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The genetics of the Lp antigen

I. Its quantitation and distribution in a sample population

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INTRODUCTION

The Lp antigen of human serum proteins, a species of β-lipoprotein, was first described by Berg (1963). Available data at that time indicated that the production of the Lp antigen was under simple autosomal dominant genetic control. Since then several reports have cited data which indicate more complex control of production of this antigen (Harvie & Schultz, 1970; Utermann & Wiegandt, 1970; Rittner & Wichmann, 1967; Ehnholm *et al.* 1971, 1972). These more recent data favour the likelihood that the genetic variation is in levels of Lp in the sera of different individuals, rather than simple presence or absence of the factor. The varying quantities of Lp antigen could represent a series of discrete levels, under the control of one or a few genes, or a continuous distribution of antigen under polygenic control. Non-genetic factors may also play a role in expression of this trait. In this communication we describe an assay for estimation of the amount of Lp antigen in human serum, and the application of this assay to a sample Caucasian population. Data based on this assay further supports the quantitative nature of the variation in Lp antigen. A bimodality of the frequency distribution is identified which is consistent with the hypothesis of control by a major gene.

MATERIALS AND METHODS

The procedure chosen for quantitation of Lp antigen in normal human serum was a radio-immune inhibition assay. Anti-Lp antibody was combined with cyanogen bromide-activated sepharose (Cuatrecasas, 1970) to form an immunoadsorbent. This immunoadsorbent was incubated first with the serum to be analysed and then with a standardized preparation of ^{125}I-labelled, partially purified Lp antigen. The amount of serum required to inhibit 50% of the maximum uptake of radioactivity by the adsorbent was taken as a measure of the Lp antigen in the unknown serum. The procedure is illustrated in Fig. 1.

Immunoadsorbent

Specific anti-Lp(a) heteroantisera were produced in rabbits against partially purified preparations of human Lp-lipoprotein, as previously described (Schultz, Shreffler & Harvie, 1968). The antisera were rendered specific by absorption with β-lipoprotein of density $< 1{\cdot}064\rho$ isolated from the serum of an Lp(a $-$) individual. This serum was identified as an Lp(a $-$) serum because, in its unconcentrated form, it did not react with a specific anti-Lp(a) antiserum in Ouchterlony

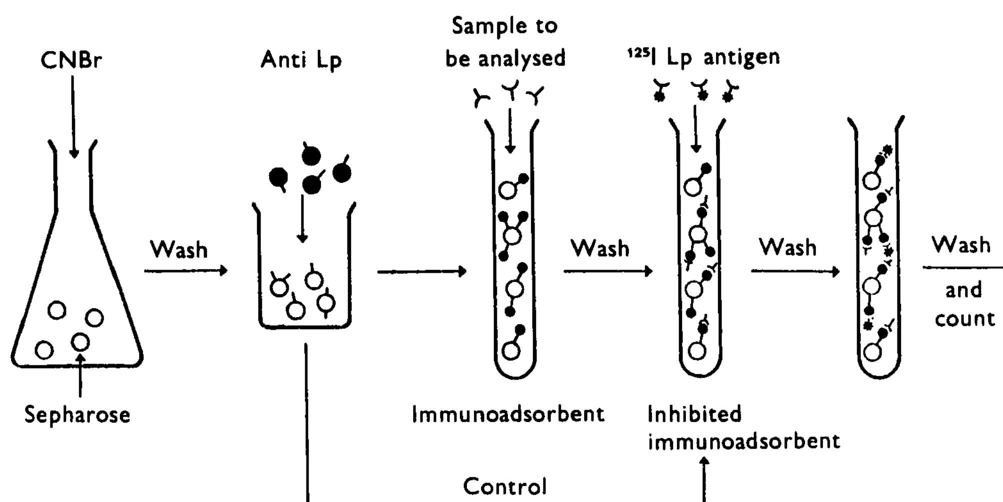

Fig. 1. Schematic representation of radioimmunoassay.

immunodiffusion. A concentrated subfraction of Lp(a –) serum containing proteins of density $> 1{\cdot}120\rho$ was also used in order to absorb any trace antibodies to these proteins. The absorbing fractions did not contain detectable Lp(a) protein. After absorption, this antiserum gave a single band on Ouchterlony immunodiffusion with Lp(a +) sera. Further details of preparation, absorption, characterization and specificity of anti-Lp(a) sera prepared in this laboratory have been previously published (Schultz *et al.* 1968). The specificity of the antiserum used in these studies was checked by comparison of its reaction against a large panel of normal sera with that of a reference antiserum. The specificity of the reference antiserum was established in cooperative studies with Dr K. Berg. Although unavailability of completely pure Lp antigen made it impossible to obtain pure anti-Lp(a) antibody by direct immunization, exhaustive absorption of the antiserum eliminated the possibility of contamination by antibodies against any serum fractions other than Lp(a). The immune globulins from this antiserum were freed of most of the contaminating proteins which might interfere with their attachment to sepharose by removal of the excess β-lipoprotein added for absorption, followed by $(NH_4)_2SO_4$ precipitation. The antisera were adjusted to a density of $1{\cdot}21\rho$ by addition of 0·32 g. of NaBr per ml. of serum and dialysed against NaBr of density $1{\cdot}21\rho$. The material was then centrifuged in a preparative ultra-centrifuge for 44 hr. at 100,000 g. The least dense fraction containing 1/5 of the total volume and all β-lipoprotein antigenic activity was removed. The antibody-rich γ-globulin was then precipitated according to a modification of the method of Kendall (1937). To the remaining β-lipoprotein-free fractions, containing 4/5 of the original serum volume, 0·5 ml. of a saturated solution of $(NH_4)_2SO_4$ was added. The mixture was allowed to stand for 1 hr. at room temperature and the precipitate that formed was separated by centrifugation.

The supernatant was removed and the precipitate redissolved in 1 ml. of phosphate buffered saline and dialysed overnight at 4° C. against phosphate buffered saline. Ouchterlony tests showed that all anti-Lp activity was found in the dissolved precipitate with none remaining in the supernatant.

This antibody preparation was attached to sepharose 4B to form the required immunoadsorbent by a modification of the cyanogen bromide activation method of Cuatrecasas (1970).

Sephararose 4B was washed three times in distilled water, allowed to settle, and the water decanted. Distilled water equal to one/half of the volume of the water removed was added, the suspension cooled to 20° C. and then one/half volume of cyanogen bromide solution containing 3·0 g./5 ml. was added to the sepharose. Temperature was maintained at 20° C. and pH adjusted to 10·5 until the pH no longer tended to drop (a maximum of 12 minutes) indicating the complete activation of the sepharose. The reaction mixture was immediately transferred to a coarse disc Büchner funnel maintained at 4° C. and washed under suction with 15 times the sepharose volume of cold 1 M-$NaHCO_3$. Suction was terminated and the partially purified anti-Lp serum (approximately 10 mg. antibody per 5 ml. sepharose) prepared as described above was immediately added to the Büchner funnel. The mixture was then transferred to a beaker and allowed to react at 4° C. for 16–20 hr. with gentle stirring.

The immunoadsorbent was washed with five 100 ml. portions of 0·1 M-$NaHCO_3$ and stored for future use in 3 vols. of 0·1 M-$NaHCO_3$ at 4° C. Before use in the assay procedure, the immunoadsorbent was diluted 1 part to 30 parts of a modified 'assay buffer' as described by Wide (1969), consisting of 80 ml. 0·5 M sodium barbital, 12 ml. 5·0 M-NaCl, and 5·3 ml. 2 N-HCl in 2 l. to which were added 3 g./100 ml. bovine serum albumin and 0·5 ml./100 ml. NP-40 detergent (Shell Chemicals, U.K., Limited, London).

Preparation of [^{125}I]Lp antigen

Lp lipoproteins were concentrated and partially purified as described elsewhere (Schultz *et al.* 1968). The concentrated solutions of Lp antigen were iodinated with ^{125}I (New England Nuclear) according to the method of Reif (1967) employing ICl. The method of Greenwood, Hunter & Glover (1963) which employs chloramine T in the iodination procedure was found to destroy the antigenic activity of the Lp protein.

Standardization of the assay

The ratio of immunoadsorbent to labelled Lp preparation that was needed in an assay varied somewhat with each batch of reagents and therefore optimal ratios were determined each time a batch of reagents was prepared.

Various amounts of immunoadsorbent suspension containing 0·1 → 1·0 ml. of a 1/30 dilution of standard suspension in assay buffer was mixed with 10 → 100 μl. amounts of labelled antigen solution. A standard curve was prepared as shown in Fig. 2. It should be noted that the curves are identical for all levels of immunoadsorbent greater than 0·3 ml., therefore 0·3 ml. was considered the optimal quantity of immunoadsorbent for this particular batch of reagent. For each batch of immunoadsorbent and each batch of iodinated Lp preparation, the amount of the latter which would give a slight excess of antigen over antibody content of immunoadsorbent was determined. The same levels of antibody preparation (immunoadsorbent) and ^{125}I-labelled antigen were then used for each determination in which a given batch of reagent was employed.

Radioimmunoassay

Reactions of unknowns with standardized immunoadsorbent were carried out in 3 ml. glass test-tubes which had been siliconized (Siliclad, Clay Adams) according to the manufacturer's directions. A standard amount of immunoadsorbent, kept in suspension by stirring on a magnetic stirrer, was pipetted into each siliconized tube followed by a volume of the serum to be

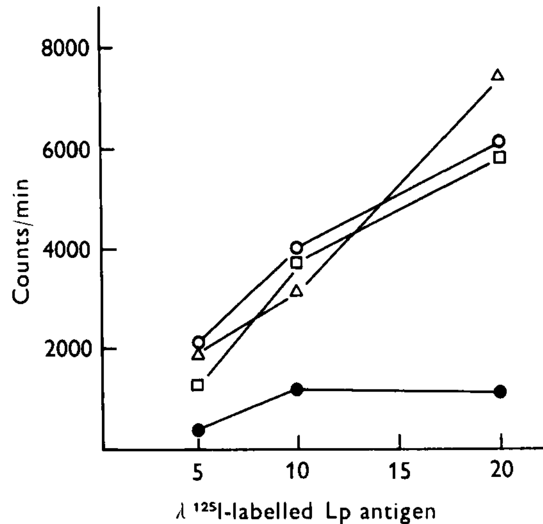

Fig. 2. Standard curve for immunoadsorbent assay. ●—●, 0·1 ml. immunoadsorbent; ○—○, 0·3 ml. immunoadsorbent; △—△, 0·5 ml. immunoadsorbent; □—□, 0·7 ml. immunoadsorbent.

quantitatively analysed as described below. The volume of the reaction mixture was then adjusted to a total volume of 1 ml. and rotated slowly for 16 hr. at 4° C. on a rotary mixer (Scientific Industries, Inc., Queens Village, N.Y.). An antigen excess quantity of ^{125}I-labelled Lp protein preparation was then added to each tube and the mixture was rotated for an additional 16 hr. Samples were washed four times with 1 ml. of 0·5 % NP-40 detergent in phosphate buffered saline and centrifuged each time in a clinical centrifuge (International Equipment Co., Boston, Mass). Samples were then counted in a gamma counter (Nuclear-Chicago Corp.).

Each serum to be analyzed was first pretested by Ouchterlony immunodiffusion and scored as a positive, weak positive or negative reactor as previously described (Schultz *et al.* 1968; Schultz, 1970). All samples were tested in the radioimmunoassay in duplicate and, with each group of samples, a sample inhibited by 25 μl. of a standard very strong Lp(a) serum and an uninhibited sample were included to find the limits of minimum and maximum uptake of ^{125}I-labelled antigen by the immunoadsorbent. The quantity of Lp(a) serum used in the minimum uptake sample was shown to be in gross antigen excess for all immunoadsorbent preparations.

A serum which was a strong Lp(a+) in Ouchterlony test was initially sampled at a level of 5 μl. of a 1:1 dilution of serum in phosphate buffered saline for use in the assay. To test a 'negative' serum 10 μl. of undiluted serum was used in the assay. Some of the samples were not on the linear portion of the inhibition curve (see fig. 2) and the determination was repeated with the sample size adjusted accordingly.

Calculations

The volume of undiluted serum required to inhibit the immunoadsorbent pickup of ^{125}I-labelled Lp protein by 50 % was used as the quantitative indication of Lp antigen in the sample. This amount of serum was estimated graphically as shown in Fig. 3. The amount of strongest positive serum to give 50 % inhibition was arbitrarily assigned a value of 1 and all other Lp 'concentrations' were based on this value. The values obtained are therefore relative rather

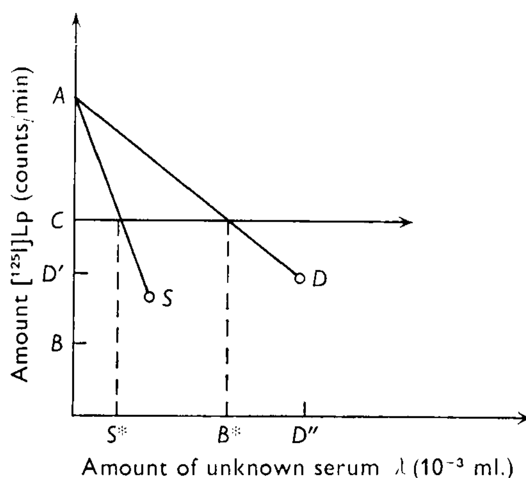

Fig. 3. Amounts of [^{125}I]Lp absorbed by immunoadsorbent after preabsorption with various amounts of unknown serum. A = Maximum capacity of immunoadsorbent (counts/min). B = Background capacity of immunoadsorbent (counts/min). $C = 1/2\ (A+B)$. D = Unknown, gives D' counts with $D''\lambda$. B^* = Interpolated amount of unknown serum that would cause a 50 % reduction in anti-Lpa-^{125}I absorption by the immuno adsorbent [intersection of lines $\overline{AD}$ and C]. S = as point D but for standard strong Lp(a) serum. S^* = as point B^* but for standard strong Lp(a) serum. Relative amount of Lp antigen in unknown sample = S^*/B^*.

than absolute values. The relative Lp units in the unknown serum were calculated according to the formula:

relative Lp units in unknown serum

$$= \frac{\lambda \text{ of strongest Lp serum needed for 50\% inhibition of immunoadsorbent } (S^*)}{B^* \text{ (see Fig. 3 legend)}}.$$

RESULTS AND DISCUSSION

One hundred and forty-six serum samples obtained from the Serology Laboratory at the University of Michigan Hospital were scored for relative amounts of Lp antigen in the manner described above. The distribution of the relative Lp units in this population is shown in Fig. 4. Relative values ranged from 0·021 arbitrary units for an Lp(a –) serum to 1·00 for the strongest Lp(a +) serum used as standard. The mean value was 0·306 and the standard deviation 0·26. The frequency distribution of this relative quantitative measure in the total sample population is positively skewed.

The measurement method described above results in a non-additive change in the ratio S^*/B^* as B^* changes linearly. Previously we have considered a frequency distribution of quantitative levels based on the ratio S^*/B^* (Schultz & Shreffler, 1973). However, the non-additive change in scale of this ratio has prompted us to consider the use of $\log_e$ of S^*/B^* as the variable of study. Since S^* is a constant, the $\log_e S^*/B^*$ ($= \log_e S^* - \log_e B^*$) will be a function of variation in B^* only. Therefore the $\log_e S^* - \log_e B^*$ results in an equal change on the scale of measurement for each increment of B^* rather than the logarithmic change which occurs in the measure S^*/B^*. The transformed data are given in Fig. 5(*a*). There is an obvious bimodality of the measure B^*.

The distributions of our measure of Lp antigen in sera which tested Lp(a +) and sera which

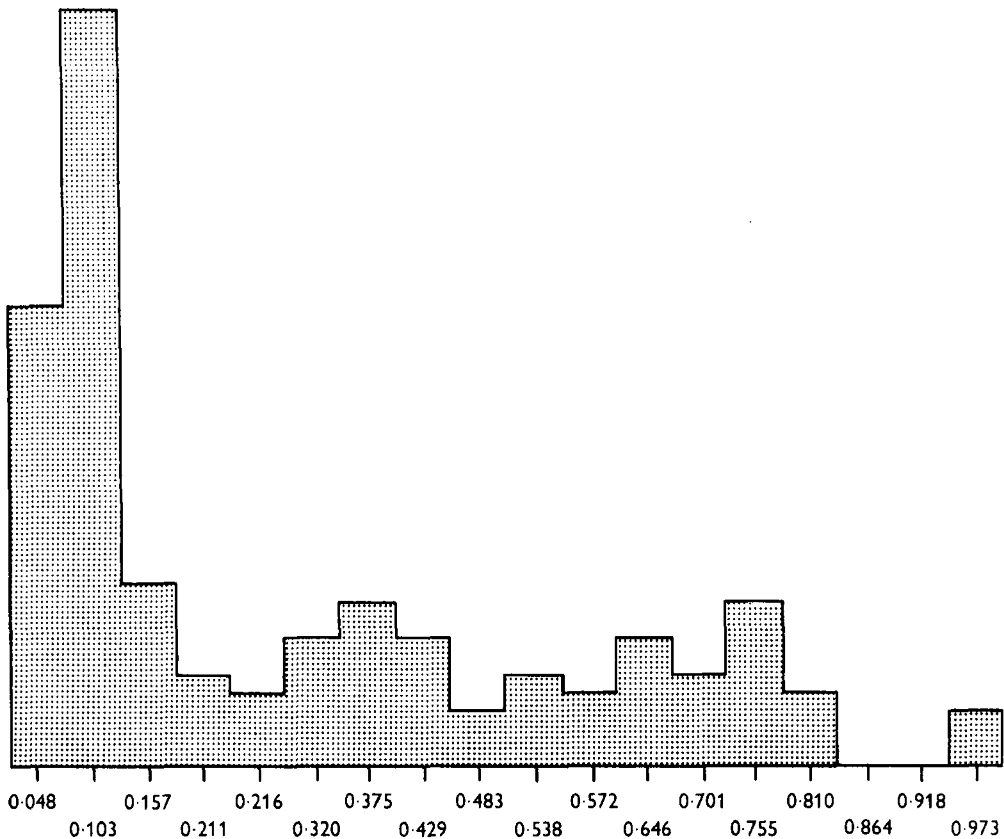

Fig. 4. The distribution of the value S^*/B^*.

tested Lp(a –) in Ouchterlony immunodiffusion when taken separately (Figs. 5*b, c*) show that the threshold level for distinguishing between positive and negative reactors lies at a value of 0·15 ($\log_e = -1{\cdot}897$). This value is also near the trough of the $\log_e$ distribution in these experiments. There are no qualitatively negative reactors who have relative Lp quantities in their sera higher than this value. There is considerable overlap of positive and negative reactions in the $\log_e$ 0·1 to $\log_e$ 0·2 range. Such a large number of individual sera with Lp quantities appearing at the threshold level could explain the wide variation in Lp(a) phenotype frequencies found by different investigators in the same or similar populations (Sing, Schultz & Shreffler, 1974). The combined effects of antisera with varying titres and individuals with varying antigen levels could have a considerable influence on phenotype frequency determinations.

It would also appear that some antigen is present in the serum of each individual as has been shown previously (Harvie & Schultz, 1970), although the lowest quantity is at most 1/50 of the highest. This quantitative variation makes it possible to explain the ability of Lp(a –) serum to elicit an immune response (Schultz *et al.* 1968) and the ability of such a serum to absorb anti-Lp(a) activity. These sera contain a small quantity of Lp(a) antigen which is not detectable on immunodiffusion assay.

In light of the quantitative variation in our measure of Lp levels the fact that two Lp(a) negative individuals, both having a level of antigen too low to be detected by Ouchterlony test, occasionally produce an Lp(a +) child can be explained by several genetic models. The overlap-

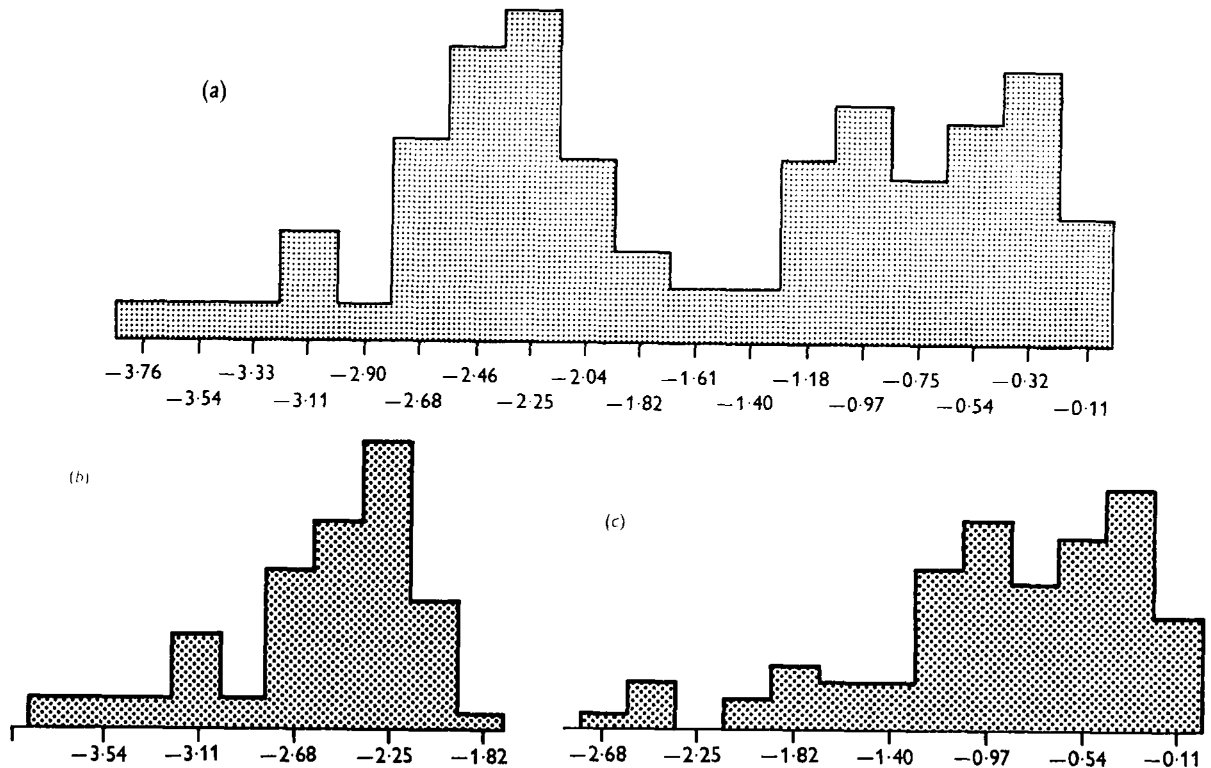

Fig. 5. The distribution of the $\log_e S^*/B^*$. (*a*) All cases; (*b*) Lp(a−); (*c*) Lp(a+).

ping bimodal distributions strongly indicate that a major genetic factor is operating to determine the modes. Variation within modes may be due to one or a combination of factors including variation in the background genotype, environmental influences and measurement error. An in-depth analysis of the relative roles of these factors is presented in the second paper of this series (Sing *et al.* 1974).

SUMMARY

The frequency distribution of the quantitative activity of the Lp antigen was found to be bimodal. It is hypothesized that a major genetic factor is operating to determine the modes.

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