Naturally Occurring Species Specific Inhibitor of Human Prothrombin in Lupus Erythematosus.* (29247)

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There have been at least thirty-three reports of a characteristic type of circulating anticoagulant appearing in patients with lupus erythematosus(1) following the report of Conley and Hartmann(2) in 1952. These anticoagulants are all probably similar if not identical in nature; they inhibit the conversion of prothrombin to thrombin by either extrinsic or intrinsic "thromboplastin" but have no effect on the generation of "thromboplastin"(3,4). They are gamma globulins, non-adsorbable on barium sulfate and precipitated from plasma between 20 and 33% saturation with ammonium sulfate(1). So far there have been few attempts to determine their mode of action using purified reagents. We have recently encountered 2 patients (T.O. and J.H.) with lupus erythematosus who have inhibitors of this type and we report here the results of our investigations on the site of action of these inhibitors.

Materials and methods. Inhibitor fractions were prepared by adsorbing the serum of each of the patients with $Al(OH)_3$. After adsorption, ammonium sulfate was added to 33% saturation. The precipitate obtained was dissolved in a volume of 0.14 M NaCl equal to half the original serum volume and dialyzed for 48 hours against 0.14 M NaCl in the cold. A normal control fraction was obtained by similarly treating normal serum. *Prothrombin* was prepared by adsorbing oxalated normal human or bovine plasma with barium sulfate (100 mg/ml plasma); after 2 washings with cold distilled water the barium sulfate was treated with 5% trisodium citrate. The eluates were dialyzed overnight against 0.14 M NaCl. Another preparation was obtained using normal human plasma that had been first treated by bentonite as in the preparation of the substrate for the factor X assay method(5); subsequent stages were as described above. The barium sulfate eluate source of bovine prothrombin was further purified by DEAE column chromatography. *Buffer.* Michaelis buffer pH 7.35 was used. *0.025 M calcium chloride* was used throughout. *Cephalin* was prepared by the method of Bell and Alton(6) and diluted 1:100 with buffer. *Plasma* was obtained by adding 9 parts whole blood to 1 part 3.8% trisodium citrate and centrifuging at 3000 rpm. *Partial thromboplastin times (PTT)* were performed by the method of Langdell, *et al*(7) modified by addition of the inhibitor or normal control fractions immediately before the final addition of the calcium. *"Product 2."* This was essentially sedimented intrinsic thromboplastin. It was prepared by preincubating 2 ml $Al(OH)_3$-adsorbed plasma diluted 1:5, 2 ml normal serum diluted 1:10 and 2 ml calcium chloride as in the performance of the thromboplastin generation test only omitting the lipid component; the serum and adsorbed plasma were both diluted with 0.14 M NaCl. When a clot formed, it was removed and 4 ml cephalin added. After 3 minutes the mixture was centrifuged at 15,000 $\times$ *g* for 30 minutes, the supernatant was poured off and the deposit rapidly rinsed twice by pouring in and out approximately 10 ml 0.14 M NaCl. The rinsed sediment, which was finally resuspended in 2 ml 0.14 M NaCl, was used within 2 hours of preparation. *Fibrinogen* source was the 0-25% ammonium sulfate precipitate of $BaSO_4$-treated bovine or human oxalated plasma. *Prothrombin times* were performed using an acetone extract of human brain. *Thrombin activity.* In the experiments with prothrombin and "product 2" described under *Results*, the thrombin generated, expressed as % total, was determined from clotting times by means of a thrombin dilution curve obtained as follows: when the substrate clotting time of the mixture containing the normal control fraction reached its minimum (8 seconds), the mixture was placed in ice water and serial

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TABLE I. Partial Thromboplastin Times of Human, Bovine and Dog Plasma After Addition of 0-33% Ammonium Sulfate Formation Prepared from Patients and Normal Plasma.

Type of plasma	Source of fraction added to test system		
	Normal	J.H.	T.O.
Human	119	275	230
Dog	103	105	92
Cow	100	97	105

TABLE II. Prothrombin Times in Seconds of Various Plasma and Plasma Mixtures After Addition of 0-33% Ammonium Sulfate Fractions Prepared from Patient (J.H.) and Normal Control.

	Source of fraction added	
	Normal control	Patient J.H.
Human plasma	15	27
Bovine plasma	37	35
9 parts bovine + 1 part human	24	34
1 part bovine + 9 parts human	16	34

dilutions made using buffer; 0.1 ml of each of these dilutions was added to 0.2 ml fibrinogen and the clotting times recorded. A curve relating clotting time to per cent activity was constructed, arbitrarily assuming the undiluted mixture to have 100% activity. All experiments were performed at 37°C.

Results. Effect of inhibitors on partial thromboplastin times (PTT) and prothrombin times. The partial thromboplastin times of human, dog and cow plasma were determined by incubating 0.1 ml of the following: citrated plasma, cephalin, inhibitor or normal control fraction and calcium. The results (Table I) show that the inhibitors prolonged the clotting times of human plasma but had no effect on dog or cow plasma. The results were similar when either "product 2" or human brain extract were substituted for the cephalin. The prothrombin times of mixtures of human and bovine plasma were then determined using human brain extract. The results (Table II) show that the addition of a relatively small amount (10%) of human to bovine plasma significantly shortened the clotting time of the bovine plasma but this shortening did not occur in the presence of the inhibitor (J.H.). Addition of a relatively small amount (10%) of bovine plasma to human plasma did not have any significant neutralizing effect on the inhibitor.

Effect of inhibitors on prothrombin to thrombin conversion by "product 2." The following reagents were incubated together: 0.2 ml human plasma eluate, 0.2 ml buffer, 0.2 ml inhibitor or normal control fraction, 0.2 ml "product 2" and 0.2 ml calcium chloride. At 30 seconds, 1, 2, 3 and 5 minutes following the final addition of calcium, aliquots were removed and added to 0.2 ml bovine fibrinogen and the clotting times recorded. The results (Fig. 1) show that both inhibitors interfere with prothrombin activation. Similar experiments were then performed using the human eluate which was prepared from bentonite-treated plasma and contained less than 5% factors X and VII. The results were similar. However, if either the bovine plasma eluate or the more purified prothrombin preparation prepared from the latter by chromatography were used, the inhibitors did not have any effect on prothrombin activation. The addition of 0.2 ml normal human serum diluted 1:10 (substituted for buffer) to either of the bovine prothrombin sources did not result in any defect in prothrombin conversion when the inhibitors were included in the incubation mixtures.

Effect of inhibitors on thrombin-fibrinogen reaction. Thrombin was prepared by incubating 0.2 ml human plasma eluate with 0.2 ml

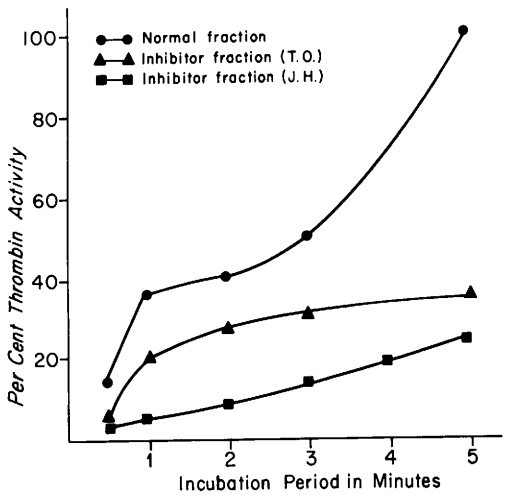

FIG. 1. Inhibition of prothrombin to thrombin conversion by inhibitor fractions. The 100% thrombin activity very roughly approximates 5 N.I.H. units.

"product 2" and 0.2 ml calcium chloride. After 20 minutes' incubation, 0.1 ml of the mixture was added to a mixture of 0.2 ml human fibrinogen and 0.1 ml of the inhibitor or normal control fractions and the clotting times recorded. The inhibitor fractions had no effect on clotting times.

Discussion. The findings that the inhibitors from both patients interfered with the reaction of the sedimented intrinsic blood thromboplastin ("product 2") with human but not with bovine prothrombin preparation suggest that the inhibitors are directed against human prothrombin. Another possible explanation is that the inhibitors interfere with some other activity in the prothrombin preparation necessary for prothrombin conversion. However, it is generally believed that "product 2" when prepared with factor V converts prothrombin to thrombin in the presence of calcium and that no other factors are needed for the conversion. If this is so, a new clotting component difficult to separate from prothrombin would have to be postulated. A third possibility is that the inhibitors require a species specific co-factor for the development of their activity; this seems unlikely as the addition of human serum to the bovine prothrombin preparation did not result in inhibition of thrombin formation by "product 2." The most logical explanation of our findings is that the inhibitors are species specific inhibitors of human prothrombin. The finding of a naturally occurring species specific inhibitor of prothrombin has not been previously reported to our knowledge. Fantl and Nance(8) claimed that one of their patients developed a species specific inhibitor against tissue thromboplastin but this finding was based on indirect evidence and their conclusions would be considered invalid in the light of more recent work(3).

Summary. Fractions were prepared by 0 to 33% saturation with ammonium sulfate from the sera of two patients with lupus erythematosus who had circulating anticoagulants. The fractions prolonged the prothrombin and partial thromboplastin times of human but not bovine or canine plasma and inhibited the conversion of human but not bovine prothrombin to thrombin by "product 2." The findings suggest that the circulating anticoagulants are species specific inhibitors of prothrombin.

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Study of Virulence and Tumorigenicity of Variants of Herpes Simplex Virus.* (29248)

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The virulence of various strains of herpes simplex virus (HSV) in newborn and young adult mice has been studied in many laboratories. Efforts to test the potential tumori-

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