*GENETIC AND ANTIGENIC STUDIES AND PARTIAL PURIFICATION OF A HUMAN SERUM LIPOPROTEIN CARRYING THE Lp ANTIGENIC DETERMINANT**

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The Lp(a) genetic variant of human serum β-lipoprotein was detected by Berg,[1] using immunodiffusion with heteroimmune precipitating antiserum. Genetic studies by various investigators[2, 3] indicated that the presence or absence of the Lp(a) factor was under the control of a single, autosomal gene and that the frequencies of the Lp^a and Lp genes were approximately 0.19 and 0.81, respectively. The Lp system is independent[4] of the Ag system.[5] Rittner and Wichmann[6] suggested that a complex of Lp genes may give rise to quantitative differences in the concentration of Lp-lipoprotein in different individuals.

Using heteroimmune antisera which distinguish the Lp(a+) and Lp(a−) phenotypes, we have developed a method for isolation and partial purification of the Lp-lipoprotein by density gradient ultracentrifugation. This method and preliminary genetic and antigenic studies are presented in this report.

Materials and Methods.—(a) *Preparation of antisera: Method 1:* Rabbits were injected intravenously every third day for four weeks with 1 ml of Lp(a+) serum. Blood was collected 7 days after the last injection. *Method 2:* A rabbit was given a 1-ml intraperitoneal injection of partially purified Lp-lipoprotein in complete Freund's adjuvant, followed by two 1-ml injections of Lp-lipoprotein without adjuvant at 2-week intervals. The rabbit was bled 1 week after the last injection. Anti-α- and anti-β-lipoprotein sera were prepared by the same procedure. Commercial rabbit antisera specific for human[4] α_1-lipoprotein and β-lipoprotein (Certified B ood Donor Service Inc., Woodbury, N. Y.) were also used.

(b) *Absorption techniques:* Antisera against Lp(a+) serum were absorbed with 1.5 vol of Lp(a−) serum at 4°C for 24 hr. Antiserum against partially purified Lp-lipoprotein was absorbed for 4 hr at 37°C and 12 hr at 4°C with β-lipoprotein purified from Lp(a−) serum (30 mg/ml antiserum) and 0.1 vol of a concentrated subfraction of Lp(a−) serum containing serum proteins with densities greater than 1.12 gm/ml. Sodium azide (1 mg/ml) was added, and antisera were stored at −20°C.

(c) *Procedures for immunological analysis.* Ouchterlony analyses[8] were performed in 0.8% agarose gel in pH 7 phosphate buffer[7] in 6-cm dishes (Consolidated Labs., Chicago, Ill.), using Feinberg[9] (Consolidated Labs) or Grafar[9] (Grafar Corp., Detroit, Mich.) well cutters. Plates were read after 24 and 48 hr incubation at 37°C and again after washing with 0.15 *M* NaCl and distilled water.

(d) *Intragel absorption tests*[10] were used to study cross-reactions between Lp-, α-, and β-lipoprotein. The concentrations of antiserum or antigen used in the absorption gel were those which gave the fastest formation of a stable, centered, precipitin line when tested on an Ouchterlony plate.

(e) *Isolation of lipoproteins:* Fresh serum was obtained from adult male donors. All preparations were kept between 2°C and 10°C and never frozen. Disodium ethylenediaminetetraacetate (Na_2 EDTA) (0.1 mg/ml) was added to serum and salt solutions. All chemicals were reagent grade. Salt solutions were made volumetrically. Densities were determined with a hydrometer or from dry weight and *International Critical Table* data. Spinco model L preparative ultracentrifuges were used.

Ultracentrifugal fractionation of lipoprotein into various density classes was done by

adjusting solvent densities with solid NaBr and/or by dialysis. The Lp factor has a density between 1.064 gm/ml and 1.12 gm/ml.

The following routine was used for preparing partially purified Lp-lipoprotein: Serum was adjusted to a solvent density of 1.064 by the addition of 8.6 gm of NaBr/100 ml of serum and centrifuged 24 hr (100,000 *g*) in no. 40 Spinco rotors. Lipoproteins with hydrated densities less than 1.064 floated into the top 2 ml of the tube and were removed using a tube cutter.[11] The infranatant solution was pooled, adjusted to a solvent density of 1.12 by the addition of 7.1 gm of NaBr/100 ml, and dialyzed overnight against a solution of NaBr (density = 1.12). This material was centrifuged for 48 hr (100,000 *g*) in the no. 40 Spinco rotor. The upper 2 ml was removed as above and pooled. This material contained the major part of the Lp activity.

The Lp-active fraction was concentrated by pervaporation at 4°C or by centrifugation and dialyzed against 1.12 density NaBr or against 1.085 density NaBr. After dialysis was complete, the sample was further purified in five-layer density gradients. In preliminary experiments (gradient method 1), the gradients were made by successively layering 1-ml amounts of NaBr of densities 1.12 and 1.10, followed by 1.0 ml of the lipoprotein-containing sample in 1.085 density NaBr and 1-ml amounts of NaBr of densities 1.064 and 1.045. These gradients were centrifuged (100,000 *g*) in the SW39 rotor for 24 hr, and fractionated into samples of known volume using a tube cutter. Depending on the experiment, as many as nine subfractions were removed. These subfractions are denoted by subscripts on the Lp designation, e.g., Lp X_i (X = Lp-lipoprotein preparation number, $i = 12 \ldots 20$). The subscript i increases with increasing density of the isolated fraction (as estimated from the known densities of the layers used in making the gradient). Each fraction was tested against specific anti-Lp-, commercial anti-α-, and commercial anti-β-lipoprotein sera, using gel diffusion. Fractions containing the Lp activity were either pooled or used individually for analytical ultracentrifugation and gel diffusion experiments. In later experiments (gradient method 2) the five-layer gradients were made as described above except that the Lp fraction was dialyzed against 1.12 density NaBr and incorporated into the bottom layer of the gradient.

Analytical ultracentrifugal flotations were done with a Spinco model E at 20°C, 30,000 rpm, with the sample in NaBr solution of 1.21 density.

Results.—Comparison of anti-Lp sera used in this study with known anti-Lp (a) serum: Two of our anti-Lp sera and a known anti-Lp(a) serum, kindly supplied by Dr. Kare Berg, gave a "reaction of identity" with Lp(a+) serum (Fig. 1*a*), indicating that our antisera were specific for the Lp-lipoprotein. This identity was supported by tests of our antisera in Dr. Berg's laboratory against a panel of sera of known Lp type, and by tests in our laboratory against a reference panel tested for us by Dr. Berg. In all tests the reaction patterns were essentially concordant. Our antisera, compared by Dr. Ch. Rittner with his anti-Lp(a) serum, gave the same strong positive reactions, but a few differences were observed in weak positive and negative reactions. There seems little question, however, that our antisera react with Lp-lipoprotein.

Genetic studies: Classifications of 674 Caucasian individuals showed a phenotype frequency for Lp(a+) of 0.501, and for Lp(a−) of 0.499. The Lp(a+) group included 75 weak reactions. In preliminary family studies in this population, 16 Lp(a-) × Lp(a−) matings resulted in 28 Lp(a−) and no Lp(a+) offspring. Lp(a+) × Lp(a−) and Lp(a+) × Lp(a+) matings gave Lp(a+) and Lp(a−) offspring in the frequencies expected with dominant inheritance of the Lp(a+) phenotype, as demonstrated by Berg.[3]

Specificity of anti-Lp sera: After absorption, the anti-Lp serum was specific in that it gave only a single precipitin band when tested against whole serum

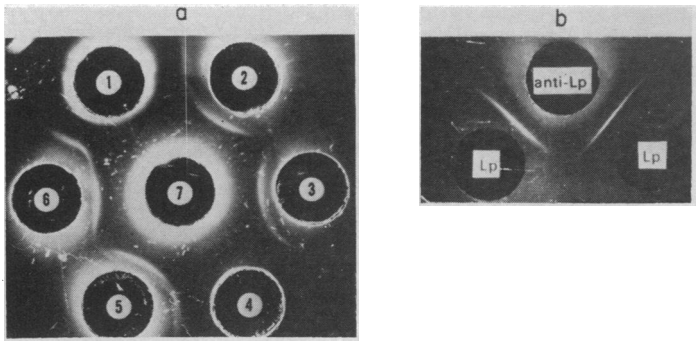

FIG. 1.—(*a*) Agarose gel diffusion tests. *Well 1*, anti-Lp prepared by method 2; *well 4*, anti-Lp prepared by method 1; *well 7*, reference anti-Lp(a); *wells 2, 3, 5, and 6*, Lp(a+) serum.
(*b*) The two Lp-antigen wells contain Lp-lipoprotein isolated from adjacent fractions of a final preparative density gradient (method 1).

(Fig. 1*a*) or partially purified Lp-lipoprotein (Fig. 1*b*). Intragel absorption tests (Fig. 2) showed that the Lp anti-Lp precipitation reaction was not affected by the presence of α- or β-lipoprotein in the gel (Figs. 2*a–d*). The α anti-α- and β anti-β-lipoprotein reactions were prevented by the homologous (Fig. 2*a, c, e, f*) and not by the heterologous (Fig. 2*b, d–f*) lipoprotein in the gel. Figure 2*d* confirms the presence of excess absorbing β-lipoprotein in the anti-Lp serum.

Antigenic relationship of Lp- to β-lipoproteins: All Lp preparations gave precipitation reactions with commercial anti-α- and anti-β-lipoprotein sera (Fig. 2*b, d*). These commercial antisera might have contained Lp(a) antibodies, so antisera were prepared against purified α(Lp a−)-lipoprotein (i.e., α-lipoprotein from an Lp(a−) serum) and β(Lp a−)-lipoprotein in order to investigate possible cross-reactivity.

An intragel absorption test (Fig. 3*a*) showed that anti-β(Lp a−) serum prevented the specific Lp anti-Lp reaction. The addition of anti-β(Lp a−) serum to preparations in wells 3 and 4 was made to ensure complete absorption, if it were to occur, and to narrow the circle of precipitation around well 3 or 4 as compared to that around well 1 or 2.

The effect of anti-β(Lp a−) serum on precipitation of Lp antigen by its specific antibody in a nonabsorbing gel is seen in Figure 3*b*. The precipitation lines between the anti-Lp serum well 7 and wells 1–3 show the variation that occurs in the amount of precipitation upon dilution of the antigen. The precipitation line between the anti-Lp serum well 7 and well 6 was due to precipitation of Lp antigen in excess of that precipitated in the well by the anti-β(Lp a−) serum and which diffused out to react with its specific antibodies. This line was absent between well 5 and well 7 because of the lack of excess Lp antigen. The precipitation line between well 4 and well 7 was caused by excess β(Lp a−)-lipoprotein antibodies which were not precipitated in well 4 due to the even greater dilution of Lp antigen preparation added to this well. The excess β(Lp a−)-lipoprotein antibodies diffused out of the well and reacted with the excess β-lipoprotein known to be present in the anti-Lp serum. The precipitation line between wells 4 and 3 cor-

FIG. 2.—The plates shown in (*a*), (*b*), and (*e*) had β-lipoprotein, isolated from Lp(a+) serum, incorporated in the agarose gel. The plates shown in (*c*), (*d*), and (*f*) had α-lipoprotein isolated from Lp(a+) serum incorporated in the agarose gel.

roborates the presence of excess β(Lp a−)-lipoprotein antibodies in well 4, since well 3 contains only antigen.

Similar absorption tests using anti-α(Lp a−)-lipoprotein serum gave results which were difficult to interpret because of the low titer of the antiserum. Further experiments are in progress.

These experiments suggest that the lipoprotein molecules that carry the Lp antigenic determinant also carry at least one antigenic determinant in common

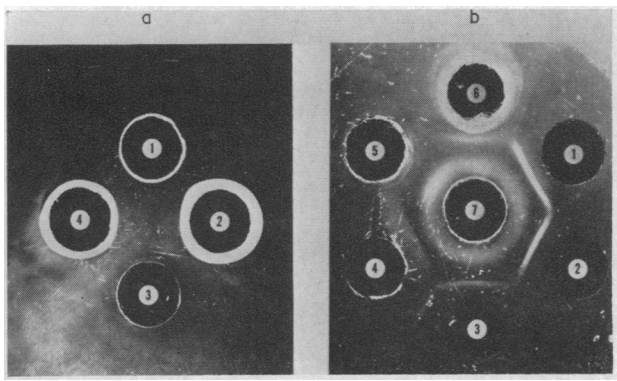

FIG. 3.—(*a*) The agarose gel contains 25% v/v of anti-β(Lp a−) serum. Wells contained Lp antigen diluted 25% v/v with 0.15 *M* NaCl (1) or with anti-β(Lp a−) serum (*3*), and anti-Lp serum diluted 25% v/v with 0.15 *M* NaCl (*2*) or with anti-β(Lp a−) serum (*4*).

(*b*) The agarose gel contained no antiserum. Wells contained concentrated (*1, 6*), 1:2 (*2, 5*), and 1:4 (*3, 4*) dilutions of Lp antigen further diluted 25% v/v with 0.15 *M* NaCl (*1–3*) or anti-β (Lp a−) serum (*4–6*). Well 7 contained anti-Lp serum.

with β-lipoprotein molecules, which themselves do not carry the Lp specificity. These data do not eliminate the possibility that the Lp-lipoprotein preparations contained α- and β-lipoproteins which do not carry the Lp determinant but which accompany the Lp-lipoprotein during the purification procedure. Also, it has not been entirely ruled out that the purified β(Lp a−)-lipoprotein used for immunization contained a lipoprotein which cross-reacted with the Lp-lipoprotein and not with β-lipoprotein.

Partial purification of Lp-lipoprotein from Lp(a+) serum: The Lp-lipoprotein has been purified from ten Lp(a+) sera, while no Lp-lipoprotein was detected in any subfraction of two Lp(a−) sera taken through the same purification procedure.

In ultracentrifuge diagrams, the Lp-lipoprotein was identified as the faster-moving peak (Fig. 4*a*, *c*, *d*, and *e*) in 1.21 density solutions, since this peak was reduced in size in weak Lp(a+) preparations (Fig. 4*d*) and was absent in Lp(a−) preparations (Fig. 4*b*). The heterogeneity of all Lp-lipoprotein preparations is evident in Figure 4. The slower-moving peak (Figs. 4*b*, *c*) gave uncorrected $s_{f,1.21,\ 20°}$ values approximating those of HDL_2 lipoproteins.[12] Gradient method 2 eliminated most of these accompanying α-lipoproteins (Fig. 4*e*, *f*), although these preparations did produce two peaks when centrifuged at solvent densities of 1.04 and 1.004.

In serum preparation 15, the Lp-lipoprotein was isolated from both the floating (Lp 15′) and the sedimenting (Lp 15) 1.064 density subfractions. The uncorrected $s_{f,1.21,\ 20°}$ values of the Lp-lipoprotein isolated from both subfractions were very similar. The appearance of Lp activity floating in 1.064 density solvent may be an artifact of preparation that is dependent on a high concentration of pre-β-lipoprotein. The density distribution of the Lp-lipoprotein is relatively broad, as shown by the results of tests on gradient subfractions.

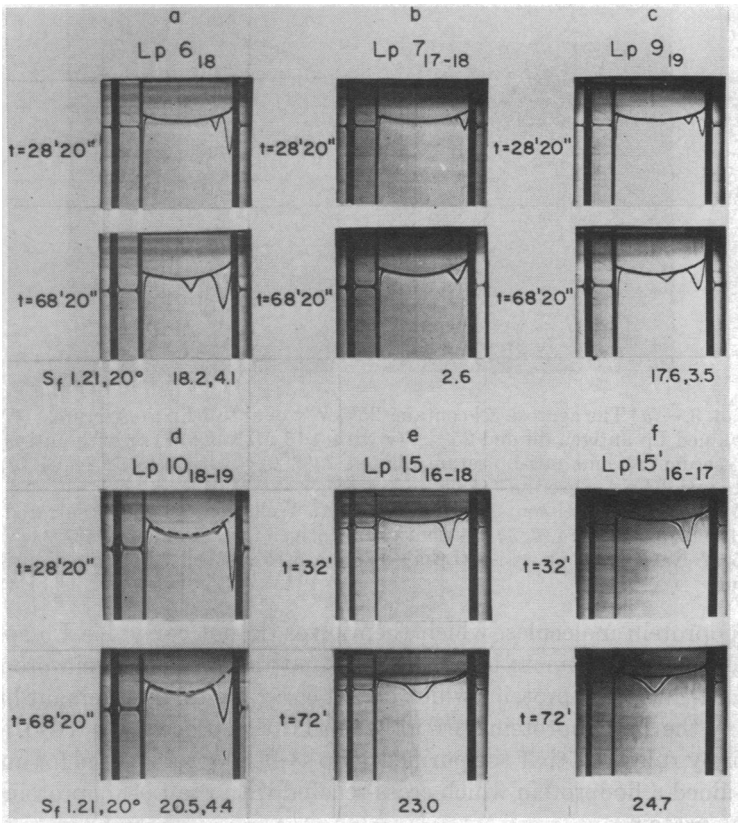

FIG. 4.—Analytical ultracentrifugation flotation patterns of various Lp-lipoprotein preparations. Lp 6, 7, 9, and 10 were isolated by gradient method 1. Lp 15 and 15′ were isolated by gradient method 2. Lp 6, 9, and 15 were isolated from sera giving strong Lp reactions. Lp 10 was isolated from serum giving a weak Lp reaction. Lp 7 was prepared from an Lp(a−) serum. *t*, corrected time; $S_{f,121,20°}$, uncorrected ultracentrifugation flotation rates in Svedberg units at 20°C and 1.21 density NaBr solution; centrifuge speed, 30,000 rpm. The baseline has been traced on the Lp ultracentrifuge patterns using the actual baseline as a template except in (*d*) where the baseline was dotted in, since it was approximated from a baseline taken at a slightly different time.

Discussion.—The specificity of the heteroimmune antisera used in this study has enabled us to assay and to trace, during partial purification, a human serum lipoprotein carrying the Lp antigenic determinant. Gel absorption experiments suggest that these Lp-lipoprotein molecules carry at least one antigenic determinant in common with non-Lp-carrying β-lipoprotein molecules. Although the gradient methods used for purification of the Lp-lipoprotein were not sufficiently refined to produce a homogeneous preparation, the major peak of Lp-lipoprotein can be separated from most of the β-lipoprotein by density gradient centrifugation. The removal of most of the non-Lp accompanying α-lipoprotein was accomplished more easily than the removal of non-Lp accompanying β-lipoprotein due to the greater molecular weight and density differences between α- and Lp- than between β- and Lp-lipoprotein. The estimated density of Lp-lipopro-

tein is about 1.07 at 20°C.[13] The uncorrected $s_{f,1.21,\ 20°}$ was about 24*S*. Both of these data are more similar to measurements of β-lipoproteins than of α-lipoproteins. Further purification and more precise characterization of the Lp-lipoprotein are underway.

Results of direct comparison of our antisera with known anti-Lp(a) serum and concordant reactions with panels of random human sera leave little doubt that the lipoprotein isolated in this study is the Lp(a)-lipoprotein. However, there are some inconsistencies. The frequency of Lp(a+) individuals in our sample is 0.50 compared with 0.33 found by Berg and others[3] in Caucasian populations. In tests of our antisera, Rittner found some discrepancies among negative and weak reactors. These discrepancies could be due to simple quantitative variations among individual sera reacting with antisera of varying titers. They might reflect qualitative variations in Lp-lipoproteins among individuals and also corresponding qualitative differences in specificities of antisera. Further chemical and immunochemical studies will be necessary to resolve this.

In any event, immunological tests of all subfractions of human sera made during the purifications procedure indicate that Lp(a−) individuals, as defined by our antisera, lack Lp-lipoprotein. Lp(a+) individuals vary with regard to the concentration of Lp-lipoprotein present. Weak positive reactors appear to have a low concentration of the same lipoprotein present in strong reactors. Analytical ultracentrifugal analysis, a less sensitive method than immunological analysis, showed that the Lp-lipoprotein from a weak reactor had an s_f value in the same range as the s_f values of Lp-lipoprotein isolated from strong reactors, but was present in a much lower concentration. Comparable subfractions from Lp(a−) sera lacked lipoprotein in this range of s_f values.

Summary.—Heteroimmune antisera were prepared and shown to be specific for the genetically determined human serum Lp-lipoprotein factor by genetic and comparative immunologic tests. This antiserum was used to follow purification of the Lp-lipoprotein. This lipoprotein, with density greater than 1.064 and less than 1.12, was separated from most of the α- and β-lipoproteins but probably has some antigenic cross-reactivity with β-lipoprotein. The data obtained from ten Lp(a+) and two Lp(a−) sera, which had been subjected to Lp-purification procedures, indicated that the basis for the genetic difference between these two types of sera was the presence of the Lp-lipoprotein in Lp(a+) sera and its absence from Lp(a−) sera.

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