Interaction of LDL, Lp[a], and reduced Lp[a] with monoclonal antibodies against apoB

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Abstract Five monoclonal antibodies (2A, 9A, 6B, L3, L7) produced in mice against human apolipoprotein B were investigated by competitive and inhibitive electroimmunoassay (EIA) for their reactivity with low density lipoprotein (LDL), lipoprotein[a] (Lp[a]), and reduced Lp[a]. All of the antibodies reacted with apoB of the different lipoproteins indicated by very similar slopes of the binding curves. None of them gave a positive reaction with apolipoprotein[a]. The amount of apoB required for 50% inhibition of antibody binding varied for the different antibodies and lipoproteins. Antibody 9A showed almost the same affinity for LDL, Lp[a], and reduced Lp[a]. Antibodies 2A and 6B bound about twofold better to LDL and reduced Lp[a] than to untreated Lp[a]. Antibodies L3 and L7 needed nearly threefold higher amounts of Lp[a]-apoB for 50% inhibition of antibody binding than of apoB of LDL and reduced Lp[a]. The amount of apoB required for 50% inhibition of antibody binding was somewhat higher in inhibitive assay than in competitive assay. We suggest that apo[a] covers certain epitopes of apoB in native Lp[a] leading to a reduced reaction with the monoclonal antibodies. However, it could also be that the binding of the [a]antigen to apoB via disulfide bridges causes profound conformational changes of the apoB region exposed to the surface.—**Gries, A., C. Fievet, S. Marcovina, J. Nimpf, H. Wurm, H. Mezdour, J. C. Fruchart, and G. M. Kostner.** Interaction of LDL, Lp[a], and reduced Lp[a] with monoclonal antibodies against apoB. *J. Lipid Res.* 1988. **29:** 1–8.

Supplementary key words monoclonal antibodies against apoB • EIA • LDL • Lp[a] • reduced Lp[a]

Apolipoprotein B (apoB) is an integral constituent of human very low density lipoprotein (VLDL) and chylomicrons and is the major protein present in low density lipoproteins (LDL) (1). ApoB is also present in lipoprotein[a] (Lp[a]) a particle of unusual structure, consisting of apoprotein[a] (apo[a]) which is linked to apoB of an LDL-like particle through disulfide bonds (2). Two main subspecies of apoB are known: VLDL and LDL contain a species with an apparent molecular weight of 549,000 called B-100 whereas, in chylomicrons, the major species shows an apparent molecular weight of 246,000 and is identified as B-48. These two species of apoB appear to be under separate genetic control (3) and, in man, B-100 and B-48 are thought to be of hepatic and intestinal origin, respectively (4). Unlike LDL, Lp[a] probably has no triglyceride-rich lipoproteins as precursors and seems to be secreted directly by the liver (5).

This particle contributes to a special lipoprotein class in the HDL_2 density fraction (1.063–1.125 g/ml). The lipid moiety, however, is almost indistinguishable from that of LDL. Because of its high atherogenicity (6–8) Lp[a] has gained increasing interest recently. Contradictory results have been reported about the clearance of Lp[a] by the LDL receptor-mediated pathway. Whereas in one study utilizing fibroblasts from normal subjects and from subjects with familial hypercholesterolemia the conclusion was reached that Lp[a] entered fibroblasts independently of the LDL receptor (9), other investigators have concluded that Lp[a] can be taken up by the same receptor site as LDL (10–13). In order to gain further insight into the structural arrangement of Lp[a], we studied the binding of several monoclonal antibodies (Mabs) against apoB to LDL, Lp[a], and reduced Lp[a] by EIA. Mabs can recognize specific epitope patterns and consequently distinguish lipoprotein particles that might be similar by conventional criteria (14).

METHODS

Isolation of plasma lipoproteins

Apparently healthy students, aged 18–25 years, were screened for Lp[a] immunoreactivity by Laurell electrophoresis (15). Lp[a] was prepared from three different

Abbreviations: Lp[a], lipoprotein[a]; EIA, enzyme immunoassay; Mabs, monoclonal antibodies; LDL, low density lipoproteins; VLDL, very low density lipoproteins; PBS, phosphate-buffered saline.

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serum pools obtained from five or six fasting Lp[a]-positive donors, each as described previously (16). For comparison LDL was isolated from the same sera from the d 1.020-1.050 g/ml fraction.

The purity of the prepared lipoproteins was checked by agarose gel electrophoresis (17), SDS-PAGE (18), and immunochemical reaction against antisera to Lp[a], apoB, apoA-I, apoA-II, apoC, and apoE as described (19). Antisera were prepared in our own laboratory (20).

Reduction of LDL and Lpa

The term "reduced Lp[a]" is used throughout this report for an LDL-like Lp[a]-particle which has been separated from apo[a]. Reduction of native Lp[a] was performed with dithiothreitol according to Armstrong, Walli, and Seidel (13). Both apo[a] and reduced Lp[a] were isolated by affinity chromatography on heparin-Sepharose (13) and tested for purity as described above.

For control experiments, freshly prepared LDL (native LDL) was reduced with dithiothreitol by the same procedure. Each of the purified fractions was dialyzed under pressure and kept under nitrogen at 4°C.

Chemical analysis

Total and free cholesterol, triglycerides, and phospholipids were determined enzymatically with commercial kits from Boehringer-Mannheim, FRG, and from Bio-Mérieux, France. The concentration of protein was measured according to Lowry et al. (21) using human serum albumin as standard. The content of apoB of the different lipoprotein samples was determined by Laurell electrophoresis using a polyclonal antibody to apoB (15) before and after reduction with dithiothreitol. Purified LDL (d 1.025-1.055 g/ml) was used as standard. The apoB content of this standard was determined according to Egusa et al. (22) as described in detail by Zechner, Moser, and Kostner (20).

Production and characterization of monoclonal antibodies[1]

Monoclonal antibodies against apoB were prepared by fusing spleen cells of immunized male Balb/c mice with a myeloma cell line. Five Mabs against apoB were used in this study. They were designated as 2A, 9A, L3, L7, and 6B.

Antibodies L3 and L7 were produced and characterized by the Research Center Clin Midy (Montpellier, France) (23). Antibodies 2A, 9A, and 6B were produced and characterized as described by Marcovina et al. (24) (Istituto Scientifico S. Raffaele, Milan, Italy). In this report they were named A, B, and C, respectively.

These five Mabs were all of the IgG 1 subclass (using specific anti-mouse immunoglobulins) and recognized different epitopes on the apoB molecule (as evidenced by competition experiments using radiolabeled antibodies). As assessed by the Western immunoblotting technique, the five Mabs bound to apoB of LDL and VLDL and none of them bound to nitrocellulose transfers containing apolipoproteins A-I, A-II, or C (23, 24). Investigated by the same technique, the clones 9A and 6B showed a strong reactivity to both apoB-100 and apoB-48, whereas 2A, L3, and L7 appeared to react only with apoB-100. The binding of L3 to LDL was not temperature-dependent; the other four antibodies bound maximally to LDL at 4°C (23, 24).

Binding assays

Binding analyses were performed by competitive and inhibitive EIA. For the competitive assay, 96-well microtiter plates (Costar, Cambridge, MA) were coated with 100 μl of LpB (d 1.040-1.050 g/ml) at a concentration of 5 μg/ml apoB in phosphate-buffered saline (PBS), pH 7.4, and incubated for 24 hr at room temperature. The plates were washed, and unoccupied binding sites were blocked by adding 100 μl of PBS containing 5% of bovine serum albumin (PBS-BSA) and incubation for 30 min at 37°C.

For measurement of the reactivity with the Mabs, each of the lipoproteins was diluted to 1 mg of apoB/ml PBS-BSA. Serial further dilutions (1/3-1/300) were prepared and assayed in duplicate. Mabs were used at the following concentrations: 2A (13 μg of protein/ml), 9A (5 μg of protein/ml), L3 (5 μg of protein/ml), L7 (3 μg of protein/ml), and 6B (7 μg of protein/ml).

Fifty μl of sample and 50 μl of the Mab-solution were applied to each well. The assay was incubated for 1 hr at 37°C. After washing, 100 μl of peroxidase-conjugated sheep anti-mouse IgG (Institut Pasteur, Paris) was added at a dilution of 1/50,000 in PBS-BSA and the mixture was allowed to stand for 1 hr at 37°C. Excess enzyme-labeled antibody was washed away and the amount of peroxidase fixed to the tubes was determined using H_2O_2 as substrate and O-phenylendiamine as hydrogen donor (25). After 30 min at room temperature in the dark, the reaction was stopped by adding 100 μl of 1 N HCl and the yellow color was measured at 492 nm. The steps of coating, washing, dosage of antibodies, addition of conjugate, addition of HCl, and spectrophotometric reading were done automatically by an ELISA processor (Behring A. G., Marburg, FRG).

For the inhibitive assay, the Mabs were diluted half as much as for the competitive assay. Fifty μl of the different lipoproteins and 50 μl of the antibodies both diluted with PBS-BSA were incubated for 24 hr at 4°C in

[1]Antibodies L3 and L7 are available from the Research Center Clin Midy (Montpellier, France). Antibodies 2A, 9A, and 6B are available from the Immunochemistry and Hybridoma Branch, Istituto Scientifico S. Raffaele, Milan, Italy.

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TABLE 1. Chemical composition of LDL, Lp[a], and reduced Lp[a]

	Cholesteryl Ester	Free Cholesterol	Triglyceride	Phospholipid	Protein
			% by weight		
LDL	39.0 ± 1.6[a]	10.9 ± 1.4	4.3 ± 1.4	21.8 ± 2.0	24.9 ± 2.1
Lp[a]	32.1 ± 1.9	9.5 ± 1.1	5.1 ± 1.9	18.7 ± 1.6	31.5 ± 2.8
Lp[a] reduced[b]	39.3 ± 1.2	11.2 ± 1.3	4.9 ± 1.7	22.2 ± 1.9	24.3 ± 1.8

[a]Mean ± SD; n = 5.
[b]Reduced Lp[a] was isolated by affinity chromatography over heparin-Sepharose.

polystyrene tubes. Afterwards 100 μl of this mixture was added to the microtiter plates (Costar, Cambridge, MA) precoated with 100 μl of LpB solution (5 μg of apoB/ml of PBS) and incubated for 1 hr at 37°C (26). Further steps were identical to those described above.

RESULTS

Reaction of the monoclonal antibodies with LDL, Lp[a], and reduced Lp[a]

The chemical composition of LDL, Lp[a], and reduced Lp[a] prepared from fasting sera with Lpa concentrations >50 mg/dl is listed in **Table 1**.

On double immunodiffusion, LDL and reduced Lp[a] only gave positive reactions with antiserum to apoB. Native Lp[a] reacted with anti-B as well as with anti-[a]. No reaction could be observed with anti-A-I, anti-A-II, anti-C, and anti-E. Apo[a] separated from Lp[a] showed immunoreactivity only with anti-[a]. As shown by agarose gel electrophoresis **(Fig. 1)** LDL and Lp[a] were not contaminated with each other. LDL, Lp[a], and reduced Lp[a] were further investigated by SDS-PAGE using 3.75% gels in presence and absence of 2-mercaptoethanol. The electrophoresis patterns show that neither LDL and Lp[a] nor reduced Lp[a] and apo[a] were contaminated with each other. There were only trace amounts of degradation products visible **(Fig. 2)**.

The content of apoB in LDL determined by Laurell electrophoresis using a polyclonal antiserum to apoB amounted to 95 ± 2% (n = 5) of the total protein mass. In reduced Lp[a] 98 ± 1% (n = 5) and in unreduced Lp[a] 65 ± 3% (n = 5) of the total protein were found to be apoB. Very similar results with respect to the apoB content in LDL were found using the isopropanol procedure as described in detail by Egusa et al (22). No differences in the content of apoB could be evaluated from the rocket areas whether or not DTT was added to LDL or Lp[a].

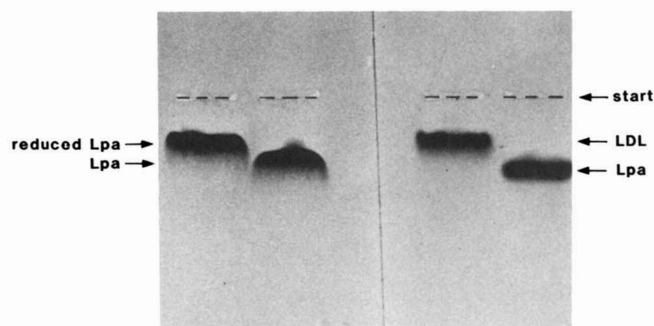

Fig. 1. Agarose gel lipoprotein electrophoresis of LDL and of Lp[a] before and after reduction with DTT (as described in Methods). From left to right: lane 1, Lp[a] reduced with DTT, after separation on heparin-Sepharose; lane 2, native Lp[a]; lane 3, native LDL; lane 4, native Lp[a].

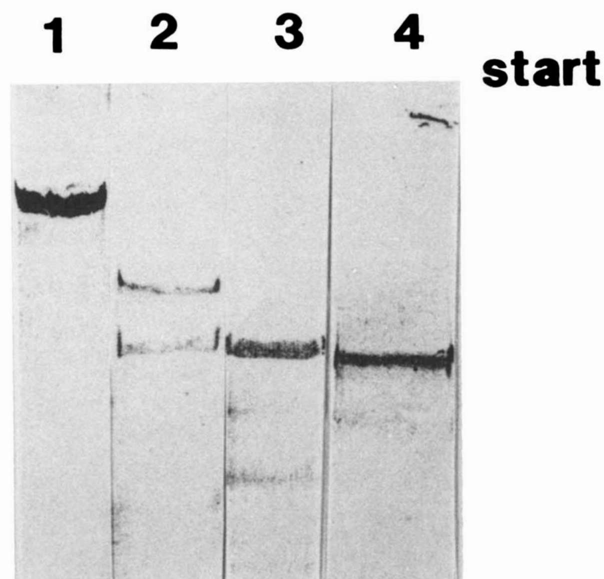

Fig. 2. SDS-PAGE (3.75% polyacrylamide gels) of Lp[a] and its reduced components. Lane 1, unreduced Lp[a]; lane 2, Lp[a] after reduction with 2-mercaptoethanol in the presence of SDS; lane 3, reduced Lp[a] (reduction was performed with dithiothreitol), reduced Lp[a] and apo[a] were separated by affinity chromatography over heparin-Sepharose; lane 4, native LDL. Twenty μg of protein was applied to each gel.

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TABLE 2. Reactivity of different monoclonal antibodies against apoB with apo[a] and LDL-apoB determined by electroimmunoassay

	2A	9A	6B	L3	L7
Apo[a]	0.017[a]	0.028	0.026	0.085	0.018
ApoB	1.051	2.050	1.842	1.434	0.777

To each well coated with 0.5 μg of apo[a] or LDL-apoB was added 2 μg of the respective antibody.
[a]Values represent mean absorbance at 492 nm (n = 3).

The specificity of the Mabs for apolipoprotein B used in this study was also investigated by EIA. Wells coated with apolipoprotein B showed highly positive reactions whereas wells coated with apo[a] showed the reaction equal or slightly higher than the background **(Table 2)**.

To establish possible differences between the immunoreactivity of apoB in LDL and Lp[a] against the monoclonal antibodies, we compared their displacement curves in the EIA. Each of the five monoclonal antibodies reacted with both LDL and Lp[a]. The slopes of the titration curves measured for one antibody were very similar for the two different lipoproteins **(Fig. 3)**.

The concentrations required to obtain a 50% inhibition of antibody binding calculated from the plots %B/Bo versus concentration of apoB are represented in **Table 3** and **Table 4**. Antibodies 2A and 9A exhibited a relatively high affinity for LDL whereas 6B, L3, and L7 needed about threefold higher concentrations of apoB for 50% inhibition of antibody binding in the competitive assay.

In the inhibitive assay the concentrations of LDL-apoB needed for 50% inhibition of antibody binding were

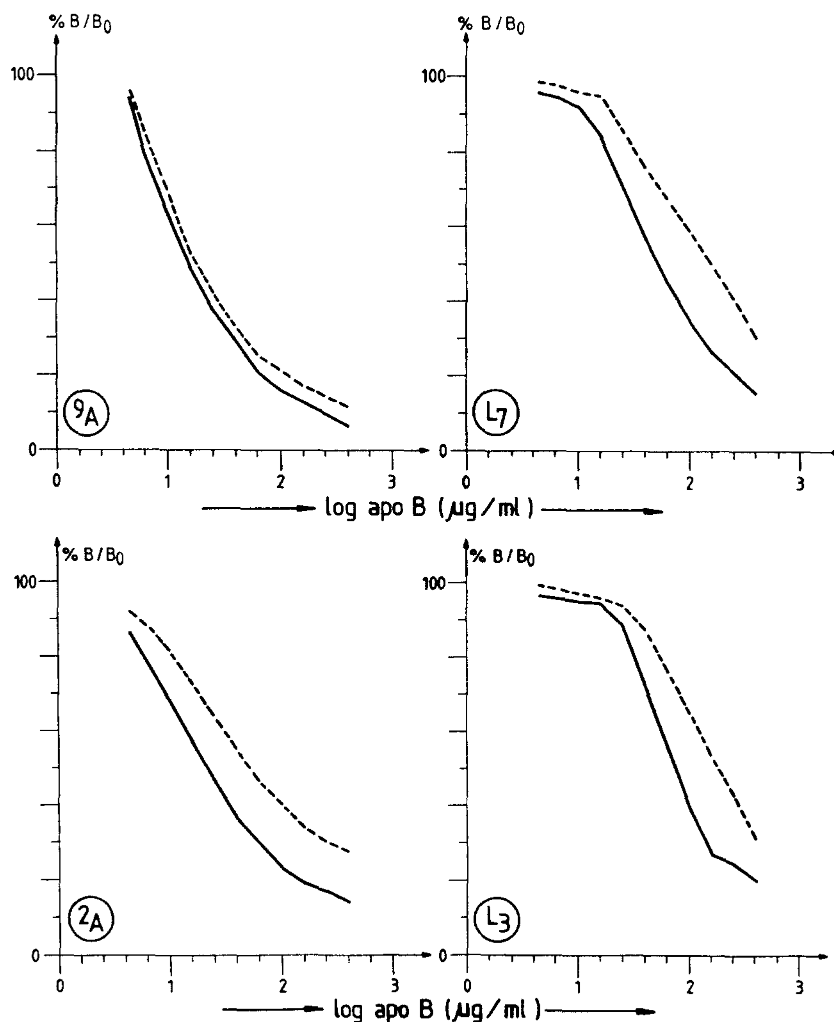

Fig. 3. Binding of monoclonal antibodies to LDL (——) and Lp[a] (-----) determined by competitive EIA. Concentrations of apoB were determined by Laurell electrophoresis. Results are expressed as percent inhibition in the EIA system and represent means of three experiments.

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TABLE 3. Specificity of monoclonal antibodies for LDL, Lp[a], and reduced Lp[a]

Mab	ApoB-LDL	ApoB-Lp[a]	ApoB-Lp[a] Reduced
		μg/ml	
2A	21.9 ± 2.8[a]	42.5 ± 7.8[b]	21.8 ± 1.7
9A	15.8 ± 2.4	17.8 ± 4.2	14.7 ± 1.9
6B	46.2 ± 5.1	73.4 ± 9.9[b]	41.7 ± 6.6
L3	70.4 ± 7.6	170.2 ± 19.0[b]	70.8 ± 11.8
L7	52.5 ± 8.3	145.2 ± 15.7[b]	46.8 ± 10.4

Results of competitive displacement curves are expressed as concentrations of protein required for 50% inhibition of antibody binding. ApoB concentrations in LDL were 95 ± 2% (n = 5), in reduced Lp[a] 98 ± 1% (n = 5), and in Lp[a] 65 ± 3% (n = 5) of total protein.
[a]Values are means ± SD, n = 5.
[b]Test of significance between two sample means in column 1 against column 2 or column 3 (Student's *t*-test); $P < 0.01$.

TABLE 4. Specificity of monoclonal antibodies for LDL, Lp[a], and reduced Lp[a]

Mab	ApoB-LDL	ApoB-Lp[a]	ApoB-Lp[a] Reduced
		μg/ml	
2A	37.0 ± 4.2[a]	55.8 ± 5.8[b]	34.9 ± 3.1
9A	28.7 ± 3.7	31.5 ± 10.3	28.6 ± 5.2
6B	43.2 ± 5.3	70.5 ± 9.9[b]	40.2 ± 3.9
L3	109.6 ± 10.6	213.8 ± 18.0[b]	114.8 ± 15.5
L7	74.1 ± 14.2	173.8 ± 13.8[b]	75.9 ± 10.5

Results of inhibitive displacement curves are expressed as concentrations of protein required for 50% inhibition of antibody binding. ApoB concentrations in LDL were 95 ± 2% (n = 5), in reduced Lp[a] 98 ± 1% (n = 5), and in Lp[a] 65 ± 3% (n = 5) of total protein.
[a]Values are means ± SD, n = 5.
[b]Test of significance between two sample means in column 1 against column 2 or column 3 (Student's *t*-test); $P < 0.01$.

somewhat higher for all antibodies. This fact may be explained by the very different methodological principles. In the competitive assay the determinant for the reaction seems to be the affinity of the antibody to the soluble antigens, whereas in the inhibitive assay part of the antibody is blocked by the formation of insoluble complexes.

Competitive and inhibitive assays performed with Lp[a] showed that for the antibodies 2A, 6B, L3, and L7 significantly higher amounts of Lp[a]-apoB are necessary for 50% inhibition of antibody binding as compared to LDL, indicating that those antibodies recognize apoB of Lp[a] to a lesser degree than that of LDL, whereas antibody 9A reacts in nearly the same way with Lp[a] as with LDL. In further experiments we separated apoprotein[a] from Lp[a] by reduction of the disulfide bonds with dithiothreitol and performed the same assays with reduced Lp[a]. Competitive and inhibitive displacement curves performed with antibody 9A showed very little differences between LDL, Lp[a], and reduced Lp[a] but also the antibodies 2A, 6B, L3 and L7 recognize reduced Lp[a] like LDL (**Fig. 4** and **Fig. 5**). The same results are obtained by calculation of the concentrations of apoB which are needed for 50% inhibition of antibody binding.

Results in Tables 3 and 4 show that the concentrations

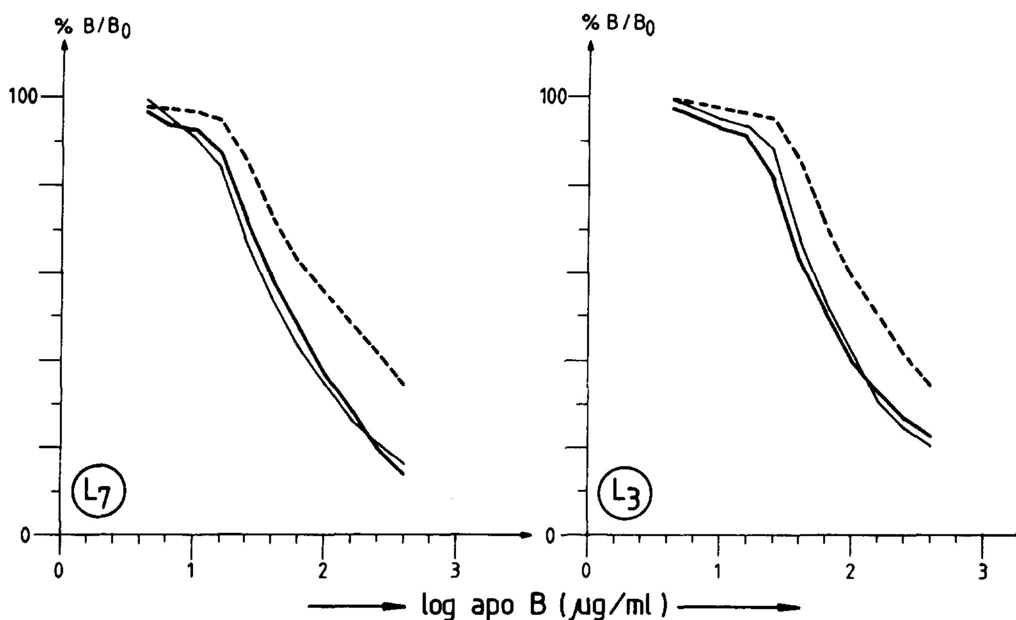

Fig. 4. Competitive curves using Mabs L7 and L3. Soluble LDL (▬), Lp[a] (-----), and reduced Lp[a] (—). The curves represent means of three experiments.

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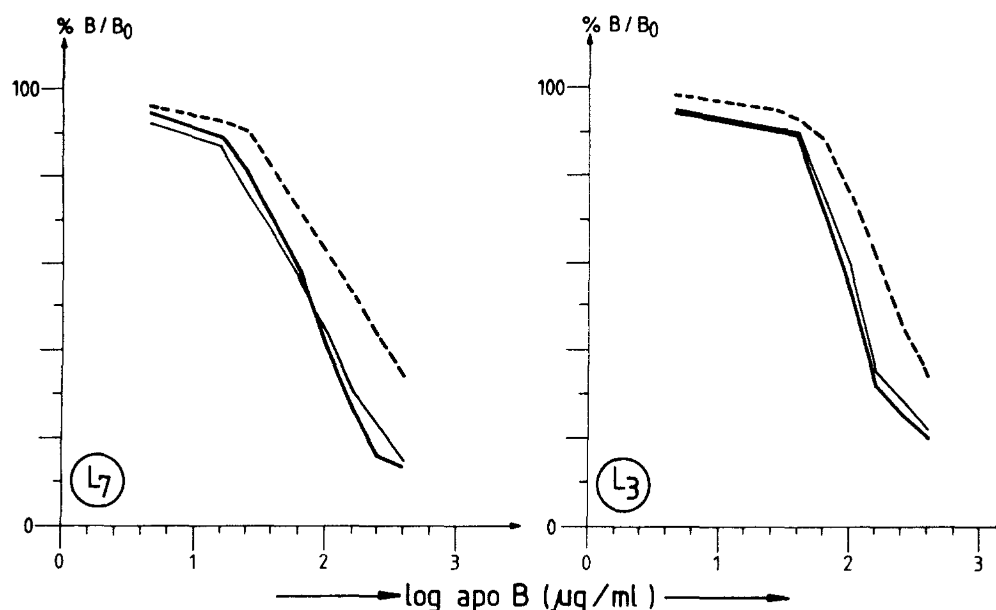

Fig. 5. Inhibition of the binding of monoclonal antibodies L7 and L3 to immobilized LDL by LDL (▬), Lp[a] (-----), and reduced Lp[a] (—) measured by inhibitive EIA. The curves represent means of three experiments.

of apoB from LDL and from reduced Lp[a] required for 50% inhibition of antibody binding are almost the same, whereas from unreduced Lp[a] up to threefold higher concentrations of apoB are necessary for 50% inhibition of antibody binding.

In control experiments native LDL was compared with reduced LDL in a competitive assay using the Mabs 2A, L3, and L7. As shown in **Fig. 6** both native and reduced LDL gave nearly identical displacement curves.

DISCUSSION

Previous investigations showed that normal unreduced Lp[a] is taken up by fibroblasts through the B/E-receptor-mediated endocytosis but shows poorer specificity for the receptor than LDL (12). The remaining lipoprotein particle from reduced Lp[a] lacking apo[a] however, was specifically bound as efficiently as LDL (13). There are several possibilities that may cause these differences. *a*) Certain "domains" close to the binding domain to the B/E-receptor are covered by apo[a]. *b*) Apo[a] causes steric hindrance in the interaction of Lp[a] with the B/E-receptor. *c*) Apo[a] is not attached to apoB at the binding site to the receptor but causes conformational changes in the binding region of apoB.

In order to investigate these open questions we prepared native and reduced Lp[a] from three different serum pools and studied the binding to different Mabs against apolipoprotein B in comparison with LDL prepared from the same serum. Each of the five antibodies that were studied recognized apoB of LDL as well as of Lp[a], indicated by very similar slopes of the binding curves (Fig. 3). There was no antibody that failed to react with native Lp[a]. As none of the antibodies gave any cross-reaction with apo[a] on direct EIA (Table 2), a binding of Mabs to the [a]-antigen of Lp[a] could be excluded. Using antibody 9A the amount of apoB needed for 50% inhibition of antibody binding was almost identical for LDL and native Lp[a]. The antibodies 2A and 6B needed an approximately twofold concentration of Lp[a]-apoB, and antibodies L3 and L7 an approximately threefold concentration for 50% inhibition of antibody binding in the competitive and inhibitive EIA (Figs. 4 and 5 and Tables 3 and 4). Since each of the lipoproteins was studied at identical apoB concentrations, we excluded the possibility that the lower reactivity of the antibodies with Lp[a] might be caused by a lower concentration of apoB.

The assumption that certain regions on apoB of Lp[a] could be different from those on LDL was confirmed by the results obtained by competitive and inhibitive EIA assays performed with reduced Lp[a]. As shown in Tables 3 and 4 and in Figs. 4 and 5 there were only small differences between the amount of apoB of LDL and reduced Lp[a] required for 50% inhibition of antibody binding indicating that epitopes on Lp[a] similar to those of LDL were exposed after separation of apo[a]. This phenomenon is in agreement with results published by Armstrong et al. (13) who showed a poorer binding of Lp[a] to the B/E-receptor in contrast to LDL and reduced Lp[a].

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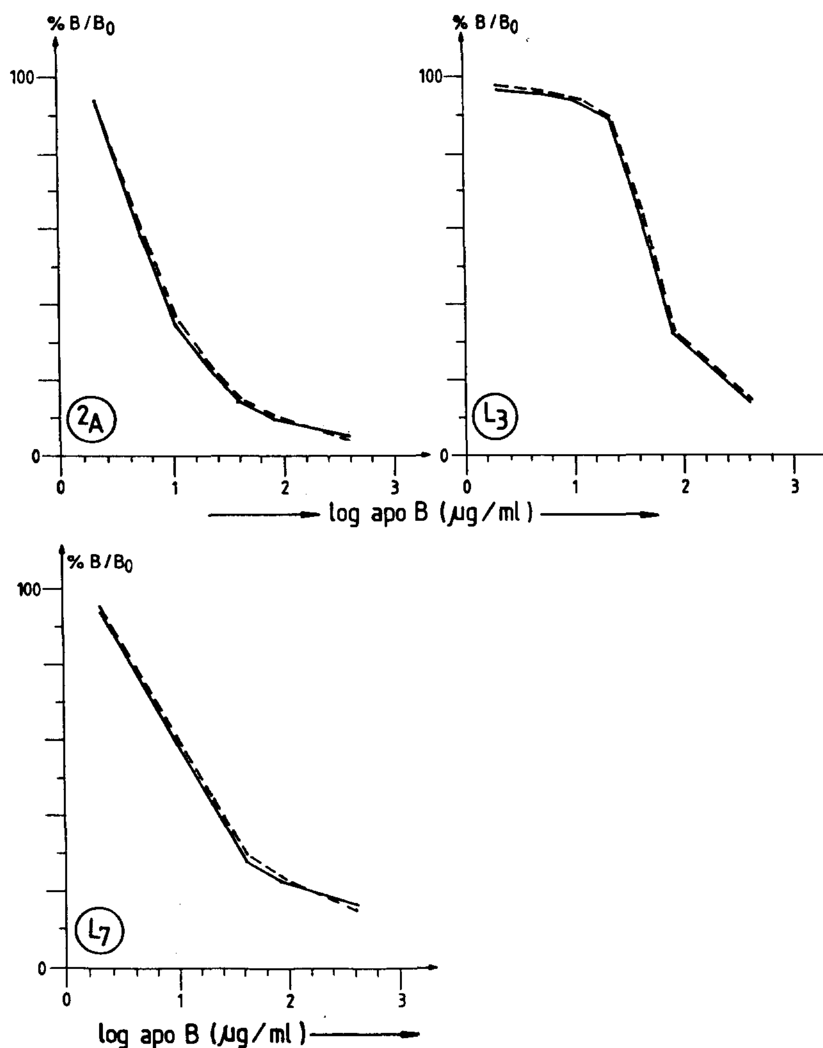

Fig. 6. Competitive displacement curves using Mabs 2A, L3, and L7. Native LDL (——), reduced LDL (-----). The curves represent means of three experiments.

In summary, we favor the idea that apo[a] probably covers certain epitopes of apoB in native Lp[a] although none of the investigated antibodies failed to react with Lp[a]. In addition, we speculate that the binding of the [a]-antigen to apoB via disulfide bridges also causes profound conformational changes of the apoB-region exposed to the surface. Otherwise one would not expect that four of the five Mabs that were studied would react well with intact Lp[a] but with a significantly lower affinity. This latter assumption, however, needs further support by physicochemical studies which are currently being undertaken in our laboratory.

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