Precipitation of Soluble Fibrin Monomer Complexes by Lysosomal Protein Fraction of Polymorphonuclear Leukocytes* (33875)

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(Introduced by M. Glenn Koenig)

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Considerable evidence indicates that polymorphonuclear leukocytes (PMN) are involved in the pathogenesis of intravascular clotting in the generalized Shwartzman reaction (GSR). Thomas and Good first postulated that a hypothetical PMN factor may interact with altered fibrinogen to produce the essential coagulum (1). Support for this hypothesis was afforded by the recent demonstration that isolated PMN granules, given with a single endotoxin injection, produce lesions of the GSR (2). The nature of the clot-promoting principle(s) of PMN origin is unknown.

Although endotoxin does not apparently interact with fibrinogen *in vitro*, it induces significant changes in fibrinogen *in vivo*. After an endotoxin injection, soluble fibrinogen derivatives are demonstrable in the circulation, indicating that an alteration in fibrinogen has occurred. One such derivative, called cryofibrin, a complex of fibrin monomer and fibrinogen, is clottable by thrombin, cold-precipitable with heparin, and precipitable by Liquoid and similar large molecular weight acidic polymers (3–5).

A different soluble fibrinogen derivative, described by Lipinski *et al.* (6) and called soluble fibrin monomer complexes (SFMC), is thought to be composed of fibrin monomers in complex with fibrinogen or fibrin molecules which have been partially degraded by fibrinolytic enzymes. The SFMC can be generated *in vitro* when thrombin and plasmin act simultaneously on fibrinogen. These soluble complexes are not clottable by thrombin. The SFMC is present in the circulation in trace amounts under physiological conditions, and its level rises significantly after an injection of endotoxin (7). Protamine, a basic protein, precipitates fibrin monomers from SFMC preparations in a nonenzymatic reaction which has been called paracoagulation (6, 8). In addition to protamine sulfate, other factors including platelet factor IV (9), staphylococcal clumping factor (10), and several physicochemical agents (8) are also able to precipitate SFMC.

Since PMN granules are rich in basic proteins (11–13) and since it has been postulated that a constituent of PMN granules may interact with altered fibrinogen in the generalized Shwartzman reaction, it was of interest to determine if material derived from isolated PMN granules would interact with SFMC. The present report describes the precipitation of soluble fibrin monomer complexes by a protein fraction obtained from rabbit PMN granules.

Materials and Methods. The lysosomal protein fraction (LPF) was prepared from rabbit PMN granules which had been obtained from sterile peritoneal exudate leukocytes as previously described (2, 14). Granules were lysed by treatment with 0.1 *N* HCL, the insoluble residue was spun down at 8200 g for 10 min, and the clear supernatant was dialyzed overnight against 0.145 *M* NaCl buffered with 0.05 *M* Tris–maleate buffer at pH 6.8. The precipitate which formed during dialysis was discarded after centrifugation, and the clear supernatant (LPF) was used for the experiments. The LPF contained 1.4 mg of protein/ml, which represents about 70% of the protein content before dialysis.

Soluble fibrin monomer complexes (SF MC) were prepared as described previously (10).

The reaction between LPF and SFMC was

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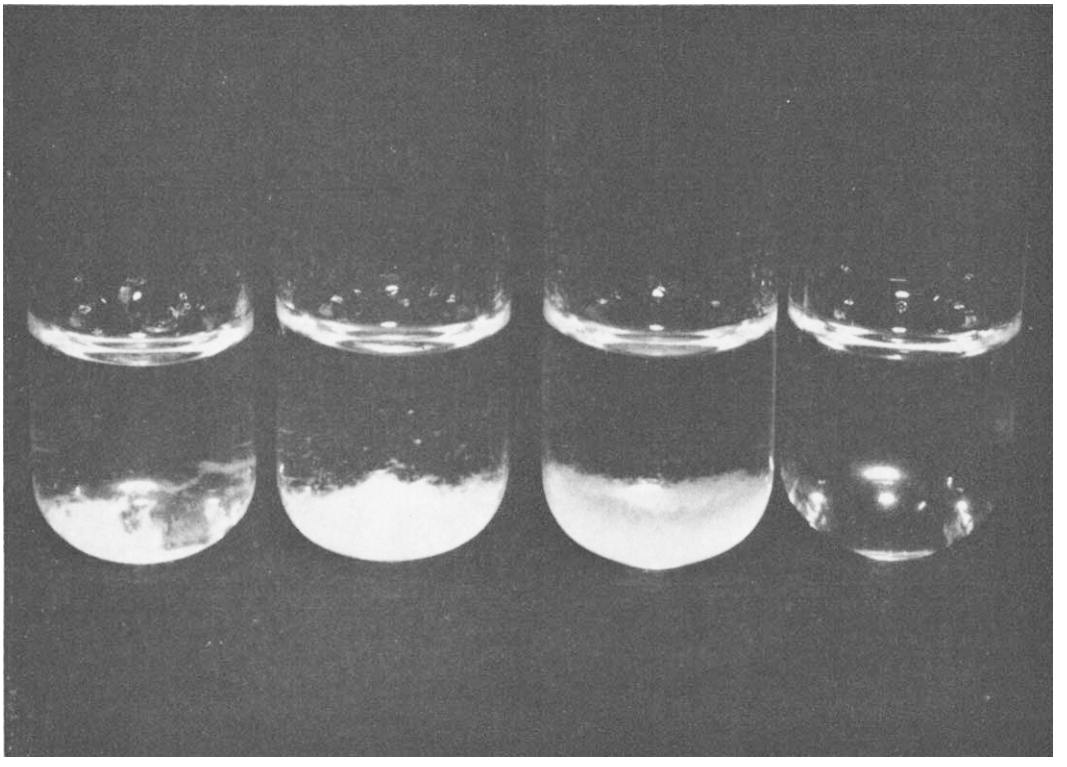

FIG. 1. Precipitation of soluble fibrin monomer complexes by lysosomal protein fraction, protamine sulfate and Liquoid. From left to right are tubes in which LPF, protamine sulfate, Liquoid and thrombin have been incubated with SFMC. Note the precipitate in first three tubes, and absence of precipitate in the sample with thrombin.

performed as follows: 0.1 ml of LPF was added to 0.9 ml of SFMC. The mixture was incubated 20 min at room temperature. The resulting precipitate was spun down, washed twice with Tris–NaCl buffer (pH 7.4), and redissolved in 1 ml of 40% urea solution in 0.2 *N* NaOH. Protein concentration was determined by absorbance at 280 mμ and calculated from a standard curve prepared from crystalline lysozyme (Armour). Plasma heparin-thrombin time was tested according to Saba *et al.* (15).

Fibrinogen was obtained commercially (Warner-Chilcott) or prepared from human plasma according to the method of Kekwick *et al.* (16). Plasmin was obtained as Lysofibrin from Novo Laboratories, Copenhagen, Denmark. Thrombin (bovine origin) was obtained from Parke, Davis and Co. Soybean trypsin inhibitor and protamine sulfate, grade I, were purchased from Sigma Chemical Co. Trypsin, 2× crystallized, was supplied by Worthington Biochemical Corporation. Sodium polyanethol sulfonate (Liquoid) was obtained from Hoffmann-LaRoche. Heparin sodium USP was purchased from Connaught Medical Research Laboratories, Toronto, Canada.

Results. Addition of lysosomal protein fraction (LPF) from PMN leukocytes to soluble fibrin monomer complexes (SFMC) caused the formation of a precipitate. The reaction was similar to that induced by 0.1% protamine sulfate. Polyanethol sulfonate (Liquoid) also produced a precipitate. Figure 1 illustrates tubes containing SFMC to which the LPF, protamine sulfate, Liquoid, or thrombin were added. Only thrombin did not cause precipitation. The paracoagulating activity of LPF preparations was about 70%

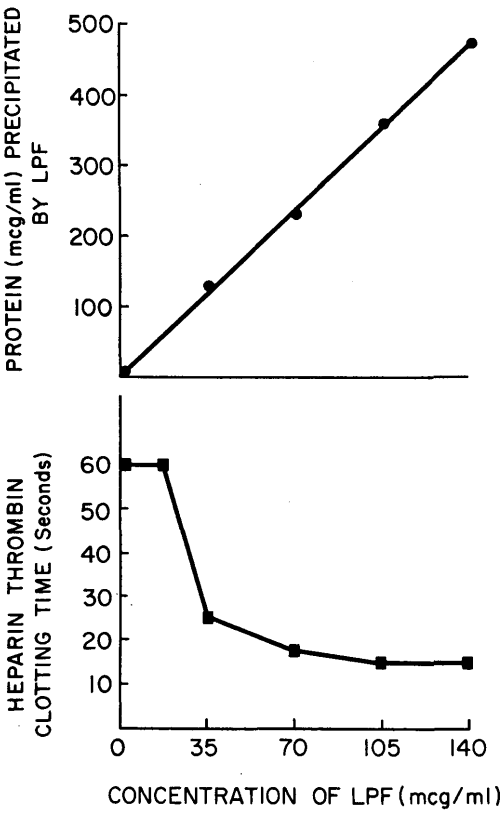

FIG. 2a (upper). Precipitation of SFMC by different concentrations of LPF; Fig. 2b (lower), the effect of different concentrations of LPF on plasma heparin-thrombin time.

that of protamine sulfate solutions when they were compared on the basis of protein concentration.

The reaction between LPF and SFMC was proportional to the concentration of lysosomal protein (Fig. 2a). Since the amount of protein in the precipitate was much greater than that in the added LPF, it is clear that the precipitate was derived in large part, and possibly entirely, from SFMC.

The LPF exhibited antiheparin activity comparable to that found in a lysosomal cationic protein by Saba *et al.* (15). The effect of LPF on plasma heparin-thrombin time is illustrated in Fig. 2b. In this determination, 70 μg of LPF inhibited approximately 1 unit of heparin.

The LPF retained its precipitating activity after heating over a wide temperature range. Samples of lysosomal fractions were heated for 30 min at different temperatures, then centrifuged and tested with SFMC. Precipitating activity was stable up to 80°. However, heating at 90° reduced that activity to 82%, and the fibrin precipitating activity of LPF was completely destroyed at 121°.

The effect of proteolysis on the precipitating activity of LPF was tested by incubation of this fraction with trypsin (0.2 mg/ml) or with plasmin (0.2 mg/ml) for 1 hr at 37°. Proteolytic digestion was stopped by addition of soybean trypsin inhibitor (0.1 mg/ml) and precipitating activity was determined (Table I). The results indicate that the activity of LPF was completely destroyed by trypsin and partially abolished by plasmin. Soybean trypsin inhibitor protected LPF against the proteolytic action of both trypsin and plasmin.

Heparin in a final concentration of 100 U/ml did not inhibit precipitation of soluble complexes. However, precipitation was prevented by urea in a final concentration of 1 *M*. After absorption of the lysosomal protein fraction on a cationic ion exchanger (SE Sephadex) at pH 4.6, the precipitating activity was removed from the solution. In contrast, an anionic ion exchanger (DEAE Sephadex) equilibrated at pH 8.3 did not absorb the fraction responsible for precipitating activity.

Discussion. The present data indicate that a lysosomal protein fraction (LPF) obtained from rabbit PMN leukocyte granules can interact with soluble fibrin monomer complexes

TABLE I. Effect of Proteolysis on the SFMC Precipitating Activity of a Lysosomal Protein Fraction (LPF).

Components added to SFMC	Precipitated protein (μg/ml)	Percentage of initial activity[a]
LPF + trypsin	0	0
+ plasmin	181	55
+ STI[b] + *trypsin*	330	100
+ STI + plasmin	330	100
+ STI	330	100

[a] Expressed as percentage of protein precipitated by LPF pre-incubated with buffer.

[b] Soybean trypsin inhibitor.

(SFMC) to form a precipitate. Because this property of LPF is inactivated by proteolytic enzymes, it is clear that its activity depends on the presence of protein. For the following reasons it seems unlikely that precipitation of soluble fibrin monomer complexes by the lysosomal protein fraction is enzymatic. First, soluble fibrin monomer complexes, the substrate for this reaction, were not clottable by thrombin or other proteolytic enzymes. Secondly, precipitation of SFMC by lysosomal protein fraction resembles that caused by protamine sulfate, a protein devoid of any enzymatic activity. Finally, under conditions used in this study, the direct relation of the amount of precipitate to the concentration of LPF is not typical for an enzymatic reaction. In addition, the lysosomal fraction involved in this phenomenon is relatively thermostable, also suggesting it acts in a nonenzymatic fashion.

The mechanism of interaction of the LPF and SFMC remains uncertain, as does the mechanism of paracoagulation produced by protamine sulfate. The reaction may depend on a change of the isoelectric point of the fibrin monomers, causing their subsequent precipitation, since both protamine sulfate and lysosomal proteins have a basic charge (13). However, it seems likely that the basic charge of the compounds causing paracoagulation is not the only characteristic responsible for this reaction, since Liquoid, a strongly acidic polymer able to induce the generalized Shwartzman reaction (17), also produces paracoagulation. Thus, paracoagulation may occur when a change in the isoelectric point of the substrate is engendered by either a strongly acidic or basic substance. Hydrogen bonds may be involved in the precipitate formation, since urea inhibits LPF-induced paracoagulation.

It is noteworthy that some analogy exists between the PMN lysosomal protein fraction and platelet factor IV. Like platelet factor IV (9), LPF is a potent paracoagulating agent. Both factors exhibit antiheparin activity as tested by the heparin-thrombin time (9, 15), but heparin does not inhibit the paracoagulating activity of platelet factor IV (9) or the lysosomal protein fraction.

The present demonstration that PMN lysosomal protein is capable of eliciting a paracoagulation reaction with SFMC is of particular interest in relation to the hypothesis that the PMN granule is the source of a clot-promoting factor operative in the GSR (1, 2). It appears that, *in vivo*, endotoxin leads to increased production of fibrinogen derivatives which are particularly susceptible to precipitation by highly charged polymeric molecules, since cryoprofibrin is precipitable by acidic polymers such as heparin and Liquoid, and since SFMC is precipitable by protamine, by lysosomal basic protein fraction, and by Liquoid. It is remarkable that granules of heterophil leukocytes contain both an acidic polymer (11) resembling chondroitin sulfate and a group of strongly basic proteins with a variety of activities (11–13). It was recently shown by electron microscopy that following endotoxin injection, there is extensive intravascular fragmentation of PMN's in the lung capillaries, presumably affording a mechanism for release of granule constituents to the circulation (18). Thus it seems that the PMN granule is a rich source of material capable of interaction with fibrinogen derivates to produce an insoluble coagulum. It remains to be demonstrated whether such lysosomal constituents actually interact with altered fibrinogen *in vivo* in the development of the generalized Shwartzman reaction and in other pathological states characterized by intravascular fibrin deposition.

Summary. A lysosomal protein fraction (LPF) isolated from rabbit PMN leukocytes is able to interact with soluble fibrin monomer complexes to form a precipitate. The reaction seems to be nonenzymatic and similar to that induced by polycationic protamine sulfate and polyanionic polyanethol sulfonate (Liquoid). The fibrin precipitating activity of LPF is thermostable and destroyed by proteolysis. The reaction between LPF and soluble fibrin monomer complexes is inhibited by 1 *M* urea. Heparin in a concentration of 100 U/ml has no effect on precipitate formation. The fibrin precipitating activity of LPF suggests that this material may be important in producing intravascular fibrin

deposition in those situations in which there is destruction of PMN leukocytes and a concurrent rise of soluble fibrin monomer complexes in the circulation.

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Comparison of Bacteriolysis, Passive Hemolysis, and Bacterial Adherence Colony Formation for Detecting Antibdy-Forming Cells* (33876)

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Considerable information is available concerning the nature of cells involved in antibody formation to antigens such as foreign erythrocytes, serum proteins and chemical haptens (1, 2). However, less consideration has been given to individual antibody forming cells to lipopolysaccharide antigens derived from bacteria. In this regard, most studies concerning immunity to microorganisms have dealt with serum antibody titers. From such studies it has been concluded that antibody to bacterial somatic antigens are mainly IgM, rather than IgG globulins (3–9). Even multiple injections of somatic antigens from gram-negative bacteria generally stimulate formation of IgM antibody. In contrast, serum proteins, haptens, and similar antigens appear to elicit mainly IgG antibody, often after a more or less transient macroglobulin response (1, 2).

Studies concerning cells involved in formation of antibody to various antigens have been facilitated in recent years by development of procedures which permit rapid detection of specific antibody plaque forming cells in semisolid medium such as soft agar or

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