Anti-Heparin Activity of Lysosomal Cationic Proteins from Polymorphonuclear Leukocytes

By Hussain I. Saba, Harold R. Roberts and John C. Herion

CATIONIC PROTEINS are important constituents of polymorphonuclear (PMN) leukocyte lysosomes. These proteins are rich in arginine and lysine. This accounts for their strong electro-positivity.1-3 These proteins are without apparent enzyme activity; nevertheless they possess a variety of biological activities.1,4,6 We have recently described an anticoagulant activity of these proteins that is probably related to their electro-positive charge.7 The lysosomal cationic proteins (LCP) appear to interfere with the role of negatively charged phospholipid micelles, or possibly platelet membranes, in the reactions involving Factor V (proaccelerin), Factor X (Stuart factor) and calcium.7 Thus, they inhibit clotting by a mechanism different from that of heparin.8,9

In view of previous reports that crude extracts of leukocytes have an anti-heparin effect,10,11 and the known anti-heparin action of various other cationic substances like protamine,12,13 synthetic polylysine14,15 and polybrene,16 this study was undertaken to determine if PMN lysosomal cationic proteins also possess anti-heparin activity.

Our results show that LCP from polymorphonuclear leukocytes do inhibit heparin but lose their own anticoagulant effect in the process. Further, an apparent inhibition of antithrombin III by LCP is most likely the result of a clot-promoting effect of LCP on fibrinogen or the reactions leading to fibrin formation.

MATERIALS AND METHODS

Polymorphonuclear leukocytes (PMN). Rabbits were given, intraperitoneally, 150 ml. of 0.25 percent glycogen in saline containing 68,000 units of penicillin and 94 mg. of streptomycin per liter; the sterile, granulocyte-rich exudates were harvested 12-14 hours later and pooled in ice-chilled, sterile flasks. The leukocytes were isolated from the exudates by centrifugation, and were washed three times with cold sterile isotonic saline.7

PMN leukocyte lysosomes and lysosomal cationic proteins (LCP). The washed granulocytes, suspended in 0.34 M sucrose, were ground gently (Duell tissue grinder K8948, From the Departments of Medicine and Pathology, University of North Carolina School of Medicine, Chapel Hill, North Carolina.

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Kontes, Vinland, N. J.) and the lysosomes isolated by differential centrifugation. The isolated lysosomes were washed with 0.34 M sucrose, thrice extracted in the cold with 0.2 N H₂SO₄ and the insoluble material removed by centrifugation. The LCP were precipitated from the acid extract of lysosomes by adding cold absolute ethanol to a final concentration of 20 percent (v/v). The LCP were then dissolved in 0.01 N HCl, dialyzed overnight against 0.01 N HCl and then against distilled water for 6-8 hours. This product was then either lyophilized and dissolved in barbital buffer just before use, or, when used immediately, dialyzed directly against the barbital buffer rather than distilled water. When prepared in this manner, LCP contained no detectable amounts of the known lysosomal enzymes.

Lysosomal enzymes. Acid phosphatase, alkaline phosphatase, RNA-ase, DNA-ase, β-glucuronidase (all obtained from Sigma Chemical Co., St. Louis, Mo.) and lysozyme (Nutritional Biochemical Corp., Cleveland, Ohio) were dissolved in barbital buffer and tested for anti-heparin activity.

Heparin, obtained commercially (liquaemin sodium, Organon Inc., West Orange, N. J.), was diluted to appropriate concentrations in 0.15 M saline just before use.

Human citrated plasma was prepared by collecting venous blood from normal human donors in 3.2 percent sodium citrate (1 part to 8 parts of blood) followed by centrifugation at 2,000 g for 30 minutes at 4 C. This plasma was pooled and either used immediately or stored at −20 C. and thawed just before use.

Thrombin, obtained commercially (bovine, topical, Parke Davis Co., Detroit, Mich.) was diluted to desired concentrations with 0.15 M saline in silicone coated tubes and used immediately.

Fibrinogen (bovine, Behringerwerke, Lloyd Brothers, Inc., Cincinnati, Ohio) was dissolved in barbital buffer, pH 7.35, to a concentration of 250 mg. percent. Antithrombin-poor canine fibrinogen was prepared according to the method of Wagner et al.

Cephalin was a commercial preparation (Thrombofax, Ortho Diagnostics, Raritan, N. J.).

Inosithin (Associated Concentrates Corp., Long Island, N. Y.), 0.05 percent in 0.15 M saline, was the phospholipid used in some of the thromboplastin generation tests.

Antithrombin was prepared by heating fresh, normal plasma at 56 C. for 3 minutes followed by centrifugation in the cold at 2,000 g for 10 minutes. The defibrinated supernate, containing the antithrombin activity, was either used immediately or frozen in small aliquots at −20 C. and thawed at 37 C. just before use. In this study, antithrombin preparations containing no heparin are designated Antithrombin III; the same antithrombin preparations containing heparin (0.2 ml. of heparin solution, containing 0.5 U/ml. to 0.4 ml. defibrinated plasma) is designated Antithrombin II.

TAAME (p-Toluene Sulfonyl-L-Arginine Methyl Ester hydrochloride, A grade, Calbiochem., Los Angeles, Calif.) was dissolved in distilled water and used as substrate for assaying thrombin by the method of Sherry and Troll.

Barbital buffer (pH 7.35, ionic strength 0.154) was prepared by Owren's method.

Methods. The following procedures were performed at least in duplicate with appropriate controls: partial thromboplastin time (PTT); thrombin-plasma clotting time; and thromboplastin generation tests (TGT). Antithrombin III was assayed by the method of Hensen and Loeliger; Antithrombin II was assayed by the same method.

RESULTS

The interaction of heparin and LCP in the partial thromboplastin time (PTT) system. Both heparin and LCP, added separately to the clotting mixture in sufficient amounts, prolong the PTT (Fig. 1, a and b). In Fig. 1a, however, it can be seen that the anticoagulant effect of 130 µg. LCP is virtually completely abolished by 0.25 units of heparin: a ratio of 0.52 mg. LCP per unit of heparin. In the reverse procedure, the anticoagulant effect of 0.1 unit of heparin is completely neutralized by approximately 16 µg. LCP: a ratio of 0.16 mg. LCP per unit of heparin (Fig. 1b). When the same batch of LCP
Fig. 1.—Interaction of heparin and LCP in a modified PTT system. Each PTT mixture contained: 0.2 ml. plasma; 0.1 ml. saline or heparin; 0.1 ml. buffer or LCP; 0.1 ml. 0.04 M CaCl₂.

A. Each clotting mixture contained 130 μg. LCP and varying amounts of heparin as shown on the abscissa.

B. Each clotting mixture contained 0.1 unit of heparin and varying amounts of LCP as shown on the abscissa.

was used in similar experiments, the LCP-heparin neutralization ratios were approximately the same.

It is evident from both Fig. 1a and b, that an excess of either LCP or heparin, in a mixture of the two, is reflected by a prolongation of the PTT.
Fig. 2.—Interaction of heparin and LCP in a thrombin-plasma clotting time system. The clotting mixture contained 0.3 ml. titration mixture (calcium-acacia-imidazole-saline), 0.1 ml. thrombin (2.5 units/ml.), 0.1 ml. plasma.

A. Each clotting mixture contained 53 μg. LCP and varying amounts of heparin as shown on the abscissa.

B. Each clotting mixture contained 0.1 unit of heparin and varying amounts of LCP as shown on the abscissa.

The interaction of heparin and LCP in the thrombin-plasma clotting time system. Heparin, alone, prolongs the thrombin-plasma clotting time. Lysosomal cationic proteins do not (Fig. 2, a and b). When 53 μg. LCP are added to the plasma, between 0.4–0.5 unit of heparin is required to significantly prolong the thrombin clotting time (Fig. 2a). In the absence of LCP, much smaller quantities of heparin exert a much greater anticoagulant effect. In the reverse pro-
The interaction of heparin and LCP in the thromboplastin generation test (TGT). Both LCP and heparin, when added singly to a thromboplastin generation mixture at zero time, inhibit the generation of intrinsic thromboplastin activity (intrinsic prothrombin activator) (Fig. 3, curves d and e). When added together, and in appropriate concentrations, however, the anticoagulant effect of both agents is completely abolished (Fig. 3, curve a). In this and all similar experiments, the LCP-heparin neutralization ratio was about 0.4 mg. LCP per unit of heparin.

Fig. 4 shows the effects of LCP, heparin, and mixtures of the two, when
Fig. 4.—Interaction of heparin and LCP in the TGT system after generation of maximum thromboplastin activity. Incubation mixtures contained 0.3 ml. of each of the following at zero time: Al(OH)₃ adsorbed plasma (diluted 1:5 with saline); aged serum (diluted 1:10 with saline); 0.05% Inosithin; 0.025 M CaCl₂. After 8 minutes incubation, 0.3 ml. of either buffer alone or buffer containing 390 µg. LCP, and 0.3 ml. saline alone or saline containing varying amounts of heparin were added to the various incubation mixtures. Beginning 1 minute later and at intervals of 2 minutes, 0.1 ml. aliquots of the incubation mixtures were added to 0.2 ml. of substrate plasma and the mixture clotted by adding 0.1 ml. 0.025 M CaCl₂. The units of heparin added to the incubation mixtures are indicated on the graph.

added to a TGT incubation mixture 8 minutes after recalcification (when maximal intrinsic thromboplastin activity is present). The addition of either LCP or heparin alone results in progressive lengthening of the clotting times (Fig. 4, c, d, and e). When added together, in a ratio of 2.6 mg. LCP per unit of heparin, the anticoagulant effect of both agents is abolished (curve a). In all experiments involving the addition of LCP and heparin to the TGT after 8 minutes incubation, the LCP-heparin neutralization ratio was about six times that observed when these two agents were added to the TGT at zero time. The higher requirement for LCP in the 8 minute TGT was not related to either the different preparations of LCP or the order of addition of LCP and heparin to the TGT incubation mixture.

Effect of LCP on antithrombin II activity. Antithrombin alone (antithrombin III) progressively inactivates thrombin, and the fibrinogen clotting effect of the latter decreases proportionately (Fig. 5, curve 1). The addition of heparin to antithrombin (antithrombin II) greatly accelerates the rate of inactivation of thrombin especially in the early stages of incubation (curve 2). LCP, in appropriate concentrations, completely inhibits the antithrombin co-factor activity of heparin, particularly in the early phases of the incubation period.
Fig. 5.—Effect of LCP on Antithrombin II.

Curve 1. The incubation mixture contained: 0.4 ml. defibrinated plasma (source of Antithrombin III); 1.2 ml. buffer; and 0.4 ml. thrombin (50 U/ml.)

Curve 2. The incubation mixture contained: 0.4 ml. defibrinated plasma; 1.0 ml. buffer 0.2 ml. heparin (0.5 U/ml.) and 0.4 ml. thrombin (50 U/ml.). Heparin + Antithrombin III is referred to as Antithrombin II.

Curve 3. The incubation mixture is identical to that used to obtain curve 2 except that the buffer contained 0.7 mg. LCP.

Curve 4. The incubation mixture contained: 0.4 ml. thrombin (50 U/ml.) and 1.6 ml. buffer.

At intervals of 5 minutes, 0.2 ml. samples from the incubation mixtures described above were added to 0.3 ml. of 300 mg% fibrinogen and the clotting time recorded.

(curve 3). It can also be seen that the clotting times in the presence of LCP are even shorter than those observed with antithrombin III alone.

In control experiments, LCP appeared to protect the clot-promoting effect of thrombin from the action of antithrombin. Therefore, the following experiments were performed to explore this effect of LCP.

Effects of LCP on antithrombin III activity. The effects of antithrombin on the clotting and esterase activities of thrombin are shown in Fig. 6a, curve 1, and 6b, bar graph 1. It can be seen that both the clotting and esterase activities of thrombin are decreased, a reflection of thrombin inactivation.
Fig. 6.—Effect of LCP on Antithrombin III.

A. Antithrombin III Assay. Curve 1. The incubation mixture contained 0.4 ml. defibrinated plasma, 1.2 ml. buffer and 0.4 ml. thrombin (50 U/ml.).

Curves 2, 3a, 4. The buffer added to the incubation mixtures contained the amount of LCP shown on the graph.

Curve 3b. Lysosomal cationic proteins (120 μg) were added to the fibrinogen substrate rather than to the incubation mixture. The final concentration of LCP in the fibrinogen substrate was, therefore, the same as in curves 3a and 3b. At intervals, 0.2 ml. samples from the various incubation mixtures described above were added to 0.3 ml. fibrinogen (300 mg%) and the clotting time recorded.

B. Residual thrombin measured by TAME assay. Bar graphs 1, 3a, and 5 were obtained with incubation mixtures identical to those used to obtain curves 1, 3a and 5 (Fig. 6a). Bar graph 6 is a control incubation mixture containing thrombin plus 1.8 mg. LCP. After 25 minutes incubation, 0.2 ml. aliquots of the various incubation mixtures were added to the TAME solution and assayed by the method of Sherry and Troll.19

The addition of LCP to a thrombin-antithrombin incubation mixture, however, apparently protects the clot-promoting effect of thrombin (Fig. 6a, curves 2, 3a, 4). The protective effect of LCP is concentration dependent; 1.8 mg. LCP almost completely masks antithrombin activity (curve 4), whereas lower concentrations are less effective (curves 2, 3a).

To further define the clot-promoting effect of LCP in the anti-thrombin-thrombin system, the following experiments were performed. First, experiments were carried on to determine whether the clot-promoting effect of LCP was due to inhibition of antithrombin. Therefore, LCP were added to the fibrinogen substrate rather than the thrombin-antithrombin incubation mixture. The results are illustrated by curve 3b, Fig. 6a. It is evident that the incubation of thrombin and antithrombin for 25 minutes should have resulted in thrombin inactivation, and longer clotting times similar to those shown in curve 1 should have been obtained. However, it can be seen that the clotting times in this
Table 1.—Clotting of Antithrombin-Poor Fibrinogen by Small Amounts of Thrombin*

<table>
<thead>
<tr>
<th>Thrombin Units†</th>
<th>LCP µg†</th>
<th>Clotting Time Seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0</td>
<td>315</td>
</tr>
<tr>
<td>0.005</td>
<td>40</td>
<td>165</td>
</tr>
<tr>
<td>0.03</td>
<td>0</td>
<td>147</td>
</tr>
<tr>
<td>0.03</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

*The clotting mixture contained 0.1 ml thrombin solution, 0.1 ml buffer or LCP in buffer, 0.3 ml of calcium-imidazole-saline-acacia mixture and 0.1 ml antithrombin-poor, canine fibrinogen (300 mg%).

†Values represent amount in the final clotting mixture.

The experiment are much shorter (curve 3b). Since LCP were not present in the thrombin-antithrombin incubation mixture, it seems unlikely that the clot-promoting effect of LCP was due to antithrombin inhibition. Further evidence suggesting that antithrombin is not inhibited by LCP was obtained by assaying residual thrombin esterase activity in thrombin-antithrombin incubation mixtures with and without LCP. The results are shown in Fig. 6b, bar graphs 1, 3a). It is evident that thrombin esterase activity is decreased by antithrombin both in the presence of LCP (bar graph 3a) and in the absence of LCP (bar graph 1). Since thrombin esterase activity is related to thrombin clotting activity, these experiments suggest that the clot-promoting effect of LCP is not due to inhibition of antithrombin III.

To determine whether LCP exerted their clot-promoting effect by potentiating the action of thrombin, the esterase activity of the latter was measured in the presence of LCP. The results show that thrombin esterase activity is no greater in the presence of LCP (bar graph 6) than in the absence of these proteins (bar graph 5). Thus, it seems reasonable to conclude that the clot-promoting effect of LCP is not due to a primary action on thrombin.

The effects of LCP on the clotting of antithrombin-poor fibrinogen by small amounts of thrombin are depicted in Table 1. It may be seen that as little as 40 µg. LCP markedly enhance fibrinogen clotting by as little as 0.03 or 0.005 unit of thrombin.

DISCUSSION

We have previously reported data that LCP inhibit clotting by interfering with the role of phospholipid, or possibly platelet membranes, in reactions involving Factor X, Factor V, and calcium. Heparin, on the other hand, has antithrombin activity and is also thought to interfere with endogenous thromboplastin by inhibiting Factor XII (Hageman factor), Factor IX (PTC), and Factor V (proaccelerin). The data reported here clearly demonstrate that LCP neutralize the anticoagulant properties of heparin. Conversely, heparin also neutralizes inhibitory effects of LCP on the blood clotting mechanism. Presumably the mutual neutralization of LCP and heparin occurs when the strongly basic groups of LCP react with the strongly acidic groups of heparin. In this respect LCP
resemble other highly cationic substances which interact with the poly-
anion, heparin.13,14,25,26

We have not attempted to define precisely the LCP-heparin neutralization ratio since the ratio varies depending upon the potency of different preparations of LCP. In most instances, however, between 0.16 and 0.5 mg. of LCP are required to neutralize 1 unit of heparin. On the other hand, significantly larger amounts of LCP are required to neutralize heparin when the two are added to TGT's after generation of maximal intrinsic thromboplastin activity. The explanation for the higher requirement for LCP in this test is unknown. Since LCP are thought to react with negatively charged phospholipid micelles during the generation of intrinsic thromboplastic activity, it is possible that there is a less amount of LCP to react with heparin in this circumstance. Other explanations are, of course, possible.

Zeya and Spitznagel have shown that the antibacterial activity of LCP are also neutralized by heparin but they did not define the neutralization ratio.3 The neutralization of heparin by LCP cannot be compared with the neutralization of heparin by other cationic substances. The protamine-heparin neutralization ratio in vitro has been reported to vary between 8:113 1:1.26 In these studies, however, heparin was measured not in units, but in milligrams.

Various fractions of granulocytes have not been systematically tested by us for anti-heparin activity. Some investigators, however, have reported that the insoluble sediment remaining after saline extraction of leukocytes can neutralize heparin.27 Anti-heparin activity has also been found in crude extracts of leukocytes.10,11,27 Our studies suggest that at least part of the anti-heparin activity of leukocytes may be due to lysosomal cationic proteins.

The effects of LCP in thrombin-antithrombin systems are of particular interest. The experiments suggest that the clot-promoting effect of LCP in these systems is not due to inhibition of antithrombin or potentiation of thrombin activity. Rather, the data suggest that LCP act primarily on fibrinogen, or the reactions leading to fibrin formation. In this respect, LCP resemble protamine which has been reported to exert a clot-promoting effect in reactions involving fibrin polymerization.28

The anticoagulant effect of LCP, described previously by us, resembles the anticoagulant activity described in some cases of disseminated lupus erythematosus.29 Perry has reported that blood obtained from a number of patients with leukemia, who had bleeding unrelated to thrombocytopenia, failed to generate thromboplastin normally.30 Since we have found that LCP from both normal and leukemic human granulocyte lysosomes, like those from rabbit granulocyte lysosomes, possess anticoagulant activity in vitro, it is possible that LCP may account for some of the unexplained bleeding seen in patients with abnormalities involving the granulocytes. Whether heparin would be of benefit in such hypothetical cases is a question that must be answered by further careful clinical investigation.

**Summary**

The lysosomal cationic proteins (LCP) of rabbit polymorphonuclear leukocytes have previously been shown to inhibit the formation of intrinsic
ANTI-HEPARIN ACTIVITY OF LYSOSOMAL CATIONIC PROTEINS

prothrombin activator. That they also have anti-heparin activity is now demonstrated in a thrombin-plasma clotting system, the partial thromboplastin time test and the thromboplastin generation test.

In thrombin clotting systems that contain antithrombin, LCP exhibit a clot-promoting effect. This activity does not appear to represent inhibition of antithrombin II or III or potentiation of the enzymatic action of thrombin. Rather it may be a direct effect on fibrinogen, or the reactions leading to fibrin formation.

SUMMARIO IN INTERLINGUA

Esseva previemente observate que le proteinas cationic lysosomal (PCL) de leucocytos polymorphonucleari de conilio inhibi le formation de intrinsec activator de prothrombina. Que illos etiam ha activitate anti heparina ha nunc essite demonstrate in un sistema coaguladori de thrombina e plasma per le test del partial tempore de thromboplastina e le test del generation de thromboplastina.

In systemas coaguladori a thrombina que contine antithrombina, PCL manifesta un effecto promotori in le formation del coagulo. Il pare que iste activitate non representa un inhibition de antithrombina II o III o un potentiation del action enzymatic de thrombina. Il es plus probable que illo es un effecto directe super fibrinogeno o super le reactiones que resulta in le formation de fibrina.

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