such conditions,

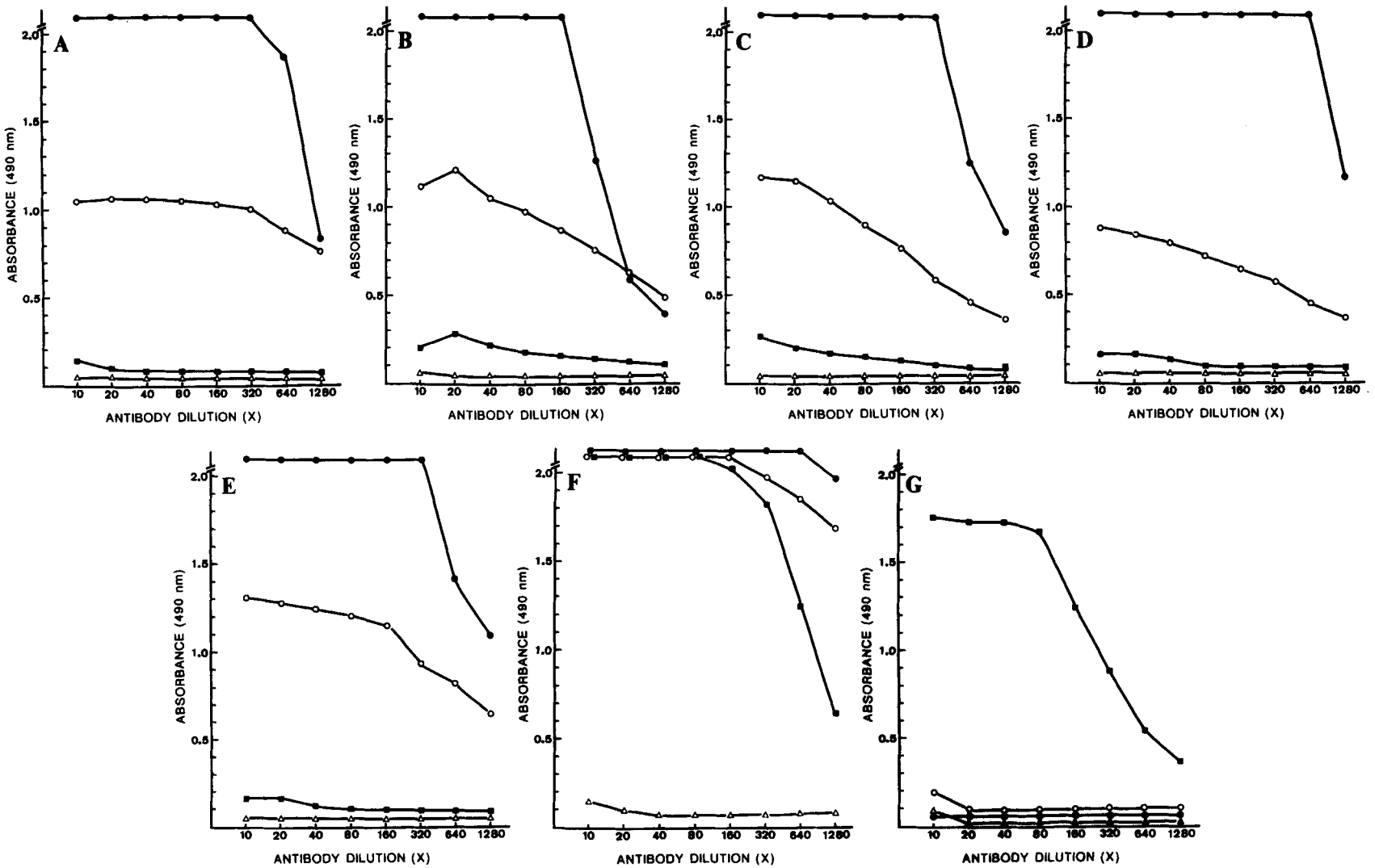

Fig. 1. Determination of the binding to various antigens of monoclonal and polyclonal antibodies to human apo[a] and to Lp[a]. Progressive dilutions of hybridoma supernatants containing monoclonal antibodies to apo[a] were tested in an ELISA assay (see Methods) for their ability to bind to purified human apo[a] (●−●), native Lp[a] (■−■), Lp[a] reduced with DTT (○−○) and LDL (△−△), which had each been separately adsorbed to polystyrene Removawells. Aliquots containing 5 μg protein/ml of each antigen were taken for coating. Patterns are: A) 14A12, B) 39A1, C) 53A9, D) 73A7, and E) 128A6; in F), a sheep polyclonal antibody to human apo[a] was used, and in G) hybridoma supernatant LHLP-1, containing monoclonal antibodies to Lp[a]. Each experimental point represents the mean of triplicate determinations.

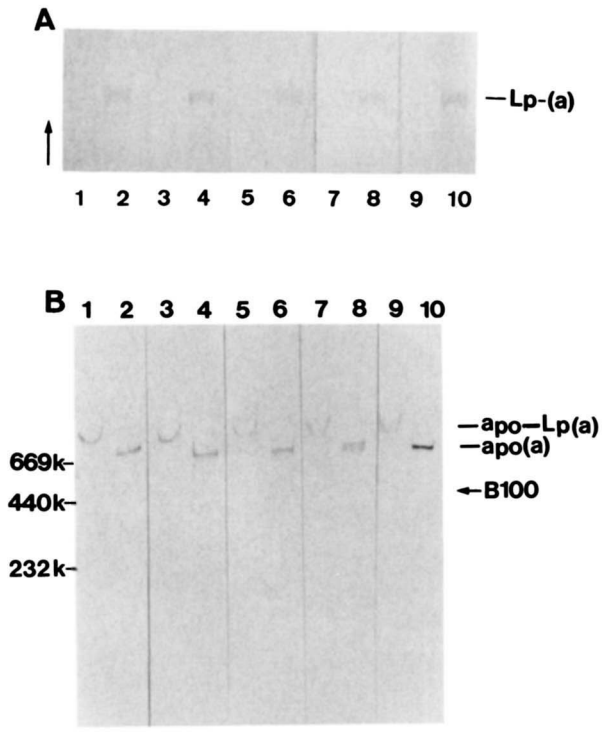

Fig. 2. Pattern A: immunoblots of purified Lp[a] and LDL (d 1.024–1.050 g/ml) following electrophoresis in agarose gel films. Electrophoretic transfer to nitrocellulose and immunoblotting were performed as indicated in Methods. Nitrocellulose transfer sheets were individually immunoblotted with dilutions (1:10) of each hybridoma supernatant containing antibodies to apo[a]. Lanes 1, 3, 5, 7, and 9, LDL (2 μg protein/well); lanes 2, 4, 6, 8, and 10, Lp[a] (2 μg protein/well). Hybridoma supernatants used for immunoblotting were lanes 1 and 2: 14A12; lanes 3 and 4: 39A1; lanes 5 and 6: 53A9; lanes 7 and 8: 73A7; and lanes 9 and 10: 128A6. Pattern B: electroimmunoblot of native and of reduced Lp[a]. After electrophoresis in SDS-polyacrylamide (2.75%)-agarose (0.5%) gels, proteins were electrophoretically transferred to nitrocellulose and immunoblotted with dilutions (1:10) of hybridoma supernatant. Lane 1: antibody 14A12; lanes 3 and 4: 39A1; lanes 5 and 6: 53A9; lanes 7 and 8: 73A7; and lanes 9 and 10: 128A6. Samples loaded in lanes 1, 3, 5, 7, and 9 were native apoLp[a] and in lanes 2, 4, 6, 8, and 10, apoLp[a] after reduction with 10 mM DTT at 37°C for 15 min. At left are shown the molecular weight values determined from standard markers electrophoresed in parallel, and at right, the marker position of migration of purified apoB-100 in such gels.

apo[a] is present in limiting amounts, a necessary condition for these studies (39).

In a typical experiment, and with apo[a] coated to the plate wells, the combination of antibodies 14A12 and 39A1 gave a plateau whose height was greater than that obtained with either of the antibodies individually (data not shown). This observation suggested that their respective epitopes might be distinct. By contrast, when 39A1 and 53A9 were added simultaneously, the plateau (measured as absorbance at 490 nm in our ELISA assay) was no higher than that when they were added separately; in this case, their respective epitopes are either identical, overlap, or are situated closely together.

Table 2 summarizes our cotitration data. On the one hand, antibodies 14A12, 73A7, and 128A6 appear to correspond to a group of related epitopes, whereas antibodies 39A1 and 53A9 appear to be identical or to interact with each other but not with the former group.

The interrelationships between these epitopes were also explored in competition ELISA assay (**Table 3**). Similar results were obtained, but with some differences. Thus, partial interaction was detected between the epitopes recognized by 39A1 and by those of 14A12, 73A7, and 128A6. Furthermore, the epitope recognized by 53A9 also appeared to show minor interaction with those of 14A12 and 128A6. A high degree of interaction was again seen between the determinants recognized by 53A9 and 39A1; this is equally true for the epitopes of 73A7 and 128A6, and indeed these epitopes may be identical.

As originally indicated by Atassi (55), the reactivities of antibodies with chemically modified antigens may facilitate the partial definition and distinction of their intramolecular specificities. Using our ELISA assay, we therefore evaluated the effect on the immunological reactivities of our antibodies of a series of chemical modifications of apo[a] (**Table 4**). Modification of cysteine residues by iodoacetamide, or of lysine residues by reductive methylation, did not significantly affect the binding of our antibodies to apo[a]; this was also the case after neuraminidase treatment to remove sialic acid. By contrast, marked reduction in the binding of our antibodies was seen upon modification of the arginine residues of apo[a] by cyclohexanedione. The greatest effects were seen for antibodies 14A12, 39A1, and 53A9, in which the initial binding was decreased by ~51, 32, and 34%, respectively.

Size heterogeneity of apolipoprotein[a]

Determination of the size of apo[a] in the serum of different individuals by electrophoresis in SDS-polyacrylamide-agarose gels followed by electroimmunoblotting

TABLE 1. Affinity constants for the binding of monoclonal antibodies to purified human apo[a]

Monoclonal Antibody	Affinity Constant ($\times 10^{-8} \cdot M^{-1}$)
14A12	1.14
39A1	1.5
53A9	0.8
73A7	1.23
128A6	1.11

The antigen, at various concentrations (40×10^{-8} M to 1.2×10^{-8} M) was mixed with a constant amount of antibody in 0.9% NaCl containing 1% BSA. After incubation for 18 hr at 20°C, 100 μl of each mixture was transferred and incubated for 1 hr at 20°C in the wells of a microtitration plate previously coated with apo[a]. The bound immunoglobulins were then determined by ELISA assay (see Methods), and the affinity constant was calculated (40). The molecular weight of apo[a] was taken arbitrarily as 600,000.

TABLE 2. Cotitration of monoclonal anti-apo[a] antibodies

Monoclonal Antibody	Absorbance (490 nm)				
	14A12	39A1	53A9	73A7	128A6
14A1	0.84 ± 0.04	1.72 ± 0.11	1.49 ± 0.10	1.08 ± 0.04	1.10 ± 0.04
39A1		1.10 ± 0.05	1.08 ± 0.03	1.34 ± 0.05	1.65 ± 0.05
53A9			1.12 ± 0.06	1.43 ± 0.05	1.58 ± 0.05
73A7				0.76 ± 0.02	0.92 ± 0.03
128A6					1.02 ± 0.05

Cotitration studies were performed as described in the Methods section. Values are means ± SD taken from a series of five experiments; duplicate determinations were performed for each antibody alone or in combination in each experiment. The coating antigen was purified apo[a].

revealed marked heterogeneity (**Fig. 3**). At least six distinct size species were detected by our five monoclonal antibodies (Fig. 3; bands 1–6); four of these species (denoted bands 3–6) exhibited an apparent molecular weight that was greater than that of apoB-100 (as indicated by arrows), one species was similar in migration to B-100 (band 2) and thus of $M_r \sim 550{,}000$, while the sixth species (band 1) was of smaller size than B-100. The largest apo[a] species, band 6, was a single component, i.e., single band phenotype (Fig. 3, lane a), whereas all the other patterns showed two major components. Thus, bands 2 and 5, 1 and 2, 2 and 4, and 2 and 3, occurred as double band phenotypes (Fig. 3, lanes b, c, d, and e, respectively). While these patterns have been consistently observed in analyses of the same sera, and do not therefore appear to result from proteolytic degradation, we did, however, detect differences in the relative staining intensities of the respective bands; the greatest variation in reactivity appeared to involve the band 2 form of apo[a].

Our polyclonal antibody reacted with all six size forms; the reaction was weakest with band 6. Only the serum sample in lane a appeared to lack the band 2 component which comigrated with apoB-100; this band failed to appear in immunoblots using both monoclonal and polyclonal antibodies.

Immunoblotting with antibody 1.8.C4 to apoB-100 confirmed the position of this protein (pattern G).

Cross-reactivity of anti-apo[a] antibodies with plasminogen

Since the extensive homology between plasminogen and apo[a] has been documented recently (27–29), it appeared relevant to evaluate the potential cross-reactivity of our monoclonal and polyclonal antibodies to apo[a] with plasminogen.

Thus, electroimmunoblotting after electrophoresis in SDS-polyacrylamide gradient gels (**Fig. 4**) showed that each of the monoclonal antibodies reacted with apo[a] in serum (Fig. 4; lanes A1, B3, C5, D7, and E9). However, each antibody failed to detect a serum component with M_r similar to plasminogen ($\sim 90{,}000$); the plasminogen content of the serum sample applied to each well was in the range of 0.2–0.5 μg protein. By contrast, when each of these antibodies was blotted to purified plasminogen

TABLE 3. Competition of monoclonal anti-apo[a] antibodies

Monoclonal Antibody	Absorbance (490 nm)					
	14A12	39A1	53A9	73A7	128A6	Polyclonal Antibody
14A12	0.004 ± 0.001	2.803 ± 0.018	2.204 ± 0.063	0.287 ± 0.024	0.450 ± 0.010	3.257 ± 0.090
39A1	0.819 ± 0.036	0.012 ± 0.008	0.012 ± 0.004	1.121 ± 0.041	1.288 ± 0.043	1.241 ± 0.065
53A9	0.775 ± 0.028	0.015 ± 0.005	0.004 ± 0.001	1.185 ± 0.046	1.353 ± 0.026	1.358 ± 0.004
73A7	0.239 ± 0.014	0.721 ± 0.045	0.824 ± 0.012	0.023 ± 0.008	0.000 ± 0.000	0.843 ± 0.097
128A6	0.081 ± 0.008	1.301 ± 0.074	1.110 ± 0.033	0.000 ± 0.000	0.000 ± 0.000	1.473 ± 0.054

Competition studies were performed as described in Methods; microtiter plate wells were first coated with the purified antibody shown at left. Absorbances are means ± SD from a series of five experiments. Values for our polyclonal antibody (shown at right) correspond to absorbances determined in the presence of a single antibody (as indicated at left) to which a preincubated mixture of apo[a] and our polyclonal sheep antibody to apo[a] had been added; the amount of polyclonal antibody bound was revealed by addition of a rabbit antibody to sheep IgG labeled with peroxidase. Color was revealed as before (see "ELISA assay" in Methods). In this way, the absorbance yield for each combination of a monoclonal antibody (indicated at left) with the polyclonal antibody to apo[a] was taken to correspond to combinations of two antibodies whose determinants were independent and noninteracting.

TABLE 4. Immunologically measurable apo[a] in chemically modified apo[a]

Monoclonal Antibody	Treatment			
	Iodoacetamide	Cyclohexanedione	Reductive Methylation	Neuraminidase
	% of starting reactivity			
14A12	100.9 ± 4.8	48.7 ± 4.6	93.7 ± 3.7	98.6 ± 2.5
39A1	98.9 ± 3.7	68.1 ± 4.6	90.6 ± 6.9	99.8 ± 2.1
53A9	97.2 ± 1.8	65.6 ± 3.7	107.0 ± 10.0	99.8 ± 3.4
73A7	99.4 ± 4.4	80.2 ± 2.0	103.8 ± 7.4	99.8 ± 2.4
128A6	108.8 ± 4.4	82.4 ± 3.6	97.1 ± 11.7	94.7 ± 6.1
LHLP1	98.3 ± 6.2	21.3 ± 4.1	110.4 ± 7.2	98.6 ± 7.2

Purified human apo[a] was chemically modified as indicated in Methods. Determination of the immunoreactivity of the modified antigen was made by ELISA assay, and compared to that of the unmodified starting material. Values are means of triplicate determinations ± 1 SD and are taken from a representative experiment.

(Fig. 4; lanes A2, B4, C6, D8, and E10), a faintly stained band could be distinguished with size approximating 90,000 daltons. Finally, the sheep anti-apo[a] polyclonal antibody reacted strongly to reveal both apo[a] and plasminogen bands in whole serum (Fig. 4, lane F11). The strong reaction of this antibody with plasminogen was confirmed by the detection of an intensely stained band of $M_r \sim 90{,}000$ upon reaction with the purified protein (Fig. 4; lane F12).

Dot immunobinding confirmed these results demonstrating the lack of significant reactivity of anti-apo[a] monoclonal antibodies with plasminogen (data not shown); as above, the polyclonal antibody was distinguished by its strong reactivity with both apo[a] and plasminogen, giving an intensely stained dot in each case. For the five monoclonal antibodies, the average lower limit of detection for apo[a] was 31 ± 6.2 ng and for plasminogen 320 ± 29.7 ng. By contrast, the polyclonal antibody detected a similar limiting amount of apo[a] (~28 ng), but was some eightfold more sensitive to plasminogen (40 ng).

In order to evaluate the cross-reactivity of our MABs with apo[a] on the one hand and plasminogen on the other, we undertook competitive inhibition assays (**Table 5**). These data show that the concentrations of plasminogen required for 50% inhibition of the binding of each of the MABs to apo[a] are some 37- to 50-fold greater than those of apo[a] itself. The markedly lower ability of plasminogen than that of apo[a] to compete for binding to anti-apo[a] MABs is thus apparent. A similar ratio (~41:1) for the competitive capacities of plasminogen versus apo[a] was also seen for our polyclonal antibody to apo[a] (Table 5), although in this case, the absolute amounts of apo[a] and of plasminogen required for 50% inhibition were 1.4-fold greater than the average amount of each needed for inhibition of MAB binding.

Enzyme-limited immunoassay of Lp[a]

A calibration curve for the estimation of Lp[a] levels in serum by our ELISA method is shown in **Fig. 5**; this curve was linear in the range of ~1 to 50 ng/ml Lp[a] protein. When plasminogen was estimated by this same MAB-based sandwich assay, the lower limit of detection of this protein was ~100 ng/ml, suggesting a significantly higher sensitivity (~100-fold greater) for Lp[a] over the effective working range of the assay. These results clearly attest to the availability of the epitopes for MABs 14A12 and 53A9 on native Lp[a] which did not interact with a plastic surface.

Our sandwich ELISA assays gave values that were highly correlated with those obtained in the immunonephelometric assay (**Fig. 6**), the correlation coefficient being 0.98. It is noteworthy that values obtained in our ELISA-MAB assay were ~10% lower than those using the immunophelometric method and based on a polyclonal antibody to Lp[a].

DISCUSSION

Considerable interest has developed of late not only in the structure and metabolism of apolipoprotein[a], but also in its potential role in atherogenesis when present as an integral component of the native Lp[a] particle (56). Both the stoichiometric and spatial relationships of the [a] protein to apoB-100 in the Lp[a] particle remain obscure. Furthermore, the remarkable homology between apo[a] and plasminogen (27-29) suggests that overlap of antibody specificities may pose considerable problems in the reliable immunological assay of Lp[a] in plasma or serum when polyclonal antisera are used (2-8, 19, 30). It is for these and other reasons that we have presently developed

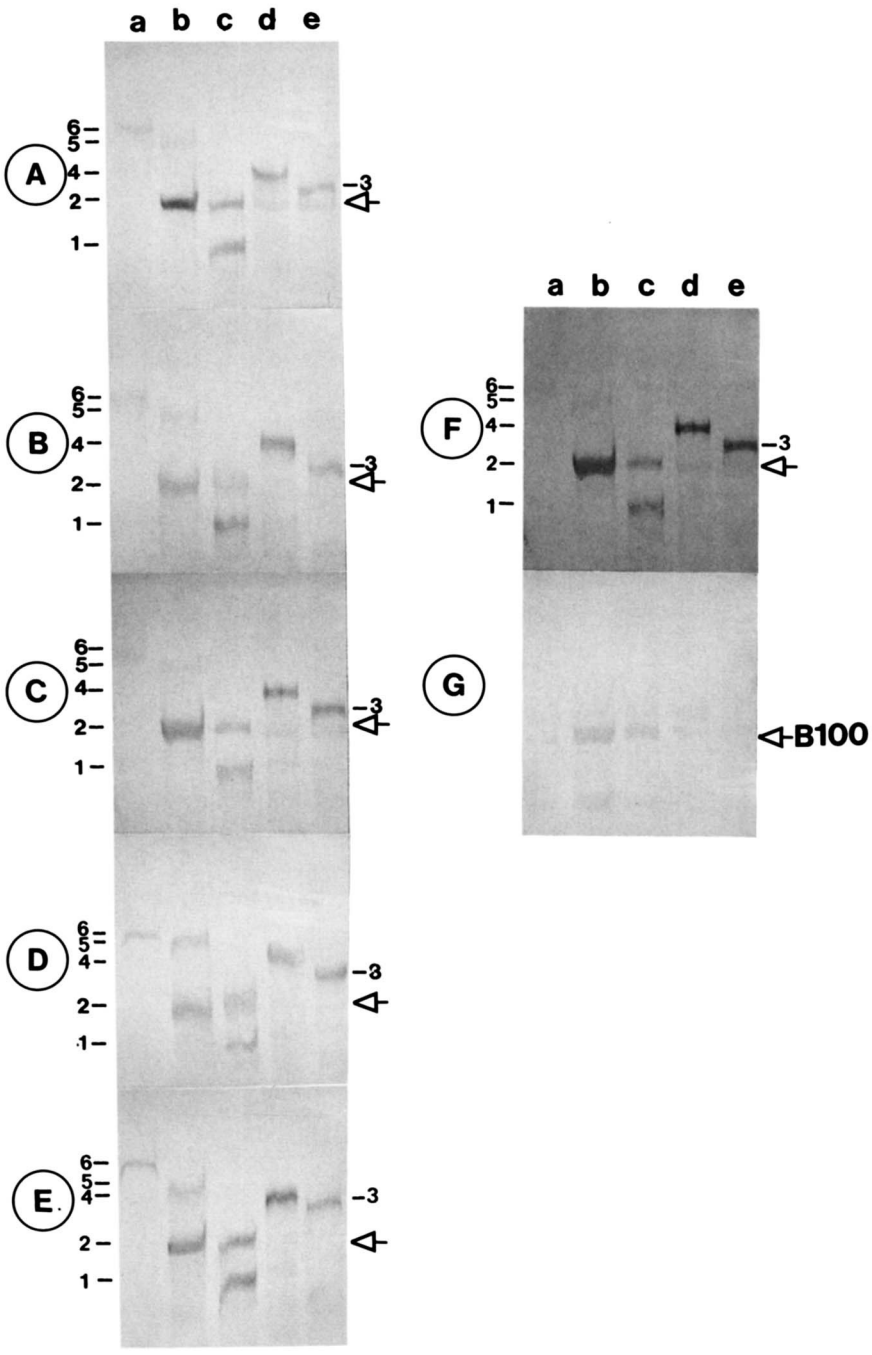

Fig. 3. Electroimmunoblots of apo[a] in sera from individuals with different single- and double-band phenotypes. Serum was reduced with DTT, electrophoresed in SDS-polyacrylamide (2.75%)-agarose (0.5%) gels and electroblotted to nitrocellulose. Apo[a] was revealed by incubation with either monoclonal or polyclonal anti-apo[a] antibodies, followed by a peroxidase-labeled anti-mouse (or anti-sheep) IgG. In patterns A–E, mouse anti-apo[a] monoclonal antibodies were applied and are 14A12, 39A1, 53A9, 73A7, and 128A6, respectively. In pattern F, a sheep polyclonal antibody to apo[a] was used, and in G, a mouse monoclonal antibody to apoB-100 (1.8.C4). Aliquots (2 μl) of sera from five individuals, from different families, were applied to wells a to e. At left are indicated the positions of the different molecular weight forms of apo[a], which are numbered 1 to 6 in order of increasing size; the arrow at the right of each gel indicates the position of apoB-100. Note the differing band intensities given by each monoclonal antibody in patterns A–E.

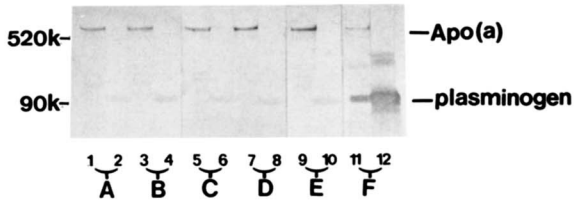

Fig. 4. Comparison of the immunoreactivity of monoclonal and of polyclonal anti-apo[a] antibodies with human apo[a] and plasminogen by electroimmunoblotting. After reduction with DTT, an aliquot of serum (equivalent to 8 μg apo[a] protein) was loaded into wells 1, 3, 5, 7, 9, and 11, and aliquots of purified human plasminogen (15 μg protein) were placed in wells 2, 4, 6, 8, 10, and 12. Electrophoresis was then performed in SDS-polyacrylamide gradient (2–16%) slab gels, and the proteins were electroblotted to nitrocellulose. Patterns A–E were developed with mouse anti-apo[a] monoclonal antibodies 14A12, 39A1, 53A9, 73A7, and 128A6 followed by a peroxidase-labeled mouse anti-IgG; pattern F was developed with a sheep polyclonal antibody to apo[a] and revealed with a peroxidase-labeled anti-sheep IgG.

and characterized a series of five monoclonal antibodies to human apolipoprotein[a].

Our antibodies, 14A12, 39A1, 53A9, 73A7, and 128A6, resembled each other in their reactivities with the various antigens studied; in addition, these reactivities were modulated similarly when the antigen was presented in either soluble or insoluble form. Moreover, our antibodies recognized only one apolipoprotein, i.e., apo[a], binding both the purified protein and that dissociated from Lp[a] by disulfide reduction (Fig. 1). These antibodies lacked significant reactivity with native Lp[a], however, when the antigen was coated onto plastic in ELISA assays (Fig. 1). Nonetheless, they bound to Lp[a] to a sufficient degree to allow its detection in immunoblots of agarose gels (Fig. 2), and in addition, they recognized apo[a] in immunoblots of delipidated Lp[a] from SDS-polyacrylamide gels (Fig. 2). In view of the recognition of native Lp[a] by the monoclonal antibodies in our sandwich-type ELISA assay (Figs. 5 and 6), these findings suggest that adsorption of Lp[a] to plastic either alters epitope structure or renders them inaccessible, thereby diminishing their reactivity. Similar observations have been previously reported, and apparently involve the induction of conformational changes in the antigen upon adsorption to plastic (57).

These patterns of reactivity served to distinguish our five MABs from both LHLP-1 and from our sheep polyclonal antibody to apo[a]. Thus, LHLP-1 failed to recognize apo[a] when presented in free, non-apoB-100-bound form (present data and ref. 22). Only our polyclonal anti-apo[a] antibody then was able to recognize apo[a] in all forms, these being independent of the state of association of apo[a] (free or bound) with apoB-100.

In a first approach to the epitopic mapping of apolipoprotein[a], we applied both cotitration and competition ELISA assays (Tables 2 and 3). While minor differences in the patterns of reactivities were evident in these two experimental systems, we were able to construct an epitopic map (not shown). This map suggested a close spatial relationship of the five epitopes in apo[a], an observation attesting to the high degree of homogeneity of the immunizing antigen. The five epitopes appear to be organized into two groups. The first, comprising 14A12, 73A7, and 128A6, consists of three epitopes that are related to the extent that 128A6 appears to overlap significantly with the epitopes of 14A12 and 73A7; the mutual interaction of 14A12 and 73A7 is less (Table 3). Of this first group, only 14A12 interacts to any significant degree with the second, comprising epitopes 53A9 and

TABLE 5. Comparison of the binding of five monoclonal antibodies and of a polyclonal antibody to human apo[a] and to plasminogen

MAB	IC_{50}		Ratio Plasminogen/Apo[a]
	Apo[a]	Plasminogen	
	μg/ml		
14A12	25 ± 2.3	950 ± 14.6	38:1
39A1	28.5 ± 1.9	1412 ± 29.7	49.5:1
53A9	31 ± 3.2	1304 ± 110.2	42:1
73A7	20.5 ± 2.8	780 ± 50.3	38:1
128A6	26.5 ± 4.2	978 ± 28.8	37:1
Polyclonal antibody	36.5 ± 5.3	1510 ± 131.6	41:1

Binding analyses were performed by competitive ELISA (see Methods). Plastic (96-well) microtiter plates were coated with 100 μl of purified apo[a] at a concentration of 1 μg/ml in coating buffer (0.05 M Na_2CO_3, pH 9.6). For measurement of antibody reactivity, 50-μl aliquots of solutions containing a range of concentrations of apo[a] or plasminogen and 50 μl of antibody solution (3 μg protein/ml) were added to each well. Results, derived from antibody displacement curves and expressed as IC_{50}, correspond to the amounts of protein required for 50% inhibition of antibody binding. Values are means ± SD, n = 3.

39A1. Slight interaction may occur however between 39A1 and 73A7 (Tables 2 and 3). Epitopes 39A1 and 53A9 show a high degree of overlap, and in addition, possess arginine residues in their determinants (Table 4) as suggested by chemical modification. The comparable reduction in the binding of the 14A12 epitope upon reductive methylation is consistent with the contribution of lysine residues to this epitope and in addition to those of 39A1 and 53A9. The epitope recognized by MAB 128A6 appears to be entirely independent and distinct from the determinants of both 53A9 and 39A1. Finally, despite the high proportion of carbohydrate in Lp[a] and apo[a] (9, 13–17), removal of sialic acid was without effect on the immunoreactivity of the latter (Table 4).

The recognition of apo[a] by our MABs was not limited to one molecular weight form, i.e., that used as the immunizing antigen ($M_r \sim 570{,}000$) but rather they reacted with all six major size forms detected in a random sampling of sera from normo- and hypercholesterolemic subjects (Fig. 3). Of these six forms, one (denoted band 1) was smaller in size than apoB-100, while a second appeared to comigrate with apoB-100 (i.e., band 2, Fig. 3); components in bands 3–6 all displayed lower electrophoretic mobilities and thus higher molecular weights than apoB-100. These data generally confirm the recent findings of Utermann et al. (26), who similarly found apo[a] phenotypes with comparable mobilities to apo[a] in SDS-polyacrylamide gels.

In order to define more precisely the potential applications of our MABs to the study of apolipoprotein[a], we compared their reactivity with apo[a] to that with the homologous protein, plasminogen. Thus, while our MABs reacted strongly with apo[a] in Western blots of serum samples electrophoresed in SDS-polyacrylamide

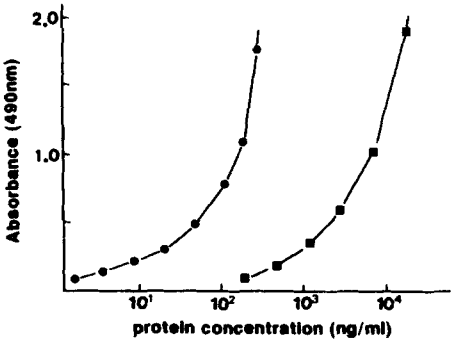

Fig. 5. Representative curve for quantitation of human Lp[a] by an MAB-based sandwich ELISA assay (see Methods), and evaluation of the cross-reactivity of plasminogen in this assay. Data points are the means of duplicate determinations from two separate assays performed simultaneously; symbols are Lp[a] (●) and plasminogen (■). Absorbance is plotted on the ordinate against Lp[a] and against plasminogen concentrations (ng/ml) on the abscissa on a log scale.

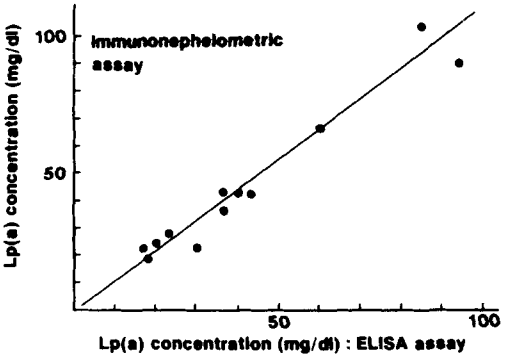

Fig. 6. Comparison of the quantitation of Lp[a] by an MAB-based ELISA assay and by an immunonephelometric method (see Methods). Serum samples (n = 12) were selected to contain a wide range of Lp[a] concentrations (~15–95 mg/dl).

gels, they failed to detect the small amounts (0.2–0.5 μg) of plasminogen present (Fig. 4, lanes 1A, 3B, 5C, 7D, and 9E). Furthermore, only a faint reaction of our MABs with this plasma protein was visible when larger amounts (15 μg) of plasminogen were present (as the purified protein) (Fig. 4, lanes 2A, 4B, 6C, 8D, and 10E). By contrast, our polyclonal antibody to apo[a] reacted strongly with plasminogen, either as a component of serum or as the purified protein (Fig. 4, F11 and 12).

We then proceeded to quantitate the cross-reactivity of these MABs with plasminogen by competitive ELISA assay (Table 5). These data clearly demonstrated that the ratio of the reactivity of our antibodies with apo[a] on the one hand, and with plasminogen on the other, was in the range of 37:1 to 50:1. In the case of antibody 39A1, a 50-fold lesser amount of apo[a] antigen than of plasminogen was required for 50% inhibition of antibody binding (Table 5). Larger amounts of each antigen, i.e., apo[a] and plasminogen, were required for 50% inhibition of the binding of the polyclonal antibody to apo[a] as compared to that for each MAB, thereby attesting to the lower average affinity of the various antibody molecules in this mixture as compared to the higher binding affinity of each MAB. These findings also reflect the presence in the polyclonal preparation of antibodies to numerous distinct antigenic determinants in apo[a], and which would be expected to require greater amounts of this antigen (or of plasminogen) to attain 50% inhibition of antibody binding.

The apparent inconsistency between our ELISA data (Table 5) and that on immunoblotting (Fig. 4F) for the binding of the polyclonal antibody to plasminogen and to apo[a] might be explained on the basis of a higher reactivity of plasminogen in the reduced and denatured form with this antibody (Fig. 4F). Thus, denatured plasminogen may express epitopes that are recognized more readily by the polyclonal rather than by the monoclonal antibodies, so producing a band of greater intensity as compared to that of the apo[a] band (Fig. 4).

Clearly the five epitopes that we have presently detected are distinct from the determinant of LHLP-1 (22). Their expression is independent of the cleavage of the disulfide bond(s) linking apo[a] to B-100, and thus they recognize both free, unbound apo[a] as well as apo[a] in native Lp[a]. Expression of these epitopes is, however, markedly diminished by the interaction of Lp[a] with plastic surfaces, a finding that necessitated our development of a sandwich-type ELISA assay for quantitation of native Lp[a]. The precise identification of the epitopes in apo[a] that are specific to our MABs is currently under investigation.

In conclusion, our monoclonal antibodies have provided the basis for a highly specific and accurate quantitative assay of Lp[a] in biological samples, and by selection of the appropriate dilution, can be free of interference from plasminogen. In addition, our antibodies should permit precise determination of the stoichiometry of apo[a] to apoB-100 in different Lp[a] particles, and particularly those containing different isoforms of apo[a].

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