On the Antigenic Identity of Human Serum Beta and Alpha-2 Lipoproteins, and Their Identification in the Aortic Intima

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CONSIDERABLE attention has centered around the relationship between low-density serum lipoproteins and the role which each may play in the development of atherosclerotic lesions. These topics have recently been reviewed. In general, there are two distinct theories about the nature of the interrelationships of these lipoproteins. According to one, there are at least two different kinds of protein within this class. These are classified as alpha-2 and beta lipoproteins, from their electrophoretic mobilities, or as very-low-density and low-density lipoproteins, from their rates of flotation in the ultracentrifuge. Indeed, some suggest that there may be several kinds of protein, perhaps an entire spectrum, that can be called by these names. According to the second theory, there is only one kind of protein within this class. The physical properties of this protein may vary widely strictly as a result of the amount and type of fat which happens to be bound to it.

With the advent of the technique of immunoelectrophoresis, a sensitive tool has been provided for studying this problem, as well as the problem of identifying these materials in the arterial intima. The present paper deals with our initial findings resulting from the application of this tool.

Methods

Immunoelectrophoresis

Immunoelectrophoretic characterization of both purified and intimal-derivative lipoproteins was carried out by the method set forth by Grabar. Appropriate reservoirs and troughs were cut in a layer of 1.75 per cent agar gel previously washed, buffered to pH 8.4, and plated out on ordinary 3-1/4 by 4-inch lantern slide glasses.

The antigen preparations were added and the slides were immediately subjected to 200 volts with 15 ma. or 20 volts/cm., for a period of two hours. At this point, electrophoretic migration was considered complete, the appropriate antisera were delivered to the troughs, and the slides were stored in humidity chambers at 4 C. until precipitin-line formation was optimal. The electrophoresis itself was also performed in the cold (4 C.) during most of the study because excessive heating had produced slight distortion in the earlier slides.

Preparation of Antigens

Pure lipoproteins were prepared by the technique described by Havel et al.. Outdated human blood was obtained from the blood bank, and the plasma separated. This was then spun at 4,400 r.p.m. for 45 minutes to remove the chylomicrons. The calculated protein-free specific gravity was then adjusted to 1.018. The plasma was spun at 78,410 G for 18 hours, and the top 3 ml. was collected from each tube. These samples were then pooled and dialyzed for 48 hours at 4 C. against normal saline. This preparation will be referred to as alpha-2 lipoprotein. The subnatant from the last spin was then adjusted to a specific gravity of 1.063, spun under the same conditions, and dialyzed. This preparation will be called beta lipoprotein. These two classes of protein taken together will be referred to as low-density lipoprotein.

Preparation of Antisera

The alpha-2 and beta lipoproteins obtained in the manner described were then given to rabbits by the multiple-portal technique for three weeks,
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In the bottom well was placed whole human serum, and in the top aortic intimal extract. The trough contained antiserum against whole human serum. The slide has been stained with Sudan black.

the first injection being intramuscular with Freund's adjuvant. The rabbits were bled three times thereafter at weekly intervals, and the antisera thus obtained were used separately. Antiserum against whole human serum prepared in horses was obtained from the Pasteur Institute in Paris.5

Preparation of Extracts of Aortic Intima

Human aortas were obtained at autopsy, usually 6 to 12 hours after death. They were then rinsed, with gentle rubbing, in running tap water to free the intima of adherent blood. The intima with a little underlying media was then carefully stripped with a sharp knife and forceps. Areas grossly free of all atheromatous involvement were kept separate from areas with fatty streak and early yellow plaques, and these in turn were kept apart from the yellow gruel expelled from advanced plaques. These samples were weighed, then homogenized in a Potter-Elvehjen glass homogenizer after addition of 4 volumes of normal saline (assuming a tissue specific gravity of 1.006). The homogenate was then spun at 4,400 r.p.m. for 45 minutes, and the colloidal suspension lying between the sediment and the floating material was carefully pipeted off. This nearly opaque suspension was then stored at 4°C until analysis.

Results

Figure 1 demonstrates one type of result obtained by this technique. After whole human serum had been placed in the bottom oblong well in the agar and electrophoresed, the antiserum against whole human serum was placed in the central trough, and the two were permitted to diffuse toward each other for several days. In this manner, a separate arc is formed for each antigen for which the antiserum contains a precipitin. The specific component can be identified by its position with respect to both the trough and the well, and by the configuration of the arc. The slide in figure 1 had actually developed almost 20 different arcs, but only the most prominent can be seen in this photograph. The picture, taken after staining for fat with Sudan black, shows clearly that two protein components of serum contain fat. That which migrates a short way toward the anode, giving the appearance of a horn off the well of origin, is the beta lipoprotein. The other, the alpha-1 lipoprotein, in agar migrates with the albumins. Other arcs take up the Sudan very slightly, in a nonspecific manner typical of protein. We have been impressed by our consistent inability to find in normal serum a separate arc in the alpha-2 region which is positive with a Sudan stain.

The upper well in figure 1 was filled with an extract of early plaque material. The prominent arc in the albumin region can be seen to have a configuration different from that for the alpha-1 lipoprotein of serum, and takes up only a trace of the lipid stain. This arc, seen in only a few of the intimal extracts, seems to be inversely related to the thoroughness of preliminary washing of the intima.
We consider it to be a contaminant from serum. The arc in the beta region which corresponds to the beta lipoprotein of serum is deeply stained with Sudan and is broad and indistinct. This is due partly to the fact that beta lipoprotein interacts with the agar medium, and partly to changes which occur with time. This particular extract had been stored for over a month before use in this determination. It gave a much sharper arc when it was fresh (fig. 5).

Figures 2, 3, and 4 present the results relevant to the relationship between the alpha-2 and beta lipoproteins. In the top trough of figure 2, the antiserum prepared in rabbits against lipoproteins isolated in the density range 1.018 to 1.063 is reacted against the corresponding pure lipoprotein, above, and against whole serum, below. These reactions are indistinguishable from each other and from the reaction produced with antiserum prepared against lipoproteins isolated in the density range 1.006 to 1.018, in the next trough down. However, the sample in the third well, containing the pure alpha-2 material, is quite distinct, forming an arc much farther from the trough and closer to the anode (actually in the alpha-2 region). It appears, therefore, that the alpha-2 lipoproteins have a faster electrophoretic rate and a much slower diffusion rate in agar than the beta lipoproteins. These are differences in physical properties, in keeping with the predictions of either of the two theories put forth at the outset.

Figure 3 is an exact repetition of figure 2 except that the antisera to beta and alpha-2 lipoproteins have been interchanged, to disclose the cross reactions. Just a glance is sufficient to show that there has been no change in pattern. It was therefore possible to interchange the two antisera without affecting the outcome. It is of interest to note in the final reaction at the bottom of figures 2 and 3 that both antisera react with whole serum in an identical manner, producing a fusion of the arc with that formed by reaction with antihuman serum. This fusion of arcs is used as a criterion of antigenic identity. It is therefore evident that each antiserum is selectively active against only one component of whole serum, and that this component is the same in both cases.

Anti-beta and anti-alpha-2 refer to antisera prepared in rabbits by immunization with human serum beta lipoprotein and alpha-2 lipoprotein, respectively. The prefix "P" refers to antisera prereacted with the cross antigens, anti-beta prereacted with alpha-2 lipoprotein, and anti-alpha-2 with beta lipoprotein. Lp means lipoprotein, whole ser. is whole human serum, and Non-pl. intima refers to the extract of aortic intima in which there were no grossly visible plaques.
Results obtained by cross absorption with each of the antigens are presented in figure 4. To the antiserum against alpha-2 lipoprotein was added four volumes of beta lipoprotein, producing the preparation referred to as P-anti-alpha-2. To the antiserum against beta lipoprotein was added four volumes of alpha-2 lipoprotein, to give P-anti-beta. The unreacted antisera were each diluted with four volumes of saline to keep all concentrations comparable. This resulted in two pre-reacted (P) solutions, both in the range of antigen excess. Referring to the bottom two troughs in figure 4, it can be seen that the excess beta lipoproteins in the P preparation has reacted with the anti-alpha-2 serum to produce a straight line from one end of the slide to the other. This line of reaction has been interrupted in the range in which the anti-alpha-2 has reacted with the alpha-2 lipoprotein which was electrophoresed in the central well, and has fused with the arc formed by that reaction.* Moreover, the P-

*The actual chronology of development of this pattern is important to the interpretation of this figure. The first line of precipitate to form was the arc of reaction between the alpha-2 in the well and the anti-alpha-2. Later, the straight line of reaction between the anti-alpha-2 and the excess beta from the P-anti-alpha-2 trough appeared. At first this straight line was totally interrupted in the region of alpha-2 lipoprotein (Lp) from the well. When the alpha-2 Lp became fully reacted and excess antibody began to diffuse up from the bottom trough through the arc which it had formed with alpha-2 from the well, it was then possible to continue formation of precipitate with excess beta from the preparation above. In this way, the straight line of cross reaction became continued, although deflected downward and containing less precipitate. Thus, the last line to form was the deflected continuation of the straight-line cross reaction into the region of alpha-2 from the well. This line should therefore not be mistaken for a second reaction of alpha-2 with P-anti-alpha-2. The persisting evidence for this is that the line of reaction of anti-alpha-2 with excess beta lipoprotein in the P preparation, formed from antibody diffusing from below, would not fuse with a second arc in the region of alpha-2 lipoprotein from the well if that arc were formed from antibody diffusing down from above. The fact that this fusion exists is clear evidence that there is only one antigen-antibody system involved in the formation.

When anti-beta, anti-alpha-2, and anti-whole-serum antisera were used to analyze the serum protein constituents of aortic intimal extracts, the results were identical, except that the anti-whole-serum was able to identify sporadic contamination. Thus, the result
Figure 5

Same as figure 4, but prepared from an aorta with less severe sclerosis, the top well containing extract from plaques and the bottom from plaque-free areas, reacted with anti-whole serum.

shown in figure 5, obtained with anti-whole serum, was duplicated with each of the specific antisera, showing positive identification of the material in the intimal extract as serum low-density lipoproteins.

The electrophoretic mobility and diffusion rates of the low-density lipoproteins found in aortic extracts are not strictly characteristic of either pure alpha-2 or pure beta lipoproteins. This might well be the result of changes occurring post mortem, during extraction, or during storage.

A semiquantitative estimate of the concentration of low-density lipoproteins in the aortic extracts can be obtained by evaluating the intensity of staining of the arc by Sudan black. Results of this procedure are illustrated in figure 5. The arc formed above, against an extract of early plaque material, contains considerably more stainable fat than that from the plaque-free areas, determined in the arc below. This indicates that the protein precipitate either contained much more fat or was present in greater quantity. Since the arcs have the same position and configuration, indicating identical physical properties, the latter explanation is more likely to be correct. The black material around the well is colloidal atheromatous material which could not be removed by centrifugation.

The aorta shown in figure 5 was taken from a middle-aged man who had been a juvenile diabetic and who died with carcinomatosis. That seen in figure 6 was from a postmenopausal woman who also died of carcinomatosis. The first aorta was severely sclerotic, having nearly half of its surface involved with ulcerated, hemorrhagic, or calcified lesions. The second was moderately involved, with only about half of its intimal surface included in atheromatous change, mostly of the fatty-plaque and fatty-streak variety. Comparing the two figures (5 and 6), it can be seen that in each, the concentration of low-density lipoproteins is greater in the fatty plaques and streaks than in the uninvolved areas of intima. In our total series of six aortas, this tendency occurred consistently. A second feature to notice is that the plaque areas of the more severely involved aorta contain a greater concentration of lipoprotein than do the corresponding areas in the less severely involved vessel. The intimal areas that contain no grossly visible atheromatous change also have less lipoprotein in the less severely involved vessel. This also has been a consistent trend in our small series. Indeed, an artery with only occasional plaques may have barely enough of this material for detection. An aorta obtained from a three-year-old child who died of leukemia had not even a single fatty streak, and in this aorta there was no detectable low-density lipoprotein. This is the only aorta in which we have failed to detect the material.

When extracts of the fatty gruel taken from the centers of advanced plaques were analyzed, it was impossible to demonstrate the presence of low-density lipoproteins. Whether they have become physically bound in some manner as to make them undetectable or whether they are actually absent cannot be determined from these studies. It is intriguing to speculate that they have broken down to form free lipids of various sorts.

Discussion

The antigenic relationship between the two major classes of low-density lipoproteins has
been the subject of investigation in several laboratories. In general, it has become clear that the alpha-1 and low-density lipoproteins are separate kinds of protein, differing chemically as well as physically. Within the class of serum proteins with the specific gravity range of 1.006 to 1.063, antigenic heterogeneity has been demonstrated in only one study. That study utilized the technique of tanned-red-cell agglutination. It showed that all the antigenic groups of the Sf 3 to 9 molecules were contained in the Sf 10 to 400 class, but that the latter had some additional groups. That this may be due to a difference in coiling of the protein chains in the presence of more or less fat cannot be ruled out. It is difficult to explain why our study has failed to confirm this report. When anti-alpha-2 antisem was prereacted with beta lipoprotein, then tested against alpha-2 lipoprotein (fig. 4, fourth well, fourth trough), no additional reactivity was found. It should be re-emphasized here that the broad band of precipitate seen in figure 4 is the result of marked antibody excess, reacting with excess beta lipoprotein from the upper trough, and not a second reaction of P-anti-alpha-2 with alpha-2.

The present study has confirmed the physical differences which exist between the alpha-2 and beta lipoproteins. The alpha-2 proteins migrate faster toward the anode in electrophoresis, float faster in the ultracentrifuge, and diffuse more slowly in agar. We have also confirmed the finding that these two classes of molecule are very similar antigenically. In fact, with the techniques employed, the results all support the concept that the protein portion of the molecules in each of the two fractions is identical. For practical purposes, at least, an antiserum to one is the same as an antiserum to the other.

In the extracts of aortic intima, the lipoprotein which was found demonstrated physical properties more like those of the beta than those of the alpha-2 lipoproteins. In the photographs presented, this can be seen most clearly by comparing the upper part of figure 5 with figure 2 or 3. The arc for alpha-2 lipoprotein is straight or even upside down when compared with that for beta lipoprotein. It is considerably farther from the trough, and the center of its range is farther to the right, indicating a faster electrophoretic mobility. In figure 5, although the configuration of the arc formed by the extract is not identical with that of beta lipoprotein, it is more like the beta lipoprotein arc than the alpha-2 lipoprotein arc with regard to the characteristics listed above. This finding is in contrast with the ultracentrifugal studies of aortic extract by Hanig et al., in which the greater portion of the lipoproteins isolated had the flotation properties of very-low-density (alpha-2) lipoproteins.

The various patterns of reaction which have been presented are summarized in diagrammatic form in figure 7. Those features of each reaction pattern which are typical from one determination to another were used in constructing these diagrams, rather than a precise copy of the actual pattern from any particular determination. In this way, an attempt has been made to provide a guide for making comparisons on the photographs of the raw data.

Immunoelectrophoretic demonstration of low-density lipoproteins in the aortic wall has been reported previously. Figure 5 is essentially the same as that published by Ott.
in 1958. However, we have been able to eliminate many of the other serum protein contaminants by washing the aortas before extraction. Moreover, semiquantitative estimate of the concentration of the material in the intimal extract has suggested a tendency for increasing severity of atherosclerosis to be associated with correspondingly larger amounts of lipoprotein in the fatty streak and plaque. This association, however, is incompletely documented at present, and apparently it does not hold for the complicated lesion with abundant atheromatous gruel.

What has become clearly evident from this study is that the serum beta lipoprotein, alone of all the serum proteins, is selectively concentrated and bound in the aortic intima. The intima appears to concentrate the beta lipoprotein from the serum. Furthermore, the lipoprotein is there before a fatty streak or plaque is evident grossly. Whether it is present before there is stainable lipid remains to be determined.

Summary

By the use of immunoelectrophoresis, it was demonstrated that ultracentrifugally isolated, very-low-density (alpha-2) lipoproteins migrate faster toward the anode in electrophoresis and diffuse more slowly in agar than do low-density (beta) lipoproteins. However, these two lipoprotein fractions were found to be antigenically indistinguishable with this technique. Extracts of the intima of aortas were shown to contain material with all the physical properties consistent with those of the low-density (beta) lipoproteins. Furthermore, this was the only serum protein which was consistently found in high concentration in washed aortas. Beta lipoprotein was frequently found in plaque-free intimae. The impression was obtained that this lipoprotein was present in greater concentration in early atheromatous plaques than in plaque-free intimae, and that it was much more concentrated in the intimae of severely sclerotic aortas than in those of moderately or uninvolved aortas. However, it has not been demonstrated in the gruel from far-advanced plaques.

References

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Circ Res. 1961;9:472-479
doi: 10.1161/01.RES.9.2.472

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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