Immunochemistry of human Lp[a]: characterization of monoclonal antibodies that cross-react strongly with plasminogen

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Abstract Forty different monoclonal antibodies were produced from hybridomas that were raised against human Lp[a]. Of these, 14 strongly cross-reacted with plasminogen on ELISA screening assays while 16 clearly did not and 10 were only marginally cross-reactive. We took advantage of the homology between plasminogen and apo[a] to define the epitopes of 8 strongly cross-reacting monoclonal antibodies. We were able to subdivide these into four general categories based upon site competition assays (using both plasminogen and Lp[a]), and their reactivity with elastolytically derived plasminogen fragments. Group A monoclonal antibodies (F1 1E3, F2 3A3) recognized epitopes within the kringle 5 and protease domains (miniplasminogen) of plasminogen. The group B monoclonal antibody (F6 1A3) reacted solely with plasminogen kringle 4-like domains and appeared to recognize a limited number of sites on Lp[a]. Group C monoclonal antibodies (F6 1B5, F6 1G9) recognized a second, more frequently distributed site within these kringle 4-like domains. The final group, D, monoclonal antibodies (F6 2C3, F6 2G2, F6 3F4) reacted with a cluster of sites found associated with kringle 4-like domains but also reacted with the miniplasminogen domain. Interestingly, only the members of this group were able to interfere with the proteolytic activity of plasmin. Neither periodate treatment of Lp[a] nor incubation of Lp[a] with ϵ-aminocaproic acid affected the binding of any of our monoclonal antibodies. —**Lafferty, M. A., A. M. Salamon, and D. C. Usher.** Immunochemistry of human Lp[a]: characterization of monoclonal antibodies that cross-react strongly with plasminogen. *J. Lipid Res.* 1991. **32:** 277–292.

Supplementary key words lipoprotein[a] • apolipoprotein[a]

The discovery of a significant correlation between Lp[a] and the risk of atherosclerotic disease (1–4) has resulted in an intense effort to characterize and examine this lipoprotein. Much of this research has been in characterization of apolipoprotein[a], the distinguishing moiety between LDL and Lp[a] which is thought to be responsible for the atherogenic nature of Lp[a] (5–8).

Amino acid and cDNA sequencing of apo[a] have shown that apo[a] has remarkable homology to plasminogen (7, 8), a 92 kDa blood plasma serine protease that has a crucial role in fibroinolysis (9, 10). Plasminogen is comprised of five domains called kringles, and a prospective serine protease domain that is responsible for the dissolution of fibrin clots (10). The homology between plasminogen kringle domains is approximately 40% and all contain six conserved Cys residues that form three disulfide bonds. These disulfides help to stabilize the characteristic triple loop structure of the kringles (8, 10).

The one apo[a] gene that has been sequenced was found to contain two types of plasminogen-like kringle domains and a serine protease domain. The amino terminus of this apo[a] isoform contains 37 repeats of a 114 amino acid domain that are 75% to 85% homologous to plasminogen kringle 4 (7, 8). The 37 domains have been further divided into 11 different groups based on size, sequence homology, and ligand binding (benzamidine, ω-aminocarboxylic acids). One of these groups, apo[a] kringle type 2, is repeated up to 27 times with 100% homology, while apo[a] kringle types K1 and K3–K11 are found in single copies (7, 8, 11). Following the repeated kringle 4-like domains is a single domain that is 91% homologous to plasminogen kringle 5 (7, 8) and a carboxyl terminal serine protease domain that is 94% homologous to the protease of plasminogen (7, 8). Within the protease domain the common serine protease catalytic triad of Ser, His, and Asp is conserved. However, activators of plasminogen (urokinase, streptokinase, and t-Pa) fail to activate apo[a] due to a critical amino acid change involving the replacement of Arg_{560} by Ser (7, 8).

The finding of several isoforms of apo[a], with sizes ranging from approximately 300,000 to 900,000 daltons,

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; apo, apolipoprotein; Lp[a], lipoprotein[a]; tPa, tissue plasminogen activator; PBS, phosphate-buffered saline.

has resulted in speculation that the variation is size may be due to the number of kringle 4-like repeats found in apo[a] isoforms (7, 8, 11–15). To account for the size variations of apo[a], the ratio of kringle 4 to kringle 5-like domains would be expected to range from approximately 15 for the lower isoforms to 40 for the higher isoforms. In one study, DNA probes were used to determine the ratios of kringle 4 to kringle 5-like domains of human apo[a] by quantitative Southern blot analysis (12). The ratios of kringle 4 to kringle 5 correlated with apo[a] apparent molecular weight (12). However, the ratios were considerably lower than expected, ranging from 0.7 to 15.7. In other studies, a correlation between messenger RNA size and serum isoform size has been found in both human and baboon hepatic apo[a] mRNA by Northern blot analysis. Koschinsky et al. (15) examined liver samples from seven human patients and found that transcript sizes from 8.0 to 12 kb correlated with apo[a] isoforms ranging from 590 to 850 kDa. Likewise, data collected on 22 baboons led Hixson et al. (13) to conclude that variations in apo[a] glycoprotein isoform sizes (ranging from 350 kDa to 800 kDa) were due to structural differences in apo[a] transcripts (6.9 kb to 11.2 kb). These studies acknowledge that post-translational modifications may also contribute to the variations in the mass of apo[a] isoforms (12, 13, 15).

A further understanding of the structure of Lp[a] has been attempted using immunochemistry (7, 11, 14, 16, 17). Eaton et al. (7) has shown that a polyclonal antiserum to apo[a] cross-reacts with plasminogen, while a polyclonal antiserum to plasminogen and one monoclonal antibody to the kringle 4 domain of plasminogen both cross-react with apo[a]. Guo et al. (16) raised five monoclonal antibodies against purified apo[a]. All five antibodies were found to react strongly against purified apo[a] and yet had limited reactivity with plasminogen. These antibodies also had limited reactivity with native Lp[a] in ELISA. Furthermore, the binding sites of these antibodies were not affected by amino acid modifications or carbohydrate removal (16). In another study, Duvic et al. (17) isolated a monoclonal antibody raised against Lp[a] (17). This antibody was shown to cross-react with plasminogen and Lp[a] but not with apo[a] when it is dissociated from Lp[a] (16, 17). The authors suggest that it recognizes a particular conformational epitope that requires intact disulfide bonds (17). These results show, as expected, that the immunological characteristics of plasminogen and apo[a] are related.

To gain further insight into the binding requirements of monoclonal antibodies raised against Lp[a], we directed our attention to antibodies that strongly cross-react with Lp[a] and plasminogen. We took advantage of the homology between plasminogen and apo[a] to define the epitopes of eight monoclonal antibodies through the use of elastolytically derived plasminogen fragments.

METHODS

Antisera

Rabbit anti-human Lp[a] antiserum was purchased from Calbiochem. This antibody, when tested by ELISA and Western immunoblot analysis, did not react with apoB. Goat anti-plasminogen antiserum was purchased from Sigma. The monoclonal antibody to the carbohydrate of *Chlamydomonas* flagellar protein was a gift from R. A. Bloodgood, Dept. of Anatomy, University of Virginia School of Medicine.

Isolation of lipoproteins and purification of apo[a]

Plasma was obtained from previously screened donors by plasmapheresis. Na_2EDTA (0.01%), sodium azide (0.02%), and Trasylol (100 kallikrien inhibiting units (KIU)/ml) (Mobay Chemicals) were later added to the plasma.

Lp[a] was isolated using the method of Fless, Zum-Mallen, and Scanu (5) with slight modifications. The density of the plasma was adjusted to 1.21 g/ml by the addition of solid NaBr. The adjusted plasma was then centrifuged at 55,000 rpm at 15°C for 48 h. The lipoprotein layer was collected and adjusted to a density of 1.41 g/ml with solid NaBr. A 5-ml aliquot of lipoprotein was placed into a 25-ml polycarbonate ultracentrifuge tube and 20 ml of a 0–30% NaBr gradient containing 0.01% Na_2EDTA and 0.02% azide was layered over it. The gradient was spun in the Ti60 rotor at 55,000 rpm and 15°C for 90 min. Fractions from the gradient were collected while continuously monitoring the protein content. The fraction containing VLDL, LDL, and Lp[a] was dialyzed against three changes of 0.01% Na_2EDTA and 0.02% azide in distilled water, and then adjusted to 7.5% CsCl (w/w) with solid CsCl. After centrifugation at 55,000 rpm at 15°C for 24 h, the Lp[a]-containing fractions from the gradient were pooled and dialyzed against phosphate-buffered saline, pH 7.4, containing 0.01% Na_2EDTA and 0.02% azide and 100 KIU/ml of Trasylol. The purity of Lp[a] was determined by SDS-PAGE.

Preparation of plasminogen fragments

Digestion of plasminogen with elastase was carried out as described by Sottrup-Jensen et al. (10). Human plasminogen (14.5 mg) (Sigma) was dissolved in 0.1 M NH_4HCO_3, pH 8.3, containing 7000 units of Trasylol to prevent the conversion of plasminogen into an active form. The molarity was raised to 0.3 M with NH_4HCO_3 prior to the addition of 450 μg of elastase. The reaction was terminated with 0.1% phenylmethylsulfonyl fluoride after incubation for 3 h and 15 min. The molarity of the carbonate buffer was then raised to 0.55 M with NH_4HCO_3, stirred for 30 min, and lyophilized overnight.

Limited elastase digestion of plasminogen resulted in four cleavage products: the amino terminal prepeptide (7

kDa); lysine binding site I (kringles 1–3, 33–39 kDa); lysine binding site II (kringle 4, 12 kDa); and miniplasminogen (k5-protease, 38 kDa), which could be further separated by lysine–Sepharose column chromatography. The elastase digestion products were redissolved in 5 ml of 0.1 M NH_4HCO_3, pH 8.3, and applied to a lysine–Sepharose column equilibrated to 0.1 M NH_4HCO_3, pH 8.3. After collection of the flow-through volume, which contained miniplasminogen, the column was washed thoroughly and lysine-binding sites I and II were eluted with 0.2 M ϵ-aminocaproic acid. Although gel filtration column chromatography was used to further purify lysine-binding site I from lysine-binding site II (10), we also purchased these fragments from Sigma. The individual fractions were then aliquoted and lyophilized overnight. The purity of the fractions was examined by discontinuous SDS-PAGE using lysine-binding sites I and II (Sigma) and prestained protein molecular weight markers (66,000–11,200 kDa, Sigma) as standards.

Production of monoclonal antibodies

Balb/c mice were injected initially with 200 μg Lp[a] in complete Freund's adjuvant. This intraperitoneal injection was followed 2 weeks later with another in incomplete Freund's. Thereafter the mice were boosted at 3-week intervals with Lp[a] in saline until sera taken from the mice tested positive against Lp[a] in ELISA. Mice were then given a final intravenous saline Lp[a] injection 4 days prior to the fusion.

The fusions were carried out according the method of Kennett (18) using 5×10^7 myeloma cells (X63-Ag8.653) and $1–2 \times 10^8$ spleen cells. After fusion, cells were resuspended in Iscove's Modified Dulbecco's Medium (GIBCO) supplemented with pyruvate (50 mg/ml), thymidine (7.6 mg/ml), aminopterin (0.18 mg/ml), and hypoxanthine (13.6 mg/ml), and then distributed to wells of 96-well microtiter plates (Falcon). Approximately 2 weeks later, when colonies were visible, the medium was tested by ELISA against Lp[a], VLDL, and plasminogen. We chose to screen for B-100 cross-reactivity with a VLDL fraction rather than a LDL fraction because it was less contaminated with apo[a].

Lp[a]-reactive hydridomas were selected and grown in 24-well microtiter plates. If the hybridomas again tested positively, they were cloned by limiting dilution in Iscove's medium supplemented with thymidine and hypoxanthine. Only wells containing single colonies that tested positive were selected for further cloning. The stability of a hybridoma was determined after the third round of cloning be testing 48 wells containing 1–3 colonies; all 48 wells had to contain Lp[a]-positive clones. One was then chosen for further study.

Ascites fluid, the source of IgG, was produced by the method of Mueller, Hawes, and Jones (19). Mice were primed with compete Freund's adjuvant 2 to 3 days prior to being injected intraperitoneally with 10^6 hybridoma cells in 0.5 ml saline. Approximately 10 days later the ascites was drawn and Na_2EDTA (0.01%), sodium azide (0.02%), and Trasylol (100 kallikrien inhibiting units (KIU)/ml) (Mobay Chemicals) were added. The ascites was then clarified by low speed centrifugation and stored at 4°C or −90°C.

Quantitation of IgG found in the ascites was determined by radial immunodiffusion assay. Five ml of 2% agarose in saline borate buffer (138 mM NaCl, 163 mM boric acid, 35 mM NaOH, pH 8.0) was boiled and allowed to cool to 55°C. After cooling, 5 ml of prewarmed (55°C) saline borate buffer containing 250 μl of goat anti-mouse sera (Miles Scientific) was added and the mixture was used to coat radial immunodiffusion plates (Millipore). The plates were allowed to cool for 1 h at room temperature and holes were cut with a 2.0-mm O.D. punch (LKB). Serial dilutions (one-half fold) of purified IgG (Miles Scientific), starting at 12 μg per well, were used to generate a standard curve. The concentration of antibody in our ascites was determined by applying 5 μl of a 1:10 dilution of each of our ascites per well. These were allowed to diffuse for 5 days at 4°C. The plates were then dialyzed against normal saline (0.85%) for 3 days with multiple changes. On the fourth day the plates were dialyzed against distilled water with two changes. The plates were then allowed to dry at room temperature. Once dried the plates were incubated with Coomassie Blue for 30 min, followed by destaining (5% methanol, 7% glacial acetic acid) until the precipitation bands were visible. The areas of the precipitation rings were measured and areas of unknown samples were extrapolated from the curve generated by our standards. Each test contained an ascites from mice bearing myeloma MOPC 21 (IgG1, κ). The amount of IgG in this ascites was determined by another method. This sample served as an internal control.

Biotinylation of antibodies

Biotinylation of antibodies was done according to manufacturer's instructions (BRL). Antibody-containing solutions, ascites or serum, were adjusted to pH 9.0 by the addition of 0.5 M sodium carbonate buffer at pH 9.0. Biotin-N-hydroxysuccinimide ester in dimethylformamide (100 mg/ml) was added to give a biotin-N-hydroxysuccinimide ester to protein ratio of 1:2 (wt:wt). The solution was gently mixed for 4 h at room temperature. The reaction mixture was then dialyzed overnight against PBS to remove unreacted biotin-N-hydroxysuccinimide ester. Titration of biotinylated antibodies was carried out by ELISA (see below). Additionally, nonspecific absorbance of biotinylated antibodies was prevented by preincubation of Immulon I 96-well microtiter plates (Dynatech, Alexandria, VA) in PBS which contained 1% casein, 0.1 mM EDTA at room temperature for 1–2 h.

ELISA

Titration of all antibodies was carried out on Immulon I 96-well microtiter plates (Dynatech, Alexandria, VA). Wells were coated with 500 ng of antigen in 100 μl of carbonate-bicarbonate buffer, pH 9.6. After an overnight incubation at 4°C, the coated plates were washed with PBS containing 0.1% Tween 20 (PBS Tween) using a Flow Laboratories microtiter plate washer. The monoclonal antibodies were serially diluted in PBS Tween the 100 μl of each dilution was added to triplicate wells of the plates. After another overnight incubation at 4°C, the plates were washed and 100 μl of an appropriately diluted secondary antibody, either rabbit anti-mouse IgG, human anti-goat IgG, or goat anti-rabbit IgG labeled with alkaline phosphate (Sigma), was added to the wells. (Biotinylated antibody titrations were done with avidin-conjugated alkaline phosphatase (Sigma).) After a 2-h incubation at 4°C, the plates were again washed and 1 mg/ml *p*-nitrophenol phosphate substrate solution was added. The *p*-nitrophenol phosphate (Sigma) was dissolved in a buffer containing 1% diethanolamine, 1 mM $MgCl_2 \cdot 6H_2O$, and 3 mM NaN_3, pH 9.8. Thirty minutes later the absorbance (405 nm) was measured on a Dynatech MR 5000 automated plate reader. Control wells were included on every plate to assure that there was not any nonspecific absorption of the primary or secondary antibodies.

The appropriate titer of individual antibodies was determined by finding the inflection point of the absorbance curves based on the average of three trials.

Competition ELISAs were carried out by serially diluting a competitor (Lp[a], plasmin, plasminogen, LDL, miniplasminogen, lysine-binding site I, lysine-binding site II, or ϵ-ACA) in PBS-Tween. After addition of 50 μl of these dilutions to Immulon I microtiter plates coated with either plasminogen or Lp[a] at 5 μg/ml, 50 μl of a constant amount of appropriately titered monoclonal antibody was added. After overnight incubation at 4°C, the secondary antibody and substrate were added as described previously.

Site competition assay

Antibody blocking assays were performed on 96-well microtiter plates by a modified version of the assay described by Oi and Herzenberg for ELISA (20). After preincubation with PBS which contained 1% casein, 0.1 mM EDTA at room temperature for 1–2 h, unlabeled monoclonal antibody was serially diluted in 1% casein, 0.1 mM EDTA, and 0.1% Tween-20 (casein diluent), and 100 μl was added to triplicate wells of either Lp[a]- or plasminogen-coated plates.

After overnight incubation at 4°C, the plates were washed and 100 μl of appropriately diluted biotinylated antibody was added in casein diluent for a 90-min incubation at 4°C. The plates were then washed and avidin-conjugated alkaline phosphatase (Sigma) was added to each well and incubated for 90 min at 4°C. After this incubation, the plates were washed a final time and substrate was added. Absorbance readings (405 nm) were taken 30 min later.

Polyacrylamide gel electrophoresis and immunoblotting

Most proteins were analyzed using 20% T: 0.5% C (acrylamide: bis acrylamide) gels in 0.075 M Tris (2-amino-2-hydroxymethylpropane-1,3-diol), 0.16 M glycine, 0.1% SDS at pH 8.3. All samples were heated at 90°C for 5–10 min in sample buffer containing 0.075 M Tris, 15% glycerol, 0.0005% bromophenol blue, 0.1% SDS with (reducing gels) or without (nonreducing gels) 4% mercaptoethanol. Prestained molecular markers were used as standards. Gels were run at a constant 20 mAMP at 4°C until the dye front reached the bottom of the gel. Analytical gels were then fixed and stained with either Coomassie Blue R250 or silver (21).

Electroblotting from polyacrylamide gels to nitrocellulose membrane (Bio-Rad) was carried out in a TransBlot apparatus (Bio-Rad) using 0.075 M Tris, 0.16 M glycine, pH 8.3, at 30 volts overnight at 4°C. The membrane was then cut into 3-mm-wide strips for reaction with various antibodies or for staining with India ink (22).

Membrane strips to be analyzed with antibodies were blocked for 2 h at room temperature with 10% Carnation low-fat powdered milk in Tris-buffered saline, pH 7.5 (TBS-milk). The antibody was appropriately diluted in TBS-milk containing 0.3% Tween 20 and then incubated with membrane strips overnight at 4°C. Unbound antibody was removed by three 10-min washes of TBS-Tween. The strips were then incubated with anti-mouse antiserum conjugated to horseradish peroxidase (Bio-Rad) diluted in TBS-milk containing 0.3% Tween 20 for 2 h at room temperature. The strips were washed and developed using HRP-Reagent (4-chloro-1-naphthol) (Bio-Rad).

Isotyping of Lp[a] was accomplished using a Mini Protean II gel apparatus (Bio-Rad) for SDS-PAGE. The use of piperazine diacrylamide (PDA, Bio-Rad), in place of bis-acrylamide and Tris-citrate running buffers, gave us our most consistent results. Thus, Lp[a] was electrophoresed using a separating gel that consisted of 4% T: 2.7% C (polyacrylamide (Sigma): PDA) in 0.19 M Tris, 0.018 M citrate, and 0.2% SDS and a 3.5% T: 2.7% C stacking gel in 0.0375 M Tris, 0.0045 M citrate, and 0.2% SDS. The tank buffer contained 0.065 M Tris, 0.013 M boric acid, 2 mM EDTA, and 0.2% SDS.

Determination of enzyme activity of plasminogen and miniplasminogen

To determine whether the zymogens, plasminogen and miniplasminogen, were activated we used Chromozym PL® (Boehringer Mannheim). Microtiter plates were

coated at 5 μg/ml with either plasminogen (Sigma), miniplasminogen, kringle 4 (Sigma), or plasmin (Boehringer Mannheim). The plates were washed 3 times using solution 1 (50 mM Tris, 100 mM NaCl, pH 8.2). After washing, 64 μl of solution 1, 8 μl of a 0.9% NaCl solution, and 8 μl of a solution that contained 0.5% (w/v) polyethylene glycol 6000, 50 mM glycine, pH 2.5, were added to each well. Sixteen μl of Chromozym PL®, dissolved at 1.9 mg/ml in a solution that contained 100 mM glycine, 0.02% Tween 20, was then added to each well. *p*-Nitraniline formation was monitored at 405 nm.

Reduction and alkylation of plasminogen

Plasminogen (10 mg/ml) was incubated with 50 mM DTT at room temperature for 2 h. Nitrogen was then passed through the reaction mix for 30 min. The solution was made up to 0.12 M iodoacetate, put on ice for 1 h, and dialyzed against PBS-Tween overnight at 4°C. The efficiency of the reduction was monitored by SDS-PAGE.

Monoclonal antibody ability to interfere with the active site of plasmin

The ability of our monoclonal antibodies to interfere with the proteolytic activity of plasmin was investigated by measuring the ability of plasmin to convert Chromozym PL® to a colored product in the presence of varying amounts of monoclonal antibody. Immulon I plates were coated with 5 μg/ml of plasmin in 100 μl of carbonate-bicarbonate buffer, pH 9.6. After an overnight incubation at 4°C the plates were washed 3 times using solution 1 (50 mM Tris, 100 mM NaCl, pH 8.2). The monoclonal antibodies were serially diluted in solution 1 (50 mM Tris, 100 mM NaCl, pH 8.2) and 100 μl of each dilution was added to triplicate wells. After an overnight incubation at 4°C the proteolytic activity of plasmin was determined as described earlier. The binding of monoclonal antibody binding to plasmin was determined using alkaline phosphatase-conjugated anti-mouse antibody, as previously described. All assays were performed in triplicate.

Periodic acid-treated ELISA plates

To determine whether any of the antibodies were specific for a carbohydrate epitope, either Lp[a] or plasminogen bound to wells of ELISA plates was treated with periodic acid by the method of Woodward, Young, and Bloodgood (23) prior to the addition of the antibody. The antigen was incubated in 50 mM sodium acetate buffer, pH 4.5, for 30 min at room temperature. Half of the samples were subjected to 20 mM periodic acid in acetate buffer, pH 4.5, for 1 h at room temperature; the other half was left in acetate buffer alone. At the end of the treatment, all the samples were incubated for 30 min at room temperature with 50 mM sodium borohydride in PBS to stop the reaction. After washing, the antibody to be tested was serially diluted and added to treated and untreated triplicate wells.

RESULTS

Identification of monoclonal antibodies with reactivity to plasminogen and Lp[a]

From a series of four fusions we isolated 40 stable hybridomas, with each fusion yielding an average of 7 stable hybridomas per 10^8 spleen cells. Medium from cultures of the 40 hybridomas were tested by ELISA on plates coated with Lp[a]-negative VLDL, Lp[a], or plasminogen. Antibody in the medium from 16 hybridoma cultures reacted only with Lp[a]. The medium from 10 hybridomas had moderate cross-reactivity with Lp[a] and plasminogen while 14 showed strong cross-reactivity between the two. None of the 40 reacted with VLDL. Eight monoclonal antibodies that showed strong cross-reactivity between Lp[a] and plasminogen were the subject of this study.

In order to assure that the monoclonal antibodies we chose were reacting to apo[a] and not apoB-100, we challenged each with serially diluted LDL or Lp[a] on microtiter plates coated with Lp[a]. Competition curves, similar to those illustrated in **Fig. 1**, were attained for all eight monoclonal antibodies. Binding of each of the monoclonal antibodies was inhibited by Lp[a] but not by LDL, which contains B-100 but not apo[a]. These experiments suggest that the binding was to the apo[a] portion of Lp[a].

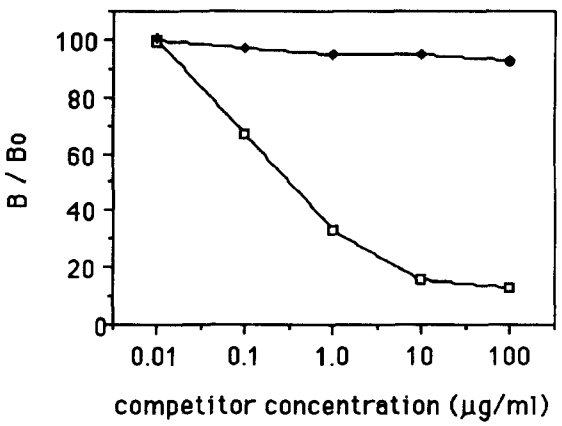

Fig. 1. Analysis of monoclonal antibody specificity by direct competition assays using LDL and Lp[a]. Serially diluted LDL or Lp[a] was applied to microtiter plates coated with Lp[a] at 5 μg/ml. A constant concentration of F6 1A3 monoclonal antibody was then added and incubated overnight. Antibody that bound to the Lp[a]-coated plates was detected using anti-mouse alkaline phosphatase. The results depicted represent the average of three trials and are typical of the response found for all of the monoclonal antibodies in this study. Monoclonal F6 1A3 results are illustrated here; (♦), LDL; (□), Lp[a].

To further confirm the specificity of each antibody, we examined them by Western immunoblot analysis. Plasminogen, LDL, and Lp[a] were first separated on SDS polyacrylamide gels under reducing and nonreducing conditions and then electroblotted to nitrocellulose membrane. Monoclonal antibody F6 1D1, previously shown not to cross-react with plasminogen (Usher, D. C., S. Silberman and F. Vella, unpublished results) and polyclonal antibodies against Lp[a], LDL, and plasminogen were included as controls.

While all monoclonal antibodies recognized apoB-100 under either conditions (**Fig. 2, Table 1**), none of the antibodies recognized apoB-100 under either condition. The reactions to plasminogen varied. F6 1A3, F6 2G2, F2 3A3, and F6 3F4 recognized plasminogen under both reducing and nonreducing conditions, while F6 1B5, F1 1E3, and F6 1G9 failed to recognize plasminogen under either condition. Monoclonal antibody F6 2C3 only recognized plasminogen under nonreducing conditions. Interestingly, while the goat polyclonal antibody specific for plasminogen could recognize both reduced and nonreduced plasminogen, the rabbit polyclonal antibody specific for apo[a] was only able to recognize nonreduced plasminogen.

Due to the variation of the responses of our monoclonal antibodies to plasminogen in Western blot analysis we decided to investigate the ability of plasminogen to compete for our antibodies on Lp[a]-coated plates. To assure that the plasminogen used in these assays was not activated into its proteolytic form, we examined the ability of plasminogen to cleave Chromozym PL® (Boehringer

TABLE 1. Summary of Western blot analysis using Lp[a] and plasminogen

	Lp[a]		Plasminogen	
Antibody	Nonreduced	Reduced	Nonreduced	Reduced
Pab-Lp[a]	+	+	+	–
Pab-Pg	+	+	+	+
F6 1D1	+	+	–	–
F6 1A3	+	+	+	+
F6 1B5	+	+	–	–
F1 1E3	+	+	–	–
F6 1G9	+	+	–	–
F6 2C3	+	+	+	–
F6 2G2	+	+	+	+
F2 3A3	+	+	+	+
F6 3F4	+	+	+	+

Lp[a] and plasminogen were separated on SDS polyacrylamide gels under reducing and nonreducing conditions and then electroblotted to nitrocellulose membrane. Strips of nitrocellulose were incubated overnight at 4°C with a 1:1000 dilution of the antibody to be tested. F6 1D1, a previously characterized monoclonal antibody that does not cross-react with plasminogen was induced as a control. Pab-Lp[a]: polyclonal antibody specific for Lp[a]; Pab-Pg: polyclonal antibody specific for plasminogen; (+): positive reaction; (–): no reaction.

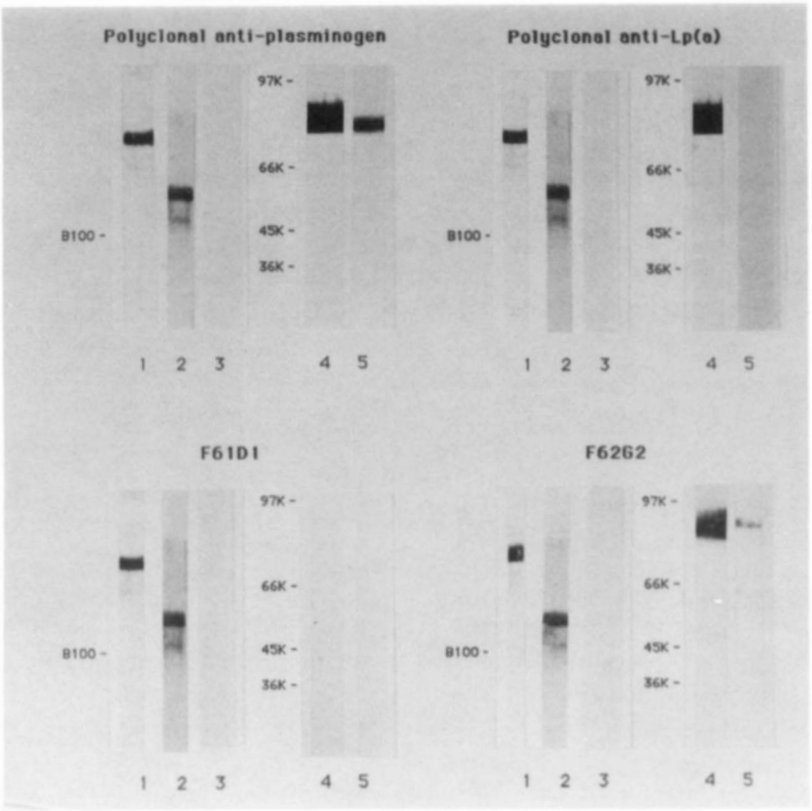

Fig. 2. Western blot analysis of Lp[a], LDL, and plasminogen under both reducing and nonreducing conditions. Reduction was accomplished by heating samples at 90°C for 10 min in sample buffer containing 4% mercaptoethanol. Lp[a] and LDL were electrophoresed in a 4% SDS-polyacrylamide gel and electroblotted to nitrocellulose as described in Methods. The migration position of apoB-100 in such gels is indicated at the left. Plasminogen samples were electrophoresed in a 20% SDS-polyacrylamide gel and electroblotted to nitrocellulose. Prestained molecular markers were used as standards. Three-mm-wide strips of nitrocellulose transfer sheets were incubated with a 1:1000 dilution of the polyclonal or monoclonal antibody listed above each section. The specific antigen was revealed by incubation with horseradish peroxidase-labeled anti-mouse antiserum for our monoclonal antibodies, by anti-goat antiserum for the polyclonal anti-plasminogen, or by anti-rabbit antiserum for the polyclonal anti-Lp[a]. The strips were developed using HRP-Reagent (4-chloro-1-naphthol). The lane designations are: 1, nonreduced Lp[a]; 2, reduced Lp[a]; 3, nonreduced LDL; 4, nonreduced plasminogen; 5, reduced plasminogen. The antibody tested on the plots is indicated at the top of each section of the figure. F6 1D1 is a control monoclonal antibody that does not cross-react with plasminogen.

Mannheim), a substrate for plasmin. We performed these assays both in the presence and absence of each of our monoclonal antibodies. In all cases plasminogen was unable to cleave the substrate, whereas plasmin, which was included as a positive control, was able to cleave the substrate (similar assays were also carried out on our elastolytically derived plasminogen fragments to assure that they were not proteolytically active).

Once assured that the zymogen was not activated, we serially diluted plasminogen on Lp[a]-coated plates. As the competition curves in **Fig. 3** indicate, eight of the nine monoclonal antibodies were inhibited by serially diluted plasminogen. Only our control monoclonal antibody F6 1D1, which did not react with plasminogen, was not inhibited by plasminogen. Similar results were found when serially diluted reduced and alkylated plasminogen was used a competitor (data not shown). Monoclonal antibodies that recognized reduced and nonreduced plasminogen in Western blot analysis (e.g., F2 3A3), and those that did not recognize either form (e.g., F6 1G9) were all inhibited in the ELISA.

Since the hybridomas that we produced were generated from injections of Lp[a] from a single individual with a single isoform, we also wanted to determine whether our antibodies could recognize different isoforms of apo[a]. We screened out antibodies against apo[a] from individuals who expressed various titers and several isoforms in Western blot analysis. Each of our monoclonal antibodies reacted with all tested isoforms of apo[a] (**Fig. 4**).

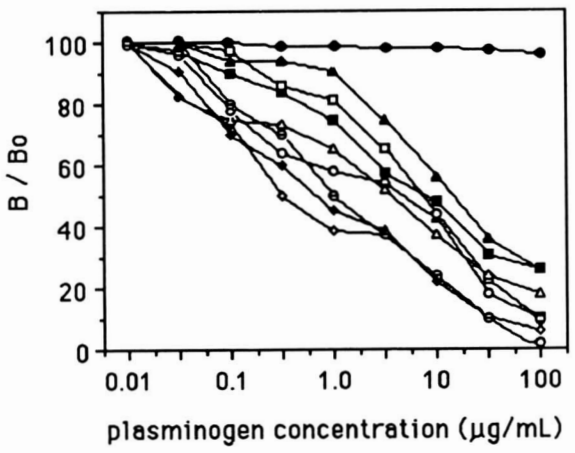

Fig. 3. Analysis of monoclonal antibody specificity by direct competition assays with plasminogen. Serially diluted plasminogen was added to triplicate wells of microtiter plates coated with Lp[a] at 5 µg/ml. A constant concentration of antibody was then added across the plate and incubated overnight. Antibody that bound to the plates was detected using anti-mouse alkaline phosphatase. Monoclonal antibody F6 1D1, which recognizes Lp[a] but not plasminogen, was included as a negative control; (♦), F6 1A3; (○), F6 1B5; (●), F6 1D1; (◇), F1 1E3; (■), F6 1G9; (□), F6 2C3; (▲), F6 2G2; (△), F2 3A3; (○), F6 3F4.

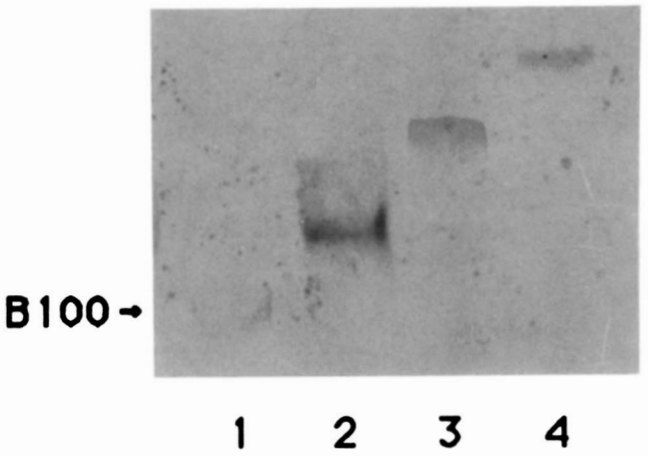

Fig. 4. Recognition of isotypes by F6 2C3. Two µl of serum samples collected from four different individuals were electrophoresed in a 4% SDS-polyacrylamide gel after reduction with 4% mercaptoethanol for 10 min. After electroimmunoblotting to nitrocellulose a 1:1000 dilution of F6 2C3 was incubated with the membrane overnight. Antibody recognition was determined using anti-mouse peroxidase and HRP color developer. The relative migration position of apoB-100 is indicated.

Determination of the number of epitopes recognized by the monoclonal antibodies

To determine the number of epitopes recognized by our monoclonal antibodies, site competition assays were performed using microtiter plates coated with Lp[a] at 5 µg/ml. The monoclonal antibodies interacted in four distinctly different ways in this assay (**Fig. 5**). Several antibodies, such as F2 3A3 and F6 1A3, did not interact, suggesting that they recognized different epitopes. Unlabeled F2 3A3 did not block the binding of biotinylated F6 1A3, nor did F6 1A3 block the binding of biotinylated F2 3A3 (Fig. 5A, B). Others, such as F6 1B5 and F6 1G9 reciprocally blocked each other's binding to Lp[a]. Unlabeled F6 1B5 inhibited biotinylated F6 1G9 and unlabeled F6 1G9 inhibited biotinylated F6 1B5's ability to bind to Lp[a] (Fig. 5C, D). This pattern suggests that the two monoclonal antibodies recognize the same or topologically related epitopes.

Two other patterns of interaction suggest that monoclonal antibodies bind to different epitopes but that their epitopes are related topologically. The first pattern is illustrated by F6 1G9 and F6 1A3 (Fig. 5B, D). Unlabeled F6 1G9 completely blocked the binding of biotinylated F6 1A3; however, unlabeled F6 1A3 was unable to block the binding of F6 1G9. The second pattern is illustrated by F6 2G2 and F6 1A3. F6 2G2 was able to partially compete for the binding of F6 1A3 and resulted in a reduced binding of the biotinylated monoclonal antibody (Fig. 5B), while F6 1A3 failed to block the binding of biotinylated F6 2G2 (Fig. 5E).

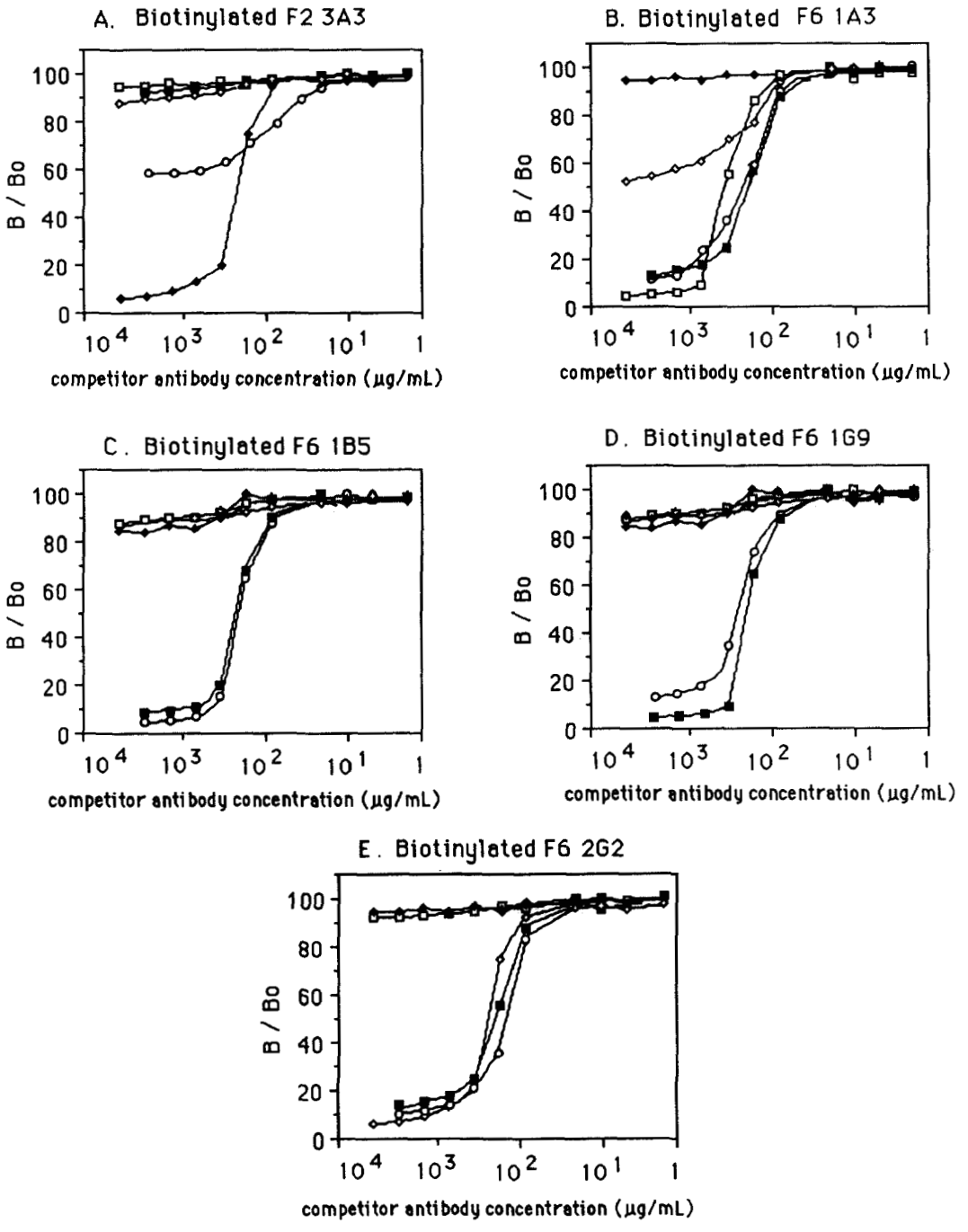

Fig. 5. Site competition analysis of monoclonal antibodies on Lp[a]-coated plates. Lp[a] was coated on microtiter plates at 5 μg/ml and incubated overnight at 4°C with serially diluted unlabeled monoclonal antibody. After washing, 100 μl of biotinylated monoclonal antibody was added at the appropriate dilution for 90 min. The binding of biotinylated antibody was detected using avidin-conjugated alkaline phosphatase. The results show the average of three independent trials. The following unlabeled monoclonal antibodies were used: (□), F6 1A3; (○), F6 1B5; (■), F6 1G9; (◇), F6 2G2; (◆), F2 3A3.

Similar experiments were carried out for all combinations of monoclonal antibodies and are summarized in **Table 2**. Although the patterns are complex, the eight monoclonal antibodies can be subdivided into four general groups. A representative of each of the four groups and their interactions with each of the other groups is depicted in Fig. 5. Group A includes monoclonal antibodies F1 1E3 and F2 3A3. These monoclonal antibodies compete reciprocally and are unable to block the binding of any other biotinylated monoclonal antibody. In addition, F6 1B5, F6 1G9, and F6 3F4 partially block their binding to Lp[a].

TABLE 2. Summary of Lp[a] site competition studies

Biotinylated Monoclonal Antibody	Unlabeled Monoclonal Antibody							
	A		B	C		D		
	F1 1E3	F3 3A3	F6 1A3	F6 1B5	F6 1G9	F6 2C3	F6 2G2	F6 3F4
F1 1E3	+ +	+ +	−	+	+	+	−	+
F2 3A3	+ +	+ +	−	+	+	−	−	+
F6 1A3	−	−	+ +	+ +	+ +	+	+	+ +
F6 1B5	−	−	−	+ +	+ +	−	−	−
F6 1G9	−	−	−	+ +	+ +	−	−	−
F6 2C3	−	−	−	+ +	+ +	+ +	+ +	+ +
F6 2G2	−	−	−	+ +	+ +	+	+ +	+ +
F6 3F4	−	−	−	+ +	+ +	+	+ +	+ +

See the legend for Fig. 5 for a description of the method. (+ +): The binding of the biotinylated antibody was completely inhibited by the blocking antibody. (+): The binding of the biotinylated antibody was only partially inhibited by the blocking antibody. (−): The binding of the biotinylated antibody was not inhibited by the blocking antibody. A, B, C, D: monoclonal antibody groupings as described in Results.

Unlabeled monoclonal antibody F6 1A3 is unable to block any of the other monoclonal antibodies and therefore appears to recognize a separate site and is categorized as a group B monoclonal antibody. Numerous examples of nonreciprocal competition are found with this monoclonal antibody as biotinylated F6 1A3 is completely blocked by unlabeled F6 1B5, F6 1G9, and F6 3F4 and partially blocked F6 2C3 and F6 2G2.

A third pattern of binding is found with F6 1B5 and F6 1G9 (group C monoclonal antibodies). When used as a blocking antibody, each of these monoclonal antibodies is able to interfere to some extent with the binding of all of the biotinylated antibodies. This indicates that they are able to bind to many more sites on Lp[a] than members of group A or B. However, when they are biotinylated, they are only blocked by themselves.

F6 2C3, F6 2G2, and F6 3F4 make up group D monoclonal antibodies. When unlabeled, these antibodies are able to reciprocally block each other's binding. Their different interactions with F1 1E3, F2 3A3, and F6 1A3 indicate that they may not bind to an identical epitope but may represent a cluster of sites.

Western blot analysis of the eight monoclonal antibodies with proteolytic plasminogen fragments

To determine the binding domains of the four groups of monoclonal antibodies, we analyzed them with elastolytically derived plasminogen fragments in Western blot analysis. Lysine-binding site I (kringles 1, 2, and 3), lysine-binding site I (kringle 4), and miniplasminogen (kringle 5 and protease domain) were separated on SDS-polyacrylamide gels under reducing and nonreducing conditions and then electroblotted to nitrocellulose membranes. Monoclonal antibody F6 1D1, which does not cross-react with plasminogen, and polyclonal antibodies against Lp[a] and plasminogen were included as controls. Polyclonal antibody specific for Lp[a] was able to recognize lysine-binding site II under both reducing and nonreducing conditions (**Fig. 6, Table 3**). However, it was only able to recognize miniplasminogen under nonreducing conditions and was unable to recognize lysine-binding site I.

Western blot analysis with proteolytic plasminogen fragments indicated that there were three types of responses. Group A (F1 1E3, F2 3A3) monoclonal antibodies were only able to recognize nondenatured miniplasminogen. Upon reduction of miniplasminogen the ability of these monoclonal antibodies to recognize their epitopes was disrupted, a result similar to that seen for the polyclonal antibody specific for Lp[a]. They also failed to recognize nonreduced or reduced lysine-binding sites I or II. Group B (F6 1A3) and C (F6 1B5, F6 1G9) monoclonal antibodies reacted with nonreduced and reduced lysine-binding site II. They did not react with lysine-binding site I or miniplasminogen under either condition. Group D (F6 2C3, F6 2G2, F6 3F4) monoclonal antibodies reacted with nonreduced and reduced lysine-binding site II as well as nonreduced miniplasminogen. In addition, F6 2G2 recognized nonreduced lysine-binding site I.

Direct competition assays using elastolytically derived plasminogen fragments

Serially diluted plasminogen fragments were incubated with each of the monoclonal antibodies on Lp[a]-coated microtiter plates. Analysis of monoclonal antibody that bound to the plates allowed us to determine how successfully the fragments competed for the monoclonal antibodies. The results obtained for one member of each group is depicted in **Fig. 7**. Serially diluted lysine-binding site II was able to compete for binding of monoclonal antibodies in groups B, C, and D. Group A monoclonal antibod-

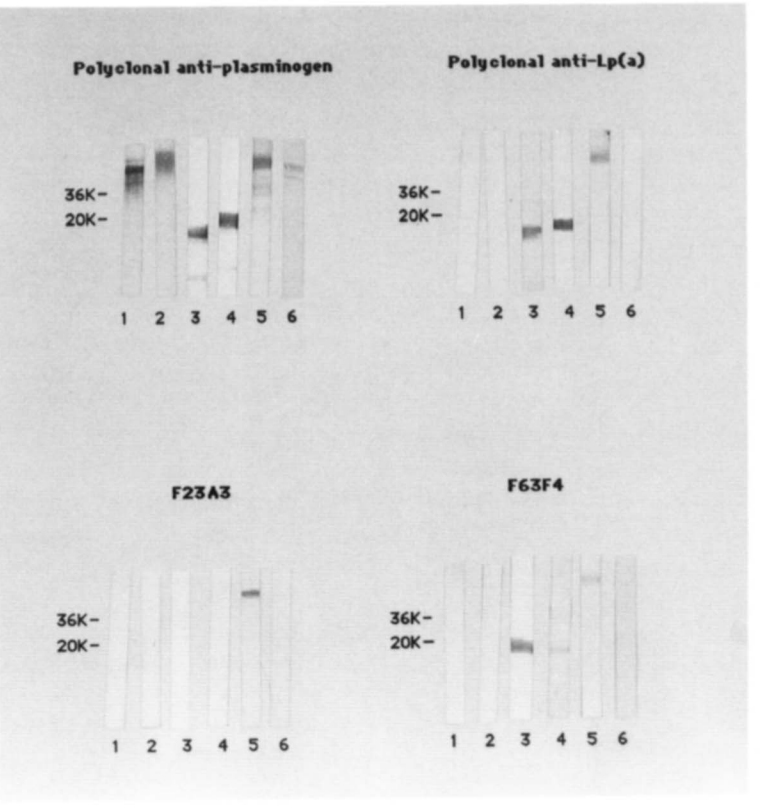

Fig. 6. Western blot analysis of elastolytically derived plasminogen fragments under both reducing and nonreducing conditions. Plasminogen samples were electrophoresed in a 20% SDS-polyacrylamide gel and electroblotted to nitrocellulose. Three-mm-wide strips of nitrocellulose transfer sheets were incubated with a 1:1000 dilution of the polyclonal or monoclonal antibody listed above each section. The specific antigen was revealed by the methods as described in the legend of Fig. 2. The lane designations are: 1, nonreduced lysine-binding site 1; 2, reduced lysine-binding site 1; 3, nonreduced lysine-binding site II; 4, reduced lysine-binding site II; 5, nonreduced miniplasminogen; 6, reduced miniplasminogen. The relative position of the relevant prestained markers is indicated.

ies were not competed by lysine-binding site II (Fig. 7, **Table 4**).

When miniplasminogen was used as the serially diluted competitor, monoclonal antibodies in groups B and C were not inhibited in their binding to Lp[a]-coated plates. On the other hand, there was strong competition for group A monoclonal antibodies. The monoclonal antibodies found in group D were partially competed for by miniplasminogen but the response was considerably less than the response to lysine-binding site II.

In all four groups of monoclonal antibodies direct competition with serially diluted lysine-binding site I did not result in significant competition. Since the monoclonal antibodies were raised against Lp[a], which does not contain plasminogen like kringles 1–3, direct competition with lysine-binding site I would not be expected to have an effect. Surprisingly, monoclonal antibody F6 2G2, which recognized nonreduced lysine-binding site I in Western blot analysis, was not competed for either. Although we are unable to explain this discrepancy, it is

TABLE 3. Western blot analysis of elastolytically derived plasminogen fragments

	Lysine Binding Site I		Lysine Binding Site II		Miniplasminogen	
Antibody	Nonreduced	Reduced	Nonreduced	Reduced	Nonreduced	Reduced
Pab-Lp[a]	–	–	+	+	+	–
Pab-Pg	+	+	+	+	+	+
F6 1D1	–	–	–	–	–	–
F1 1E3	–	–	–	–	+	–
F2 3A3	–	–	–	–	+	–
F6 1A3	–	–	+	+	–	–
F6 1B5	–	–	+	+	–	–
F6 1G9	–	–	+	+	–	–
F6 2C3	–	–	+	+	+	–
F6 2G2	+	–	+	+	+	–
F6 3F4	–	–	+	+	+	–

Pab-Lp[a]: polyclonal antibody specific for Lp[a]; Pab-Pg: polyclonal antibody specific for plasminogen; (+): positive reaction; (–): no reaction.

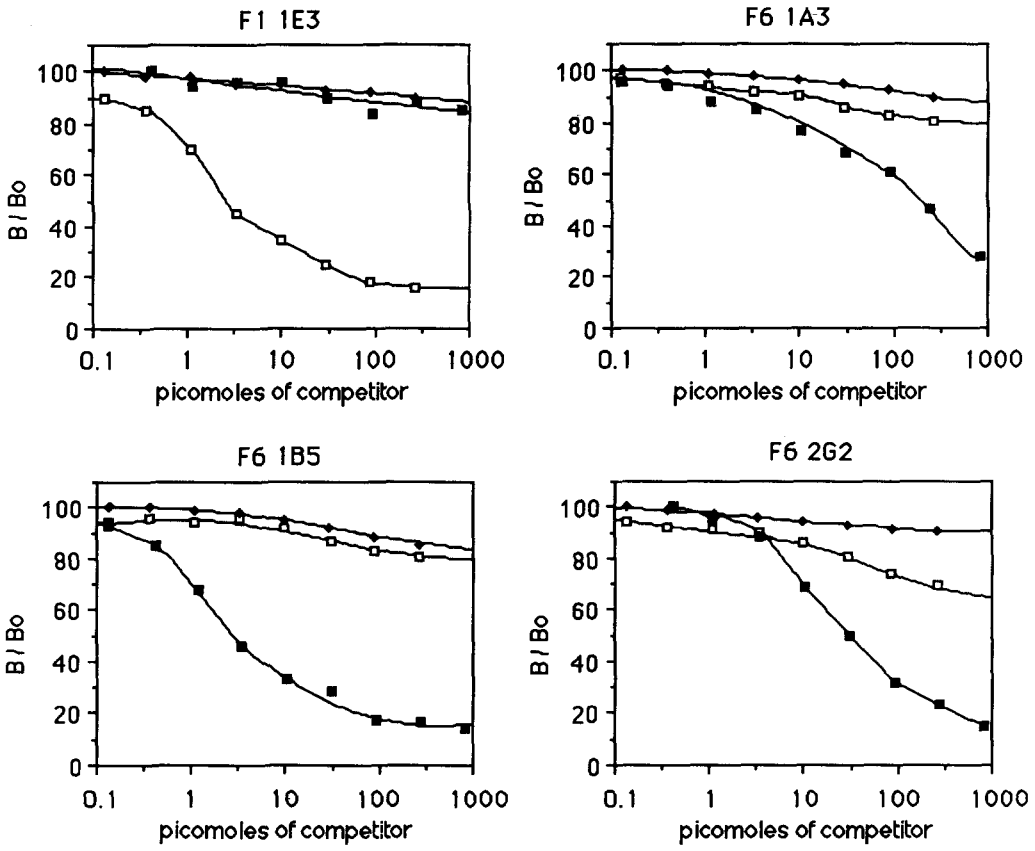

Fig. 7. Elastolytically derived plasminogen fragment competition. Plasminogen fragments were used to challenge monoclonal antibodies for binding to microtiter plates coated at 5 μ/ml Lp[a]. Each of the four groups of monoclonal antibodies is represented: Group A (F1 1E3), Group B (F6 1A3), Group C (F6 1B5), and Group D (F6 2C3). The results are from the average of three independent trials; (♦), lysine-binding site I; (□), lysine-binding site II; (□), miniplasmingoen.

possible that exposure of lysine-binding site I to SDS may have resulted in exposure of a normally hidden site during Western blot analysis.

Site competition using plasminogen-coated plates

In order to further characterize our monoclonal antibodies, we repeated the site competition assays on plasminogen (Table 4). We wanted to determine whether the epitope groupings attained using apo[a] with its multiple repeats of kringle 4 would be altered when plasminogen, with its single kringle 4 domain, was used.

Group A (F1 1E3, F2 3A3) monoclonal antibodies were reciprocally blocked by each other and were not capable of blocking any of the other biotinylated antibodies (Table 4), the same result as found when Lp[a]-coated plates were used. All of the members of group D were able to partially compete for the binding of biotinylated F1 1E3. Additionally, F6 3F4 was able to partially interfere with biotinylated F2 3A3's ability to bind to plasminogen.

Group B monoclonal antibody (F6 1A3) was able to block the binding of biotinylated F6 1G9 and F6 3F4 as well as itself on plasminogen, unlike tests using Lp[a] where it was only able to block itself. Further its binding was interfered with by all monoclonal antibodies found in groups C and D. This suggests that the F6 1A3 site is present on a subset of the Lp[a] kringle 4 domains bearing group C sites.

Site competition assays using miniplasminogen and group D antibodies

The results of the Western blot and direct competition analysis using elastolytically derived plasminogen fragments showed that group A antibodies (F1 1E3, F2 3A3) react only with miniplasminogen while group D monoclonal antibodies recognized both lysine-binding site II and miniplasminogen. Group D (F6 2C3, F6 2G2, F6 3F4) monoclonal antibodies were able to partially compete for the binding of biotinylated F1 1E3 in the site competition analysis using plasminogen as the coated antigen. F6 3F4 was able to interfere with the binding of biotinylated F2 3A3. This suggests that either the group D sites on miniplasminogen are close to those recognized by group

TABLE 4. Plasminogen site competition analysis

Biotinylated Monoclonal Antibody	Unlabeled Monoclonal Antibody							
	A		B	C		D		
	F1 1E3	F3 3A3	F6 1A3	F6 1B5	F6 1G9	F6 2C3	F6 2G2	F6 3F4
F1 1E3	+ +	+ +	−	−	−	+	+	+
F2 3A3	+ +	+ +	−	−	−	−	−	+
F6 1A3	−	−	+ +	+ +	+ +	+ +	+ +	+ +
F6 1B5	−	−	−	+ +	+ +	+ +	+ +	+ +
F6 1G9	−	−	+	+ +	+ +	+ +	+ +	+ +
F6 2C3	−	−	−	+ +	+ +	+ +	+ +	+ +
F6 2G2	−	−	−	+ +	+ +	+ +	+ +	+ +
F6 3F4	−	−	+	+ +	+ +	+ +	+ +	+ +

The procedure used was the same as that described in the legend to Fig. 5 except that microtiter plates were coated with 5 μg/ml plasminogen. (+ +): The binding of the biotinylated antibody was completely inhibited by the blocking antibody. (+): The binding of the biotinylated antibody was only partially inhibited by the blocking antibody. (−): The binding of the biotinylated antibody was not inhibited by the blocking antibody. A, B, C, D: monoclonal antibody groupings as described in Results.

A monoclonal antibodies or that the binding of group D monoclonal antibodies to kringle 4 results in a conformational change in the antigenic determinants of group A monoclonal antibodies.

To further study the effects of the group D monoclonal antibodies on group A monoclonal antibodies, we used purified miniplasminogen in site competition analysis. Unlabeled group D (F6 2C3, F6 2G2, F6 3F4) monoclonal antibodies, and group A (F1 1E3, F2 3A3) monoclonal antibodies were serially diluted on a microtiter plate coated with miniplasminogen. The ability of biotinylated group A (F1 1E3 and F2 3A3) monoclonal antibodies to bind to their epitopes was then examined. **Fig. 8** illustrates the competition curves obtained with biotinylated F1 1E3. The results were identical to those obtained for biotinylated F2 3A3. Biotinylated F1 1E3 and F2 3A3's binding was not affected by the presence of either F6 2C3 or F6 2G2. However, both group A biotinylated antibodies were partially inhibited when unlabeled F6 3F4 was preincubated with miniplasminogen, indicating that F6 3F4 recognizes a site on miniplasminogen that adjoins that of the group A monoclonalantibodies.

Effect of ϵ-aminocaproic acid on antibody binding

In order to further characterize the recognition sites of our monoclonal antibodies, we used ϵ-aminocaproic acid as a competitor of antibody binding to Lp[a]-coated plates. ϵ-Aminocaproic acid interferes with antibody binding to the lysine binding sites on Lp[a] and plasminogen (24). Although Lp[a] bound to lysine-Sepharose can be eluted with approximately 1.8 mM ϵ-aminocaproic acid, concentrations of up to 200 mM ϵ-aminocaproic acid were ineffective in preventing the binding of any of our monoclonal antibodies (data not shown).

Effect of monoclonal antibody binding on plasmin activity

To determine whether the binding of our monoclonal antibodies affected the proteolytic activity of plasmin, we serially diluted monoclonal antibody on plasmin-coated plates. After an overnight incubation the ability of plasmin to cleave Chromozym PL® was determined. In addition, the ability of each of our monoclonal antibodies to bind to plasmin was determined. Monoclonal antibody F6 1D1 was included as a control. All monoclonal antibodies, except F6 1D1, were able to bind to plasmin (data not shown). All of the antibodies that only recognized kringle 4 of plasminogen (F6 1B5, F6 1G9, F6 1A3) were

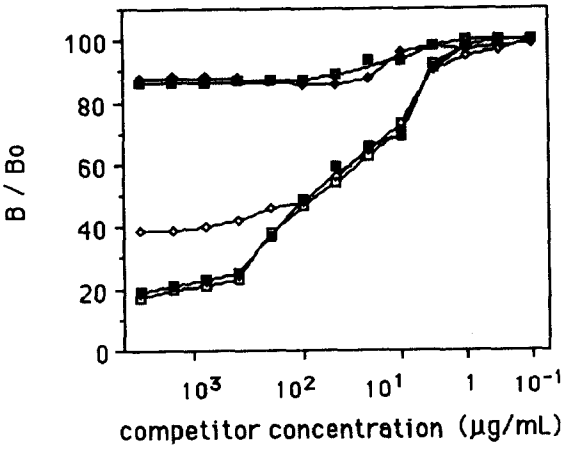

Fig. 8. Site competition assays were performed as previously described using 5 μg/ml miniplasminogen as the coated antigen. The ability of biotinylated F1 1E3 to bind to miniplasminogen after preincubation with serially diluted unlabeled F1 1E3, F2 3A3, F6 2C3, F6 2G2, or F6 3F4 was detected using avidin-conjugated alkaline phosphatase. The results given are from the average of three independent trials; (□); F1 1E3; (♦), F6 2C3; (□), F6 2G2; (◇), F6 3F4; (■), F2 3A3.

unable to inhibit plasmin activity (data not shown). Similarly, those antibodies that only recognized miniplasminogen (F1, 1E3, F2 3A3) did not inhibit plasmin activity (**Fig. 9**). However, all of the monoclonal antibodies that cross-reacted with kringle 4 and miniplasminogen (F6 2C3, F6 2G2, F6 3F4) were able to interfere with plasmin's ability to cleave the substrate (Fig. 9).

Effect of periodic acid treatment on monoclonal antibody binding

The method of Woodward et al. (23), which uses a mild periodate oxidation of antigens bound to ELISA plates to destroy carbohydrate epitopes without altering epitopes associated with proteins, was utilized. As a control a monoclonal antibody that was directed against a flagellar glycoprotein of *Chlamydomonas* was used. A crude extract of *Chlamydomonas* containing flagellar glycoprotein (a gift from Dr. R. A. Bloodgood, University of Virginia School of Medicine) was treated with 20 mM periodic acid for 60 min, whereupon the binding of the antibody was eliminated (data not shown). The same treatment of Lp[a]-coated plates failed to affect the binding of our monoclonal antibodies. Likewise, all antibodies except F1 1E3 were unaffected by treatment of plasminogen. Surprisingly, plasminogen treated with 20 mM periodic acid bound F1 1E3 poorly (**Fig. 10**). Our previous studies characterized F1 1E3 as recognizing an epitope within

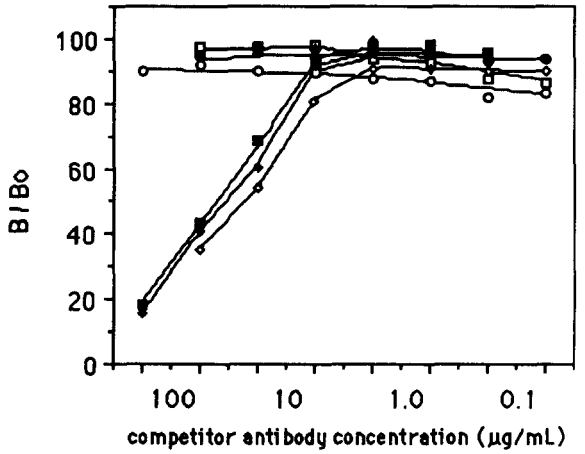

Fig. 9. The ability of our monoclonal antibodies to interfere with the proteolytic ability of plasmin was investigated by serially diluting each monoclonal antibody on a plate that was coated with 5 µg/ml of plasmin (Boehringer Mannheim). After an overnight incubation at 4°C the plates were washed 3 times using solution 1 (50 mM Tris, 100 mM NaCl, pH 8.2). After washing, 64 µl of solution 1, 8 µl of 0.9% NaCl, and 8 µl of a solution that contained 0.5% (w/v) polyethylene glycol 6000, 50 mM glycine, pH 2.5, were added to each well. Sixteen µl of Chromozym PL®), dissolved at 1.9 mg/ml in a solution that contained 100 mM glycine, 0.02% Tween 20, was then added to each well. *p*-Nitraniline formation was monitored at 405 nm. All assays were performed in triplicate; (○), control, no monoclonal antibody added; (□), F1 1E3; (●), F6 2C3; (■), F6 2G2; (◇), F6 3F4.

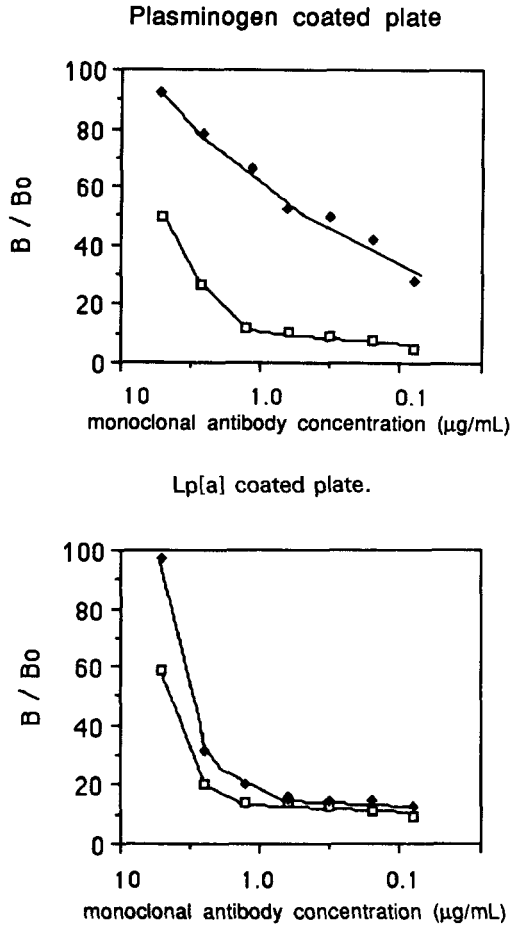

Fig. 10. The effects of periodic acid treatment on monoclonal antibody F1 1E3's ability to recognize epitopes. Plasminogen or Lp[a] (coated at 5 µg/ml) microtiter plates were pretreated for 30 min with 50 mM sodium acetate buffer, pH 4.5. One half of the lanes were then incubated with 20 mM periodic acid in acetate buffer, pH 4.5, for 1 h at room temperature. After washing, serially diluted F1 1E3 was added. The results are from the average of six independent trials; (□), periodate-treated samples; (◆), untreated samples.

miniplasminogen. Since plasminogen is glycosylated at two sites found within the lysine-binding site I but not in miniplasminogen, we repeated the experiment with miniplasminogen to determine whether this effect was caused by the periodate oxidation of a sensitive amino acid. However, this treatment had no effect on the binding of F1 1E3 (data not shown).

DISCUSSION

The kringle domains of plasminogen have been characterized with the use of elastolytically derived plasminogen fragments. The fragments generated from limited elastolysis of plasminogen include lysine-binding site I (kringles 1, 2, and 3), lysine-binding site II (kringle 4), and miniplasminogen (kringle 5 - protease domain) (10). This

treatment does not affect the abilities of these fragments to interact with fibrin, benzamidine, and ϵ-aminocaproic acid (10, 2–27). In addition, the immunoreactivity of these fragments is maintained. Further immunological studies have shown that polyclonal and monoclonal antibodies, raised against one elastolytically derived plasminogen fragment, are able to distinguish it from the others in spite of the 35% homology between domains of plasminogen (28).

In this study, we took advantage of the strong homology between apo[a] and plasminogen as well as the immunological stability of elastolytically derived plasminogen fragments to identify the binding sites of monoclonal antibodies that were raised against Lp[a]. After selecting monoclonal antibodies that cross-reacted with plasminogen, we analyzed their reactivity with elastolytically derived plasminogen fragments in Western blot analysis. Three different types of reactivity were observed. One set of monoclonal antibodies recognized an epitope on miniplasminogen, a second set only recognized kringle 4, and the third set recognized both kringle 4 and miniplasminogen.

Plasminogen site competition assays distinguished further differences among the monoclonal antibodies. The monoclonal antibodies that recognized miniplasminogen (group A: F1 1E3, F2 3A3) showed reciprocal inhibition in site competition studies and yet were affected differently by the monoclonal antibodies that reacted with both miniplasminogen and kringle 4 in Western blot analysis. This suggests that F1 1E3 and F2 3A3 recognize neighboring sites on miniplasminogen. Further support for this came from studies using periodate-treated plasminogen. This treatment disrupted the ability of F1 1E3 to recognize its epitope while it had no effect on F2 3A3.

Further division of the monoclonal antibodies that recognized kringle 4 alone and those that reacted with both kringle 4 and miniplasminogen resulted when plasminogen site competition assays were analyzed. Although group C (F6 1B5 and F6 1G9) monoclonal antibodies only recognized kringle 4 in Western blot analysis, they showed complete reciprocal interference with all antibodies that reacted with both kringle 4 and miniplasminogen (group D: F6 2C3, F6 2G2, F6 3F4). When site competition assays using miniplasminogen were performed (data not shown). F6 1B5 and F6 1G9 were not able to interfere with the binding of F6 2C3, F6 2G2, and F6 3F4. This suggests that the binding of monoclonal antibodies to kringle 4 epitopes of plasminogen is such that they are able to sterically inhibit the binding of monoclonal antibodies to miniplasminogen epitopes.

F6 1A3, which recognized kringle 4 alone, was only able to partially inhibit F6 1G9 and F6 3F4 and had no effect on the other monoclonal antibodies studied. Therefore, F6 1A3 appears to recognize a separate plasminogen site. In a similar manner, when the monoclonal antibodies that react with kringle 4 and miniplasminogen (group D) were analyzed in miniplasminogen site competition assays, F6 3F4, but not F6 2C3 or F6 2G2, was able to block biotinylated F1 1E3 or F2 3A3. This suggests that the F6 3F4 binding site is distinct from those of F6 2C3 and F6 2G2.

It was expected that the use of Lp[a] as the antigen in site competition assays would result in a more complicated pattern due to the multiple kringle 4-like domains of apo[a]. Despite the homology found between the multiple repeats, 11 different types of kringle 4-like domains have been characterized in apo[a] based upon sequence and ligand binding analysis (7). Thus, monoclonal antibodies that recognize a kringle 4-like domain in apo[a] may recognize epitopes in all of these domains or a subset of these domains.

The group D monoclonal antibodies, which reacted with miniplasminogen in Western blots, showed nearly identical site competition results with either plasminogen or Lp[a]. However, a more complex pattern of competition was observed when the groups of monoclonal antibodies that recognized kringle 4 were used in Lp[a] site competition assays. These assays allowed us to group our monoclonal antibodies based upon the subsets of kringle 4-like domains they recognized. F6 1A3 (group B), which was able to partially interfere with F6 1G9 (group C) and F6 3F4 (group D) binding to plasminogen, was not able to interfere with the binding of these monoclonal antibodies to Lp[a]. This suggests that F6 1A3 recognizes an epitope that is only found on a limited number of kringle 4-like repeats of apo[a]. On the other hand, group C monoclonal antibodies (F6 1B5 and F6 1G9) interfere with the binding of all other antibodies on Lp[a], indicating that they recognize a binding sequence found on many kringle 4-like domains of apo[a]. Their ability to partially interfere with monoclonal antibodies F1 1E3 and F2 3A3, which react with the miniplasminogen-like domain of apo[a], may be due to either conformational changes to the miniplasminogen-like domain of apo[a] caused by the binding of these antibodies to the multiple repeats of kringle 4-like domains of apo[a], or by direct steric hindrance of the bound monoclonal antibody with the antibodies which recognize miniplasminogen.

Group D monoclonal antibodies (F6 2C3, F6 2G2, and F6 3F4) were able to reciprocally interfere with each other's binding on Lp[a] but did not completely interfere with the binding of biotinylated F6 1B5 or F6 1G9. Due to the varied response of these three monoclonal antibodies with F6 1A3, F1 1E3, and F2 3A3, we predict that they represent a cluster of sites rather than a single epitope.

Our finding that monoclonal antibodies F6 2C3, F6 2G2, and F6 3F4 (group D) were able to recognize an epitope on kringle 4 and miniplasminogen may indicate that they recognize a site which is homologous to kringle 4 and kringle 5. These antibodies did not react with kringles

1→3. Therefore, despite the 40% homology found between the kringle domains of plasminogen, these monoclonal antibodies appear to be able to differentiate two of the five kringle domains of plasminogen.

We used ϵ-aminocaproic acid inhibition studies to further characterize the binding regions of each of our monoclonal antibodies. Plasminogen binds to fibrin and α_2-antiplasmin through interactions of the ligands with the kringles' lysine-binding domains (9). Previous studies have shown that ϵ-aminocaproic acid can prevent some monoclonal antibodies from recognizing their epitopes (9, 25, 27). We found that ϵ-aminocaproic acid does not interfere with the binding of any of our monoclonal antibodies to either plasminogen or Lp[a], indicating that our monoclonal antibodies do not recognize lysine binding sites.

Investigation of the ability of our monoclonal antibodies to inhibit the proteolytic activity of plasmin enabled us to further characterize the binding domains of our monoclonal antibodies. We found that groups B and C (the kringle 4-recognizing monoclonal antibodies) and group A (the miniplasminogen-binding monoclonal antibodies) were not able to interfere with the active site of plasmin. On the other hand, group D monoclonal antibodies (F6 2C3, F6 2G2,, F6 3F4), which cross react with kringle 4 and miniplasminogen, were able to interfere with the proteolytic ability of plasmin. The interference of activity by these monoclonal antibodies may be due to steric hindrance of substrate binding or a conformational alteration of the active site.

A major difference between plasminogen and apo[a] is the amount of carbohydrate present. Carbohydrate makes up to 25–30% of the mass of apo[a] but only 1.5% of the mass of plasminogen (5, 10). Plasminogen can be glycosylated at two sites found within lysine-binding site I, a galactosamine-based oligosaccharide attached to Thr 345 and a glucosamine-based oligosaccharide at Asn 288 (10). The absence or presence of these carbohydrate moieties results in different isoforms of plasminogen. In apo[a], the amino acid sequence between the kringle 4-like repeats contains six potential sites for glycosylation and each kringle contains one, giving rise to 253 potential glycosylations sites (5). It is not known which of these sites are actually modified (5).

An experiment designed to determine whether our monoclonal antibodies recognized carbohydrate demonstrated that none of our monoclonal antibody epitopes were affected by periodate treatment of Lp[a]. This result is not surprising since monoclonal antibodies to Lp[a] carbohydrate would not be expected to cross-react with plasminogen lysine-binding site II or miniplasminogen, which lack carbohydrate. What was surprising was that periodate treatment of plasminogen affected the binding of F1 1E3. F1 1E3 binds to miniplasminogen, a region that is not glycosylated. When we used periodate-treated miniplasminogen to further investigate this finding, we found that there was no effect on F1 1E3's binding. Thus, it is plausible that carbohydrate stripping of plasminogen causes a conformational change in the miniplasminogen domain that affects the binding of the antibody. Perhaps the relative position of the carbohydrate-sensitive sites on Lp[a] are such that the miniplasminogen-like domain is not altered.

The results of these experiments suggest that all the epitopes recognized by our monoclonal antibodies are protein in nature. However, not all carbohydrate epitopes are sensitive to periodate treatment. Epitopes containing O-acetyl sialic acids appear to be resistant to the treatment and epitopes consisting of linear sugar chains with linkages at carbon 3 are only partially destroyed by periodate (23). Nevertheless, most monoclonal antibodies that react with carbohydrate are directed against nonreducing terminal carbohydrate structures (23). These are quite sensitive to periodate treatment.

Western blot results indicate that all of our monoclonal antibodies that react with nonreduced miniplasminogen fail to react with reduced miniplasminogen. This was also true for our polyclonal antibody directed against Lp[a]. It was able to recognize nonreduced Lp[a], plasminogen, and miniplasminogen as well as reduced Lp[a], but failed to react with reduced miniplasminogen or plasminogen. In contrast to this, all of the monoclonal antibodies that recognized kringle 4 reacted with both reduced and nonreduced kringle 4. This latter result was similar to that of Plow and Collen (28). They investigated monoclonal and polyclonal antibodies raised against plasminogen kringle 4 and found that they were able to recognize kringle 4 in either a reduced or nonreduced state (28).

The inability of some of our anti-Lp[a] monoclonal and polyclonal antibodies to recognize reduced and denatured miniplasminogen and plasminogen in Western blots led us to investigate the ability of reduced and alkylated plasminogen to act as a competitor in ELISA. We found that our monoclonal and polyclonal antibodies bound equally to plasminogen and to reduced and alkylated plasminogen. This suggests that the antigenic nature of the epitopes is maintained after reduction and that the inability of our antibodies to recognize their epitopes in Western blot analysis is due to the subsequent denaturation in SDS-PAGE or during transfer to nitrocellulose. It should be mentioned that caution should be taken in drawing conclusions about plasminogen cross-reactivity from Western blot analysis of reduced and denatured samples.

In conclusion, we have identified a number of different epitopes on apo[a] that are also found on plasminogen. Two monoclonal antibodies react with different, yet neighboring, sites on the kringle 5 - protease domain. Three monoclonal antibodies react with two different epitopes on the kringle 4 domain and three monoclonal anti-

bodies were found that apparently recognize epitopes of kringle 4 that are shared by kringle 5. Since these epitopes are found on all Lp[a] isoforms tested, we now hope to utilize them as probes to aid in the determination of the structural basis for apo[a] isoform differences.

The research on which this project is based was financed in part by the State of Delaware, grant DRP-88-17, authorized by the State Budget Act of Fiscal 1988, and by a grant from Terumo Medical Corporation.

Manuscript received 27 July 1990 and in revised form 25 October 1990.

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