

# An Alternative Pathway for Fibrinolysis

## I. THE CLEAVAGE OF FIBRINOGEN BY LEUKOCYTE PROTEASES AT PHYSIOLOGIC pH

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**ABSTRACT** An alternative fibrinolytic system, active at physiological pH, is present in peripheral blood leukocytes. The fibrinolytic proteases localized predominantly in the leukocyte granules are capable of degrading both fibrinogen and fibrin, and plasmin activity does not contribute significantly to this proteolytic event. The specificity of the alternative fibrinolytic proteases for fibrinogen and the characteristics of the derivative cleavage fragments are clearly distinguishable from the classical plasmin system. The high molecular weight derivatives of fibrinogen, generated by the alternative system, under physiological conditions, are larger than the plasmin-generated X fragment, exhibit immuno-electrophoretic mobility comparable to native fibrinogen, and are not coagulable by thrombin. Analysis of the constituent polypeptide chains of the fragments reveals cleavage of the A $\alpha$ , B $\beta$ , and  $\gamma$  chains of fibrinogen. The lower molecular weight derivatives of fibrinogen, generated by the alternative system, are structurally distinct from previously described fibrinogen degradation products and exhibit potent anticoagulant activity. This anticoagulant activity can be attributed to interference with normal fibrin polymerization. The proteases of the alternative fibrinolytic systems are actively secreted by leukocytes when stimulated to undergo a nonlytic release reaction. These results provide direct evidence for a fibrinolytic system resident in leukocyte granules that is associated with the leukocyte release reaction and is capable of generating unique fibrinogen cleavage fragments.

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## INTRODUCTION

Leukocytes are observed in intimate association with fibrin deposits in two fundamental biological processes. Within the confines of the vascular compartment, leukocytes frequently accumulate in complex thrombi consisting of fibrin and platelets (1-5); whereas in extravascular sites, the migration of leukocytes to sites of injury where fibrin has accumulated is a central feature of the inflammatory response (5-9). In both circumstances, the interaction of these cells with fibrin may be active rather than passive, based on the morphologic identification of fibrin within leukocytes (5-9), the accumulation of leukocytes in thrombi relative to their concentration in blood (4), and the demonstration that fibrinolytic activity of leukocytes is not confined to low pH, at which many cathepsins are active, but also is observed at neutral pH (10-16).

Although elements of the plasma fibrinolytic system,<sup>1</sup> plasminogen and its proactivator, may be constituents of leukocytes (14, 17), a number of studies have demonstrated within the cells a fibrinolytic system distinct from plasmin. This conclusion is based on the differential sensitivity of the systems to inhibitors, as well as comparisons of the molecular characteristics of the enzymes (11-14). The purpose of the present study is to examine the alternative fibrinolytic system of leukocytes with respect to: (a) the relative contribution of the plasminogen and the plasminogen-independent system to the total fibrinolytic activity of the leukocytes; (b) the molecular characteristics of cleavage of fibrinogen by leukocyte proteases; (c) the biologic properties of the fibrinogen cleavage fragments generated by leukocyte protease; and (d) the release of protease from leukocytes when subjected to appropriate stimulus.

<sup>1</sup>Fibrinolysis is defined in the generalized sense as the capacity to degrade either fibrinogen or fibrin substrates.

## METHODS

**Leukocyte isolation and subcellular fractionation.** Leukocytes were isolated from citrated whole blood according to previously described procedures (13), with dextran (250,000 mol wt) separation and brief hypotonic lysis to remove residual red cells. After three to five washes of the leukocytes in phosphate-buffered saline, pH 7.3 (PBS),<sup>3</sup> the leukocytes appeared morphologically intact by light microscopy and consisted of more than 80% granulocytes.<sup>3</sup> More than 90% of the leukocytes in the starting blood sample were recovered. The washed cells were suspended at a concentration of approximately  $10^8$  cells/ml of PBS and lysed by repeated freezing and thawing (at least six cycles). The supernate obtained after centrifugation at 3,000 *g* for 20 min constituted the crude fibrinolytic leukocyte proteases (LP<sub>r</sub>) utilized in most experiments.

Subcellular components of the leukocytes were separated according to the method of Janoff and Scherer (18), utilizing 0.34 M sucrose for cell lysis, followed by differential centrifugation to obtain granular and cytoplasmic fractions. Such preparations were found to be free of intact leukocytes by microscopic examination.

**Assay of fibrinogen degradation.** Degradation of fibrinogen was assessed either by the release from radioiodinated fibrinogen of [<sup>125</sup>I]peptides soluble in trichloroacetic acid (TCA), or by prolongation of the thrombin time of fibrinogen after incubation with leukocyte extracts. In the former system, 10  $\mu$ l of [<sup>125</sup>I]fibrinogen (1  $\mu$ Ci/ $\mu$ g) in 0.2 ml of carrier fibrinogen at 2.5 mg/ml was added to 0.2 ml of the LP<sub>r</sub> preparation. The mixture was incubated at 37°C and at selected time intervals 0.4 ml of 40% TCA was added. After centrifugation, 0.2 ml of the supernate was counted and compared to control samples incubated under the same conditions but containing PBS instead of LP<sub>r</sub>. Thrombin time was measured in a system containing 0.1 ml of human fibrinogen at 2.5 mg/ml in PBS and 0.1 ml of a suitable dilution of the LP<sub>r</sub> in PBS. After incubation at 37°C for appropriate periods of time, 0.1 ml of the mixture was withdrawn, and 0.1 ml of bovine thrombin at 10 U/ml was added. The pH of this mixture remained constant at pH 7.3 throughout the entire incubation period. The thrombin time of controls consisting of purified fibrinogen and thrombin ranged from 14 to 18 s. Samples were considered uncoagulable if they did not clot within 180 s after addition of thrombin. To assess the presence of thrombin inhibitors, LP<sub>r</sub> preparations were preincubated with thrombin for as long as 1 h and then added to fibrinogen substrates. The thrombin time was identical in all assays to that of the control lacking LP<sub>r</sub>, thus excluding artifactual thrombin inhibition.

**Immunoelectrophoresis.** Immunoelectrophoretic analyses were performed on 7.5  $\times$  2.5-cm slides coated with 1% Noble Agar (Difco Laboratories, Detroit, Mich.) in 25 mM barbital buffer, pH 8.8. A volume of 10  $\mu$ l of digested fibrinogen samples at 1.25 mg/ml was electrophoresed at 6 V/cm for 75 min. A goat antiserum to human fibrinogen (100  $\mu$ l) was utilized to develop precipitin arcs at 20°C.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis was performed on 5  $\times$  75-mm gels in the

presence of sodium dodecyl sulfate (SDS) as described by Weber and Osborn (19). Under nonreducing conditions, 50  $\mu$ g fibrinogen or fibrinogen digests were applied to polymerized 5% acrylamide gels. For samples reduced with 2-mercaptoethanol, 100  $\mu$ g samples were applied to 7.5% polyacrylamide gels. The gels were subjected to electrophoresis at 8 mA/gel and stained with Coomassie brilliant blue. Molecular weights of constituent chains of the various digests were estimated in the reduced system utilizing serum albumin (68,000), the heavy (50,000) and light chains (23,500) of IgG, ovalbumin (43,000), pepsin (35,000), trypsin (23,300), and myoglobin (17,200) as standards.

**Release of leukocyte proteases.** The release of LP<sub>r</sub> from leukocytes was assessed with zymosan preincubated with fresh plasma (20, 21). Zymosan particles (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) were refluxed in PBS for 1 h and washed by centrifugation with additional PBS. The zymosan was activated by incubation of 10 mg of the particles with 1 ml of fresh citrated plasma for 30 min at 37°C. After centrifugation and three to five washes with PBS, 1 ml of isolated leukocytes at  $1 \times 10^8$  cells/ml of PBS was added to the zymosan pellet. The mixture was incubated at 37°C for 60 min with agitation and centrifuged at 800 *g* for 10 min, and the fibrinolytic activity of the supernatant fraction was tested.

**Inhibition of fibrin polymerization.** Interference with fibrin polymerization by LP<sub>r</sub> digests of fibrinogen was assessed as described by Latallo, Fletcher, Alkjaersig, and Sherry (22) but 2.0 M KI was employed for clot dissolution (23) rather than KBr. After solubilization, monomers were maintained in solution in 0.3 M KI, 0.01 M Tris, pH 8.0. Assay of polymerization was initiated by the addition of 0.2 ml of LP<sub>r</sub> digests at 0.2 mg/ml in 0.05 M NaPO<sub>4</sub>, pH 6.0, and 0.2 ml of monomer at 0.2 mg/ml. The turbidimetric reaction was monitored spectrophotometrically at 660 nm and at 22°C.

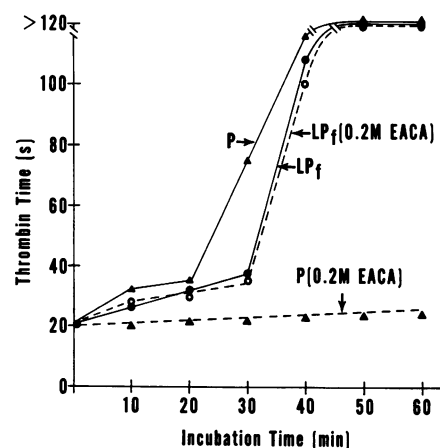


FIGURE 1 The fibrinolytic activity of leukocyte lysate in the presence or absence of EACA, as demonstrated by the prolongation of the thrombin time of fibrinogen. An eightfold dilution of LP<sub>r</sub> from  $1 \times 10^8$  cells was incubated with fibrinogen (1.25 mg/ml) in the presence (○---○) and absence (●—●) of 0.2 M EACA. At the indicated times, aliquots of the mixtures were withdrawn and the thrombin times determined. Plasmin (P) at 5  $\mu$ g/ml (activated with streptokinase) in the presence (△---△) and absence (▲—▲) of EACA is indicated as a control.

<sup>3</sup> Abbreviations used in this paper: EACA,  $\epsilon$ -aminocaproic acid; LP<sub>r</sub>, leukocyte protease(s) with fibrinolytic activity; PBS, phosphate-buffered saline, pH 7.3; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

<sup>3</sup> Lymphocytes, which constituted the remaining 15–20% of the cells in such preparations, contain no detectable fibrinolytic activity (Plow, E. F., and T. S. Edgington. Unpublished observations).

TABLE I  
The Fibrinolytic Activity of Leukocytes Isolated from Five Individual Donors

Donor	Leukocyte concentration  10 <sup>8</sup> /ml	LP <sub>f</sub> Activity				
		Fibrinolytic titer*			TCA-solubility†	
		-EACA	+EACA§	Titer/10 <sup>8</sup> cells	-EACA	+EACA
					%	
1	1.2	8	8	6.7	10.0	9.8
2	3.5	16	16	4.6	12.2	12.4
3	4.0	2	2	0.5	6.0	5.8
4	2.6	8	8	3.1	9.0	10.2
5	3.0	4	4	1.3	8.0	8.0

\* The fibrinolytic titer is the highest dilution of leukocyte lysate rendering fibrinogen uncoagulable by thrombin after a 1-h incubation at 37°C.

† TCA-solubility is expressed as the percent of [<sup>125</sup>I]fibrinogen not precipitated by TCA after a 30-min incubation with each LP<sub>f</sub> preparation.

§ A final concentration of 0.2 M EACA in the assay system.

|| Fibrinolytic titer divided by leukocyte concentration.

**Proteins.** The human fibrinogen utilized in this study was prepared by differential ethanol fractionation followed by ammonium sulfate precipitation (24, 25). Such preparations have been routinely found to be more than 95% coagulable by thrombin and to be devoid of other proteins by immunoelectrophoresis, polyacrylamide gel electrophoresis in SDS, and protein electrophoresis in cellulose acetate. When

required for specific experiments, the fibrinogen was rendered plasminogen-free by incubation with lysine attached to Sepharose 2B beads (26). Plasminogen was prepared according to the method of Deutsch and Mertz (27). Bovine thrombin was purchased from Parke, Davis & Company (Detroit, Mich.) or from Sigma Chemical Co. (St. Louis, Mo.).

## RESULTS

The fibrinolytic capacity of leukocyte lysates, LP<sub>f</sub>, is clearly demonstrated by prolongation of the thrombin time of fibrinogen, as exemplified in Fig. 1. LP<sub>f</sub> produced a progressive prolongation of the thrombin time of fibrinogen, and with the particular dilution of LP<sub>f</sub> utilized, the sample was uncoagulable after a 50-min incubation. The relative contribution of the plasminogen system to this fibrinolytic capacity of leukocytes was assessed by measuring the activity of LP<sub>f</sub> in the presence or absence of 0.2 M ε-aminocaproic acid (EACA), an inhibitor of plasminogen activation and of plasmin. As shown in Fig. 1, the prolonged thrombin time produced by LP<sub>f</sub> was not demonstrably altered by the presence of EACA. In contrast, the activity of purified plasmin was completely inhibited by this concentration of EACA. These results suggest that active plasmin did not contribute to the fibrinolytic activity of the leukocyte lysate.

The fibrinolytic activity of LP<sub>f</sub> is also demonstrable as an increase in the solubility of radioiodinated fibrinogen in TCA. As shown in Fig. 2, in the presence of LP<sub>f</sub> there was a progressive decrease in the precipitability of the radioactivity associated with fibrinogen during the incubation period, indicative of the cleavage and release of small peptide fragments from fibrinogen. After a 50-min incubation, approximately 20% of the radioactivity was nonprecipitable in the acid. This pat-

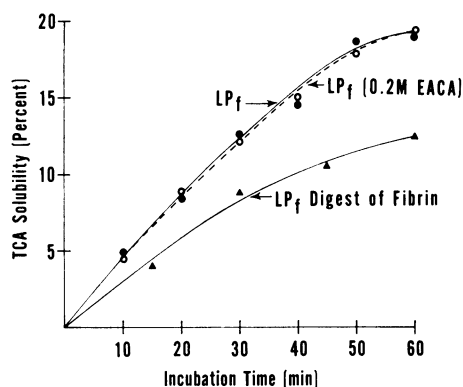


FIGURE 2 The fibrinolytic activity of leukocyte lysate in the presence or absence of EACA, as demonstrated by the increased solubility of fibrinogen and fibrin in TCA. The assay system consisted of 0.2 ml fibrinogen at 2.5 mg/ml, 0.01 ml [<sup>125</sup>I]fibrinogen, and 0.2 ml of a 1/8 dilution of LP<sub>f</sub> from 1 × 10<sup>8</sup> cells either in the presence (○---○) or absence (●—●) of 0.2 M EACA. At the indicated times, 0.4 ml of 40% TCA was added, and 0.2 ml of the supernate was counted after centrifugation. The TCA-precipitability of fibrin (▲—▲) was tested in an identical system, except that the sample was clotted with 10 U of thrombin before the addition of LP<sub>f</sub>. The clots were rimmed and removed by centrifugation, and the TCA-precipitability of the supernate was determined. Results are corrected for the TCA-precipitability of the radioactivity in the absence of LP<sub>f</sub>, which remained constant at 92% during the incubation.

tern was also unaltered in the presence of 0.2 M EACA, substantiating that plasmin did not significantly contribute to the fibrinolytic activity of the leukocyte lysate. Also shown in Fig. 2 is the progressive increase in the TCA-solubility of fibrin when incubated with LP<sub>f</sub>. Thus, in the general sense, LP<sub>f</sub> are fibrinolytic in that they are capable of cleaving both fibrinogen and fibrin substrates.

The presence of LP<sub>f</sub> activity in leukocytes prepared from five individual donors is considered in Table I. All preparations of leukocytes contained LP<sub>f</sub> activity, as detected by either progressive prolongation of the thrombin time or by a decrease in TCA-precipitability of fibrinogen. In both detection systems, LP<sub>f</sub> activity in each preparation was not measurably altered by the presence of 0.2 M EACA, further indicating the lack of participation of the plasmin system. Based on the LP<sub>f</sub> activity per 10<sup>6</sup> leukocytes, considerable variation in the LP<sub>f</sub> activity of the five preparations was observed. Such differences could arise from variable levels of the proteases within the cells or from variable levels of inhibitors of these enzymes in the leukocytes.

When leukocytes were fractionated into granular and cytoplasmic fractions, the fibrinolytic activity of LP<sub>f</sub> was predominantly associated with the granular fraction (Table II). In all preparations, the activity of the granular fractions was significantly greater than the activity of the total lysate prepared from the same number of cells. This observation suggests that an inhibitor of LP<sub>f</sub> may be present in the leukocytes, and that this putative inhibitor is segregated from the LP<sub>f</sub> during subcellular fractionation.

TABLE II

*Subcellular Distribution of Fibrinolytic Leukocyte Protease and its Putative Inhibitor*

Preparation*	LP <sub>f</sub> activity	
	Fibrinolytic titer‡	TCA-solubility§
(a) Whole leukocytes	32-64	1.02-13.5
(b) Leukocyte granules	256-512	18.0-22.5
(c) Leukocyte cytoplasm	0-8	0-5.2
(d) Granules + PBS	256	22.5
(e) Granules + cytoplasm	64	14.2

\* In experiments a, b, and c, 1.0-1.2 × 10<sup>6</sup> leukocytes were prepared from three individual donors. One half of each preparation, suspended in 2.0 ml of PBS and lysed by repeated freezing-thawing, constituted the whole leukocyte preparation; granules and cytoplasmic fractions were prepared from the other half of each preparation. Granular fractions, obtained after ultracentrifugation, were also suspended in 2.0 ml PBS and the activity was released by freezing and thawing. The cytoplasmic fractions were concentrated to a 2.0-ml volume. In experiments d and e, one granular preparation was diluted with an equal volume of either PBS or a cytoplasmic fraction containing no LP<sub>f</sub> activity.

‡ Reciprocal of the highest dilution of each preparation rendering fibrinogen uncoagulable by thrombin after a 1-h incubation at 37°C.

§ Percent of [<sup>125</sup>I]fibrinogen not precipitable by 20% TCA after a 20-min incubation with each preparation at 37°C.

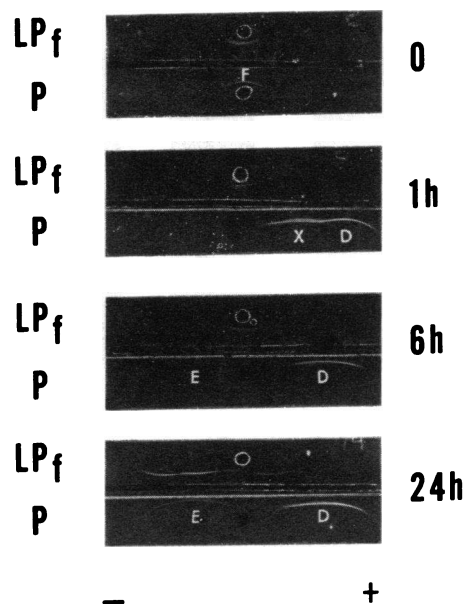


FIGURE 3 Immunoelectrophoresis in agar gels of fibrinogen cleaved by leukocyte proteases and by plasmin. Fibrinogen at 2.5 mg/ml was incubated with LP<sub>f</sub> or plasmin (P). At the indicated time, 10  $\mu$ l of the digests were removed and electrophoresed at 6 V/cm for 75 min. Precipitin arcs were developed with 100  $\mu$ l of a goat antiserum to fibrinogen. The letters superimposed on the immunoelectrophoretic patterns refer to fibrinogen (F) or to its identifiable plasmin cleavage products.

Further evidence for the existence of such an inhibitor was sought by adding either a cytoplasmic fraction containing no LP<sub>f</sub> activity or buffer to an active granular preparation. As shown in Table II, the cytoplasmic fraction significantly diminished the LP<sub>f</sub> activity of the granular preparation. While this observation may indicate the presence of specific inhibitors of the proteases within the leukocyte cytoplasm, it must alternatively be considered that the cytoplasmic fraction could contain competitive substrates.

### The cleavage of fibrinogen by leukocyte proteases

With some assurance that the LP<sub>f</sub> activity at physiologic pH is distinct from that of plasmin, the cleavage of fibrinogen substrate by LP<sub>f</sub> and by plasmin was compared. The activities of LP<sub>f</sub> and plasmin were adjusted so that the fibrinogen sample was coagulable at 30 min but would not clot at 1 h. Samples were withdrawn at a number of time intervals and compared by several analytical techniques. The results that follow are selected but characteristic. Identical cleavage patterns have been observed with six different LP<sub>f</sub> preparations.

**Immunoelectrophoretic analysis.** Immunoelectrophoresis in agar gels revealed significant differences in the fragmentation of fibrinogen by LP<sub>f</sub> as contrasted to

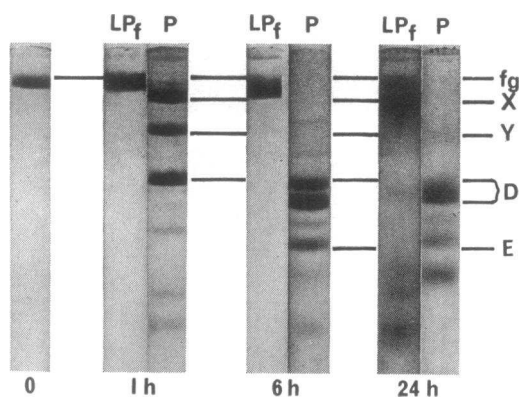


FIGURE 4 SDS polyacrylamide gel electrophoresis of fibrinogen cleaved by plasmin (P) and  $LP_1$ . Fibrinogen or fibrinogen digests (50  $\mu$ g protein), withdrawn at the indicated times, were electrophoresed on 5% gels at 8 mA/gel. Gels were stained with Coomassie brilliant blue. Identifiable plasmin cleavage fragments of fibrinogen are indicated.

plasmin (Fig. 3). Analysis immediately upon addition of either enzyme gave a pattern characteristic of native fibrinogen. After 1 h of incubation, the pattern of  $LP_1$  digests was not distinguishable from that of native fibrinogen, yet such samples were not coagulable by thrombin. In contrast, the pattern of plasmin digests at 1 h incubation was significantly altered, and the precipitin arcs characteristic of X and D fragments were recognized (28). At 6 h,  $LP_1$  digests still exhibited a fibrinogen-like arc and an additional faint secondary arc was observed. The plasmin digests of this time had reached the terminal stage of cleavage, as indicated by the presence of arcs characteristic only of the D and E fragments (28, 29). At 24 h,  $LP_1$  digests gave a complex pattern which remained distinct from that of plasmin

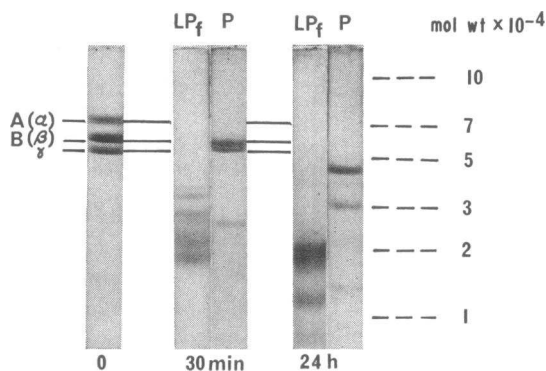


FIGURE 5 SDS polyacrylamide gel electrophoresis of reduced digests of fibrinogen produced by plasmin (P) and  $LP_1$ . Samples were withdrawn from the digests at the indicated times and reduced with 1% 2-mercaptoethanol in SDS for 1 h at 37°C. Samples (100  $\mu$ g protein) were applied to 7.5% gels and electrophoresed at 8 mA/gel. Molecular weights were established from the relative mobility of standard proteins.

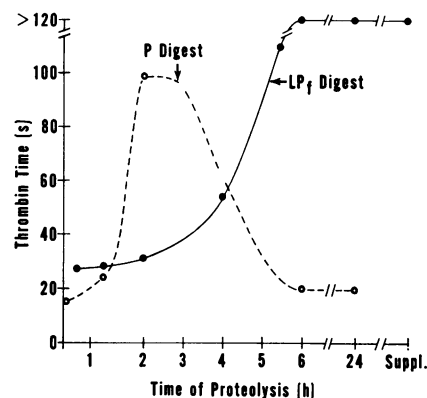


FIGURE 6 Anticoagulant properties of leukocyte protease and plasmin digests of fibrinogen. At the indicated times 100  $\mu$ l of a  $LP_1$  digest ( $\bullet$ — $\bullet$ ) or a plasmin digest ( $\circ$ — $\circ$ ) of fibrinogen (1.25 mg fibrinogen/ml) were withdrawn and inhibited with either 20 U of Trasylol ( $LP_1$  digest) or 0.2 M EACA (P digest). An equal volume of undigested fibrinogen at 2.5 mg/ml was added to each digest and the thrombin times of the mixtures were determined.

digests. When 24-h  $LP_1$  digests were supplemented with additional proteases and again incubated, this pattern was not altered.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis in SDS under nonreducing conditions revealed significant differences in the cleavage of fibrinogen by  $LP_1$  as contrasted to plasmin (Fig. 4). Preparations of native fibrinogen produced only a single protein band. At 1 h, the  $LP_1$  digest contained a large fragment, only slightly smaller than fibrinogen, as indicated by the slightly greater mobility than fibrinogen; the broadness of this band suggested the presence of a heterogeneous population of fragments. In the plasmin digest, bands characteristic of the X, Y, and D fragments were recognized (30). The fragment(s) in the 1-h  $LP_1$  digest appeared larger than the X fragment, suggesting a mol wt of over 270,000, but unlike the X fragment not coagulable by thrombin. After 6 h of enzymatic cleavage, only D and E fragments were identified in the plasmin digest; however, the  $LP_1$  digest contained a large fragment similar in size to the X fragment but somewhat smaller than the 1-h  $LP_1$ -generated fragments. At 24 h, a large fragment exhibiting considerable size heterogeneity but generally similar in molecular weight to the X fragment persisted in the  $LP_1$  digest. Additionally, a number of smaller fragments were detected at this time. One of these lower molecular weight fragments was similar in size to plasmin-generated D fragment (approximately 80,000) while the other fragments appeared smaller than plasmin-generated E fragment (less than 50,000).

Digests reduced with 2-mercaptoethanol and subjected to analytical acrylamide gel electrophoresis on 7.5% gels

in 1% SDS are shown in Fig. 5. Fibrinogen gave three bands characteristic of the constituent  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. After 30 min of plasmin cleavage, partially degraded  $\alpha$  and  $\beta$  chains were observed, but the  $\gamma$  chain was not recognizably altered. It is well established that the gamma chain remains insensitive to plasmin cleavage until late stages of digestion, and in the 24-h digests the  $\gamma$ -chain derivative was only slightly smaller than the original  $\gamma$  chain of fibrinogen (30–33). In contrast, in the 30-min  $LP_r$  digest, which is still coagulable, all three chains, including the  $\gamma$  chains, were cleaved. At least six discrete polypeptide bands were recognized, with mol wt ranging from approximately 36,000 to 18,000. In the 24-h digest, all constituent chains were of mol wt less than 21,000.

### Anticoagulant properties of fibrinogen digests

The anticoagulant properties of the fragments generated by  $LP_r$  and by plasmin are compared in Fig. 6.  $LP_r$  and plasmin digests of fibrinogen were withdrawn after varying periods of incubation, and the capacity of the digests to prolong the thrombin time of intact fibrinogen was assessed. In plasmin digests, it has been reported that maximum anticoagulant activity is associated with the Y fragment (34, 35). In the particular plasmin digest utilized, the anticoagulant activity present in the 2–4-h samples corresponded to the appearance and disappearance of the Y fragment (see Fig. 4); whereas digests containing only the terminal fragments D and E (6 h) exhibited little anticoagulant activity.  $LP_r$  digests also exhibited anticoagulant activity, but its time

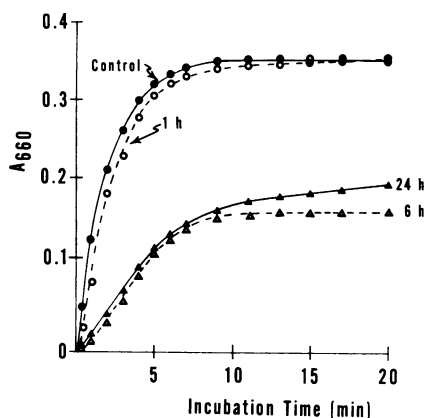


FIGURE 7 Inhibition of fibrin polymerization by  $LP_r$  digests of fibrinogen. The reaction was initiated by the addition of 0.2 ml of the  $LP_r$  digests at 0.2 mg/ml in 0.05 M  $NaPO_4$ , pH 6.0, to fibrin monomers at 0.2 mg/ml. The reaction was monitored at 660 nm at 22°C. The  $LP_r$  digests, identical to those shown in Fig. 6, are 1-h digest (○---○), 6-h digest (△---△), 24-h digest (▲—▲), and a control (●—●), monomers diluted with buffer alone.

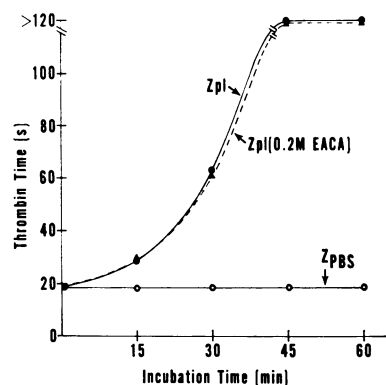


FIGURE 8 Release of fibrinolytic proteases from leukocytes. Leukocytes at  $1 \times 10^8$  cell/ml were incubated for 1 h at 37°C with zymosan particles which had been pretreated with either fresh plasma ( $Z_{p1}$ , ●—●) or PBS ( $Z_{pBS}$ , ○—○). After centrifugation at 800  $g$  for 10 min, the supernates were incubated with an equal volume of fibrinogen at 2.5 mg/ml. At various times portions of the mixtures were withdrawn and the thrombin time was determined. EACA at a 0.2 M concentration did not affect the prolongation pattern produced by the supernate of cell stimulated with activated zymosan [ $Z_{p1}$ -(EACA ▲---▲)].

course and the extent of anticoagulant activity differed markedly from those of the plasmin digest. Anticoagulant activity of the  $LP_r$  digests was recognized in the 4-h samples. This activity increased in the 6-h digest, persisted in the 24-h digest, and was not diminished by supplementation of the digest with additional  $LP_r$ . Incubation of the  $LP_r$  digests with thrombin before the addition of fibrinogen did not reduce thrombin activity. Also, when the thrombin concentration was increased from 1 to 10 U, producing a thrombin time of the control fibrinogen sample of less than 3 s, the mixture of the digests and fibrinogen was still uncoagulable. These observations suggest that the anticoagulant properties of the  $LP_r$  digests may arise predominantly from interference with the polymerization of fibrin monomers rather than with interference with thrombin or the release of fibrinopeptides.

The interference of  $LP_r$  digests with fibrin monomer polymerization is directly demonstrated in Fig. 7. The polymerization reaction was observed spectrophotometrically in the presence or absence of selected  $LP_r$  digests generated in Fig. 6. Addition of the 6-h or 24-h digests to fibrin monomers significantly altered the polymerization reaction, affecting the initiation, the rate, and the final extent of polymerization. Such effects are consistent with a complexing of  $LP_r$ -generated fragments with fibrin monomers that in turn produced defective and incomplete polymerization. Addition of the 1-h  $LP_r$  digest, which exhibited minimal anticoagulant activity, to the fibrin monomers produced only minimal effects on polymerization. The consistency of these re-

TABLE III

*Nonlytic Release of Fibrinolytic Proteases from Leukocytes\**

Stimulus	Fraction	LP <sub>t</sub> activity‡	LDH activity§
Zymosan + plasma	Supernate	2	28
	Cells	2	2,318
Zymosan + PBS	Supernate	0	106
	Cells	4	2,256

\* Washed zymosan particles pretreated with plasma or PBS were suspended in 1.0 ml PBS containing 10<sup>8</sup> leukocytes. After a 1-h incubation at 37°C, the mixtures were centrifuged at 800 g for 10 min. The supernates were removed and tested directly for LP<sub>t</sub> activity. The pellets, containing the cells and zymosan, were resuspended in 1.0 ml PBS and subjected to six freeze-thaw cycles. The LP<sub>t</sub> activity released from the cells following freezing and thawing constituted the activity of the cells.

‡ Reciprocal of highest dilution rendering fibrinogen uncoagulable after a 1-h incubation at 37°C.

§ Lactic dehydrogenase was measured at pH 8.55 and 37°C by kinetic analysis at 340 nm of the rate of NAD reduction. Results are expressed in IU (38).

sults with those observed in Fig. 6 suggests that the anticoagulant activity of LP<sub>t</sub> digests arise primarily if not entirely from inhibition of the polymerization phase of the fibrin transition.

#### Nonlytic release of fibrinolytic proteases from leukocytes

A number of studies have demonstrated that leukocytes actively secrete or release enzymes from granules when subjected to appropriate stimuli through a nonlytic mechanism (20, 21, 36, 37). To evaluate specifically the release of LP<sub>t</sub>, zymosan particles activated by incubation with fresh plasma were utilized to stimulate leukocytes to undergo the release reaction (20, 21, 36, 37). After incubation of the activated zymosan with 1 × 10<sup>8</sup> cells for 1 h, the mixture was centrifuged, and the supernatant medium was assayed for fibrinolytic activity. The supernatant medium from cells incubated with activated zymosan produced a characteristic prolongation of the thrombin time, and after 45 min the fibrinogen was uncoagulable (Fig. 8). This activity was not inhibited by EACA, ruling out a contribution from plasmin. The supernate from control cells that had been incubated with nonactivated zymosan exhibited no fibrinolytic activity. The LP<sub>t</sub> activity of the supernate from stimulated cells represented approximately 50% of the fibrinolytic activity obtained by freezing and thawing the same number of cells (Table III). Only 2% of the total lactic dehydrogenase activity, a cytoplasmic and nonreleasable enzyme (36, 37), appeared in the supernatant medium from cells stimulated by activated zymosan, indicating that the cells had not been nonspecifically lysed by the stimulus. A similar percentage (4.2% of the lactic dehydrogenase activity) was detected in the supernatant medium from the control cells incubated with nonactivated zymosan.

## DISCUSSION

Leukocytes are capable of proteolytic degradation of both fibrinogen and fibrin by an intrinsic enzymatic system distinct from plasmin. The failure of EACA to alter the fibrinolytic activity of LP<sub>t</sub> indicates that active plasmin was not present in the leukocyte lysates. This observation is consistent with the results of other studies (11–13) in which plasmin activity and the antigenic determinants of plasmin were not demonstrable in leukocytes. In contrast, Prokopowicz not only found evidence for the presence of the plasminogen system in leukocytes but also isolated plasminogen from leukocytes (14, 17). Our observations do not resolve these apparent inconsistencies; but in preliminary observations, utilizing sensitive radioimmunochemical approaches (25, 29), we have detected very low levels of plasmin-related antigens within several leukocyte preparations (nanogram levels per 10<sup>8</sup> cells). If the material in the leukocytes antigenically related to plasmin was entirely present as the inactive zymogen, plasminogen, or if an inhibitor of plasmin intrinsic to the leukocytes completely masked plasmin activity, plasmin would still not significantly contribute to the fibrinolytic activity of the cells.

The manner in which LP<sub>t</sub> cleaves fibrinogen is clearly distinct from plasmin. The high molecular weight fragments generated by LP<sub>t</sub> in early digests (up to 6 h) exhibit electrophoretic patterns similar to fibrinogen but are of lower molecular weight than the parent molecule. These fragments exhibit lower mobilities in SDS polyacrylamide gels under nonreducing conditions than the X fragment generated by plasmin, suggesting an apparent molecular weight of greater than 270,000 (28). The observation that these high molecular weight fragments are not clotted by thrombin, in contrast to the X fragment, suggests that cleavage by LP<sub>t</sub> is not confined to the COOH-terminal aspects of the A $\alpha$  and B $\beta$  chains of fibrinogen, sites of early plasmin cleavage (30, 32). This interpretation is supported by SDS polyacrylamide gel electrophoretic analyses after reduction of the LP<sub>t</sub> digests, which demonstrate early cleavage of all three primary constituent chains of fibrinogen.

The capacity of the LP<sub>t</sub> to cleave all three chains rapidly with retention of a high molecular weight species suggests restricted specificities of the proteases for the fibrinogen substrate and preservation of molecular structure by disulfide bonds. This restricted specificity suggests the presence of only a limited number of fibrinolytic enzymes in the leukocyte, as well as a limited number of specific peptide bonds susceptible to LP<sub>t</sub>. Ohlsson observed that the fibrinolytic activity of the leukocyte lysates was confined to a single, rather homogeneous peak on molecular exclusion chromatog-

raphy (13). More recent studies have suggested the presence of at least two fibrinolytic leukocyte enzymes, but differences in specificity between the two have not been established (12, 39). The ability of the leukocyte proteases to cleave the gamma chain of fibrinogen rapidly distinguishes this system not only from plasmin but also from trypsin, thrombin, and a number of bacterial proteases (40, 41). Because of this unusual specificity, the fibrinolytic leukocyte proteases may provide a unique probe for structural studies of the fibrinogen molecule.

Although the subject of relatively limited study, it is attractive to consider that the alternative fibrinolytic system may be of physiological significance (5, 9). Activation of the coagulation system leads to release of fibrinopeptides A and B and the formation of fibrin. Leukocytes are subject to chemotaxis by fibrinopeptide B (4) and, once localized, they may be induced to release LP $\epsilon$  by various mechanisms, including phagocytic or nonphagocytic reactions (36). Release may also be evoked by immunologic effector systems including antigen:antibody complexes and complement activation (36). The fibrinolytic and anticoagulant properties arising from this system suggest a significant role in physiological homeostasis of the coagulation system. Further investigation will be required to provide a broader and sounder perspective as to the significance of this system.

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