Biochimica et Biophysica Acta, 401 (1975) 6–14

BBA 77022

A CRITICAL EVALUATION OF THE PROPOSAL THAT SERUM APOLIPOPROTEINS ARE THE MAJOR CONSTITUENTS OF THE HUMAN ERYTHROCYTE MEMBRANE*

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(Received February 7th, 1975)

SUMMARY

1. The EDTA and Triton X-100 extracts of human erythrocyte ghosts gave no precipitin lines in double diffusion analyses with antibodies to either lipoprotein A, lipoprotein B, lipoprotein C, lipoprotein D, Lp(a) lipoprotein or arginine-rich apolipoprotein of normal human serum (for nomenclature for serum lipoprotein families and apolipoproteins, see Alaupovic, P., Kostner, G., Lee, D.M., McConathy, W. J. and Magnani, H.N. (1972) Expo. Annu. Biochem. Med. 31, 145–160 and Alaupovic, P., Lee, D. M. and McConathy, W. J., (1972) Biochim. Biophys. Acta 260, 689–707.) These membrane preparations also reacted negatively with commercially available antisera to α- and β-lipoproteins.

2. The normal serum very low density, low density and high density lipoproteins formed no precipitin lines with antibodies to either intact or EDTA-extracted ghosts.

3. The serum apolipoproteins and their constitutive polypeptides (A-I, A-II, B, C-I, C-II, C-III, D and arginine-rich apolipoprotein) reacted negatively with antibodies to intact or EDTA-extracted ghosts. The EDTA and Triton X-100 extracts of erythrocyte ghosts gave no reaction with monospecific antibodies to serum apolipoproteins and their constitutive polypeptides.

4. Ghosts dissolved in 2 % sodium dodecyl sulfate gave positive immunoprecipitin lines with antisera to α- and β-lipoproteins. However, the sodium dodecyl sulfate solution in concentrations greater than 0.1 % also formed precipitin lines with antisera to the same lipoproteins.

5. These results do not support the suggestion (Langdon, R.G. (1974) Biochim. Biophys. Acta 342, 213–228) that serum apolipoproteins are integral protein constituents of human erythrocyte ghosts. The immunoprecipitin lines observed in the latter study might have been due to the presence of trace amounts of serum lipoproteins loosely attached to the cellular surfaces or, more probably, resulted from nonspecific interactions between the proteins and the sodium dodecyl sulfate used as the solubilizing agent.

* A preliminary report of this study was published in the Abstract of Papers, American Society of Hematology, Dec. 7–10, 1974, Atlanta, Georgia, p. 106.

INTRODUCTION

The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis [1, 2] and crossed immunoelectrophoresis [3] of human erythrocyte membranes indicated the presence of approx. 20 polypeptide bands or immunoprecipitates, but only four proteins have so far been isolated and partially characterized [4, 5]. Two of these proteins accounting for 50–55 % of the total membrane protein have been designated as spectrin [6] and component a [7] or protein E [8]. The chemical nature and functional role of these two major proteins have not yet been fully established [4, 5]. The other two partially characterized proteins represent minor constituents. One of these was recognized as the glycolytic enzyme D-glyceraldehyde-3-phosphate dehydrogenase [9] and the other was identified chemically as a sialoglycoprotein and functionally as the carrier of blood group antigens [10, 11]. The former constitutes 5–6 % and the latter 2–3 % of the total membrane protein [5]. Topographical studies have shown that both the component a and sialoglycoprotein extend across the membrane, whereas spectrin and glyceraldehyde-3-phosphate dehydrogenase are located at the inner membrane surface [3, 4]. In view of the many uncertainties and unresolved questions regarding the structural and functional properties of most of the erythrocyte ghost proteins, the recent report [12] suggesting that serum apolipoproteins are the major intrinsic protein constituents of erythrocyte membranes has added a new direction to the studies on the characterization of membrane proteins and possible relationship between the red blood cells and circulating lipoproteins. On the basis of immunochemical evidence and quantitative determination of terminal amino acids, Langdon [12] has concluded that protein moieties of α- and β-lipoproteins may account for approximately one half of the total protein content of erythrocyte membranes.

Although in the past most of our studies on the lipid-protein interactions in biological systems have been concerned with the chemical and immunological characterization of the soluble plasma lipoproteins [13], we have initiated recently a similar study on the macromolecular constituents of the erythrocyte ghosts. To confirm and further explore Langdon's suggestion, we have tested the erythrocyte ghosts with antisera to all known human plasma lipoproteins and apolipoproteins available in our laboratory. Results of these studies do not support the suggestion that serum apolipoproteins are integral protein constituents of human erythrocyte ghosts.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company, St. Louis, Mo.

Preparation and solubilization of membranes

Blood was freshly drawn from healthy human donors. The erythrocyte ghosts prepared by the method of Dodge et al. [14] were essentially free of hemoglobin. From each unit of blood (450 ml), 120–150 ml of packed ghosts were obtained with a protein concentration of 3–4 mg/ml. The erythrocyte ghosts were solubilized with Triton X-100 at a final concentration of 5 % (v/v) in 10 mM phosphate buffer, pH 7.4, or with sodium dodecyl sulfate at a final concentration of 0.1 % (w/v) in 10 mM sodium phosphate buffer, pH 7.4.

Extraction of membranes with EDTA

One part of packed erythrocyte ghosts (3–4 mg protein/ml) was mixed with 10 parts of 1.0 mM EDTA, pH 9.5, and the suspension was allowed to stand at room temperature for 1 h [15]. The ghost suspension was centrifuged at $27000 \times g$ for 30 min ($8.1 \cdot 10^5\ g \cdot$ min) in a Sorvall centrifuge, type RC-2B, and the clear supernatant was removed by aspiration. The supernatant was concentrated by ultrafiltration (PM-10 filter, Amicon Corp., Lexington, Mass.) to a final protein concentration of approx. 10 mg/ml.

Isolation of lipoprotein density classes

Plasma samples for the isolation of lipoproteins were collected from healthy young men and women by plasmapheresis. The isolation of very low density lipoproteins ($d < 1.006$ g/ml), low density lipoproteins ($d = 1.006$–1.063 g/ml) and high density lipoproteins ($d = 1.063$–1.21 g/ml) was carried out by sequential ultracentrifugation as previously described [16].

Polyacrylamide gel electrophoresis

The polyacrylamide gels consisted of 5.6 % acrylamide, 1 % sodium dodecyl sulfate, 8 M urea, 0.05 M sodium phosphate, 0.02 % *N,N'*-methylenebisacrylamide, 0.075 % ammonium persulfate and 0.025 % *N,N,N',N'*-tetramethylethylenediamine, pH 8.6. Samples were dissolved in 2 % sodium dodecyl sulfate, 0.005 M sodium phosphate, 8 M urea and 10 % β-mercaptoethanol. The final protein concentration ranged from 0.3 to 1.0 mg/ml. To 100 μl of sample, 50 μl of glycerol and 3 μl of tracking dye (0.05 % Bromophenol Blue in water) were added. After mixing, the solution was carefully layered on the top of the gel.

Electrophoresis was carried out in a battery of 12 gels, each 75 mm long, in a Canalco polyacrylamide gel apparatus (Rockville, Md.) at a constant current of 5 mA per gel for approx. 2 h, employing a buffer consisting of 0.05 M sodium phosphate and 0.1 % sodium dodecyl sulfate, pH 8.6.

The gels were fixed and stained overnight in a solution of 0.04 % Coomassie Brilliant Blue, 25 % isopropyl alcohol and 10 % glacial acetic acid. Destaining was performed after several changes of a solution consisting of 10 % isopropyl alcohol and 10 % glacial acetic acid until the gel background was clear.

Immunological methods

Double immunodiffusion analyses [17] were carried out in 1 % agarose (Seakem Agarose, Bausch and Lomb, Inc., Rochester, N.Y.) employing a barbital buffer, pH 8.4, containing 0.1 % sodium azide. Hemagglutination inhibition tests were performed according to a modified procedure [18] of Takatsy.

The preparation and characterization of individual antisera to human serum lipoprotein families A, B and C and the constitutive polypeptides A-I, A-II, C-I, C-II and C-III were done as previously described [16]. The antiserum to apolipoprotein D was prepared and characterized as described in a recent communication from this laboratory [19]. The rabbit antiserum to human Lp (a) lipoprotein was generously provided by Professor K. Berg, University of Oslo, Norway. The arginine-rich apolipoprotein was isolated from a patient with type III hyperlipoproteinemia according to the procedure described by Shelburne and Quarfordt [20]. The anti-

serum to arginine-rich apolipoprotein obtained by immunizing a sheep with two intraperitoneal injections of purified antigen dispersed in complete Freund's adjuvant (5 mg/ml) was generously provided by Dr W. J. McConathy, Oklahoma Medical Research Foundation, Oklahoma City. This antiserum gave a single precipitin line with purified arginine-rich apolipoprotein. Goat anti-human β-lipoprotein serum (Lot No. 22051: 11.2 mg protein/ml) was purchased from Miles Laboratories, Inc., Elkhart, Ind. and rabbit antibodies to human α-lipoproteins (Batch No. 23486) and rabbit antibodies to human β-lipoproteins (Batch No. 2076U) were obtained from Behring Diagnostics, Woodbury, N.J. The commercially available antiserum to β-lipoproteins is comparable but not equivalent to anti-lipoprotein B, while the antiserum to α-lipoproteins is comparable but not equivalent to anti-lipoprotein A. The antisera to α- and β-lipoproteins reacted with both lipoprotein A and lipoprotein B.

Antisera to whole human erythrocyte ghosts and EDTA-extracted ghosts were prepared by immunizing rabbits with the appropriate antigens at weekly intervals for 4 weeks [15]. 1 ml of suspended ghosts (4 mg protein/ml) or 1 ml of EDTA extracted ghosts (10 mg protein/ml) were mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected intraperitoneally into white New Zealand rabbits. One week after the last injection, the rabbits were bled by cardiac puncture. The antisera gave positive precipitin lines with corresponding antigens dissolved in 5 % Triton X-100.

RESULTS

Double diffusion analyses of erythrocyte ghosts and plasma lipoproteins

The EDTA extraction of erythrocyte ghosts resulted in solubilization of 20–25 % of the total protein content. The polyacrylamide gel electrophoresis of EDTA extract revealed the presence of a number of bands identified according to the provisional classification of Fairbanks, Steck and Wallach [2, 5] as components 1, 2, 4.1, 4.2, 5 and 6 (Fig. 1B). The supernatant fraction obtained by ultracentrifugation of Triton X-100 extract contained approx. 60 % of the ghost protein; the polyacrylamide gel electrophoresis of this soluble fraction showed the presence of components 3, 4.1, 4.2, 6 and 7 as well as small amounts of components 1, 2 and 5 (Fig. 1C). It also contained the sialoglycoproteins. Comparison of these electrophoretic patterns with those of ghosts solubilized in a sodium dodecyl sulfate β-mercaptoethanol solution (Fig. 1A) and pellet fraction of Triton X-100 extract (Fig. 1D) indicated that all major and, most probably, all minor proteins were present in EDTA- and Triton X-100-soluble extracts of ghosts.

The EDTA and Triton X-100 extracts of erythrocyte ghosts gave no precipitin lines in double diffusion analyses with antibodies to either lipoprotein A, lipoprotein B, lipoprotein C or apolipoprotein D. Fig. 2 shows that these two membrane preparations also reacted negatively with commercially available antisera to α-lipoproteins and β-lipoproteins. The serum very low density, low density and high density lipoproteins formed no precipitin lines with antibodies to either intact or EDTA-extracted ghosts (Fig. 3). However, both the ghost preparations and serum lipoproteins reacted positively with their corresponding antisera (Figs 2 and 3).

To further test the antigenic components of erythrocyte ghosts and serum

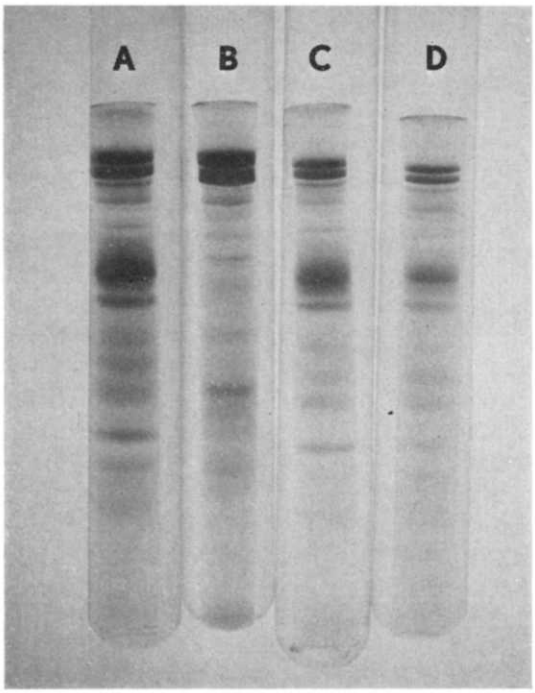

Fig. 1. Polyacrylamide gel electrophoresis of ghost proteins. A, whole ghosts; B, EDTA-extracted ghosts; C, supernatant fraction of ghosts solubilized in 5% Triton X-100; D, pellet fraction of ghosts solubilized in 5% Triton X-100. The supernatant and pellet fractions of ghosts solubilized in 5% Triton X-100 were obtained by ultracentrifugation at 105 000 × *g* for 2 h. All samples applied to the gels were dissolved in a solution consisting of sodium dodecyl sulphate, sodium phosphate, urea and β-mercaptoethanol as described in the Materials and Methods.

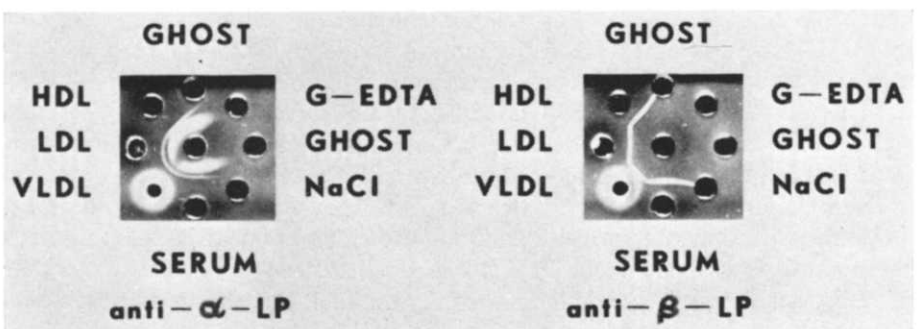

Fig. 2. Immunodiffusion patterns of ghost proteins and serum lipoprotein density classes. Antigens are placed in the outer wells and the antisera in the inner wells. GHOST, ghosts solubilized in 5% Triton X-100; G-EDTA, EDTA extract of ghosts; SERUM, fresh human serum; VLDL, very low density lipoproteins; LDL, low density lipoproteins, HDL, high density lipoproteins; anti-α-LP, antiserum to α-lipoproteins; anti-β-LP, antiserum to β-lipoproteins.

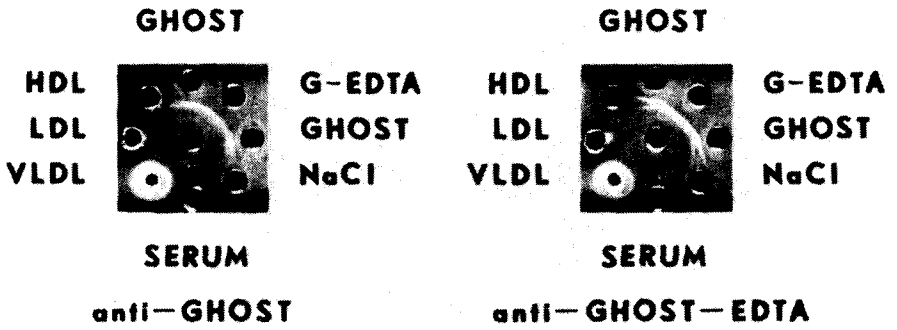

Fig. 3. Immunodiffusion patterns of ghost proteins and serum lipoprotein density classes. Antigens are placed in the outer wells and the antisera in the inner wells. The abbreviations are the same as in Fig. 2. Anti-GHOST, antiserum to intact ghosts; anti-GHOST-EDTA, antiserum to EDTA-extracted ghosts.

lipoproteins, both groups of compounds were analyzed simultaneously by double diffusion with a mixture of equal volumes of antisera to erythrocyte ghosts and to α- and β-lipoproteins. The crossed immunoprecipitin lines (Fig. 4) characteristic of nonidentity reactions provided further evidence that erythrocyte ghosts do not contain antigenic components of serum α- or β-lipoproteins.

To eliminate the possibility that lipid constituents of serum lipoproteins may be masking the common antigenic site(s) between apolipoproteins and erythrocyte ghosts, the individual apolipoproteins and their constitutive polypeptides obtained by delipidization of the corresponding lipoprotein families were tested by double diffusion with antisera to intact and EDTA-extracted ghosts. Similarly, the EDTA and Triton X-100 extracts of erythrocyte ghosts were examined with antisera to individual apolipoproteins and their constitutive polypeptides (A-I, A-II, ApoB, C-I, C-II, C-III, ApoD, arginine-rich polypeptide and Lp(a) lipoprotein). The absence of precipitin lines in each individual reaction showed that erythrocyte ghosts and delipidized serum lipoproteins do not share common antigenic components.

Although the results of double diffusion analyses have shown that serum apolipoproteins are not the integral protein constituents of erythrocyte ghosts, we have not excluded the possibility that serum lipoproteins attached to the surface of the erythrocyte by weak interactions may have been removed during the isolation and washing procedure in our experiments. To explore this possibility, hemaggluti-

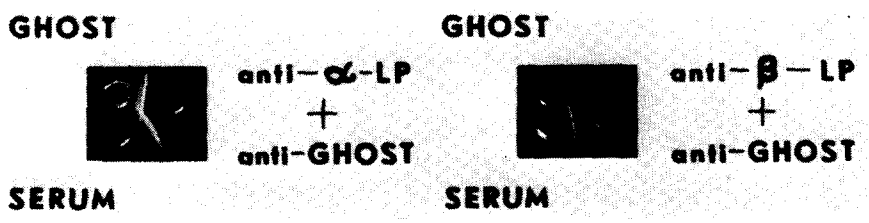

Fig. 4. Immunodiffusion patterns of ghost proteins and fresh human serum. Ghosts were dissolved in 5 % Triton X-100. The antigens are placed in wells on the left side of each pattern and the mixture of antisera in wells on the right side of each pattern. The abbreviations are the same as in Figs 2 and 3.

nation tests were carried out with all antisera described in Materials and Methods. Results of these experiments showed that red blood cells were agglutinated only with antiserum to β-lipoproteins, which indicates either a nonspecific reaction or a loose attachment of the highly hydrophobic lipoprotein B to the membrane surface. However, the negative result of hemagglutination inhibition tests carried out with the red blood cells and lipoprotein B favored the former rather than the latter explanation for the agglutination of erythrocytes by antiserum to lipoprotein B.

Effect of detergents on the immunoprecipitin reactions

Langdon's suggestion that serum apolipoproteins are the major protein constituents of erythrocyte membranes [12] was based mainly on the results of qualitative and quantitative immunoprecipitation reactions. In these experiments, the hemoglobin-free erythrocyte ghosts were dissolved in 2 % sodium dodecyl sulfate and, in some cases, this solution was dialyzed overnight at room temperature against 0.1 % sodium dodecyl sulfate in 10 mM phosphate buffer, pH 7.2. In contrast to our results with ghost preparations solubilized in EDTA and Triton X-100, ghosts dissolved in sodium dodecyl sulfate gave precipitin lines with antisera to α- and β-lipoproteins (Fig. 5). These two sets of experiments differed only in the type of detergent employed for solubilizing the erythrocyte ghosts, yet yielded quite contrary results. As indicated in several recent reports [3,21–23], the choice of detergents may be of critical importance for the outcome and interpretation of immunoprecipitation reactions. It has been shown that sodium dodecyl sulfate produces conformational changes in ghost proteins [24], and forms in a final concentration of 0.1 % or more, artificial precipitin lines with serum proteins and lipoproteins in agar diffusion and immunoelectrophoresis [22, 23]. Triton X-100 solubilizes 60–90 % of the membrane protein [3] without causing conformational changes [24] or substantial loss of enzymatic activity [25]. While efficiently solubilizing proteins and lipoproteins, this nonionic detergent does not interfere with their immunoprecipitation [3, 21]. To examine the effect of these two detergents on the immunoprecipitation reactions, we first analyzed erythrocyte ghosts according to the procedure described by Langdon [12] and then tested sodium dodecyl sulfate solutions of various concentrations in the absence of antigens. Ghosts dissolved in 2 % sodium dodecyl sulfate and dialyzed overnight at room temperature against 0.1 % sodium dodecyl sulfate in 10 mM phosphate buffer gave precipitin lines with commercially available antisera to both α- and β-lipoproteins. However, the sodium dodecyl sulfate solutions in concentrations greater than 0.1 % also formed precipitin lines

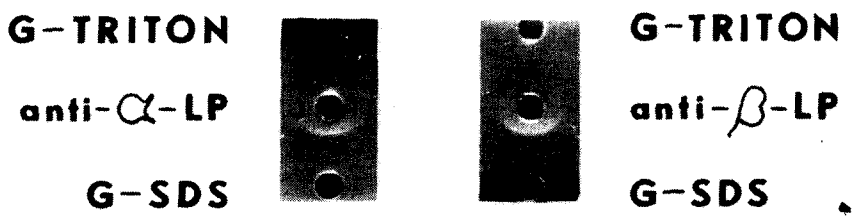

Fig. 5. Immunodiffusion patterns of ghost proteins. G-TRITON, ghosts solubilized in 5 % Triton X-100; G-SDS, ghosts dissolved in 2 % sodium dodecyl sulfate; anti-α-LP, antiserum to α-lipoproteins; anti-β-LP, antiserum to β-lipoproteins.

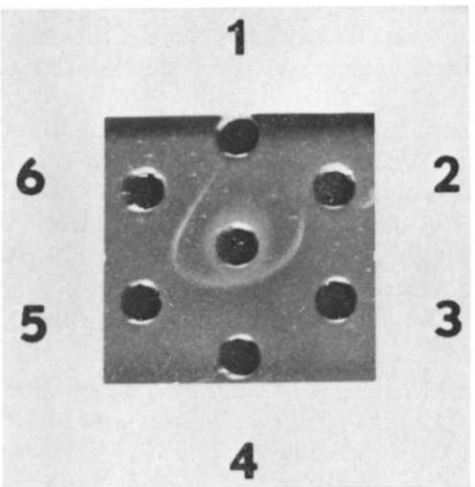

Fig. 6. Immunodiffusion pattern of various concentrations of sodium dodecyl sulfate. Well 1 contains 0.9 % NaCl; well 2 contains 0.1 % sodium dodecyl sulfate; well 3 contains 0.5 % sodium dodecyl sulfate; well 4 contains 1 % sodium dodecyl sulfate; well 5 contains 1.25 % sodium dodecyl sulfate; well 6 contains 2 % sodium dodecyl sulfate. The inner well contains commercially available rabbit antiserum to α-lipoproteins.

with antisera to α-lipoproteins (Fig. 6) and β-lipoproteins. The shape of sodium dodecyl sulfate precipitin lines depended considerably on the antisera used; they varied from sharp, well defined lines, as shown in Fig. 6, to broad, diffuse bands. It should also be emphasized that dialysis of protein samples treated with sodium dodecyl sulfate does not prevent the formation of artificial precipitin lines [23]. The application of Langdon's solubilization procedure to albumin indicated that the final sodium dodecyl sulfate concentration in ghost preparations was probably greater than 0.1 %. Bovine serum albumin was dissolved in 2 % sodium dodecyl sulfate and dialyzed overnight at room temperature against 0.1 % sodium dodecyl sulfate. After lyophilization, the gravimetrically determined sodium dodecyl sulfate concentration was 1.2 %. Control experiments carried out with another aliquot of the same ghost preparation dissolved in Triton X-100 gave negative results with antisera to both α- and β-lipoproteins.

CONCLUSION

The intriguing (from a chemical and metabolic point of view) suggestion that serum apolipoproteins are the major protein constituents of erythrocyte membrane has not been confirmed by the results of our present study. Our conclusion was based on double diffusion analyses and hemagglutination inhibition tests employing (a) antisera to all known lipoprotein families and corresponding apolipoproteins, and (b) antibodies to intact and EDTA-extracted ghosts. Ghosts solubilized in Triton X-100 or their aqueous EDTA extracts gave no precipitin lines with any of the employed antibodies. Conversely, serum samples and isolated lipoprotein density classes formed no precipitin lines with antisera to ghost preparations. The negative results of hemagglutination inhibition test excluded the presence of non-

precipitating antibodies. On the basis of these findings we have concluded that the integral ghost proteins identifiable on polyacrylamide gel electrophoresis as components 1–7 and sialoglycoproteins do not share common antigenic sites with serum lipoproteins or apolipoproteins.

One possible explanation for the discrepancy between these results and those reported by Langdon is that the immunoprecipitin lines observed in the latter study might have been due indeed to the presence of trace amounts of serum lipoproteins loosely attached to the cellular surfaces. The other more probable explanation is that the discrepancy resulted from nonspecific interactions between the proteins and the sodium dodecyl sulfate used as the solubilizing agent.

ACKNOWLEDGEMENTS

The authors are grateful to Drs D. M. Lane, R. Delaney and R. M. Hyde (University of Oklahoma Health Sciences Center) for helpful discussions and to Drs D. M. Lee and W. J. McConathy and Mr A. Suenram for preparing the serum lipoprotein density classes and antisera to apolipoproteins. We thank Mr R. Smith for his skillful technical assistance and Mrs M. Farmer and Mrs L. Stansberry for their help in the preparation of the manuscript. This study was supported in part by grant HL-6221 from the U.S. Public Health Service and by the resources of the Oklahoma Medical Research Foundation.

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