THE MECHANISM OF SPONTANEOUS RESOLUTION OF THROMBOEMBOLI

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EVIDENCE supporting the spontaneous disappearance of venous thrombi and pulmonary emboli is growing. Freiman and Wessler noted that fresh, serum-induced clots—embolized in the lung—rapidly disappear. The efficiency of this process was markedly impaired when, instead of fresh clots, aged or partially organized clots were allowed to embolize. Allison and his coworkers confirmed the rapid resolution of large, radiopaque, fresh blood clots from the pulmonary arteries. Also, the disappearance rate is fast as the findings of Springate and his associates would indicate. Ninety per cent of freshly prepared emboli disappeared from the pulmonary arterial tree in the first four hours, and only a small number of clots became organized.

This observation in animals has its counterpart in man. Sautter and his associates documented, by pulmonary arteriography, the complete resolution of massive pulmonary emboli in two patients. Similarly, Fred and his co-workers observed the spontaneous disappearance of pulmonary emboli in six out of seven patients within one to three weeks and a nearly complete resolution in the seventh patient. As no specific treatment was directed toward the thromboembolism, it was reasoned that the fibrinolytic system of the patient affected the resolution. Yet, the value of thrombolytic agents in attempting to affect their disappearance has never been clearly established. The rapid disappearance of pulmonary emboli could not be associated with enhanced fibrinolytic activity of the blood in studies by Barnard or from observations by Springate in rabbits. Wright and his associates were able to accelerate resolution with the use of anticoagulant drugs; however, they were unable to demonstrate systemic fibrinolytic activity during this process. To make matters even more complex, there is unmistakable evidence that clots sometimes fail to disappear or they only partially dissolve.

The mechanism of clot resolution and the circumstances that affect this phenomenon remain to be clarified. In the absence of demonstrable evidence of systemic fibrinolysis, the breakdown of fibrin by local processes should be considered. Hence, it has been postulated that the local release of proteolytic enzymes, or their precursors from damaged vascular endothelium, at the site of a thrombus or embolus would effect the breakdown of fibrin. This mechanism, previously postulated by Kwaan and McFadzean, is difficult to assess and, hence, will have to await further confirmation. We previously demonstrated the ability of leukocytes to affect clot resolution (7).

Although we were far from able to explain all the vagaries that surround this complex problem, during the course of previous and unrelated studies, we came upon another mechanism, which may well participate in this process. An account of these underlying processes form the subject of this article.

MATERIALS AND METHODS

Bovine thrombin, a purified preparation containing less than 5 per cent contaminat-

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ing proteins, was provided for labeling by Seegers and McCoy (18). According to the technique described by McFarlane this preparation was labeled with ^{131}I, to the extent that the iodination did not exceed the mean of one-half atom of ^{131}I per protein molecule. The free ^{131}I was removed by overnight dialysis of the protein solution in sodium Sequestrene® (edetic acid)-treated dialysis bags, as previously described by Miller and Copeland, against a continuous change of distilled water at zero degrees C. At the end of the dialysis, the supernatant fraction contained less than 0.7 per cent of the total radioactivity after precipitation of protein of an aliquot of the labeled preparation with trichloroacetic acid.

Aliquots of bovine plasma were clotted with labeled thrombin for 30 minutes. The clot was allowed to synerize prior to its washing in several changes—in some instances as many as ten—of saline solution, a sequence previously described by Jacobsson. After drying, the clots were dissolved in 40 per cent buffered urea, and aliquots of this solution were counted in a well-counter.

Unlabeled thrombin was prepared by chromatography from a commercially available bovine thrombin preparation, as described by Rasmussen. The thrombin-containing fractions were collected in siliconized tubes, pooled, lyophilized, and used for the study of the inactivation of thrombin clotting and esterase activity.

Commercially prepared heparin, Liquemin®, was used in amounts varying from 0.01 to 20 units per milliliter of reaction mixture. Lyophilized bovine fibrinogen served as the fibrinogen source. Bovine plasma was collected in ethylene diamine tetra-acetic acid, quick frozen, and kept at −20 degrees C. Alpha-p-toluene sulfonyl-l-arginine methyl ester—TAMe—was supplied for investigation.

Thrombin activity of the different preparations and reaction mixtures was determined by clotting and esterolytic techniques. Determination of thrombin-clotting activity was carried out in triplicate in siliconized test tubes by a technique previously described by Seegers (17–19). Equal quantities, 0.4 to 0.8 milliliter, of serum or heat defibrinated plasma and thrombin in buffered isotonic saline solution, pH 7.4, were added to buffered saline solution, with or without heparin, to a final volume of 2 milliliters. Simultaneous determinations were performed on normal bovine serum—or heat defibrinated plasma—heparinized bovine serum—or defibrinated plasma—and buffer without serum or plasma. The amount of thrombin added ranged from 50 to 200 National Institutes of Health units per milliliter and was the same for all three reaction mixtures.

The reaction mixtures were incubated, and at various times, aliquots were removed for determination of the residual thrombin activity.

Thrombin esterase activity for alpha-p-toluene sulfonyl-l-arginine methyl ester was performed by two different techniques. The spectrophotometer was used to determine changes in the absorption spectrum for hydrolyzed alpha-p-toluene sulfonyl-l-arginine methyl ester tromethamine at different wave lengths, as previously described by Hummel, and a pH stat was used to determine the amount of acid released during alpha-p-toluene sulfonyl-l-arginine methyl ester hydrolysis, a procedure essentially the same as previously described by Engel and his colleagues.

RESULTS

The use of increasing amounts of thrombin to clot the same amount of plasma resulted in a proportionally increased incorporation of thrombin ^{131}I into the clot. The relative amount of thrombin adsorbed, however, remained the same, no matter how much thrombin was used. It represented approximately 35 per cent of the thrombin activity initially added to the plasma. When plasma was diluted to obtain fibrinogen concentrations ranging from 1.5 to 5 milligrams per clot, the amount of

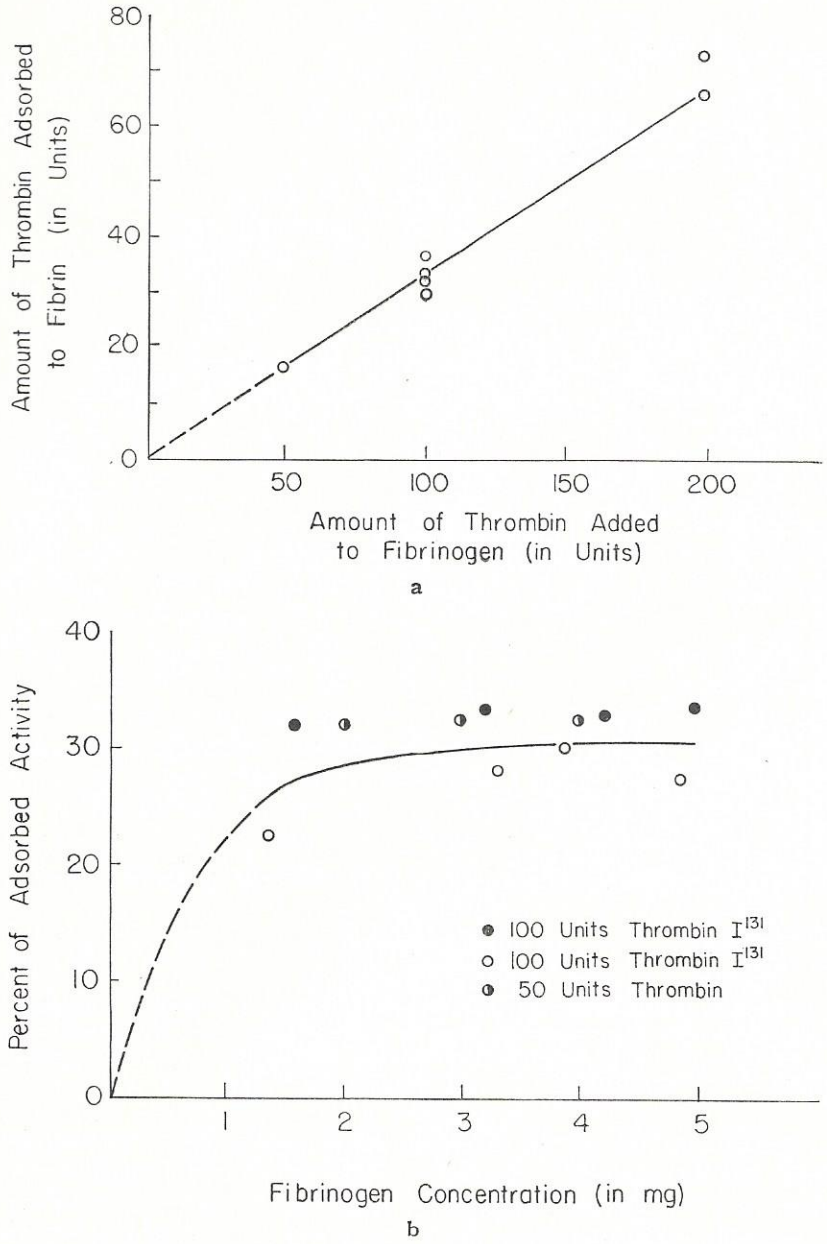

FIG. 1. Thrombin adsorption by a fibrin clot. a, The number of counts in the clot increases proportionally to the amount of radioactive thrombin added. b, The percentage of radioactive thrombin included by the fibrin is not related to the fibrinogen concentration.

thrombin included by a washed fibrin clot was the same irrespective of the quantity of thrombin added (Fig. 1).

The thrombin concentration of a thrombin-buffer solution, when compared to that of a thrombin-buffer-serum mixture at zero time, was found to be the same. Although no decline in the thrombin concentration of a thrombin-buffer mixture was observed during a five to six hour incubation period at 37 degrees C., a definite decline upon the incubation of thrombin

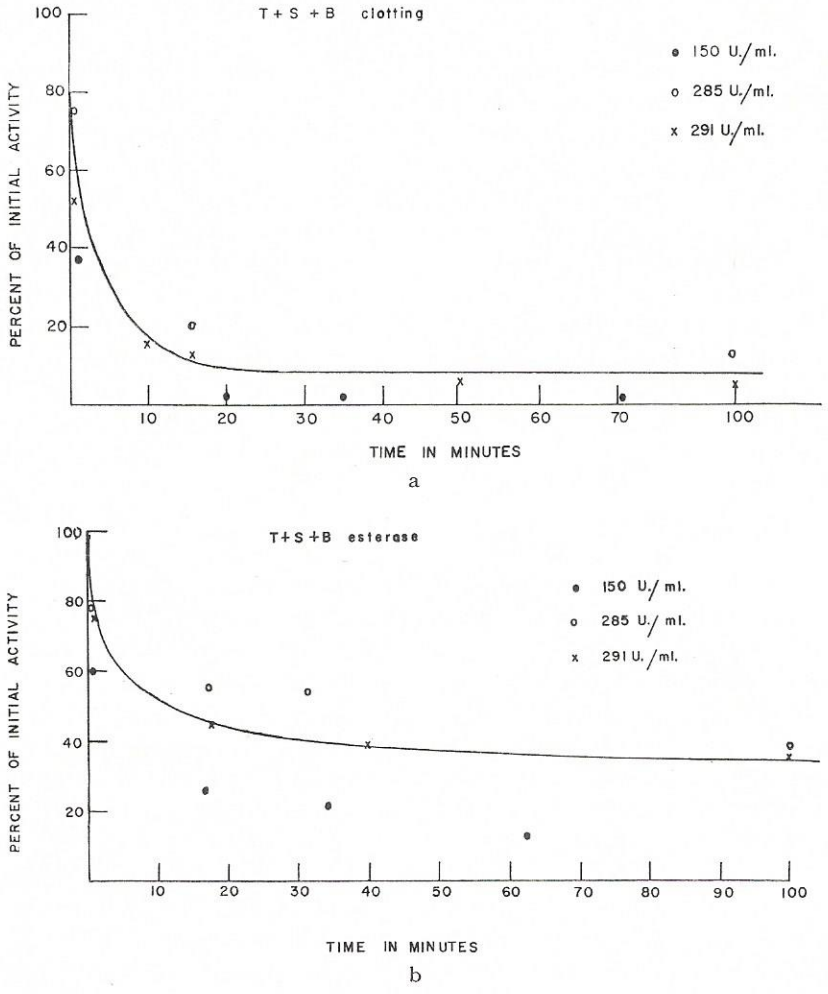

FIG. 2. In vitro disappearance of thrombin in the presence of antithrombin. a, Thrombin clotting activity. b, Thrombin esterase activity. (T+S+B=thrombin+serum+buffer.) Thrombin concentrations: 150, 285, and 291 units per milliliter of reaction mixture.

with serum or defibrinated plasma was noted. The extent of the decline of the thrombin activity is a function of the antithrombin activity of the serum sample.

After one to two hours, incubation of serum and thrombin in different concentrations at 37 degrees C., loss of clotting activity is virtually complete. In contrast, one-third to one-half of the original esterase activity usually remains intact for a considerable time (Fig. 2). This not only suggests that esterase activity is less sensitive to the antithrombin action but also that with the aid of serum or a purified antithrombin preparation, thrombin esterase and thrombin-clotting activity can be separated. The esterase-saving effect is not greatly altered by heparin (Fig. 3) which accelerates the rate of the initial disappearance of the esterase activity without affecting the total amount of enzyme neutralized. In fact, heparin which greatly accelerates the inhibition of thrombin-clotting activity in serum, affects thrombin esterase activity slightly (Fig. 4).

DISCUSSION

Thrombin is bound onto fibrin. Although previously described by Seegers (17–19) as

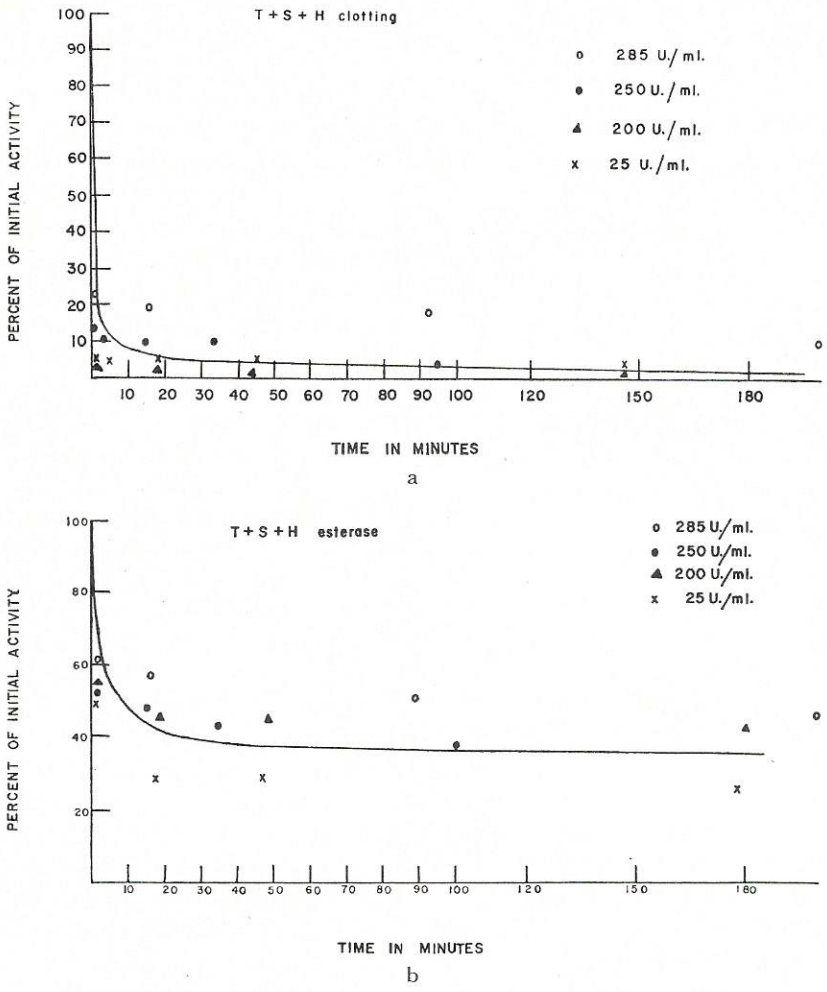

FIG. 3. In vitro disappearance of thrombin in the presence of heparin and antithrombin. a, Thrombin-clotting activity. b, Thrombin esterase activity. (T+S+H=thrombin+serum+heparin.) Thrombin concentrations: 25, 200, 250, and 285 units per milliliter of the reaction mixture.

well as by Quick and Favre Gilly, qualitative and quantitative features of this reaction remain to be established. The observation that part of the adsorbed activity can be washed from the clot, only to appear in the washing saline solution, suggests that the original quantity bound to fibrin must exceed the quantity of thrombin which we found still attached to the clot after several washings. It may, therefore, be assumed this represents thrombin that is tightly bound to fibrin. In contrast, the thrombin lost during the washing presumably represents thrombin that was loosely attached, which might also be easily lost in vivo as during clot retraction. Since the amount fixed in this manner is not dependent of the fibrinogen concentration but only upon the thrombin concentration, it would appear that the fixation of thrombin represents a property of the thrombin molecule rather than of fibrinogen or fibrin. The stickiness of the thrombin molecule to different surfaces, particularly glass, macrophages, yeast cells, and platelets, has previously been alluded to and was confirmed in the present study for fibrin. This material is presumably no longer available for clotting. In addition to binding of thrombin by

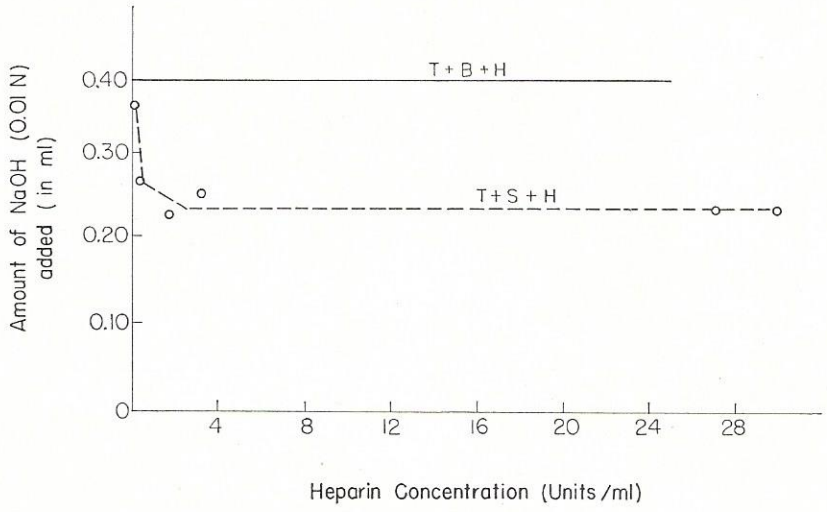

FIG. 4. Effect of heparin on thrombin esterase activity.

fibrin, thrombin activity is also neutralized by the antithrombin activity of the blood.

The results of these experiments indicate that during a one to two hour incubation period of serum with different concentrations of thrombin at 37 degrees C., thrombin-clotting activity virtually disappears. In contrast, one-third to one-half of the original esterase activity remains intact for a considerable time. The esterase-saving effect is not greatly altered by heparin which accelerates the rate of the initial disappearance of the esterase activity without affecting the total amount of enzyme neutralized. In fact, heparin which greatly accelerates the inhibition of thrombin-clotting activity in serum affects the thrombin esterase activity only slightly.

The close physical relationship between fibrinogen and plasminogen was previously observed. In fact, this association is so close that gel filtration on a Sephadex® column is required to effect their separation. This possibly explains why, under physiologic conditions, plasminogen remains intimately associated with the fibrin that forms the framework of a clot or thrombus, as was previously demonstrated by Astrup and Mullertz.

Considerable amounts of serum are sequestered in the interstices of a blood clot or thrombus. The antithrombin content of this serum is not likely to be rapidly renewed. Since there is no reason to assume that the reactions observed in the test tube are any different from those occurring in vivo, it would appear that after its adsorption onto fibrin, thrombin-clotting activity is rapidly lost after contact with the serum-containing antithrombin. In contrast, part of the esterase activity persists. Thrombin esterase activates plasminogen both in vivo, as was demonstrated by Seegers (17–19), as well as in vitro, as was subsequently shown by Engel and his colleagues. The activation of plasminogen could well represent the mechanism whereby clots and thrombi spontaneously disappear. The fact that plasma clots, formed in the test tube after the addition of thrombin, lyse without demonstrable plasminogen activator activity in the plasma lends support to this concept.

One point requires further comment. As the results of these studies indicate, the amount of thrombin adsorbed onto fibrin is proportional to the amount of thrombin generated and independent of the plasma fibrinogen concentration. Hence, it would appear that the plasma fibrinogen concentration of the patient is one significant factor in determining the rate of clot resolution. Rapid disappearance would be expected to

occur in clots formed in plasma with relatively low fibrinogen levels, while conversely, slower disappearance could be anticipated if the plasma fibrinogen level of the patient was to be high. Since plasma fibrinogen levels vary markedly in different individuals, or even in the same individual, under different circumstances, it would seem significant to consider differences in the plasma fibrinogen concentration as a possible cause for the vagaries that surround this phenomenon. Although subject to rapid changes, it seems advisable to obtain fibrinogen determinations in patients with thromboembolism to establish further evidence for the existence of the postulated relationship. Other factors that play a role in affecting the rate of clot resolution would be the plasma antithrombin level, the plasminogen concentration, and the residual thrombin esterase activity in the clot. The fact that the described process is not adversely affected by heparin lends it practical significance. Patients who are not in a state of shock after pulmonary embolism are rapidly heparinized. This procedure accelerates the inactivation of thrombin-clotting activity only. To promote the resolution of the clot, additional attempts at urokinase therapy seem to be warranted in those instances in which fibrinogen concentrations are high.

SUMMARY

The mechanism of spontaneous resolution of thromboemboli remains to be explained. Results of our studies indicate that during clot formation part of the thrombin generated is adsorbed by fibrin. Its clotting activity is short-lived. In contrast, its esterase activity persists.

Previously, it was shown that thrombin esterase activates plasminogen. Large quantities of plasminogen are present in clots. Resolution of thromboemboli appears to result from plasminogen activation by thrombin esterase in the clot due to several factors.

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