papers and notes on methodology

A selective bi-site immunoenzymatic procedure for human Lp[a] lipoprotein quantification using monoclonal antibodies against apo[a] and apoB

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Abstract A selective bi-site ELISA assay procedure for quantification of Lp[a] lipoprotein in human plasma based on linkage of apo[a] to apoB is described. The lipoproteins referred to as apo[a]:B were captured by a mixture of two anti-apo[a] monoclonal antibodies (K07, K09) and were revealed by a mixture of six anti-apoB monoclonal antibodies coupled to peroxidase. Since apo[a] and plasminogen have striking similarities in protein structure, the selective binding of Lp[a]:B in our assay depended upon the marked difference in affinity of the K07 and K09 mixture for Lp[a]:B (K_d = 0.32 × 10^{-10}M) versus plasminogen (K_d = 0.47 × 10^{-7}M). The high sensitivity (the Lp[a]:B working range 0.06–0.40 μg/ml) and the use of anti-apoB as antibody tracer added to the selectivity of the assay. The expression of K07 and K09 epitopes determined by competitive inhibition method and the reactivity of Lp[a]:B particles measured by bi-site ELISA were similar on individual lipoproteins, independent to their plasma levels. The assay is precise, and intra- and interassay coefficients of variation were 4.7% and 9.6%, respectively. It yields quantitative Lp[a]:B values that correlate highly with Lp[a] levels obtained by electroimmunoassay with polyclonal antibody (r = 0.73) or with Lp[a] levels measured by the other bi-site ELISA using only K07 and K09 antibodies (r = 0.96). However, upon analyzing each individual plasma with an arbitrary Lp[a]-cut off of 15 mg/dl, evidence of the qualitative aspect of the lipoprotein was obtained. The group with Lp[a] < 15 mg/dl had higher frequency of subjects (65%) with the ratio Lp[a]/Lp[a]:B above 1.5. In contrast, only 19% of subjects had this high ratio in the group with Lp[a] > 15 mg/dl. This qualitative aspect of the lipoprotein depended probably on the size polymorphism of apo[a]. —**Vu Dac, N., H. Mezdour, H. J. Parra, G. Luc, I. Luyeye, and J. C. Fruchart.** A selective bi-site immunoenzymatic procedure for human Lp[a] lipoprotein quantification using monoclonal antibodies against apo[a] and apoB. *J. Lipid Res.* 1989. **30:** 1437–1443.

Supplementary key words plasminogen • bi-site ELISA • atherosclerosis

Of the human lipoproteins species, Lp[a] lipoprotein is unique with regard to structural and genetic aspects. Lp[a] is composed of glycoprotein [a] or apo[a] linked to apoB-100 through disulfide bonds (1, 2). Recent findings show that apo[a] and plasminogen, a precursor of plasmin enzyme that dissolves blood clots, have surprisingly similar gene sequence (3) and structure (4). Immunological cross-reactivity between the two proteins was also demonstrated (4–6). In addition, Lp[a] presents a marked heterogeneity both in the lipoprotein density (7, 8) and glycoprotein size (8, 9). Recent studies showed that apo[a] exhibited a size polymorphism (with apparent M_r in the range 400–700 kDa) that is associated with genetically controlled Lp[a] lipoprotein concentrations in plasma (10, 11). Clinical interest in this lipoprotein was greatly stimulated by the suggestion that high levels of Lp[a] could be an independent risk for atherosclerosis (12–14). On the basis of these recent findings, the possible interference of plasma plasminogen and the expression of antigenic determinants of Lp[a] must be firmly established when this lipoprotein has to be measured.

In order to quantify the lipoprotein containing both apo[a] and apoB, referred to as Lp[a]:B, we developed an original bi-site immunoenzymatic assay in which the mixture of two monoclonal antibodies (K07, K09) against apo[a] was used to capture apo[a]-containing lipoproteins. Thereafter the particles were revealed with a mixture of six anti-apoB monoclonal antibodies coupled to peroxidase. The differential affinity of K07 and K09 for apo[a] and plasminogen was studied, and the conditions of the assay, in which interference of plasminogen could be avoided, were established. The apparent concentrations of Lp[a]:B in plasma were compared with Lp[a] level as determined by other immunological techniques

Abbreviations: Lp[a], lipoprotein[a]; Lp[a]:B, lipoprotein[a] containing apo[a] and apoB; Mab, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; EIA, electroimmunoassay; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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that make use of monoclonal or polyclonal antibody against apo[a].

METHODS

Plasma

Plasma samples were collected from apparently healthy fasting subjects in EDTA Vacuotainers (Centre de medecine preventive Institut Pasteur de Lille). After addition of preservatives (final concentrations being: EDTA, 0.27 mmol/l; ϵ-aminocaproic acid, 0.9 mmol/l; chloramphenicol, 0.6 mmol/l; and glutathione, 0.3 mmol/l), plasmas were frozen at −20°C or stored at +4°C and assayed within 48 h.

Preparation of Lp[a] lipoproteins

Lp[a] was isolated from the density fraction (1.06–1.12 g/ml) of pooled plasma with high levels of Lp[a] by gel filtration on Sepharose CL 6B (2.6 × 100 cm) (Pharmacia Fine Chemicals).

Three peaks were obtained and the leading edge of the first peak was collected. Checks for contaminants revealed the presence of only apo[a] and apoB immunoreactivity as determined by immunoblotting. Double-decker rocket immunoelectrophoresis with polyclonal anti-apoB and anti-apo[a] showed that all of the apoB was cross-linked to apo[a]. The purified Lp[a]:B particles contained 30% protein and 61% lipid (carbohydrates were not determined) in agreement with results described by Albers and Hazzard (15). The conversion to total lipoprotein Lp[a]:B was also based on the percentage of protein (15).

Preparation of monoclonal antibodies

Antibodies K07 and K09 were obtained from the fusion between SP_2O and mouse splenic cells immunized with purified Lp[a] lipoproteins. These antibodies showed reactivity against apo[a] and cross-reacted with plasminogen as determined by immunoblotting (6).

Monoclonal antibodies against apoB were obtained from the cell fusion performed with SP_2O and splenic cells of the mouse immunized with LDL. Antibodies B04, B05, B06, B07, B18, and B19 are specific for apoB and do not react with other apolipoproteins or plasma proteins. Their epitopes are located on T1/T3 thrombin fragments of apoB-100 (16). The mixture of monoclonal anti-apoB antibodies or antibody K09 was coupled to peroxidase (EC1.11.1.7 Boehringer, Mannheim, F.R.G.) as described (17).

Affinity of monoclonal antibodies

The dissociation constants of antigen-antibody equilibria in solution were determined as described by Friguet et al. (18). We incubated 0.1 ml of antibody (30 ng/ml of K07 or K09) with 0.1 ml of Lp[a]:B or plasminogen at various concentrations. After 15 h of incubation at room temperature, 0.1 ml of the mixture was transferred and incubated for 2 h at 37°C in the well of microiter plates previously coated with purified Lp[a]:B (0.5 μg/well). The antibody bound was detected by peroxidase-labeled rabbit anti-mouse Ig. The dissociation constant K_d was calculated by the modified Scatchard equation (18). By this competitive inhibition method under the same incubation conditions (15 h of incubation is long enough for equilibrium to be reached), we also determined the expression of epitopes K07, K09 on ten individual plasma samples Lp[a]:B ranging from 1 to 40 mg/dl.

Bi-site ELISA procedures

Polystyrene microtiter plates (Costar 3590, USA) were coated overnight at room temperature with 0.1 ml of a mixture of K07 and K09 (molar ratio 1:1) at 20 μg/ml in PBS. The standard was diluted to give an Lp[a]:B particle concentration ranging from 0.01 to 0.40 μg/ml, and the plasma samples were diluted 200- to 800- to 3200-fold in PBS-1% BSA.

Plates were washed four times with PBS before adding 0.1 ml of sample or standard (in duplicate) to the wells and incubated for 150 min at 37°C. After washing with PBS, 0.1 ml of peroxidase coupled to anti-apoB at dilution 1/1000 was added and incubated for 120 min at 37°C. Then wells were washed and incubated with 0.1 ml of substrate solution (3 mg/ml of *o*-phenylenediamine and 0.64 μl/ml of concentrated H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.6) for 30 min in the dark. The reaction was stopped by addition of 0.1 ml of 1 N HCl and the absorbance was determined at 492 nm. Washing, distribution of reagents, and measure of absorbance were performed with the ELISA Processor II (Behring, F.R.G.) For the comparison study, Lp[a] was measured under the same conditions with K07 as immobilized antibody and K09 as peroxidase-conjugated antibody.

Plasminogen interference

To assess the interference of plasminogen under the conditions of our bi-site ELISA assay, competitive inhibition was studied with purified Lp[a]:B and purified plasminogen (Diagnostica Stago, France). To the well, previously coated with a mixture of K07 + K09 antibodies (2 μg/well), 0.05 ml of Lp[a] at 0.06 mg/dl and 0.05 ml of plasminogen at concentrations ranging from 0.1 to 80 mg/dl were added and incubated. The bound Lp[a]:B was then determined as described above.

Electroimmunoassay procedure

Electroimmunoassay of Lp[a] was performed according to Kostner et al. (13) with commercially available sheep anti-Lp[a] antisera and a pool of serum as secondary standard. Double-decker rocket immunoelectrophoresis was performed as previously described (2). The lower gel

to which the samples were applied contained anti-apoB antisera and the upper gel anti-Lp[a].

RESULTS

Characterization of monoclonal antibodies

Of the proteins from human plasma, immunoblotting revealed only a reactivity of the monoclonal antibodies K07 and K09 against apo[a] and plasminogen (6). To study the interaction between these antibodies and the two distinct proteins, we determined the antigen-antibody dissociation constants (K_d) of K07 and K09 alone or the mixture of K07 + K09. Based on the mean apparent M_r of apo[a] (500 kDa) and plasminogen (92 kDa) (3) we observed a marked difference in affinity of K07 and K09 for Lp[a]:B (0.87×10^{-11}M and 0.29×10^{-10}M) versus plasminogen (0.45×10^{-7}M and 0.47×10^{-7}M). The K_ds of the mixture (K07 + K09) were 0.32×10^{-10}M and 0.47×10^{-7}M for Lp[a]:B and plasminogen, respectively (**Fig. 1**). To diminish a possible influence of apo[a] heterogeneity on the expression of one epitope, we used the mixture of monoclonal antibodies (19) K07 and K09 in our ELISA assay. The concomitant expression of these epitopes was determined on 10 individual plasmas with different Lp[a] levels ranging from 1 to 40 mg/dl. The slopes of the competitive inhibition curves were similar; the data transformed by logit of antibody binding ranged from − 1.80 to − 2.15 with a mean of − 2.00; the typical slope of the pool of plasma Lp[a]:B was − 2.05 (**Fig. 2**). These results suggest that epitopes K07 and K09 were expressed similarly on Lp[a] of different individuals.

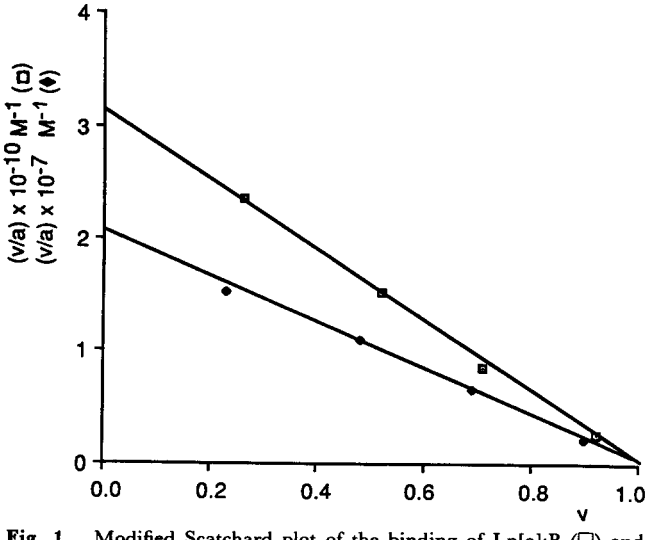

Fig. 1. Modified Scatchard plot of the binding of Lp[a]:B (□) and plasminogen (♦) to the mixture of (K07 + K09) antiapo[a] Mab; v is the fraction of bound antibody and a is the concentration of free antigen (18).

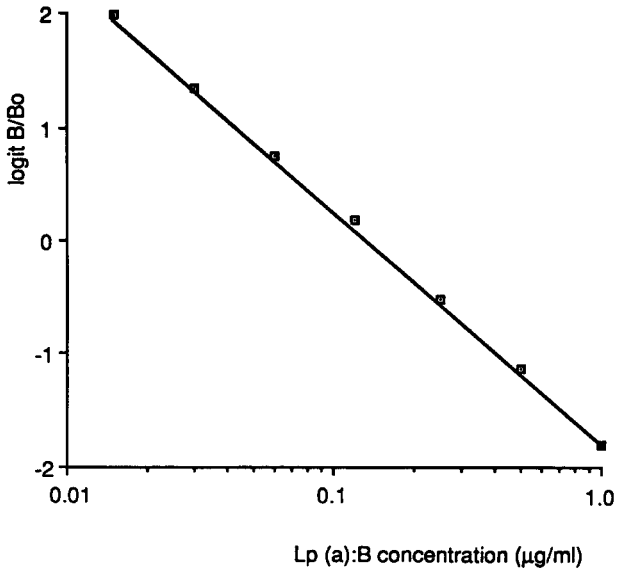

Fig. 2. Competitive inhibition of binding of the mixture (K07 + K09) Mab to immobilized Lp[a]:B by Lp[a]:B lipoproteins of the plasma pool.

To standardize the measurement of particles containing apo[a] and apoB, we used the mixture of six monoclonal antibodies anti-apoB B04, B05, B06, B07, B18, and B19 in our bi-site ELISA assay. The K_ds of this antibody mixture for apoB-Lp[a] and apoB-LDL were 2.85×10^{-8}M and 0.93×10^{-8}M, respectively, showing the lower affinity of antibodies for antigen determinants on apoB-Lp[a] than on apoB-LDL as described recently (20).

Bi-site ELISA assay

To avoid the interference of plasma plasminogen, we increased the sensitivity of the assay using a high concentration of anti-apoB peroxidase-conjugated antibodies to reveal the particles Lp[a]:B captured by immobilized antibodies K07 + K09 (**Fig. 3**). The working range of the standard curve performed with purified Lp[a]:B was between 0.06 µg/ml and 0.40 µg/ml as expressed by total lipoprotein (15). Because of the polymorphism of apo[a], we used a pool of 50 plasmas as secondary standard (11.5 mg/dl of Lp[a]:B). This standard did not contain detectable free apo[a] as determined by double-decker rocket immunoelectrophoresis. Thus, all apo[a] could be considered to be linked to apoB in this standard plasma.

To test the conditions under which plasma plasminogen could interfere with the measurement of Lp[a]:B, we performed competition experiments between a constant amount of Lp[a]:B (0.06 mg/dl) and various levels of purified plasminogen (range 0.1–80 mg/dl). Plasminogen at high concentration (40 mg/dl) could inhibit up to 56% of the binding of Lp[a]:B (**Fig. 4**). However at a concentration of 1.25 mg/dl, the inhibition was about 18%. Inasmuch as this plasminogen level corresponds generally to the 1/40 dilution of the plasma which contains relatively

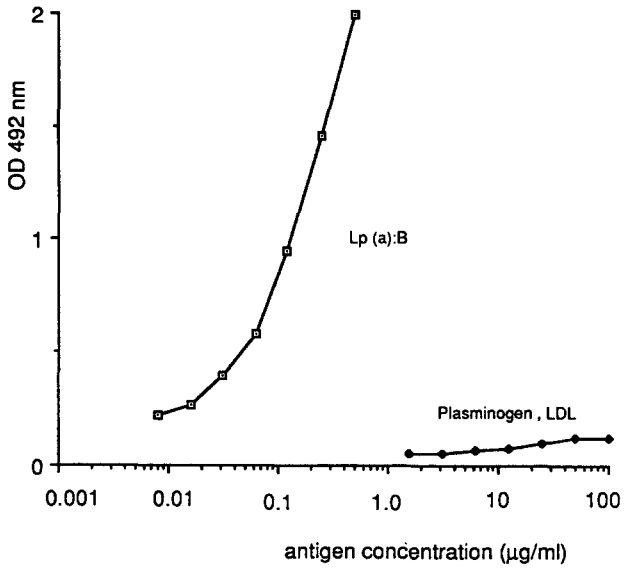

Fig. 3. Specific Lp[a]:B particles by the bi-site ELISA assay using anti-apo[a] and anti-apoB monoclonal antibodies. Purified Lp[a]:B particles (□) (µg/ml total lipoprotein), plasminogen or LDL (◇) (µg/ml protein). Typical absorbance of zero sample is 0.050.

invariant levels of plasminogen (47 ± 7 mg/dl) (21) and inasmuch as in practice we only used this dilution for the determination of very low plasma Lp[a]:B level (<0.24 mg/dl), the interference of plasma plasminogen was therefore avoided under the conditions of our assay.

To determine the immunoreactivity of individual Lp[a]:B, we measured Lp[a]:B in ten plasma samples with the Lp[a]:B range 1–40 mg/dl. The ELISA curves as expressed by logit of absorbance at 450 nm shared the slopes ranging from 1.85 to 2.30 with a mean of 2.15. The slope of the secondary standard was 2.12. To assess the accuracy of the assay, we performed a recovery experiment in which different quantities of purified Lp[a]:B (5, 10, 50 µg) were added in three plasmas with low, medium, and high Lp[a]:B levels (1,10,30 mg/dl). The recovery ranged from 85 to 100%.

The precision of the method was explored by measuring nine plasmas 10 times on the same plate to obtain the within-assay coefficient of variation (mean intraassay CV = 4.7%). These plasma samples were assayed on 3 days to determine the between-assay CV (mean interassay CV = 9.6%). The effect of storage at −20°C on plasma Lp[a]:B content was investigated with these nine plasmas on different days; the mean of variation was 9.8%.

Comparison with EID and other methods

We have compared the levels of Lp[a]:B measured by the proposed bi-site ELISA assay with those obtained by rocket electroimmunoassay (Lp[a] levels higher than 6 mg/dl, n = 30, $r = 0.73$), and by another bi-site ELISA using immobilized K07 and peroxidase-K09 antibody (all Lp[a] levels, n = 125, $r = 0.96$). High correlations were obtained between these immunological methods. The data for the correlation are summarized in **Table 1.**

Comparative study of Lp[a] and Lp[a]:B

In order to study the difference in apparent Lp[a] levels, 125 plasma samples were analyzed for Lp[a]:B concentration and Lp[a] using antibodies K07 and K09. The highest correlation was obtained with these methods when all plasma Lp[a] concentrations were taken into consideration (Table 1, **Fig. 5**). However, when individual plasma samples were analyzed, we observed a large difference between apparent Lp[a] and Lp[a]:B levels. To quantify this difference, we calculated the ratio of Lp[a] versus Lp[a]:B. At an arbitrary cut off level of 15 mg/dl (11), we could subdivide the population into two distinct groups. The first group with Lp[a] levels lower than 15 mg/dl had a high frequency of subjects with higher apparent Lp[a] versus Lp[a]:B (65% shared the ratio 1.5–3.0). In contrast, the second group with Lp[a] levels higher than 15 mg/dl had a high frequency of subjects showing similar apparent Lp[a] versus Lp[a]:B (81% presenting the ratio < 1.5). The results are shown in **Fig. 6.** To investigate the possibility that the relatively higher Lp[a] than Lp[a]:B level would mean the presence of Lp[a] free of apoB or free apo[a] in certain plasma, 8 plasmas with higher apparent Lp[a] than Lp[a]:B levels were checked by double-decker rocket immunoelectrophoresis. The results (**Fig. 7**) were negative for all samples: no free apo[a] (unassociated with apoB) was

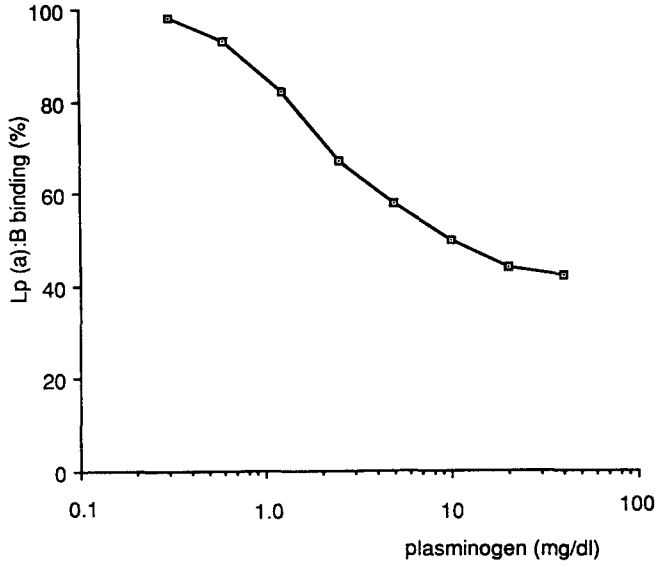

Fig. 4. Interference of plasminogen under the conditions of the bi-site ELISA for Lp[a]:B. Various levels of plasminogen (0.1–80 mg/dl) and a constant amount of Lp[a]:B (0.06 mg/dl) competed for the binding to immobilized antibodies K07 + K09. Results are expressed by % of B (presence of plasminogen competitor)/B_0 (absence of plasminogen).

TABLE 1. Comparison of methods for measuring Lp[a] lipoproteins in plasma

Y	X	n	Regression Equation	r
Bi-site ELISA Lp[a]	bi-site ELISA Lp[a]:B	125	Y = 1.09 X + 1.6	0.96
EIA Lp[a]	bi-site ELISA Lp[a]:B	30	Y = 0.85 X + 4.1	0.73

found as compared with dithiothreitol-treated samples (22) and purified apo[a]. This is in agreement with several previous reports (1, 2). Therefore, the difference in apparent Lp[a] versus Lp[a]:B seems, probably, to be linked to the polymorphism of apo[a] rather than the presence of Lp[a] without apoB or the presence of a high level of free apo[a] in the plasma.

Distribution of Lp[a]:B lipoproteins

The distribution of Lp[a] and Lp[a]:B among a group of 125 subjects from northern France was strongly skewed, with the highest frequency at low levels of these lipoproteins (Fig. 5). About 75% of the subjects had levels lower than 15 mg/dl; this is in agreement with other results obtained by other authors using different immunochemical methods (15, 23–25). Because Lp[a]:B particles were quantified by the selective and sensitive assay, it is interesting to note that all subjects had particles containing apo[a] and apoB in this population.

DISCUSSION

We have developed a selective bi-site ELISA method to quantify plasma Lp[a] based on the linkage of apo[a] to apoB present on the same particle referred to as Lp[a]:B. Because of the immunological cross-reactivity of K07 and K09 and other anti-apo[a] antibodies with plasminogen (4–6) resulting from the striking similarity of their protein structure, the selective binding of Lp[a] in our assay depends upon the marked differernce in affinity of the K07, K09 mixture for Lp[a]:B (K_d = 0.32 × 10^{-10}M) versus plasminogen (K_d = 0.47 × 10^{-7}M). The possible interference of plasma plasminogen within the conditions of our assay was investigated. To this end we performed competition studies between purified Lp[a] and plasminogen for binding to immobilized antibodies K07, K09. In fact plasminogen showed up to 56% of inhibition of Lp[a]:B binding, but only at high concentrations (40 mg/dl) corresponding to undiluted plasma which generally contains a constant plasminogen level (47 ± 7 mg/dl) (21). Inhibition of 18% was obtained with plasminogen at a concentration of 1.25 mg/dl corresponding to the plasma dilution of 1/40; in practice we did not use this dilution to measure

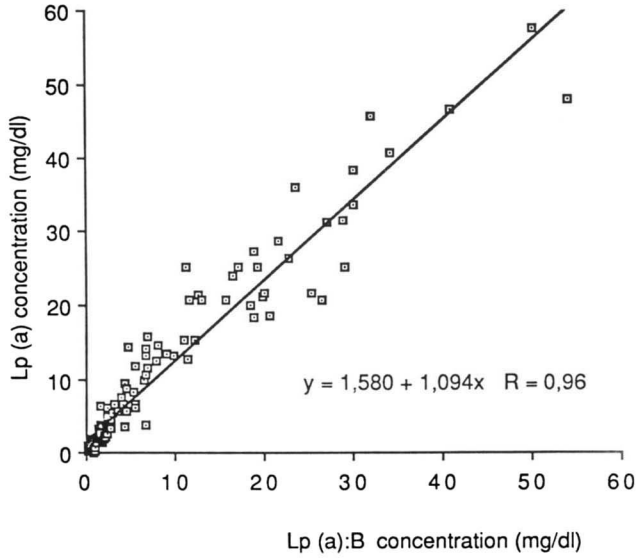

Fig. 5. Comparison of Lp[a] and Lp[a]:B plasma levels as measured by the two different bi-site ELISA assays. High correlation was obtained when all Lp[a] concentrations were taken into consideration.

plasma Lp[a]:B. Furthermore, we revealed the Lp[a]:B captured by anti-apoB monoclonal antibodies; this step added further selectivity to the method.

Since the size polymorphism of apo[a] is associated with Lp[a] levels in plasma (10, 11), the antigenic determinant recognized by monoclonal antibodies could be expressed variably on different individual samples of plasma Lp[a]. We studied the concomitant expression of K07 and K09 epitopes on 10 plasmas with various Lp[a]:B levels. The similar epitope expression of apo[a] on individual plasma lipoproteins allows the use of these monoclonal antibodies for the quantitative determination of Lp[a]:B in all plasma samples. The Lp[a]:B values determined by the bi-site ELISA correlated well with those obtained with the electroimmunodiffusion method using a polyclonal antibody. The sensitivity of our bi-site ELISA was higher than radioimmunoassay (23); its selectivity was also

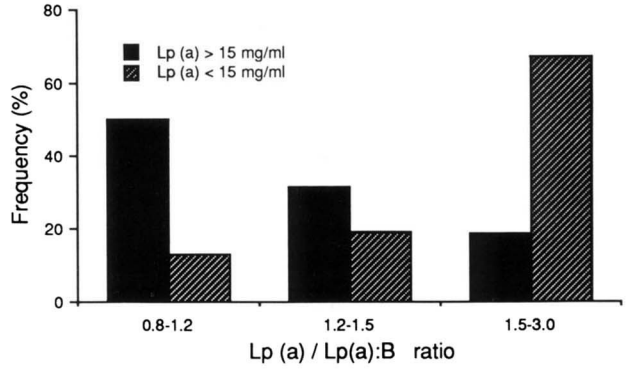

Fig. 6. Distribution of the ratio Lp[a]/Lp[a]:B as a function of Lp[a] levels. An arbitrary cut-off (15 mg/ml) was used (11) to subdivide the two groups.

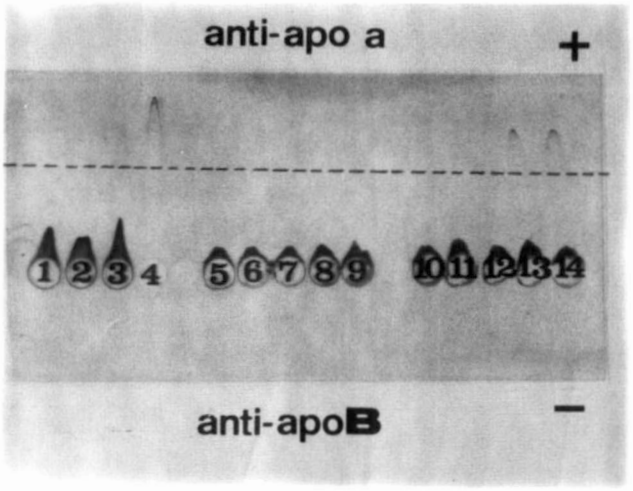

Fig. 7. Double-decker rocket immunoelectrophoresis. The lower agarose deck contains anti-apoB and the upper deck contains anti-apo[a]. Ten μl of sample was added to the well. Well 1 LDL (7 μg); wells 2 and 3, Lp[a]:B (1-7 μg); well 4, apo[a] (7 μg); wells 5-12, plasma samples showing higher Lp[a] as compared to Lp[a]:B; wells 13 and 14 dithiothreitol-treated plasma samples.

demonstrated and it allowed us to confirm the presence of Lp[a]:B lipoprotein particles in all subjects as noted by others previously (23, 25). The distribution of Lp[a]:B among the group of 125 subjects from the north of France is similar to those observed in different white populations. The distribution was strongly skewed with the highest frequency at a low level of Lp[a]:B; about 75% of subjects had levels lower than 15 mg/dl as measured by polyclonal antibodies (15, 23, 24).

From all these results, we conclude that the monoclonal antibodies K07 and K09 do not detect the conformational polymorphism of apo[a] as was observed on apoB probed by monoclonal antibodies (26, 27). However, the fact that the ratio of Lp[a] to Lp[a]:B correlates with apparent Lp[a] plasma concentration suggests a marked influence of the size polymorphism of apo[a] on the measurement of the lipoprotein particle by the bi-site ELISA assay. Although the presence of apo[a] linked to apoB could induce profound conformational changes on several apoB epitopes (16, 20), the accessibility of apoB epitopes studied with 27 monoclonal antibodies (including 4 antibodies used in our assay) was similar for apoB-LDL/apoB-Lp[a] and independent of apo[a] polymorphism (16). Thus the difference between Lp[a] and Lp[a]:B levels appears to be linked to a number of repetitive kringles that characterize the size polymorphism of apo[a] (3, 4). Phenotypes B, S_1, S_2 with an apparent low molecular weight of apo[a] associated with high plasma Lp[a] levels (>15 mg/dl) (10, 11) can offer fewer apo[a] epitopes as compared to phenotypes S_3, S_4. These latter are associated with plasma Lp[a] levels lower than 15 mg/ml, they have an apparent high molecular weight, and thus present a large number of apo[a] epitopes to which monoclonal antibodies K07, K09 bind.

In conclusion, the method described in this paper is selective, sensitive, reproducible, and quantitative. As it also shows a qualitative aspect of lipoprotein Lp[a], it promises to be a useful tool for further clinical and basic research investigations.

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