STUDIES ON THE IMMUNOCHEMISTRY OF HUMAN LOW DENSITY LIPOPROTEINS UTILIZING AN HEMAGGLUTINATION TECHNIQUE*

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Immunochemical techniques have been used extensively in studies on the chemistry and metabolism of serum lipoproteins. Several investigators (1–8) have produced antibodies against normal human lipoproteins by the injection of purified lipoproteins into rabbits, or into chickens (9). The antigen-antibody reaction between lipoproteins and their antisera has been demonstrated by precipitation (1–3, 5, 8), complement fixation (2, 3), gel diffusion (1–4, 6–8), and immuno-electrophoresis (6, 10). These investigations have suggested that low density and high density lipoprotein fractions are immunologically distinct. However, the immunologic relationships between low density lipoprotein subfractions, the S_f 3–9 and S_f 10–400 lipoproteins (11), have not been completely defined, since the immunochemical studies have been hindered by limitations of the procedures available for fractionation of low density lipoproteins and lack of immunologic techniques sufficiently sensitive for detection of heterogeneity within lipoprotein fractions low in protein. The immunologic specificity of S_f 3–9 and S_f 10–400 lipoprotein subfractions is important since metabolic studies have indicated a precursor-product relationship between the subfractions (12) while determinations of N terminal amino acid residues (13) have demonstrated heterogeneity of the protein component especially within the S_f 10–400 lipoprotein fraction.

In the present investigation, S_f 3-9 and S_f 10-400 lipoprotein fractions, prepared by the dextran sulfate-ultracentrifugal flotation procedure of Oncley *et al.* (11) and the ultracentrifugal flotation procedure of Havel *et al.* (14), were used to immunize rabbits. A modification of the hemagglutination technique of Boyden (15), using sheep erythrocytes treated with tannic acid, "tanned" cells, as the carrier for specific lipoproteins, was developed for the immunologic investigation of the low density lipoprotein system. Antisera were titrated and cross-absorption studies performed by hemagglutination procedures. Precipitin ring and gel diffusion studies were included.

Although the concentration of low density lipoproteins is elevated in atherosclerosis (16), a causal relationship between lipoprotein concentration and atherosclerosis has not been demonstrated. It is conceivable that an elevation

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in low density lipoprotein concentration may stimulate the production of antibodies and that antibodies are involved in the atherosclerotic disease process. Serum from normal and atherosclerotic subjects was investigated by hemagglutination procedures for the presence of autoantibodies against S_f 3-9 and S_f 10-400 lipoproteins; none, however, were found.

Materials and Experimental Methods

Preparation of Lipoproteins.—Low density S_f 3–9 and S_f 10–400 lipoproteins were isolated from pools of acid citrate dextrose plasma, approximately 1 liter in volume, by the dextran sulfate-density gradient method of Oncley, Walton, and Cornwell (11), and the flotation procedure of Havel, Eder, and Bragdon (14). The S_f 3–9 and S_f 10–400 lipoproteins prepared by these methods were purified by a second ultracentrifugal flotation. In this purification scheme, the S_f 3–9 lipoprotein fraction was dialyzed[1] against 15 volumes of 2 M NaCl[2] (density 1.078). Six ml. aliquots of the dialyzed lipoprotein solution were then placed in a 13.5 ml. lusteroid centrifuge tube. The tube was filled by layering 0.15 M NaCl over the lipoprotein solution and centrifuged at 100,000 *G* for 18 hours in the Spinco model L ultracentrifuge. The S_f 3–9 lipoprotein fraction formed an orange-yellow band in the center of the centrifuge tube. These bands were separated with a tube cutter, pooled, and dialyzed against 15 volumes of 0.15 M NaCl containing no EDTA.[3] The S_f 10–400 lipoprotein fraction was dialyzed against 15 volumes of 0.15 M NaCl, placed in a 13.5 ml. lusteroid centrifuge tube, and centrifuged at 9,300 *G* for 30 minutes. Chylomicrons formed a turbid layer at the top of the centrifuge tube. This layer was removed, the infranatant solution placed in a second centrifuge tube, and centrifuged at 100,000 *G* for 18 hours. The S_f 10–400 lipoprotein fraction formed a turbid band at the top of the centrifuge tube. These bands were separated with a tube cutter, pooled, and dialyzed against 15 volumes of 0.15 M NaCl containing no EDTA. Lipoprotein fractions were characterized by their nitrogen (17), phosphorus (18), and cholesterol (19) content.

A high density lipoprotein fraction (α-lipoprotein) and serum free of lipoproteins were prepared by ultracentrifugal flotation. Serum was adjusted to density 1.21 with a NaCl-KBr solution (14) and centrifuged at 100,000 *G* for 18 hours. All serum lipoproteins floated to the top of the centrifuge tube where they were separated and pooled. The infranatant serum (density 1.21) was centrifuged a second time at 100,000 *G* for 18 hours. Traces of lipoprotein were removed, the infranatant serum pooled, and dialyzed against 0.15 M NaCl containing no EDTA. This dialyzed fraction was designated as serum free of liproproteins. High density lipoproteins were then prepared from the pooled serum lipoproteins (supernatant fraction). The supernatant solution containing all lipoproteins was dialyzed against 2 M NaCl and 6 ml. aliquots placed in centrifuge tubes. Each tube was filled by layering 0.15 M NaCl over the lipoprotein solution and centrifuged at 100,000 *G* for 18 hours. The S_f 10–400 lipoproteins floated to the top of the tube, S_f 3–9 lipoproteins formed an orange-yellow band at the center of the tube, while high density lipoproteins sedimented to the bottom of the tube. The high density lipoprotein fraction was separated and adjusted to density 1.21 with a NaCl-KBr solution. Six ml. aliquots were placed in a centrifuge tube, the tube filled by layering 0.15 M NaCl over the crude high density lipoprotein fraction, and centrifuged at 100,000 *G* for 18 hours. High density lipoproteins formed an orange-yellow band in the lower third of the tube above contaminating serum pigments and proteins, while traces of the low density lipoproteins

[1] All dialysis and centrifugation procedures were performed at 4°C.

[2] Saline solutions contained 100 mg. per liter of the disodium salt of ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.0 ± 0.2 with 1 N NaOH.

[3] EDTA, ethylenediaminetetraacetic acid.

floated to the upper part of the centrifuge tube. The high density lipoprotein fraction was separated, pooled, and dialyzed against 0.15 M NaCl. In this isolation procedure, two centrifugation cycles were used in the separation of low density lipoproteins and other serum proteins from the high density lipoprotein fraction.

Immunologic Procedures.—Nine 2 to 3 months old female rabbits (1800 to 2400 gm.) were selected for the preparation of antisera. Three rabbits received 1 ml. of a fresh S_f 3–9 lipoprotein fraction containing 0.08 mg. of protein nitrogen intravenously on alternate days for a series of 6 injections. Two rabbits in this group were exsanguinated 2 weeks after the last injection. The third rabbit received a booster injection containing 0.08 mg. protein nitrogen 2 weeks after the original immunization series was completed. This rabbit was exsanguinated 1 week later. A second group of 3 rabbits was immunized in the same manner with a fresh S_f 10–400 lipoprotein fraction containing 0.08 mg. of protein nitrogen as the antigen. The third group of 3 rabbits served as a normal control.

Tannic acid–treated erythrocytes were prepared and used to adsorb lipoprotein antigens. 1 ml. of washed, packed sheep erythrocytes was added to 10 ml. of 1:10,000 tannic acid in 0.15 M NaCl and incubated for 10 minutes at 37°C. The cells were sedimented, resuspended, and washed 3 times in 0.15 M NaCl. The S_f 3–9 and S_f 10–400 lipoproteins were diluted to the same protein concentration, 0.04 mg./ml., with 0.15 M NaCl. These protein concentrations correspond to 0.18 mg./ml. of the S_f 3–9 lipoprotein fraction and 0.40 mg./ml. of the S_f 10–400 lipoprotein fraction (11). Lipoprotein concentrations were estimated from nitrogen analyses. Packed "tanned" erythrocytes, 0.5 ml., were added to 10 ml. of the diluted lipoprotein fractions and the suspension incubated from 30 minutes to 2 hours with stirring. At the completion of the incubation peroid, the cells were washed 3 times with 0.15 M NaCl and diluted to a 0.5 per cent suspension with a sodium chloride-phosphate buffer.[4]

Antiserum prepared from the immunization of rabbits was inactivated at 56°C. for 30 minutes, diluted 1:2 with 0.15 M NaCl, and 1 ml. aliquots absorbed with 0.4 ml. of washed packed sheep erythrocytes. The suspension was incubated at room temperature for 10 minutes and another 0.4 ml. volume of sheep erythrocytes added. After an additional 10 minute incubation period, the cells were centrifuged and the supernatant serum withdrawn. Doubling dilutions of the inactivated absorbed antisera were made with the sodium chloride-phosphate buffer[4] in a series of 15 agglutination tubes with 3 drop volumes according to the method of Rheins *et al.* (20). Three drops of a 0.5 per cent suspension of the "tanned" erythrocytes carrying either S_f 3–9 or S_f 10–400 lipoprotein fractions were added to each tube. The tubes were incubated at 37°C. for 30 minutes in a water bath and then examined for agglutination either by pattern or after a 1 minute centrifugation at 1000 R.P.M. in a Servall angle head centrifuge. A diluent control was included with each experimental series.

Immune and normal rabbit sera were titrated to determine whether the immune sera displayed a specificity for erythrocytes of different animal species. Antisera and sera from 2 normal rabbits were inactivated by incubation at 56°C. for 30 minutes. Doubling dilutions of antisera and normal rabbit sera were made with the sodium chloride–phosphate buffer[4] throughout a series of 10 tubes in 3 drop volumes. Three drops of a 0.5 per cent suspension of washed test erythrocytes were added to each tube. All tubes, including a diluent control, were incubated at 37°C. for 30 minutes and examined for agglutination.

The immunologic specificity of S_f 3–9 and S_f 10–400 antisera was determined by cross-absorption studies with S_f 3–9 and S_f 10–400 lipoproteins carried on "tanned" cells. An S_f 3–9 antiserum (No. 691) was inactivated, pre-absorbed with sheep erythrocytes, and diluted 1:32 with the sodium chloride-phosphate buffer.[4] The S_f 3–9 antiserum, 0.5 ml., was placed

[4] The sodium chloride–phosphate buffer contained 7.65 gm. of Na_2HPO_4, 1.81 gm. of KH_2PO_4, and 8.50 gm. of NaCl in 1 liter of deionized water (pH 7.4 $\pm$ 0.2).

in a centrifuge tube and 0.5 ml. of packed "tanned" erythrocytes carrying S_f 10–400 lipoproteins added. This suspension was incubated from 15 to 30 minutes at 37°C., an additional 0.5 ml. of the "tanned" cells carrying S_f 10–400 lipoproteins added and again incubated. At the completion of several absorption cycles, the suspension was centrifuged and the supernatant serum removed. Aliquots of absorbed and unabsorbed antisera were then titrated against S_f 3–9 and S_f 10–400 lipoprotein fractions by the hemagglutination procedure. The highest dilution in which a 4+ agglutination occurred was considered as the antibody titre for the antiserum. An S_f 10–400 antiserum (No. 498) was absorbed with sheep erythrocytes, diluted 1:16 with the sodium chloride-phosphate buffer,[8] and absorbed in a similar manner with S_f 3–9 lipoproteins carried on "tanned" erythrocytes. The S_f 3–9 and S_f 10–400 antisera were absorbed with their homologous antigens carried on "tanned" erythrocytes in control experiments.

The specificity of lipoprotein antisera and the purity of lipoprotein antigens used to immunize rabbits was confirmed by several experimental procedures. Lipoprotein antisera were examined for cross-reactivity against antigens consisting of the high density lipoprotein fraction, albumin (Cohn V), γ-globulins (Cohn II), and serum free of lipoproteins, by hemagglutination, precipitin ring, agar diffusion plate (21), and agar diffusion tube methods (22).

TABLE I

The Antibody Titre of S_f 3–9 and S_f 10–400 Antisera Titrated against S_f 3–9 and S_f 10–400 Lipoproteins Carried on "Tanned" Erythrocytes

Antiserum	Lipoprotein and titre	
	S_f 3–9	S_f 10–400
Anti S_f 3–9 (492)	1:4096	1:512
Anti S_f 3–9 (493)	1:1024	1:256
Anti S_f 10–400 (498)	1:512	1:256
Anti S_f 10–400 (499)	1:256	1:256

RESULTS

Since low density lipoproteins vary in their protein content, equal amounts of protein nitrogen rather than lipoprotein were used in the immunization procedures. The immunization studies revealed that an injection of 0.08 mg. of protein nitrogen from either S_f 3-9 or S_f 10-400 lipoprotein fraction was sufficient to stimulate antibody production in rabbits. This amount of nitrogen corresponded to 2.3 mg. of the S_f 3-9 lipoprotein fraction or 5.0 mg. of the S_f 10-400 lipoprotein fraction. The rabbit antisera obtained did not react with high density lipoproteins, albumin, γ-globulins, or lipoprotein-free serum, in systems employing "tanned" cells, in precipitin ring titrations, on agar diffusion plates, or in agar diffusion tubes. Hemagglutination titers for S_f 3-9 antisera against S_f 3-9 lipoproteins carried on "tanned" cells varied from 1:1024 to 1:4096.

Hemagglutination titers for an immunization series are recorded in Table I. The titer for S_f 10-400 antisera against the S_f 10-400 lipoprotein fraction was 1:256 (Table I).

In an experiment to test completeness of adsorption an S_f 3–9 lipoprotein fraction was adsorbed on "tanned" cells, and then the supernatant solution was withdrawn and re-adsorbed with "tanned" cells. A 0.5 per cent suspension of the latter cells in sodium chloride-phosphate buffer[4] did not hemagglutinate upon the addition of S_f 3–9 antiserum. The supernatant

TABLE II

Titration of S_f 3–9 Antiserum, S_f 10–400 Antiserum, and Normal Rabbit Sera against Erythrocytes of Different Blood Groups and Animal Species

Serum	Erythrocyte and titre										
	Human				Sheep	Guinea pig	Dog	Calf	Pig	Horse	Chicken
	O	A	AB	B							
Anti S_f 3–9 (492)	1:2	1:4	1:2	1:2	1:1	1:2	1:1	1:1	1:16	1:4	1:2
Anti S_f 10–400 (498)	1:2	1:1	1:1	0	1:2	0	1:1	1:2	1:4	1:1	0
Normal rabbit (496)	1:1	1:2	1:2	1:8	1:1	1:1	1:1	1:1	1:2	1:1	1:2
Normal rabbit (497)	1:1	1:1	0	1:2	1:2	1:1	1:2	1:1	1:16	1:4	0

TABLE III

Cross-Absorption Studies with S_f 3–9 and S_f 10–400 Antisera

Antiserum	Lipoprotein antigen and titre				Absorption cycles
	Unabsorbed		Absorbed		
	S_f 3–9	S_f 10–400	S_f 3–9	S_f 10–400	
A. Titre of S_f 3–9 Antiserum Before and After Absorption with S_f 10–400 Lipoproteins Carried on "Tanned" Erythrocytes					
Anti S_f 3–9	1:512	1:256	1:32	0	3
Anti S_f 3–9	1:512	1:256	1:32	0	4
Anti S_f 3–9	1:256*	1:128*	1:32*	0*	3
B. Titre of S_f 10–400 Antiserum before and after Absorption with S_f 3–9 Lipoproteins Carried on "Tanned" Erythrocytes					
Anti S_f 10–400	1:128	1:256	±	±	2
Anti S_f 10–400	1:512	1:256	0	0	3
Anti S_f 10–400	1:128*	1:128*	0*	0*	3

* Lipoprotein fractions prepared by the ultracentrifugal flotation procedure of Havel *et al.* (14) and repurified.

solution after 2 adsorption cycles gave no evidence of residual lipoprotein in agar diffusion or precipitin ring tests. The experiments thus indicated that the lipoprotein fraction had been adsorbed completely by the first group of "tanned" cells.

The S_f 3-9 and S_f 10-400 antisera and sera from two normal rabbits were titrated against erythrocytes of the four human blood groups and against

erythrocytes obtained from sheep, guinea pig, dog, calf, pig, horse, and chicken. The antisera gave no titers outside the range of normal antibodies when compared to normal rabbit serum. These results are summarized in Table II.

The antibody titers for an S_f 3-9 antiserum before and after absorption with an S_f 10-400 lipoprotein fraction carried on "tanned" cells, and the antibody titers for an S_f 10-400 antiserum before and after absorption with an S_f 3-9 lipoprotein fraction carried on "tanned" cells are recorded in Table III. Antibodies specific for the S_f 3-9 lipoprotein fraction remained in the S_f 3-9 antiserum after three and four absorption cycles with S_f 10-400 lipoproteins carried on "tanned" cells. No antibodies specific for either S_f 3-9 or S_f 10-400 lipoprotein fractions remained in the S_f 10-400 antiserum after three absorption cycles with an S_f 3-9 lipoprotein fraction carried on "tanned" cells. The S_f 3-9 and S_f 10-400 lipoprotein fractions obtained by the ultracentrifugal flotation procedure of Havel *et al.* (14) reacted no differently from lipoprotein fractions prepared by the dextran sulfate flotation procedure of Oncley *et al.* (11).

Sera from 5 individuals with no history of heart disease and sera from 3 patients with recent myocardial infarction and transaminase levels above 40 units were titrated against an S_f 3-9 lipoprotein fraction carried on "tanned" erythrocytes. No circulating antibodies were demonstrated in the subjects investigated.

DISCUSSION

Boyden (15) showed that tannic acid–treated erythrocytes adsorb proteins from solution and demonstrated that erythrocyte-protein complexes act as hemagglutinins in antigen-antibody reactions. This procedure is probably the most sensitive technique available for serologic studies *in vitro* (23). Low density lipoproteins, high molecular weight molecules low in protein content, are adsorbed from solution and react as hemagglutinins in the same manner as other proteins. This fact suggests that the protein moiety is on the surface of the lipoprotein molecule and so is available for adsorption on the "tanned" erythrocyte. Although the structure of the lipoprotein molecule is unknown, the euglobulin solubility (11), combination with sulfated polysaccharides (11), and adsorption on "tanned" erythrocytes, all support the concept of a protein surface and lipide core. The structure recently proposed by Zollner (24)—a lipid surface and protein core—is not consistent with the formation of lipoprotein hemagglutinins.

The present investigation confirmed the fact that the hemagglutination reaction is a sensitive method for the measurement of antibody titer. It appears to be superior to other immunologic procedures for the demonstration of antibody specificity since specific antibodies in a lipoprotein antiserum can be absorbed completely by repeated cross-absorption cycles with antigens

carried on "tanned" erythrocytes. This technique also eliminates consideration of the equivalence phenomenon since excess antigen is removed by sedimentation. Furthermore, hemagglutination is superior to quantitative precipitin methods for studies of lipoproteins since lipoprotein molecules in the upper region of the S_f 10-400 lipoprotein spectrum are turbid, and molecules with a very low density, the S_f 10-400 lipoprotein fraction, may float rather than sediment upon aggregation in antigen-antibody complexes (25). When S_f 10-400 antisera were absorbed with S_f 3-9 lipoproteins carried on "tanned" cells, all agglutinins directed against both the S_f 10-400 and S_f 3-9 lipoprotein fractions were removed. The data indicate that the S_f 3-9 lipoprotein fraction contains the same antigens as the S_f 10-400 lipoprotein fraction. When S_f 3-9 antisera were absorbed with S_f 10-400 lipoproteins carried on "tanned" cells, all agglutinins directed against the S_f 10-400 lipoprotein fraction were removed, whereas some agglutinins directed against the S_f 3-9 lipoprotein fraction remained in the antiserum. Since low density lipoprotein antisera did not react with high density lipoproteins, albumin, γ-globulins, and serum free of lipoproteins, the low density lipoproteins were apparently highly purified. Furthermore, the short immunization schedule and immunization with lipoproteins rather than an alum precipitate (2, 3) presumably tended to minimize the production of antibodies against trace impurities. The S_f 3-9 lipoprotein fraction, therefore, contained one or more antigens in addition to the antigens in S_f 10-400 lipoprotein, and it seems reasonable to assume that the additional antigenicity was lipoprotein in nature.

Other investigators (1, 2) have reported that S_f 3-9 lipoproteins and various lipoprotein bands within the S_f 10-400 lipoprotein spectrum were indistinguishable immunologically. The present studies confirm these observations and suggest that lipoprotein antigens throughout the S_f 10-400 lipoprotein spectrum are identical with lipoprotein antigens in the S_f 3-9 lipoprotein fraction, although the S_f 3-9 lipoprotein fraction may contain additional antigens not present in the S_f 10-400 lipoprotein fraction. It should be emphasized that antisera directed against S_f 10-400 and S_f 3-9 lipoproteins contained no antibodies directed against the high density lipoprotein fraction. Thus, the immunologic studies support the investigations of Gitlin *et al.* (12) who demonstrated the conversion, *in vivo*, of S_f 10-400 to S_f 3-9 lipoproteins. Gitlin *et al.* (12) were unable to demonstrate the conversion of low density lipoproteins to high density lipoproteins, an antigenically distinct lipoprotein fraction.

The immunologic specificity of the S_f 10-400 lipoprotein fraction is not consistent with N terminal amino acid studies (13) in which additional amino acid end groups were demonstrated in the S_f 10-400 lipoprotein fraction. The discrepancy between N terminal amino acid studies and immunologic studies may be resolved in several ways: (*a*) The concentration of some lipoprotein antigens in the S_f 10-400 lipoprotein fraction might have been insufficient to

stimulate antibody production; (*b*) Low density lipoproteins isolated from subjects with idiopathic hyperlipemia and used in the N terminal amino acid studies might have been different from the lipoproteins present in normal individuals; (*c*) Lipoprotein fractions prepared for N terminal amino acid analysis might have contained protein impurities.

Antibodies prepared against the S_f 3-9 lipoprotein fraction may be used to detect both S_f 3-9 and S_f 10-400 lipoproteins; however, these antibodies will not distinguish between the low density lipoprotein fractions. Although an immunologic estimation of low density lipoproteins has been proposed (25), this method will not estimate the concentration of specific low density lipoprotein fractions elevated in disease processes such as myxedema or nephrosis (12, 16). The S_f 3-9 lipoprotein fraction may be used to detect antibodies against S_f 3-9 and S_f 10-400 lipoproteins. In the present investigation, no circulating antibodies against low density lipoproteins were demonstrated in the serum of 5 normal subjects or 3 subjects with recent myocardial infarctions by the hemagglutination of S_f 3-9 lipoproteins carried on "tanned" cells.

SUMMARY

Hemagglutination is a specific and sensitive technique for investigating the purity of lipoproteins and the immunologic relationships between low density lipoprotein fractions.

The S_f 10-400 and S_f 3-9 lipoprotein fractions, isolated from human serum by dextran sulfate-density gradient centrifugation procedure and repurified by centrifugation appeared to contain only lipoprotein antigens since these fractions did not stimulate the production of antibodies against other serum proteins. Cross-absorption experiments with lipoproteins carried on "tanned" cells demonstrated that the S_f 3-9 lipoprotein fraction contains all the antigenic components of the S_f 10-400 lipoprotein fraction together with additional antigenic components not found in the S_f 10-400 lipoprotein fraction. Thus S_f 3-9 and S_f 10-400 lipoprotein fractions are immunologically similar but not identical. Low density lipoproteins contain no antigens in common with the high density lipoproteins.

An S_f 3-9 antiserum can be used to detect both S_f 3-9 and S_f 10-400 antigens. The S_f 3-9 lipoprotein fraction used as an antigen will detect antibodies against both S_f 3-9 and S_f 10-400 lipoprotein fractions. The S_f 3-9 and S_f 10-400 antisera did not contain immune antibodies against erythrocytes of the different blood groups or against sheep, guinea pig, dog, calf, pig, horse, and chicken erythrocytes.

Normal subjects and subjects with recent myocardial infarctions had no circulating autoantibodies against the S_f 3-9 and S_f 10-400 lipoprotein fractions.

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