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Purification of Opsonically Active Human and Rat Cold-Insoluble Globulin (Plasma Fibronectin)†

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ABSTRACT: Two different affinity chromatographic procedures were developed for the purification of opsonin from plasma or serum, utilizing either heparin or specific antibody containing columns. Activities were measured in liver tissues by the uptake of ^{125}I-labeled latex particles to which gelatin was covalently coupled. Regardless of whether the proteins were derived from human or rat blood, opsonic potencies of the purified products were very similar to that of human cold-insoluble globulin (CIG or fibronectin) obtained as a byproduct in the course of factor XIII fractionation by the method of Lorand & Gotoh [Lorand, L., & Gotoh, T. (1970) *Methods Enzymol. 19*, 770–782]. As judged by electrophoretic and immunological criteria, all procedures yielded materials of similar purity. Furthermore, human and rat opsonins cross-react immunologically. The hepatic system appears to be quite specific for gelatinized particles, because no uptake could be accomplished when fibrinogen, fibrin, or serum albumin was coupled to latex. In competition assays, only gelatin or collagen added in solution caused an inhibition of the uptake of gelatinized particles, while fibrinogen or serum albumin had no effect. In addition to the opsonic protein, some constituent of commercial heparin, other than that responsible for the anticoagulant activity of this mucopolysaccharide mixture, is required for promoting the hepatic uptake of gelatinized latex particles. Further support for the identity of opsonin with CIG, first suggested by Blumenstock and co-workers [Blumenstock, F. A., Saba, T. M., Weber, P., & Loffin, R. (1978) *J. Biol. Chem. 253*, 4387–4291], was obtained by showing that the opsonic activity of a solution could be reduced considerably by the process of specific cross-linking by factor $XIII_a$ in the presence of fibrin.

Phagocytosis is the specialized function of certain cells in higher organisms involved in host defense with the purpose of ingesting and digesting harmful particles such as bacteria, antigen–antibody complexes, tissue debris, and colloidal pollutants. It has many similarities to the transport of low molecular weight nutrients (salts, amino acids, and sugars) and, in addition, it depends on humoral recognition factors (opsonizing factors) which give the process selectivity and efficiency (DiLuzio et al., 1971). So far at least two opsonizing systems have been recognized. One is related to the removal of antibody-coated antigenic particles, often involving the participation of a complement (Gigli & Nelson, 1968; Morelli & Rosenberg, 1971; Moreau & Skarnes, 1975). The other system, relating to the subject matter of our work, seems to be specific for the phagocytosis of collagen- or gelatin-coated

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particles. A plasma protein, henceforth called opsonin, and heparin, an acidic mucopolysaccharide (Murray, 1963; Filkins & DiLuzio, 1966), are the humoral factors involved in this process. The latter type of "opsonin" is not related to the antibody–complement system (McLain et al., 1976).

It was shown that purified rat serum opsonin behaved as a single protein similar in size and electrophoretic mobility to α_2-macroglobulins of human and rabbit plasma (Molnar et al., 1977). Purification procedures similar to ours were also used by Blumenstock et al. to isolate opsonin from rat (1976) as well as human sera (1977). These workers made the significant observation that human cold-insoluble globulin (CIG or plasma fibronectin) showed immunological identity with their human opsonin preparations and had great similarities in amino acid composition as well (Blumenstock et al., 1978). Albeit recognizing the fact that proof of biological identity would also require a demonstration that the CIG purified by well established methods was opsonically active, thus far no evidence could be obtained in this regard because isolation of CIG by ethanol fractionation (Mosesson et al., 1975) yielded an inactive product (Blumenstock et al., 1978). It will be shown in this paper that the highly purified CIG, prepared on a rather large scale during the course of isolating the fibrin stabilizing factor zymogen (coagulation factor XIII) from human plasma, as described by Lorand & Gotoh [1970; see also Curtis & Lorand (1976)], displays extremely good opsonic activity in supporting the phagocytic uptake of gelatinized test particles in an in vitro hepatic cell system.

Two affinity chromatographic procedures will also be described for purifying (human and rat) opsonin. One of these, a specific antibody containing column, could be used to isolate opsonin directly from plasma or serum in a single step of operation. The other, Sepharose-bound heparin, could be employed for the further purification of opsonin from plasma or serum concentrates. In addition, the present study deals with the interaction of opsonin with fibrin under cross-linking conditions by the fibrin-stabilizing enzyme, factor $XIII_a$, and also with questions relating to the specificity of collagen for the coating of test particles, as well as the heparin requirement for phagocytosis.[1]

Materials and Methods

Covalent Binding of Gelatin to Latex Particles. Carboxylated latex (2 mL) (0.4-μm beads, 10% dry weight; Dow Chemical Co., Indianapolis, IN) was mixed with 10 mg of gelatin (Nutritional Biochemicals Co., Cleveland, OH) in 1 mL of water and a 500-μL solution containing 4 M sodium chloride and 0.8 M sodium borate (adjusted to pH 8 with hydrochloric acid). A trace amount of gelatin which was previously labeled with ^{125}I (chloramine-T method; Hunter & Greenwood, 1962) was also included to be able to measure binding efficiency. Finally, 50 mg of *N,N'*-dicyclohexylcarbodiimide (Aldrich Chemical Co., Milwaukee, WI) in 0.5 mL of ethanol was added, and the mixture was kept at room temperature for 3 h and at 4 °C overnight. Then 1 mg of tyrosine hydroxamide (Calbiochem, La Jolla, CA) was admixed, and the suspension was brought to room temperature for 1 h, following which it was diluted to 40 mL with a solution (buffer A) containing 0.15 M sodium chloride and 10 mM sodium borate at pH 8. The latex particles were harvested by centrifugation at 15000 rpm for 10 min in a Sorvall RC 2B centrifuge, using a SS-34 rotor; they were washed with 40 mL of buffer A and collected again by centrifugation. This washing procedure was repeated twice, and the latex particles were then suspended in 10 mL of buffer A. About 50–70% of the radioactivity added as the tracer gelatin (10^6 cpm) remained attached to the particles, indicating that a total of 25–35 μg of gelatin became incorporated into 1 mg of latex particle (dry weight).

Radioiodination of Gelatin-Coated Latex Particles. The latex-bound gelatin suspension (2 mL), prepared as described above, was mixed at room temperature with 0.1 mL of 20 mM sodium iodide, as well as with 100 μL (100 μCi) of ^{125}I-labeled sodium iodide (Amersham/Searle, Arlington Heights, IL) and 25 μL of chloramine-T (1 mg/mL; Aldrich Chemical Co., Milwaukee, WI). Another 25 μL of the chloramine-T solution was added 10 min later, and the reaction was allowed to proceed for 10 min before 1 mg of sodium tetrathionate was admixed and the latex particles were removed by centrifugation (see above). The sediment was washed and centrifuged 7 times in succession, each time with 40 mL of buffer A containing 1 mM of sodium iodide. Then, following dialysis against two changes of 4 L of buffer A (lasting for about 12 h each), the radioactive particles were harvested by centrifugation, suspended in 10 mL of buffer A, and stored at 4 °C. The preparation contained less than 0.1% of nonsedimentable radioactivity (15000 rpm; 10 min) and had a latex content of about 4 mg/mL of suspension. Immediately prior to use in phagocytic experiments, an aliquot of the suspension was diluted 10-fold into a Krebs–Ringer phosphate buffer of pH 7.4 (Umbreit et al., 1972) and dispersed by brief sonication (10-s burst at setting 2; Model W140D Brenson sonifier, Plainview, NY).

Bioassay for the Phagocytic Uptake of ^{125}I-Labeled Gelatinized Particles by Rat Liver Slices. White male or female, Holtzmann or Sprague–Dawley rats weighing 250–350 g were sacrificed by decapitation and, following exsanguination, 0.5–1-mm thick liver sections were prepared and cut into (100 ± 30)-mg (wet weight) pieces. Each slice was immersed in a Wheton vial containing heparin (10 units of the Upjohn product, unless otherwise indicated), blood fractions, and other additions in Krebs–Ringer buffer as specified, with a total volume of 1.2 mL. The phagocytic process was initiated by the addition of 0.1 mL of the 10-fold diluted (10000–30000 cpm), ^{125}I-labeled, gelatinized latex suspension and was allowed to continue at 37 °C for 30 min, while the vials were shaken gently in a metabolic shaker (New Brunswick Instruments, New Brunswick, NJ). Following the phagocytic reaction, the tissue slices were placed in a tray containing 2 L of cold 0.1 M sodium chloride and were rinsed to remove particles which were not bound by the liver. Each slice was then transferred into a counting tube for measurement of radioactivity (Model 1085 γ counter, Nuclear Chicago, Chicago, IL). Experiments were performed in triplicates, and, after correcting for background radiation, the uptake of isotope was calculated for 100 mg of tissue and was averaged for the three measurements. Results for the opsonin-dependent uptake of particles are tabulated in terms of the percentage of the total dose of latex-bound radioactivity originally added to the tissues, subtracting the percentage uptake without extraneous opsonin.

The experiments for the uptake by liver slices of latex particles coated with bovine serum albumin, human fibrinogen, or fibrin were performed in an analogous manner. The covalent coupling of these proteins to carboxylated latex and the consecutive iodination of the modified particles were also similar to those described for gelatin. Latex-bound fibrin was obtained by treatment of latex-bound fibrinogen prior to

[1] Preliminary reports of these results have already appeared: Gudewicz et al. (1978) and Molnar et al. (1978).

radioiodination with 100 units of human thrombin in 5 mL of Krebs–Ringer phosphate buffer of pH 7.4 for 20 min at 37 °C, and the particles were washed 3 times with 40 mL each of buffer A.

Opsonin Preparations. Fresh as well as outdated acid citrate dextrose (ACD) blood bank human plasma, fresh citrated rat plasma, and fresh human and rat sera served as starting materials for isolating opsonin.

Authentic cold-insoluble globulin or plasma fibronectin (Mosher, 1975) was prepared as a byproduct of purifying the fibrin stabilizing factor zymogen (coagulation factor XIII) from 10 L of fresh or outdated human plasma according to the procedure described by Lorand & Gotoh [1970; see also Curtis & Lorand (1976)]. Separation of the zymogen from CIG was achieved by DEAE-cellulose chromatography using a linear sodium chloride gradient in a 50 mM Tris-HCl buffer of pH 7.5 containing 1 mM of EDTA. While factor XIII emerged at an ionic strength of about 0.09, the essentially pure CIG peak eluted at about 0.18. The latter (300 mL; 1 mg/ml protein) was concentrated by precipitation with ammonium sulfate (0.4 saturation; 4 °C; 60 min), and, after centrifugation, it was dissolved in 60 mL of 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.1 mL of Trasylol (10 000 Kallikrein units/mL; FBA Pharmaceuticals, New York). In order to remove possible small molecular weight contaminants, gel filtration on Sepharose 6B (Pharmacia, Sweden; 2.5 × 110 cm column) was carried out in the above buffer using a protein load of 40 mg. All material absorbing at 280 nm (ISCO detector; Lincoln, NB) emerged as a single peak at a V_e/V_0 ratio of 1.2. This material was concentrated by precipitation with ammonium sulfate, taken up in the EDTA- and Trasylol-containing Tris buffer as before, and dialyzed against the same. Typically, about 400 mg of pure CIG was obtained from 10 mL of plasma, and the preparation (8 mg of protein per mL) was stored at 4 °C.

Affinity chromatography with Sepharose 4B–heparin (kindly provided by Dr. A. J. Gray, Jr., of Abbott Laboratories, North Chicago, IL) was performed on ammonium sulfate concentrates (Allen et al., 1973) obtained from plasma as well as serum. The 2 × 10 cm column, thermostated at 4 °C, was equilibrated with 50 mM Tris-HCl and 1 mM EDTA at pH 7.4, and, following application of protein (50–70 mg in 10 mL of the above buffer, corresponding to about 100 mL of starting plasma or serum), elution was carried out with a linear salt gradient by mixing 300 mL of the Tris–EDTA buffer with 300 mL of 0.5 M sodium chloride in the same buffer. Elution rate was 0.5 mL/min, and 5-mL fractions were collected with the aid of a refrigerated fraction collector at 4 °C. Fractions were tested for protein contents by measuring absorbancy at 280 nm, and 0.2-mL aliquots were taken for assaying phagocytosis-promoting activity in the hepatic system as described. The opsonically active fractions were pooled and were concentrated 10-fold either in an Amicon Diaflo (Amicon Corp., Lexington, MA) or in a vacuum dialysis apparatus. In order to assess the separation of opsonic activity from α_1-macroglobulin in the rat and from α_2-macroglobulin in the human preparations, we also measured the trypsin binding activities which characterize these macroglobulins according to the method of Ganrot (1973) using benzoylarginine *p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) in the presence of excess soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ) by measuring the absorbancy of *p*-nitroanilide (410 nm) formed on account of the hydrolysis of substrate by the trypsin–macroglobulin complex. Chloride concentrations in the chromatographic fractions were determined by titrating with silver nitrate in the presence of potassium chromate (Sawyer, 1958).

Antibody affinity columns were used for the isolation of opsonins directly from fresh human and rat plasmas or sera. Up to 20 mL of these starting materials, mixed with 1000 units of heparin, was applied (room temperature) to a column (0.9 × 5 cm) containing antiopsonin antibodies covalently coupled to Sepharose 4B (Axen & Erback, 1971) and equilibrated with Krebs–Ringer solution. The latter buffer (ca. 30 mL) was used also for removing proteins which were not specifically retained by the column. Opsonic activity was eluted with a solution of 0.1 M glycine hydrochloride, pH 2.3, and the eluate (5 mL) was immediately neutralized by dropwise addition of 1 M potassium biphosphate.

Immunological Techniques. Opsonins for the immunization of rabbits were prepared from fresh human and rat plasmas by using a previously described procedure (Molnar et al., 1977) which included ammonium sulfate fractionation, gel filtration on Sepharose 4B, and preparative free-flow electrophoresis. Further purification was obtained by means of DE-52 cellulose (Whatman Inc., Clifton, NJ) chromatography in the presence of 4% (v/v) glycerol. Cellulose [6 mL (packed volume)] was mixed with 6 mL of Sephadex G-25 to enhance the flow rate, and the column was equilibrated with a solution of 50 mM sodium chloride in 10 mM Tris-HCl (pH 7.5) and 4% glycerol. Following the transfer of the protein sample (10 mg in 5 mL of the above buffer) to the column, a linear salt gradient was applied by mixing 300 mL of the starting buffer with a 300-mL solution of 0.2 M sodium chloride in 10 mM Tris-HCl (pH 7.5) and 4% glycerol. Opsonic activity was collected in the fractions eluted at an ionic strength of about 0.16.

Purified opsonin (1 mg) in 1 mL of buffer A was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI), and 1-mL doses were injected intramuscularly into white New Zealand rabbits on days 0, 14, 21, 28, and 35. The animals were sacrificed by decapitation 7 days after the last injection, and the blood was collected. The serum was heat-treated (56 °C) for 30 min and was assayed by immunodiffusion for reactivity against either normal human or rat serum. Initially, both human or rat opsonin antisera gave one major and two minor precipitin bands when tested against the normal serum of the same species, suggesting the presence of some minor antigenic contaminants. In order to remove nonspecific antibodies, we passed the antisera (50 mL) through columns (1 × 10 cm) containing opsonin-depleted human or rat serum proteins covalently attached to Sepharose 4B (Axen & Erback, 1971). Opsonin depletion was achieved by treating 5 mL of normal serum with 0.2 mL of heparin (200 units) and 0.5 mL of gelatinized latex (50% suspension) for 10 min at room temperature and then removing the latex particles by centrifugation (10 000 rpm; 10 min). Following this procedure, both antisera showed only single preciptin bands in immunodiffusion with regard to normal sera and could thus be used for preparing opsonin-specific IgG's in the following manner. Proteins precipitating from the antisera (2.5 mL) at 0.4 saturation of ammonium sulfate were collected, dissolved, dialyzed against 10 mM phosphate buffer at pH 7, and applied to a DE-52 cellulose column (2.5 × 30 cm) equilibrated with the same buffer. The IgG fractions passed through unretained and were then stored with 0.03% sodium azide at 4 °C. These preparations were used in all immunoassays and also in the coupling to cyanogen bromide activated Sepharose 4B (10 mg of protein per g of wet resin; Axen & Erback, 1971) for the affinity chromatographic procedure already described.

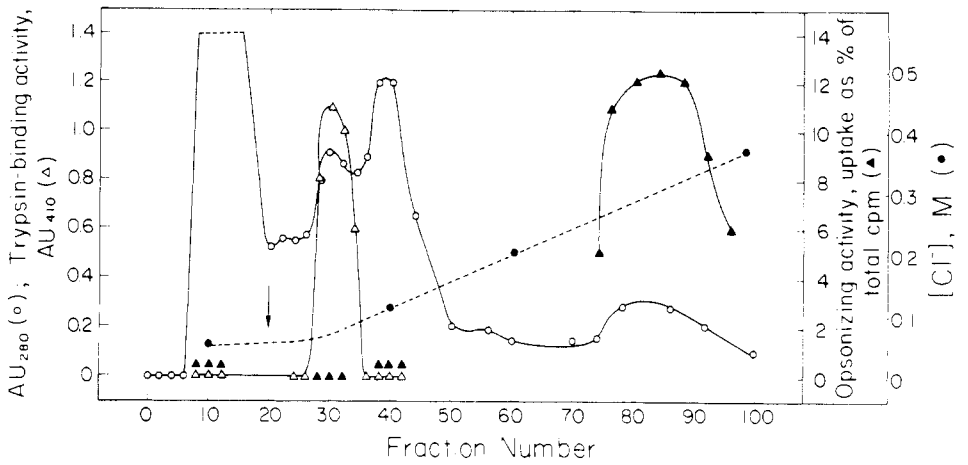

FIGURE 1: Purification of opsonizing activity by chromatography on a heparin column. Rat plasma fraction (450 mg of protein in a 20-mL solution), obtained by precipitation with 0.35 saturated ammonium sulfate and dialyzed against a 50 mM Tris-HCl buffer of pH 7.5 containing 1 mM of EDTA, was applied to a 2 × 10 cm column of Sepharose 4B-heparin and eluted with the sodium chloride gradient indicated. The vertical arrow denotes the beginning of the salt gradient. Fractions of 5 mL were collected and tested for protein (absorbancy at 280 nm; ○) and chloride (●) contents as well as for opsonic (▲) and trypsin binding activities (△) by using aliquots of 0.2 mL in the latter three determinations. For experimental details, see Materials and Methods section.

Table I: Purification of Opsonin by Ammonium Sulfate Fractionation, Followed by Affinity Chromatography on Sepharose 4B-Heparin Columns (2 × 10 cm)[a]

	protein (mg/100 mL)		total opsonin units in 100 mL (×10⁻³)		sp opsonin act. (units/mg)	
	plasma	serum	plasma	serum	plasma	serum
starting material	7000	7000	100	36	14	5
0.35 saturated $(NH_4)_2SO_4$ fractions	690	650	54	27	79	42
Sepharose 4B-heparin column fractions	35	39	34	24	980	610

[a] Data pertain to experiments similar to that presented in Figure 1, with rat plasma or serum as starting materials.

Double immunodiffusions (Ouchterlony, 1949) were performed at room temperature over 24 h in 0.8% agarose in phosphate-buffered (0.01 M sodium phosphate, pH 7.4) saline (0.85%) containing 0.1% sodium azide. Immunoelectrophoretic analysis was carried out according to Scheidegger (1955). Precipitin patterns were photographed with a camera purchased from the Cordis Corp., Miami, FL.

Polyacrylamide disc gel electrophoresis was performed on protein samples (25–200 μg) without denaturation (Rodbard & Chrambach, 1971) as well as with denaturation in sodium dodecyl sulfate (Laemmli, 1970). Reduction of disulfides, wherever indicated, was achieved by treating proteins (15 μg) with 0.2 M dithiothreitol (100 °C; 5 min) in solutions of 0.05 mL containing 1% sodium dodecyl sulfate and 3 M urea.

Other Materials and Procedures. Human fibrinogen (fraction I-4; Mosesson et al., 1967) and human α-thrombin (Fenton et al., 1977) were kindly provided by Drs. M. Mosesson and D. Galanakis of Downstate Medical Center, Brooklyn, NY, and by Dr. J. W. Fenton, II, Division of Laboratories and Research, New York State Department of Health, Albany, NY, respectively. "High"- and "low"-activity heparin fractions [see Teien et al. (1977) and Rosenberg et al. (1978)] were obtained from Kabi A.B., Stockholm, Sweden. Collagens of type I from rat skin and rat tail tendon and type IV from bovine lens capsule were gifts from Dr. A. Veis, Northwestern University Medical School, Chicago, IL.

Bovine serum albumin (BSA) and heparin (150 units/mg and 1000 units/mL) were purchased from Sigma Chemical Co., St. Louis, MO, and Upjohn Co., Kalamazoo, MI, respectively.

Fibrin-stabilizing factor (factor XIII) was isolated from blood bank plasma (Lorand & Gotoh, 1970; Curtis & Lorand, 1976), and the solution of the purified zymogen was assayed for the concentration of potentially active enzymatic sites by titration with [^{14}C]iodoacetamide (Curtis et al., 1974). By this index, the material showed nearly 100% functional purity.

Results

Opsonizing activity was measured by a modification of the procedure of Saba & DiLuzio (1965), using rat liver slices. The radiolabeled lipid emulsion or sulfur colloid (Molnar et al., 1977) was replaced by latex particles to which gelatin was covalently attached and labeled with ^{125}I. In the absence of extraneous opsonizing factor, only about 15% or less of the added radioactive dose was taken up by the liver slices. However, the presence of increasing amounts (up to 20 μL) of plasma or serum caused a linear increase in the uptake of radiolabeled particles. From the slope of the line, 1 unit of opsonizing activity could be defined as that amount of material which caused the uptake of 1% of the total dose of radioactive particles added to the liver tissue. Similar dose relationships were also obtained for the various opsonic fractions, and, for the most purified preparations, the linear range persisted up to 20 μg of opsonizing protein per test. It should be pointed out that, on account of variations of the phagocytic potencies of liver slices from one animal to another and also probably because of differences in gelatinized latex particles, this is not an absolute unit. Thus, even though there was good reproducibility on a given day, when liver slices from the same animal and the same batch of latex particles for one set of experiments were used, the actual specific activities obtained in different experiments cannot be compared.

Efforts for purifying opsonin centered on suitable affinity chromatographic procedures and, as shown in Figure 1, the Sepharose 4B-heparin column could be used to good advantage. Passage of the 0.35 saturated ammonium sulfate

Table II: Direct Isolation of Opsonin from Serum Using a Specific Antibody Containing Affinity Column[a]

	total opsonizing units	sp opsonizing act. (units/mg of protein)
starting serum (17 mL)[b]	2040	1.7
serum effluent (17 mL)[b]	0	
dialyzed glycine eluate (7 mL)	4200	1300

[a] Rat serum (17 mL) was applied to a 0.9 × 5 cm column of Sepharose 4B coupled to rabbit IgG against rat opsonin, and the column was further washed with 30 mL of Krebs–Ringer solution. Specifically absorbed protein was subsequently eluted with 0.1 M glycine hydrochloride (pH 2.3), immediately neutralized by the addition of K_2HPO_4, and dialyzed against Krebs–Ringer solution at 0 °C overnight. [b] Aliquots were kept on ice and were tested for opsonizing activity together with the dialyzed glycine eluate.

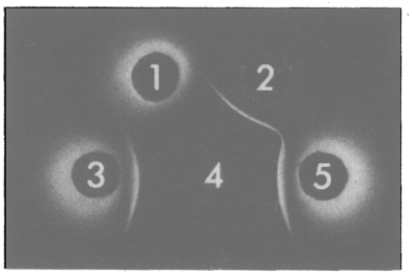

FIGURE 2: Analysis of purified rat serum opsonin by Ouchterlony immunodiffusion. (1) Deopsonized rat serum, passed through an affinity column of rabbit IgG specific against rat opsonin; (2) eluate with 0.1 M glycine hydrochloride, pH 2.3, from the same column (see Table II); (3 and 5) normal rat serum; (4) rabbit immunoserum against rat opsonin. For experimental details, see Materials and Methods.

concentrate (Allen et al., 1973) from either rat plasma or rat serum through this column produced an approximately 12-fold increase in specific opsonic activity (see Table I). It should be noted that the heparin column provided an excellent separation of opsonin from the α_1-macroglobulin, identified by its trypsin binding activity (Ganrot, 1973). The pattern obtained with the ammonium sulfate concentrate of human plasma [which was further processed through Sepharose 4B (Molnar et al., 1977)], was very similar to that shown in Figure 1, but trypsin binding activity, associated with α_2-macroglobulin in this case (Bourrillon & Razafimahaleo, 1972), was not even retarded by the column.

In search of affinity procedures immediately applicable to plasma or serum, the experiments with rabbit antibodies raised against either rat or human opsonin were both successful. When the antibody was coupled to Sepharose 4B as described under Materials and Methods, a 0.9 × 5 cm column had the capacity of absorbing all opsonin activity from 20 mL of plasma or serum, and this opsonin activity could be eluted quantitatively with a 0.1 M glycine hydrochloride buffer of pH 2.3. The findings, presented in Table II, were confirmed by Ouchterlony immunodiffusion (Figure 2).

In view of the reported immunologic cross-reactivity (Blumenstock et al., 1978) between "cold-insoluble globulin" (CIG) and opsonin, we have also tested the human CIG preparation which was obtained as a byproduct of the large-scale purification of fibrin-stabilizing factor (blood coagulation factor XIII), using the method described by Lorand & Gotoh [1970; also Curtis & Lorand (1976)]. CIG separates well from factor XIII in the DEAE-cellulose chromatographic step employed in that procedure and is eluted later in the salt gradient at a concentration of about 0.15 M

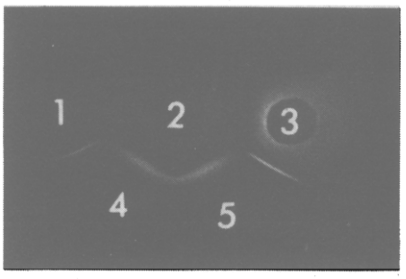

FIGURE 3: Immunological comparison by the Ouchterlony technique of opsonins prepared by various chromatographic procedures. (1) Material purified from human plasma on a Sepharose 4B–heparin column; (2) human cold-insoluble globulin obtained by chromatography on DEAE-cellulose; (3) protein isolated from rat serum by using an affinity column of Sepharose 4B containing rabbit IgG against rat opsonin; (4 and 5) rabbit immunoserum against rat opsonin. For details, see Materials and Methods.

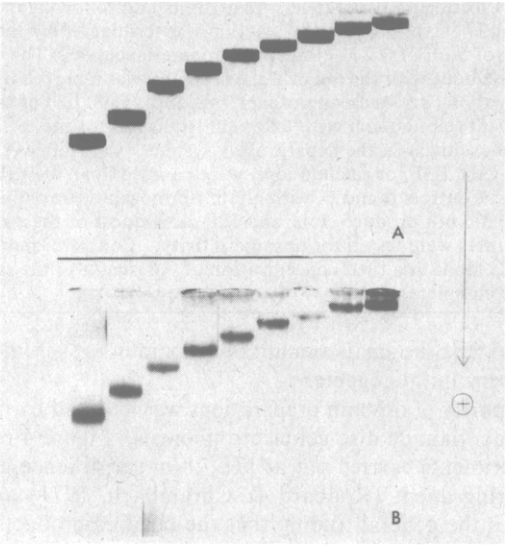

FIGURE 4: Comparison of human and rat opsonins (15-μg samples) by electrophoresis (Rodbard & Chrambach, 1971) at different concentrations of polyacrylamide. (A) CIG from human plasma; (B) rat serum opsonin isolated directly by means of affinity chromatography on an antibody-containing column. Gel concentrations, from left to right: 4.62, 5.01, 5.39, 5.78, 6.16, 6.55, 6.93, 7.32, and 7.70%.

NaCl. The collected CIG pool was mixed with Trasylol (final concentration 2% of CIG) and concentrated by precipitation with 40% of saturating ammonium sulfate.

All three methods, namely, DEAE-cellulose, heparin, or antibody affinity chromatography, produced opsonin preparations of similarly high specific activities (1900–2600 opsonizing units/mg of protein) regardless of whether human or rat plasma or serum was used as the starting material. As far as stability is concerned, the human CIG preparation obtained by salt gradient elution from DEAE-cellulose proved to be superior to the others, essentially retaining its opsonin activity for at least 6 months while stored at 4 °C. The other products deteriorated within a couple of months under similar conditions of storage.

By use of immunodiffusion to compare the preparations obtained by the three procedures, it was found that the human material gave immunological identity with rat opsonin. As illustrated in Figure 3, antirat opsonin antiserum raised in rabbits showed the same single precipitin line with the human as well as the rat preparations. All products gave a single arc in immunoelectrophoresis, corresponding to the position of α_2-globulins. No arc was observed when rabbit antiserum prepared against whole rat serum was used, suggesting that no major contamination by other serum proteins was present

Table III: Specific Removal of Opsonin Activity from Solution by the Fibrin Cross-Linking System[a]

	opsonic act. (% uptake)	
	expt 1	expt 2
clot liquor of complete mixture	3	4
clot liquor of control A, CIG omitted	0	3
clot liquor of control B, FSF omitted	10	18
clot liquor of control C, $CaCl_2$ omitted	10	18
control mixture D, fibrinogen omitted	11	18
control mixture E, thrombin omitted	10	18
control F, CIG alone	12	17

[a] The complete mixture (0.2 mL) comprised the following purified human proteins and salts at the final concentrations indicated: fibrinogen (3 mg/mL), cold-insoluble globulin (CIG; 0.45 mg/mL), fibrin-stabilizing factor (FSF; 14 μg/mL), and thrombin (2.5 units/mL) in 50 mM Tris buffer of pH 7.5, with 0.1 M NaCl and 2.2 mM $CaCl_2$. Clotting occurred in less than 1 min, and following incubation at 37 °C for 90 min, the reaction was terminated by the addition of 5 μL of 0.2 M ethylenediaminetetraacetate. The clot was pressed out with the aid of a glass rod and was removed by centrifugation (Beckman microfuge). Aliquots (100 μL) of the supernatant (clot liquor) were taken (in triplicate) for the testing of opsonic activity in the hepatic assay system. Controls A–C, without CIG, FSF, or calcium ions, were handled in an identical manner. Controls D and E, with either fibrinogen or thrombin omitted, did not produce clots, and 100-μL aliquots of these entire mixtures were tested for opsonic activity. Control F represents CIG alone at a final concentration of 0.45 mg/mL, the same as in the complete mixture.

and also that the small amount of opsonin in rat serum itself is not very immunogenic.

The purity of opsonin preparations was assessed by means of polyacrylamide disc gel electrophoresis. Figure 4 relates to experiments carried out at pH 7.8 in the absence of any denaturing agent (Rodbard & Chrambach, 1971) and illustrates the general finding that the purified products give protein profiles which, apart from some occasionally weakly staining bands, comprise only a single major component. Furthermore, under the conditions examined, the electrophoretic mobility of this component was the same regardless of whether the material was derived from plasma or serum, from either man or rat. The experiment also shows that the similarities of the electrophoretic mobilities for purified human and rat opsonins extend to all gel concentrations, indicating that these proteins are quite similar in regard to charge as well as Stokes' radii (Hedrick & Smith, 1968).

Regardless of the method of purification of the opsonic proteins, electrophoretic patterns in sodium dodecyl sulfate (Laemmli, 1970), with and without reduction from both human and rat sources, corresponded to those already given in the literature for human cold-insoluble globulin [see Mosesson (1978)]. In the absence of dithiothreitol, apart from a faint band of about 200 000 molecular weight, the 400 000 molecular weight species was the predominant one. Occasionally, another lesser band at much higher molecular weight could be seen, perhaps representing the γ-glutamyl-ε-lysine-bonded protein [see Mosher (1975)].

It is known that cold-insoluble globulin is a substrate for the fibrin-stabilizing enzyme, particularly when fibrin is also present in the medium (Mosher, 1976); in the latter case covalently cross-linked hybrid polymers of fibrin and CIG form. We utilized this property of CIG for examining the question of whether opsonic activity could also be removed from solution in a manner consistent with this behavior of CIG toward the cross-linking enzyme. The results presented in Table III prove that the residual opsonic activity remaining

Table IV: Interference by Soluble Proteins on the Opsonin (15 μg of Human CIG) Dependent Uptake of Gelatinized Latex Particles. Specific Inhibition of Hepatic Uptake by Free Gelatin

	mg of soluble protein added	% uptake
	none	12
gelatin	0.025	0
BSA	1	14.5
fibrinogen	1	16

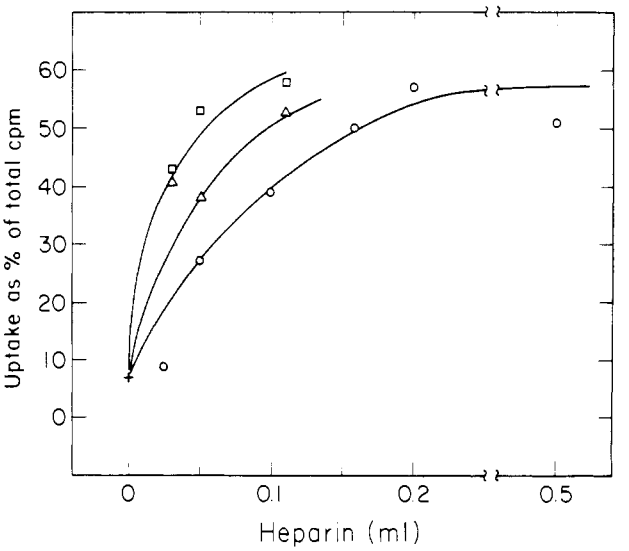

FIGURE 5: Effects of various heparin preparations on the CIG-promoted uptake of gelatinized latex particles by rat liver slices. Each reaction mixture contained 30 μg of human CIG, 40 μg of ^{125}I-labeled latex–gelatin (10 000 cpm), and a 100-mg (wet weight) slice of rat liver as well as the amount of heparin indicated on the abscissa. For experimental details, see Materials and Methods. (○) Upjohn heparin (130 μg/mL); (△) Kabi low-affinity heparin (50 μg/mL); (□) Kabi high-affinity heparin (50 μg/mL).

in a clot liquor is determined by the presence or absence of the functioning of the fibrin-stabilizing factor (FSF) in the clotting mixture. When the full system was operating, some 75% of the opsonic activity could be removed from solution. Two controls—omitting either FSF or Ca^{2+} ions—showed that fibrin formation alone, in the absence of cross-linking, would not reduce the opsonin activity of the clot liquor at all and thus strengthen the hypothesis that opsonic activity is, indeed, a characteristic property of the native CIG protein itself.

The availability of highly purified opsonin preparations made it possible to investigate the specificity of recognition of the protein coating of the latex particles by liver slices. Neither the coupling of serum albumin nor fibrinogen nor fibrin to latex generated particles suitable for phagocytosis. Inhibition experiments (Table IV), in which the phagocytosis of gelatinized particles was measured in the presence of extraneously added proteins, confirmed this specificity because only the presence of gelatin in the soluble phase could abolish hepatic uptake. Significant competition was also seen with collagen types I and IV. No inhibition of gelatin–latex uptake was observed by using human and rat sera from which the opsonins were removed by an affinity column (Sepharose 4B–antiopsonin).

As far as its anticoagulant effect is concerned, heparin can be subfractionated into high- and low-activity components (Lam et al., 1976; Höök et al., 1976; Teien et al., 1977; Rosenberg et al., 1978). We find that phagocytosis can be supported by either type of heparin (Figure 5), indicating that opsonization depends on a component of this mucopoly-

saccharide mixture different from that known to combine with the antithrombin molecule. The high-affinity preparation seemed to be slightly more effective than the low-activity one, but the difference between them may not be significant. The Upjohn preparation, however, appears to be less potent than either of the former two.

Discussion

In the framework of the present paper, the term phagocytosis is applied to describe the uptake of gelatinized particles by fresh liver slices and corresponds to the terminology of Blumenstock et al. (1978), who considered this type of uptake to be related to the functioning of the Kupffer cells themselves. In fact, the phagocytic system employed by us is quite similar to the one originally developed by Saba & DiLuzio (1965) except that, because of increased particle stability, we found it advantageous to replace the lipid test emulsion with latex particles to which the gelatin was attached in a covalent manner. The chemical coupling of gelatin to latex, as opposed to the simple coating of lipid with this protein, was also compatible with using ^{125}I for the labeling of the particles rather than the shorter lived ^{131}I isotope.

This system appears to be quite specific for gelatin (i.e., collagen), because no uptake could be accomplished when either fibrinogen, fibrin, or serum albumin was coupled to latex. Furthermore, only free gelatin (Table IV) or collagen in solution could inhibit the uptake of gelatinized particles.

In addition to an opsonizing protein, a constituent of commercial heparin preparations is essential for promoting the phagocytosis of gelatin-covered particles. However, as indicated by the results of Figure 5, the phagocytically important component of heparin is unrelated to its anticoagulant activity. Fractions of the parent mucopolysaccharide mixture stimulated phagocytosis regardless of whether their antithrombin activities were high or low.

Similarities in antigenic properties and amino acid composition suggested that the human plasma opsonic protein might be identical with the so-called cold-insoluble globulin (i.e., plasma fibronectin); thus far, however, no known CIG preparation could be shown to display opsonizing activity (Blumenstock et al., 1978). The findings in the present paper provide a rather firm support for the identity of these two proteins. CIG obtained as a byproduct of purifying fibrin-stabilizing factor (i.e., coagulation factor XIII) from plasma (Lorand & Gotoh, 1970; Mosher, 1975) showed the highest opsonizing activity. Since no organic solvents were employed, Blumenstock et al. (1978) may be correct in that the use of ethanol in purifying CIG (Mosesson et al., 1975) may have led to a loss of biological activity.

The results presented in Table III satisfy an even more stringent criterion for the identity of CIG with opsonin. Apart from fibrinogen and fibrin (Lorand et al., 1972), very few plasma proteins seem to be able to serve as substrates (Mosher, 1976) for the enzyme generated from the fibrin stabilizing factor zymogen by the action of thrombin and Ca^{2+} ions (Curtis & Lorand, 1976). CIG has the rather unique property of forming copolymers with fibrin under the influence of this enzyme (Mosher, 1976), involving perhaps the C-terminal, disulfide-rich domains of CIG and the α chains of fibrin (Iwanaga, 1978). It can be assumed that the hybrid CIG–fibrin polymer is held together by γ-glutamyl-ϵ-lysine interprotein cross-links, similar to those found in fibrin alone under such conditions [see Lorand (1972)]. We utilized this property of CIG to examine the question of whether opsonic activity itself could be removed from solution by the operation of the fibrin cross-linking system. As shown in Table III, soluble opsonic activity was indeed markedly reduced by the functioning of the complete system, but the mere act of fibrin clot formation did not lower the opsonic content of the clot liquor.

In addition to demonstrating that the CIG obtained as a byproduct of the existing method for purifying factor XIII (Lorand & Gotoh, 1970) by salt gradient elution from DEAE-cellulose was opsonically active, we have invested considerable effort in developing new affinity chromatographic procedures for the isolation of opsonin from both human and rat plasma and serum. The heparin–Sepharose column could be most successfully used in conjunction with concentrates previously obtained by precipitation with 0.35 saturation of ammonium sulfate and processed through Sepharose 4B (see Figure 1), whereas the antiopsonin antibody containing column could be employed for the direct isolation of opsonin in a single step of operation from either plasma or serum (Table II). An additional advantage of the Sepharose–heparin column was that it gave an excellent separation of rat α_1-macroglobulin and the α_2-opsonin which are difficult to separate by other means due to their close relationship in size and electrophoretic mobilities (Molnar et al., 1977). The three purification methods yielded materials of similar opsonic activities and of comparable purities.[2] Though we are still in the process of gathering information with regard to the relative stabilities of the various products, preliminary evaluation suggests that the DEAE-cellulose gradient chromatographic procedure provides the most stable preparation. The activities obtained by the affinity chromatographic methods seemed to decline within a few weeks of storage at 0 °C, but the CIG isolated on DEAE-cellulose retained considerable activity for several months, albeit the $NaDodSO_4$ electrophoretic profile indicated progressively more proteolytic degradation of the molecule.

It should be noted that the purified human and rat opsonic proteins cross-react immunologically (see Figure 3) and are quite similar in regard to charge as well as Stokes' radii. Furthermore, the amino acid compositions of the two proteins show great similarities (unpublished experiments). We have recently been able to demonstrate antigenic cross-reactivity and a comparable opsonic potency also for chicken opsonin (Molnar et al., 1979).

Finally, it should be mentioned that the uptake of gelatinized particles by the hepatic system seems to represent only the very first step in the complex process of phagocytosis, namely, recognition and binding. The radioactive particles which become bound to the cells can be released by a consecutive treatment with trypsin; thus, there could have been no internalization of the particles. However, our recent work with macrophage monolayers from rat peritoneal exudate shows (Molnar et al., 1979) that, in the very same experimental system comprising gelatinized latex, opsonin, and heparin, extensive internalization of the particles ensues. The reason as to why the liver slice system lost its ability to phagocytize the bound particles is not known.

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[2] Very recently we obtained such highly active opsonin also from a gelatin affinity chromatographic column, using arginine for elution (Vuento & Vaheri, 1978).

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