Journal of Clinical Investigation
Vol. 46, No. 4, 1967

The Anticoagulant Activity of Lysosomal Cationic Proteins from Polymorphonuclear Leukocytes *

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Summary. A cationic protein fraction from rabbit polymorphonuclear leukocyte lysosomes has been shown to exert a potent anticoagulant effect on human blood *in vitro*. The anticoagulant activity is detectable in the whole blood clotting time, the recalcification time of platelet-rich plasma, the prothrombin time, the partial thromboplastin time, and the thromboplastin generation test. The lysosomal cationic proteins do not inhibit any of the known specific procoagulants. They appear to inhibit clotting by blocking the formation of intrinsic thromboplastin possibly by interfering with the role of phospholipids in the reaction involving Factors V and X and calcium.

Introduction

For many years there have been suggestions that leukocytes may play a role in blood coagulation (1, 2). It was not until 1951, however, that Martin and Roka (3) demonstrated prolongation of the clotting time of plasma in various clotting tests by lysates of leukocytes. Subsequently, Graham, Ebert, Ratnoff, and Moses (4) observed anticoagulant activity in a saline extract of granulocytes. The specific substance or substances in the leukocyte responsible for this anticoagulant activity have not yet been identified.

Recently the specific granules of polymorphonuclear leukocytes, established by Cohn and Hirsch to be lysosomes (5), have been found to contain a number of biologically active substances. In 1963 Zeya and Spitznagel isolated from polymorphonuclear leukocyte lysosomes a group of apparently nonezymatic, highly cationic proteins that possess potent antimicrobial activity (6). Subsequent studies have demonstrated these lysosomal cationic proteins (LCP) to be pyrogenic (7) and to produce inflammation and tissue injury (8–10). Since strongly cationic proteins like protamine (11, 12) and synthetic basic polypeptides (13, 14) have been shown to inhibit coagulation, it seemed reasonable that cationic proteins of polymorphonuclear leukocyte lysosomes might exhibit similar activity. This study demonstrates that lysosomal cationic proteins from polymorphonuclear leukocytes are very potent inhibitors of blood coagulation, and that they appear to inhibit the formation of intrinsic thromboplastin (intrinsic prothrombin activator) by interfering with the role of phospholipid (possibly platelet membranes) in the last stages of blood coagulation.

Methods

Glassware. All glassware used for isolating cells and cell fractions was rendered endotoxin-free by either baking at 180° C for 3 hours or soaking overnight in butanol, followed by repeated rinsing with pyrogen-free saline (7).

Polymorphonuclear (PMN) leukocytes. Sterile peritoneal exudates were induced in normal rabbits, 12 to 14 hours before harvesting, by ip injection of 200 ml of 0.25% glycogen in saline containing 68,000 U penicillin and 94 mg streptomycin per L. Exudates were pooled in an ice-chilled flask and the cells isolated by centrifugation as previously described (7).

PMN leukocyte lysosomes and lysosomal cationic protein. The lysosomal fraction was obtained by homogenization and differential centrifugation of granulocytes in 0.34 M sucrose according to the method of Cohn and Hirsch (5).

* Submitted for publication July 7, 1966; accepted December 22, 1966.

Supported in part by U. S. Public Health Service grants AI 04925, HE 06350, and TI AM 5345.

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The lysosomal cationic protein fraction was prepared by acid extraction (0.2 N H_2SO_4) of lysosomes, followed by precipitation with cold ethanol, 20% vol/vol, as described by Zeya and Spitznagel (6). The precipitate was dissolved in 0.01 N HCl, dialyzed against 0.01 N HCl overnight, then against distilled water for 6 hours, and lyophilized. After removal of the LCP, a second fraction was obtained from the 20% ethanol supernatant by precipitation with cold ethanol (45% vol/vol). This fraction was also dissolved in 0.01 N HCl, dialyzed against distilled water, and lyophilized. Both the 20% ethanol precipitate (LCP) and the 45% ethanol precipitate were tested for the presence of the following enzymes: lysozyme (15), ribonuclease and deoxyribonuclease (16), β-glucuronidase (17), acid and alkaline phosphatases (18), and cathepsin (19). All batches of LCP tested possessed potent anticoagulant activity, but when compared in a standard partial thromboplastin time test, there was slight variation in potency among different batches probably due to loss of activity during lyophilization or from qualitative differences in the protein obtained from different batches of cells. Thus, in the experiments to be described, the amount of LCP necessary for a desired effect was not always the same. The LCP used in clotting studies were dissolved just before use in barbital buffer, pH 7.35, ionic strength 0.154 (20). Although LCP were more readily soluble in weak acid, the resulting low pH was deemed undesirable for the clotting tests.

Lysozyme,[1] ribonuclease,[2] deoxyribonuclease,[2] β-glucuronidase,[2] and acid and alkaline phosphatases [2] were obtained commercially and tested for anticoagulant activity in the partial thromboplastin time and thromboplastin generation tests in the same concentrations as those used for testing LCP.

Human citrated plasma was prepared by collecting venous blood from five normal donors into 3.2% sodium citrate (1 part to 8 parts blood) and centrifuging at 4° C for 30 minutes at 2,000 *g*. The pooled plasma was stored at −20° C and thawed at 37° C just before use. Platelet-rich plasma was prepared by centrifuging citrated venous blood at 4° C for 5 minutes at 400 *g*.

Plasmas deficient in Factors V (proaccelerin), VII [SPCA (serum prothrombin conversion accelerator), proconvertin], VIII (AHF, antihemophilic factor), IX (PTC, plasma thromboplastin component), X (Stuart factor), or XII (Hageman factor) were prepared as described above but from patients with known congenital deficiency of the specific factor; these patients had not been transfused within 2 weeks before giving blood for this study. Plasma deficient in Factor XI (PTA, plasma thromboplastin antecedent) was prepared from normal plasma by the method of Horowitz, Wilcox, and Fujimoto (21).

Cephalin was either prepared from human brains as described previously (22) or obtained commercially as Thrombofax.[3]

Inosithin, 0.05%, used as the source of lipid in some of the thromboplastin generation tests (23), was obtained commercially as the dry powder.[4]

Tissue thromboplastin was obtained commercially as Simplastin.[5]

Thrombin [6] (topical, bovine) was diluted to the desired concentration with saline in siliconized glass tubes just before use.

Russell's viper venom (RVV) was the commercial preparation, Stypven.[7]

Fibrinogen was prepared from canine plasma by $(NH_4)_2SO_4$ precipitation as described previously (24). Human fibrinogen [8] was dissolved in barbital buffer and dialyzed overnight against barbital buffer at 5° C.

Coagulation studies. The following tests were performed at least in duplicate with and without LCP: recalcification time (25); one stage prothrombin time (PT) (26); partial thromboplastin time (PTT) (27); thrombin clotting time (28); and specific assays for prothrombin (24) and Factors VIII (27), IX (29), X (30), XI (21), and XII (31). Factor V assay was performed by the technique of the Factor VIII assay except that the substrate plasma was obtained from a patient congenitally deficient in Factor V (32). The effect of LCP on Factor VII was determined by adding LCP to normal plasma, diluting the resulting mixture to 10% with buffer, and comparing its corrective effect on Factor VII–deficient plasma with a simultaneous control containing buffer without LCP. In all assays for specific factors the concentration of LCP was adjusted so as to add an amount that prolonged the PTT of undiluted normal plasma to 150 seconds or greater (control 60 to 80 seconds).

Except for the substitution of inosithin for platelets in some studies, thromboplastin generation tests (TGT) were performed by the method of Biggs and Douglas (33). LCP were added to the incubation mixture either at the beginning of incubation (zero time) or after the generation of maximal thromboplastin activity as determined by the shortest clotting time of the substrate plasma. In other experiments using the TGT, LCP were added to the substrate plasma.

The effects of LCP were also tested in a multistage sequential clotting system. All reagents for this test, except the phospholipid, were prepared according to methods previously described (34). In this system, crude Factor X, prepared from serum obtained from a Factor IX–deficient patient, was activated by Russell's viper venom, crude Factor V was prepared from hemophilic plasma, and crude prothrombin was prepared from Factor X–deficient plasma. When all reagents were supplied in the stages and sequence as shown in Figure 7, the clotting time varied from about 27 to 36 seconds. When any clotting factor was omitted, the clotting time

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Ortho Pharmaceutical Corp., Raritan, N. J.
[4] Associated Concentrates, Long Island, N. Y.
[5] Warner-Chilcott Laboratories, Morris Plains, N. J.
[6] Parke, Davis, Detroit, Mich.
[7] Burroughs Wellcome, Tuckahoe, N. Y.
[8] Merck Sharp & Dohme, West Point, Pa.

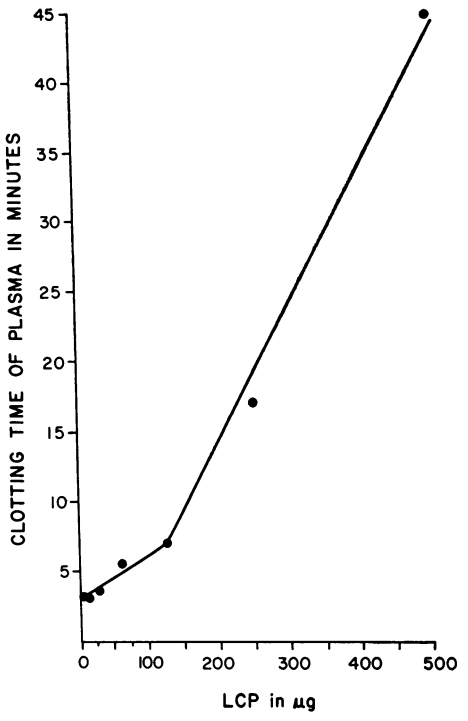

FIG. 1. EFFECT OF LYSOSOMAL CATIONIC PROTEINS (LCP) ON THE RECALCIFICATION TIME OF NORMAL PLASMA. Citrated, platelet-rich plasma, 0.1 ml, plus 0.1 ml of either buffer or buffer containing LCP was clotted by adding 0.1 ml of 0.02 M $CaCl_2$. The abscissa shows micrograms of LCP in final clotting mixtures.

was > 300 seconds except that when Factor X was omitted the clotting time was 114 seconds, presumably due to Factor X contamination of the prothrombin reagent.

The effect of LCP on the activation of Factor X was tested according to the method described by Breckenridge and Ratnoff (34), but in some experiments 0.2 ml additional phospholipid was added to the incubation mixture after the addition of LCP.

Appropriate controls for all clotting studies were performed by substituting barbital buffer alone for buffer containing LCP.

Results

The lysosomal cationic protein fraction. The lysosomal fraction obtained by precipitation with cold ethanol, 20% vol/vol, was found to be soluble in distilled water and in solutions of acid pH, but its solubility decreased in alkaline solutions. This fraction was found to be free from the known lysosomal enzymes lysozyme, ribonuclease, deoxyribonuclease, β-glucuronidase, acid and alkaline phosphatases, and cathepsins as previously shown by others (8–10, 35–37). Most of these lysosomal enzymes, however, were present in the fraction precipitated with ethanol, 45% vol/vol.

The lysosomal cationic protein fraction was found to be heterogenous when subjected to electrophoresis on cellulose acetate strips in acetate buffer, pH 4.0 for 1 hour (200 v, 0.002 amp) as reported by others (9, 10, 35–37).

Commercial preparations of lysozyme, ribonuclease, deoxyribonuclease, β-glucuronidase, and acid and alkaline phosphatases, added to clotting systems in approximately the same concentration as LCP, had no significant anticoagulant activity. All the cell fractions obtained during the processing of polymorphonuclear leukocytes for isolation of lysosomes were tested. None of these, except the lysosomal fraction, exhibited anticoagulant activity. Although most of the anticoagulant activity was found in the LCP, similar activity was detected in the fraction containing lysosomal enzymes. This was attributed to contaminating LCP and was supported by finding, upon electrophoresis of this fraction with enzymes, bands corresponding to LCP. The lysosomal membranes (washed sediment of acid-lysed granules), like all the other nonlysosomal leukocyte fractions, possessed no anticoagulant activity.

Effect of LCP on whole blood and plasma. LCP significantly prolong the clotting of whole blood in proportion to the amount added. For example, 600 µg of LCP added to 1 ml of whole blood resulted in a clotting time of 45 minutes (control 17 minutes); the clotting time was 58 minutes when 900 µg LCP was added to 1 ml whole blood. Similar results were obtained when the clotting time of whole blood was determined with the Lee–White technique. The recalcification time of platelet-rich plasma was also prolonged by LCP in proportion to the amount of LCP added (Figure 1). The anticoagulant activity of LCP was demonstrable in both the prothrombin and partial thromboplastin time as shown in Figure 2. Here, too, the prolongation was proportional to the amount of LCP added; buffer alone in the controls produced no detectable prolongation in either the PT or PTT.

Effect of LCP on specific procoagulants. To determine whether the prolonged clotting time of plasma and whole blood were due to inhibition of

one or more of the procoagulants, specific assays for Factors V, VIII, IX, X, XI, and XII were performed after adding LCP to normal plasma in a final concentration of 1.5 mg per ml. The results, shown in Figure 3, demonstrated no specific inhibition of any of the procoagulants studied even though assays were carried out on portions of plasma containing sufficient LCP to prolong the PTT of the undiluted plasma to more than 180 seconds. Although not shown in the Figure, similar experiments demonstrated that LCP did not inhibit Factor VII; a diluted sample of a normal plasma–LCP mixture had the same corrective effect on Factor VII–deficient plasma as did a diluted sample of a normal plasma–buffer mixture. In normal plasma containing 1.5 mg of LCP per ml, the concentration of prothrombin, determined by specific assay, was 300 U per ml, a value similar to that of the control plasma containing only buffer.

Effect of LCP on thromboplastin generation. When LCP were added to the thromboplastin generation mixture at the beginning of incubation, no detectable thromboplastin activity appeared even after incubation for as long as 30 minutes (Figure 4A). The same amount of LCP, added after 8 minutes of thromboplastin generation, also inhibited intrinsic thromboplastin activity, but the inhibitory effect was less marked than when LCP was added at zero time. The apparent progressive inhibitory effect of LCP when added after

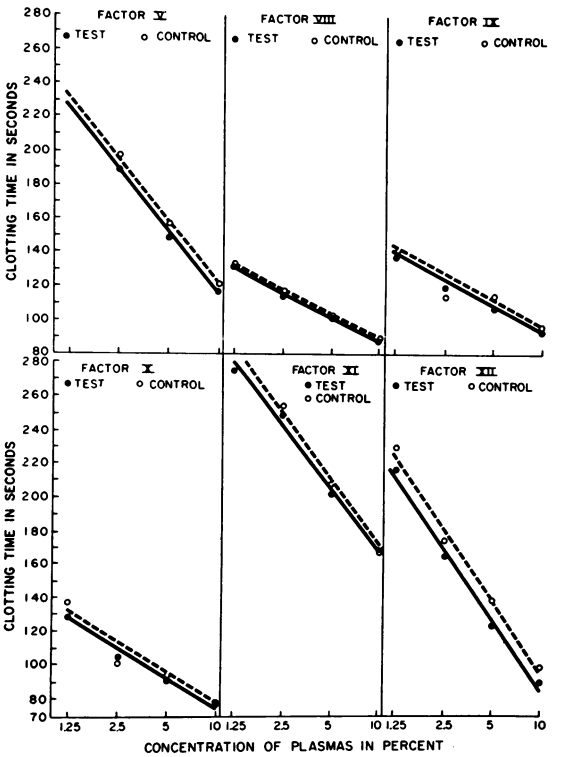

FIG. 3. EFFECT OF LCP ON FACTORS V, VIII, IX, X, XI, AND XII. One ml of either buffer or buffer containing 3 mg of LCP was incubated for 2 to 3 minutes at room temperature with 1.0-ml samples of pooled normal plasma. (Partial thromboplastin time on plasma with LCP, 180 seconds; on control, 69.2 seconds.) Test and control plasmas were assayed for specific procoagulants as described under Methods.

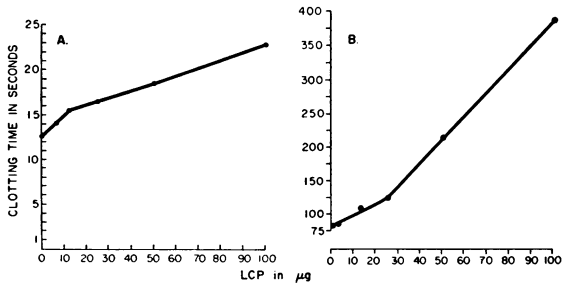

FIG. 2. EFFECT OF LCP ON A) THE PROTHROMBIN TIME AND B) THE PARTIAL THROMBOPLASTIN TIME. The abscissa shows micrograms of LCP in final clotting mixtures. A) Pooled normal plasma, 0.1 ml, plus 0.1 ml of either buffer alone or buffer containing LCP was clotted by adding 0.2 ml thromboplastin–$CaCl_2$ mixture. B) Pooled normal plasma, 0.1 ml, plus 0.1 ml of either buffer or buffer containing LCP plus 0.1 ml of cephalin was clotted by adding 0.1 ml of 0.02 M $CaCl_2$.

generation of maximal thromboplastin activity suggested that formation of thromboplastin did not continue in the presence of LCP. To further test this possibility we added LCP to the substrate plasma, rather than to the incubation mixture. Illustrative results of such experiments are shown in Figure 4B. The inhibitory effect in this circumstance is just barely detectable even though the final concentration of LCP in the substrate plasma was comparable to that of the experiments shown in Figure 4A.

Lysosomal cationic proteins not only inhibited thromboplastin activity, but the degree of inhibition was increased by greater amounts of protein. This is apparent on comparison of curves A, B, and C of Figure 5. This inhibitory effect, however, was not reversed by dilution since, if this occurred, the curves shown in Figure 5 would not

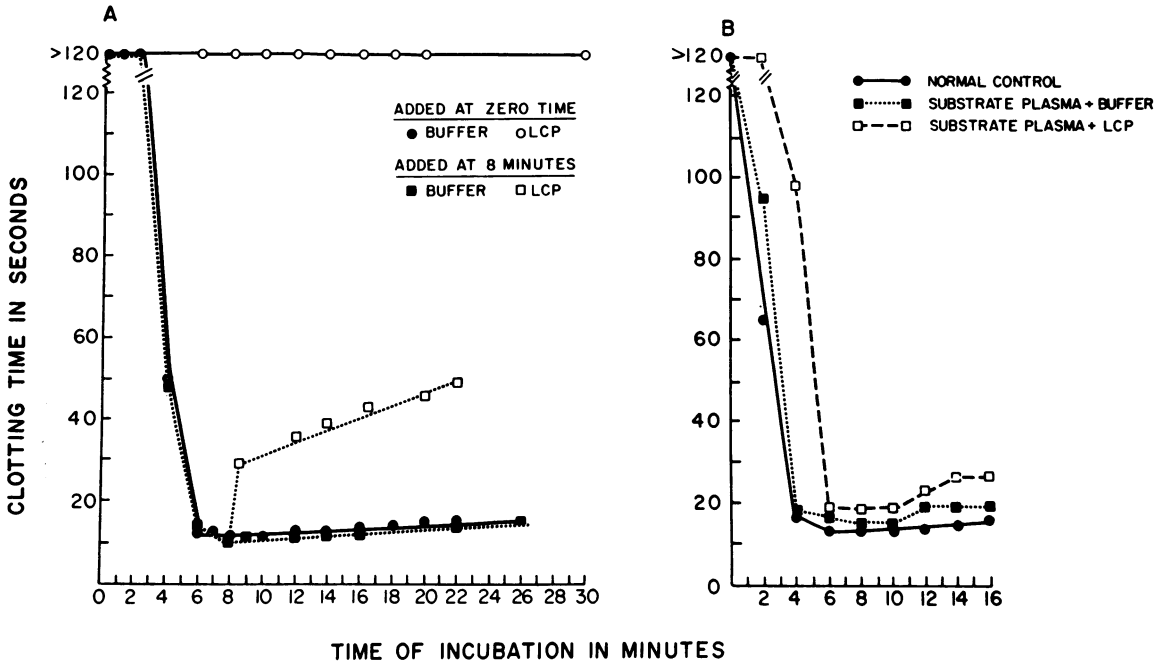

FIG. 4. EFFECT OF LCP ON THROMBOPLASTIN GENERATION. The thromboplastin generation mixtures, incubated at 37° C, contained 0.5 ml of each of the following: $Al(OH)_3$-adsorbed plasma, diluted 1:5; normal human serum, diluted 1:10; 0.05% inosithin in buffer; and 0.025 M $CaCl_2$. A) At zero time and after 8 minutes of incubation, 0.5 ml of either buffer alone or buffer containing LCP (3 mg per ml) was added to the thromboplastin generation mixture. At intervals after addition of buffer or LCP, 0.1 ml of the incubation mixture was added to 0.2 ml of normal plasma and the mixture clotted by adding 0.1 ml 0.025 M $CaCl_2$. B) At intervals beginning with zero time, 0.1 ml of the thromboplastin generation mixture was added to 0.2 ml normal plasma containing 0.1 ml of either buffer alone or buffer containing LCP (600 μg per ml) and the mixture clotted by adding 0.1 ml of 0.025 M $CaCl_2$.

be parallel, but rather would converge toward the control curve.

Effect of LCP on various stages of thromboplastin formation. Since the TGT probably reflects a composite effect of several reactions, LCP were tested in the individual reactions thought to be necessary for thromboplastin generation. The activation of Factor X is likely to be one of these (38). From the results shown in Figure 6, it is apparent that after incubation for 60 to 90 seconds, Factor X was maximally activated by RVV even in the presence of LCP. The prolonged clotting time observed in the test system seemed to be related to an effect of LCP on something other than the activation of Factor X; this effect was reversed by adding more cephalin to the incubation mixture containing LCP.

The effect of LCP on other reactions necessary for the production of prothrombin activator was measured in the five stage test illustrated in Figure 7. In this system the addition of LCP before Factor V markedly inhibited the formation of intrinsic thromboplastin as indicated by the prolonged clotting time (test A). The same amount of LCP, added after Factor V, did not inhibit the formation of thromboplastin; the clotting time approximated that of the control (test B). In repeated tests in which LCP were added after Factor V, the clotting time varied slightly with the concentration of phospholipid and LCP, but in all these tests the clotting time was nearly that of the control. It is also evident that, under the conditions of the five stage test, LCP had no detectable effect on the thrombin–fibrinogen reaction; fibrinogen clotted as promptly as in the control (test C).

The findings in the five stage test suggested that LCP inhibited the generation of intrinsic thromboplastin by interfering with one or more of the reactions involving Factor V, Factor X, phospholipid, and calcium. The formation of a fine precipitate upon mixing 300 μg of LCP with

0.1 ml of a 1:25 dilution of cephalin suggested the possibility that LCP interacted and thus interfered with the action of phospholipid in the intrinsic thromboplastin generation. The phospholipid micelles carry an electronegative surface charge (39) opposite to that of electropositive LCP. The possibility of specific electrostatic interaction between LCP and phospholipid was examined further in the TGT (Figure 8). After generation of maximal thromboplastin activity, LCP added to the incubation mixture prolonged the clotting time of substrate plasma. This effect of LCP was promptly reversed when more cephalin was added to the incubation mixture. The inhibitory effect of LCP on intrinsic thromboplastin generation also occurred when platelets, rather than platelet substitute, were used in the TGT, and it was reversible by the addition of more platelets. Complete reversal of the inhibitory effect of LCP on intrinsic thromboplastin formation was not observed when increased amounts of Factor V, Factor X, or calcium were added to the incubation mixture used for the TGT (Figure 9).

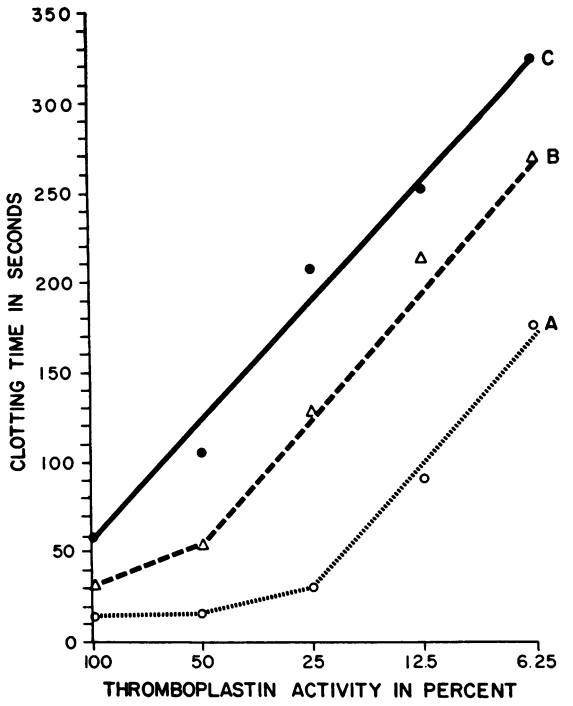

FIG. 5. EFFECT OF DILUTION ON THROMBOPLASTIN–LCP MIXTURE. Standard thromboplastin generation mixtures, prepared as described, were incubated at 37° C. After 10 minutes, when maximal thromboplastin activity was found to be present, the following reagents were added to the thromboplastin generation mixtures: test A: 0.5 ml buffer; test B: 0.5 ml buffer containing LCP (1.5 mg per ml); test C: 0.5 ml buffer containing LCP (3 mg per ml). After these additions the mixtures were placed in a melting ice bath and serial dilutions of each made with barbital buffer. After warming again to 37° C, 0.1 ml of each dilution was added to 0.2 ml of normal plasma and the mixture clotted by adding 0.1 ml of 0.025 M $CaCl_2$. The 100% values on the abscissa represent the thromboplastin activity of 0.1 ml of the undiluted incubation mixture, the 50% values a 1:2 dilution, and so on.

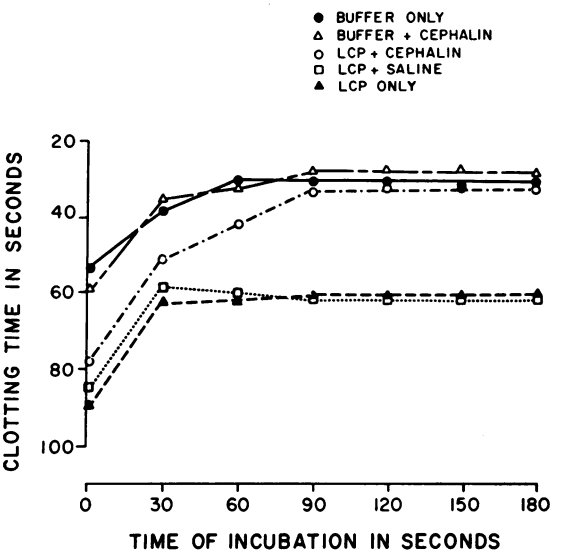

FIG. 6. EFFECT OF LCP ON THE ACTIVATION OF FACTOR X. The activation mixtures, incubated at 37° C, contained 0.1 ml of each of the following: crude Factor X, Russell's viper venom (diluted 1:200,000), cephalin, 0.025 M $CaCl_2$, and either buffer alone or buffer containing LCP (2 mg per ml). To separate activation mixtures, with and without LCP, we added 0.2 ml of either cephalin or saline in an attempt to shorten the clotting time of the system containing LCP. For all five test systems designated in the legend, a separate activation mixture was incubated for each period of time shown on the abscissa. When incubation of the mixtures was completed, 0.1 ml of Factor X–deficient plasma was added to each and the clotting time recorded.

Antithrombin activity. Antithrombin activity was assayed by adding LCP to bovine thrombin and comparing the subsequent conversion of fibrinogen to fibrin by this thrombin. In the control, LCP were replaced with buffer.

Varying amounts of LCP did not prolong the thrombin clotting time of canine fibrinogen (Table I). Actually with increased amounts of LCP the thrombin–fibrinogen clotting time was consistently shorter than the control. Similar results were

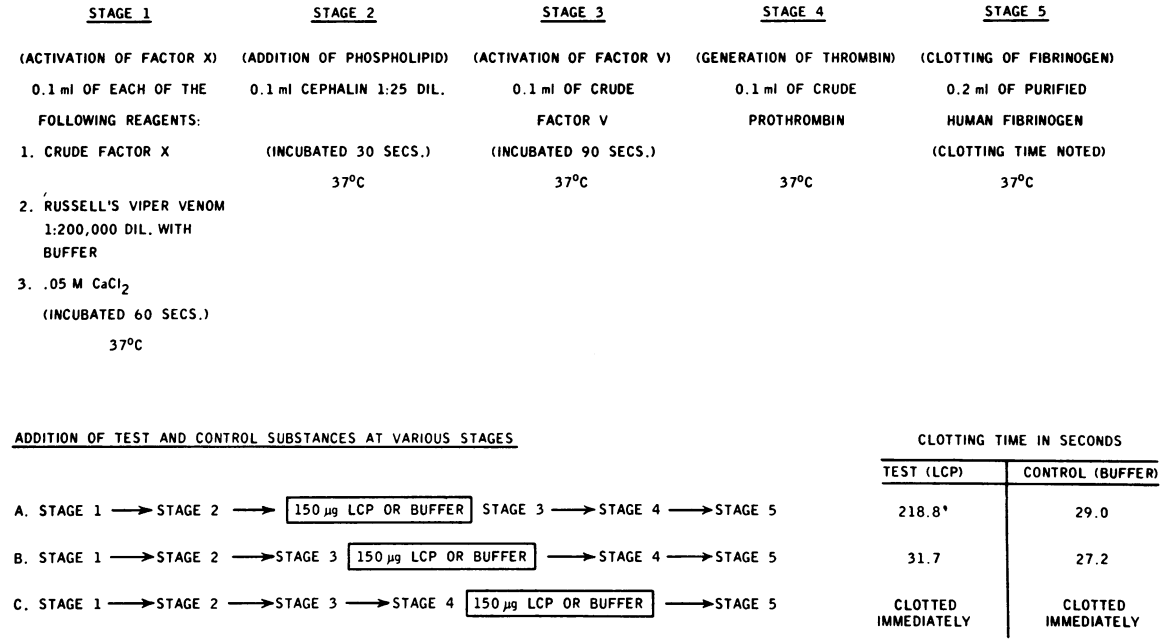

FIG. 7. EFFECT OF LCP AT VARIOUS STAGES OF A SEQUENTIAL CLOTTING SYSTEM. Factors and reagents were prepared as described under Methods. In control tests, omission of any of the factors or reagents listed under the various stages produced a clotting time greater than 300 seconds. Test mixtures were transferred to an ice bath before the addition of 0.1 ml of either buffer alone or buffer containing LCP, and afterwards returned to the water bath at 37° C for 60 seconds before completing subsequent stages.

obtained when purified human fibrinogen or pooled normal citrated plasma was substituted for canine fibrinogen.

Discussion

These studies indicate that the lysosomal cationic proteins of polymorphonuclear leukocytes possess a potent anticoagulant effect. This effect is manifested in many of the conventional tests of coagulation including whole blood clotting time, recalcification time, prothrombin time, and partial thromboplastin time. These cationic proteins, however, do not appear to act by inhibiting or inactivating any of the known plasma procoagulants, since in the assay for these factors normal plasma previously incubated with LCP is no different from control plasma. Although the possibility exists that one or more of the specific procoagulants in plasma is inhibited by LCP, and the inhibition reversed by the dilution necessary for assay, this explanation seems unlikely, since dilution does not reverse the inhibitory effect of LCP in the TGT. Rather, the results of studies using the TGT suggest that LCP interfere with the formation of intrinsic thromboplastin, thromboplastin itself, or both. The normal results obtained when LCP were added to the substrate plasma, rather than to the thromboplastin incubation mixture, make it more likely that LCP act primarily by interfering with the formation of thromboplastin. This interpretation is supported by the results ob-

TABLE I
Effect of LCP on thrombin–fibrinogen clotting system

Final concentration of LCP in test mixture*	Clotting time†
µg	*seconds*
0	39.9
10	33.3
18.7	28.6
37.5	32.9
75	31.6
150	25.6
300	25.0

* Test mixture contained 0.1 ml thrombin solution (2.5 U per ml), 0.1 ml of buffer or buffer containing LCP, and 0.3 ml of calcium–imidazole–saline–acacia mixture.

† Measured after adding to the test mixture 0.1 ml fibrinogen solution (300 mg per 100 ml).

tained in the five stage clotting system that show the anticoagulant effect of LCP to be pronounced only when it is added before the addition of Factor V. These findings, plus the demonstration that LCP seem neither to inhibit the activation of Factor X nor to possess antithrombin activity, suggest that LCP may act by interfering with the role of phospholipid in the formation of intrinsic thromboplastin. This possibility is strengthened by the observations that mixtures of cephalin and LCP, probably as a result of electrostatic interaction, form a precipitate and that only cephalin, not Factor V, Factor X, or calcium, can fully reverse the anticoagulant activity of LCP. Zipursky and co-workers (40) recently suggested that the anticoagulant properties of another cationic substance, Polybrene, might result from its ability to neutralize the negatively charged phospholipids. Fur-

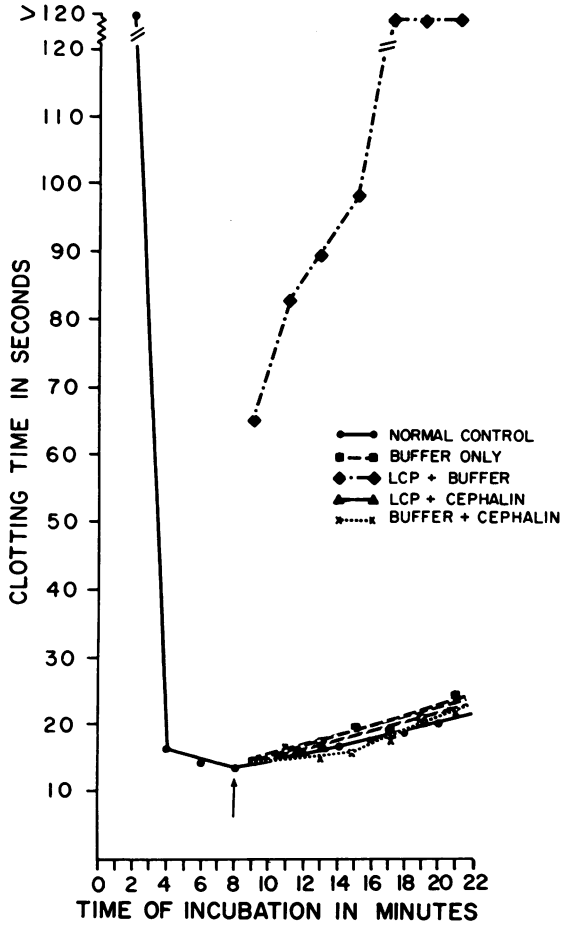

FIG. 8. THE EFFECT OF CEPHALIN IN REVERSING THE INHIBITORY EFFECT OF LCP ON THROMBOPLASTIN FORMATION. Standard thromboplastin generation mixtures, prepared as described, were incubated at 37° C. At 8 minutes, when maximal thromboplastin activity was present (arrow), either buffer alone or buffer containing LCP (2.5 mg per ml) was added, followed immediately by 0.5 ml of crude cephalin (0.15%) suspended in 0.15 M saline; in controls 0.5 ml of buffer substituted for cephalin. Incubation of the thromboplastin generation mixtures at 37° C continued, and, at intervals, 0.1 ml of each was mixed with a 0.2-ml sample of normal plasma and this mixture clotted by adding 0.1 ml of 0.025 M $CaCl_2$.

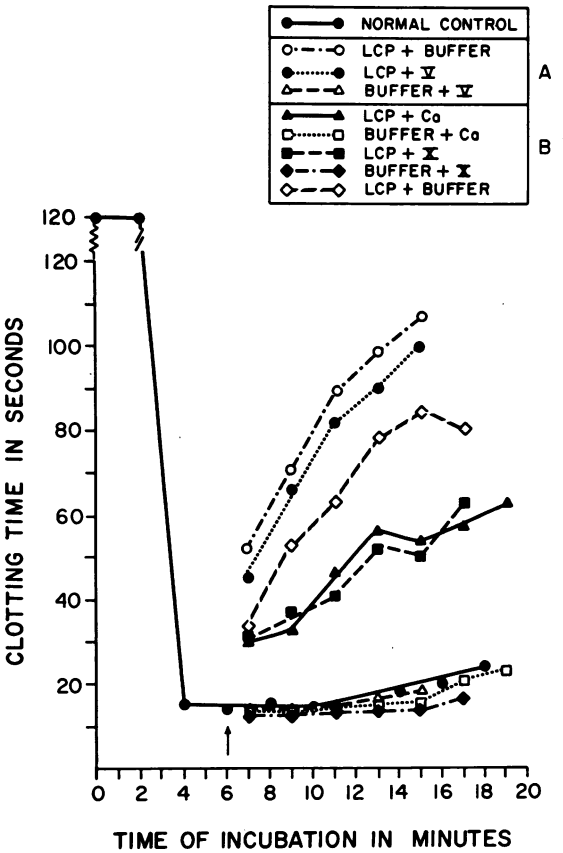

FIG. 9. THE EFFECT OF FACTORS V AND X AND CALCIUM IN REVERSING THE INHIBITORY EFFECT OF LCP ON THROMBOPLASTIN FORMATION. Standard thromboplastin generation mixtures, prepared as described, were incubated at 37° C. At 6 minutes, when maximal thromboplastin activity was present (arrow), either buffer alone or buffer containing LCP was added, followed immediately by 0.5 ml of either crude Factor V, Factor X, or 0.05 M $CaCl_2$; in controls, 0.5 ml of buffer alone substituted for these Factors or reagents. Incubation of the thromboplastin generation mixtures at 37° C continued, and, at intervals, 0.1 ml of each was mixed with a 0.2-ml sample of normal plasma and this mixture clotted by adding 0.1 ml of 0.025 M $CaCl_2$. In A, the concentration of LCP was 2.5 mg per ml; in B, the concentration was 1.5 mg per ml.

thermore, the report that platelets bear a negative surface charge (41), and the suggestion by Marcus (42, 43) that the activity of platelets in clotting may depend upon the platelet membrane, are compatible with this hypothesis.

Although these studies do not clarify the exact nature of intrinsic thromboplastin, they do point out the essential role of phospholipid in its formation and confirm that Factor X, Factor V, and calcium, in addition to phospholipid, are required for the development of full intrinsic thromboplastin activity (34, 44). It is possible that understanding the mechanism of the LCP activity found in this study will provide additional information about the nature and process of intrinsic thromboplastin formation.

The lysosomes of eosinophils and basophils have not been specifically investigated; neither have extensive studies of granulocytes of different species been carried out. Eosinophils from horses, however, have been reported to inhibit the generation of thromboplastin *in vitro* (45), and in preliminary studies we have detected anticoagulant activity in a fraction of human granulocyte lysosomes containing nonenzymatic cationic proteins.

The role of LCP as an endogenous anticoagulant has yet to be investigated *in vivo*. However, considering the huge population of polymorphonuclear leukocytes in the body, and their rapid turnover, it is possible that the lysosomal cationic proteins from these cells might be involved in the regulation of normal hemostasis as well as in some of the poorly understood hemorrhagic disorders. It is noteworthy that lysosomal cationic proteins interfere with the same stages of intrinsic thromboplastin formation as the circulating anticoagulant reported in some cases of disseminated lupus erythematosus (34).

Acknowledgments

We are indebted to Dr. H. I. Zeya, Department of Microbiology, for invaluable suggestions and help. The technical assistance of Mrs. Marva Dowdy, Mrs. Doris Sparrow, and Mr. Bruce H. Beveridge is also gratefully acknowledged.

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