The Mechanism of Fibrinolysis Induced by Bacterial Pyrogens*)

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It has been well known for many years that the intravenous injection of vaccines of certain bacteria causes an elevation of body temperature and induces fibrinolysis (16, 17, 22). The substance responsible for this action has been purified and identified as a lipopolysaccharide (24—26). The observation of induced fibrinolysis has been confirmed by many investigators (6, 7, 8, 10, 23). Hörder and Kickhöfen (12) checked it by thrombelastography, fibrin plates and by the splitting of TAMe. They concluded that active plasmin was present. Eichenberger and coworkers (6, 8, 23) already speculated that it could be a cytofibrinolysokinase which activates the fibrinolytic system after the injection of pyrogens. Leucocytes may be involved in this mechanism. Von Kaulla (13, 14) proved the presence of an activator of plasminogen in plasma after injection of pyrogens. However, the exact mechanism of induction of fibrinolysis remained unclear until now. The following experiments were performed to try and clarify this mechanism.

Materials and Methods

1. Plasma and/or serum was collected 90 minutes after the first intravenous injection of 0.4 to 0.5 μg of purified bacterial pyrogens (Pyrexal, a lipopolysaccharide from Salmonella abortus equi) from patients of the second department of gynecology, who had received pyrexal for the treatment of chronic adnexitis. This amount of pyrogen is sufficient to produce fibrinolysis with a great degree of certainty. The relation between dosage and action is discussed elsewhere (10).

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2. Thrombelastography according to the method of Hartert was used as a crude test for fibrinolysis. Samples were only used for this study when they proved to be active by this method.

3. Bovine fibrinogen was prepared according to the method of Astrup and Müllertz (2).

4. Bovine plasminogen was prepared according to the method of Remmert (21), lyophilized, and used in a concentration of 10 mg/ml.

5. Proactivator was prepared from human milk according to the method of Astrup and Sterndorff (3), lyophilized, and used in a concentration of 1 mg/ml.

6. Thrombin-Roche was used in this study.

7. Streptokinase used was the commercial preparation of Varidase-Lederle in a concentration of 300 U/ml.

8. The standard fibrin plate method of Astrup (2) and the heated fibrin plate method of Lassen (15) were used throughout this study for the determination of the factors of the lytic system. We followed the original methods with the exception that the plates were heated to 90° C for 90 minutes to be sure that the plasminogen was destroyed.

The euglobulins of plasma or serum were precipitated by dilution and acidification with 1% acetic acid to pH 5.3. The precipitate was dissolved in half the volume of barbital buffer (Owren) pH 7.2 with 1/10 volume of 1.33% sodium oxalate solution added to prevent clotting of the samples. Before being applied to the plates the euglobulin solution was diluted 3 times by barbital buffer pH 7.2 and/or the reagents necessary for the determination of the individual factors. Plasma and/or serum were examined without precipitation only in a few cases.

a) *Plasmin*: was determined on heated fibrin plates without any additions.

b) Determination of *plasminogen*. To 1 ml of the redissolved euglobulin fraction of plasma or serum 0.1 ml streptokinase (300 U/ml) and 1.9 ml buffer were added. The mixture was tested on heated fibrin plates. An increase in the lysed area compared with the area on heated plates without streptokinase added was a measure for the plasminogen, provided that there was the necessary amount of proactivator present.

c) The determination of *activator* could be performed on standard fibrin plates. This would have been easier. However, the size of the areas lysed by the same amount of plasmin on heated and on standard plates was different. This made quantitative estimations of the activities difficult. Therefore we preferred to use heated plates. A standardized amount of bovine plasminogen (10 mg/ml) was added to 1 ml of the euglobulin solution and 1 ml buffer. The mixture was tested immediately.

d) The estimation of *proactivator* was performed on heated fibrin plates after addition of plasminogen (10 mg/ml) and streptokinase (300 U/ml) to 1.0 ml of the sample. The increase in the lysed area compared with the area lysed on heated plates without streptokinase added was an estimate of proactivator.

e) The presence of a *fibrinolysokinase* was tested on heated fibrin plates after addition of human proactivator (1 mg/ml) and of bovine plasminogen (10 mg/ml) to the sample. An increase in the lysed area, as compared with the area lysed without proactivator added, was an estimate of the presence of fibrinolysokinase.

7. Each run of determinations was accompanied by the following control tests.

a) Standard plates without any test material to exclude spontaneous lytic fibrinogen.

b) Human proactivator and streptokinase was placed on heated fibrin plates to prove that heating was sufficient to destroy the plasminogen in the plates.

c) Bovine plasminogen was placed on standard plates to exclude plasminogen contaminated with plasmin or activator.

d) Human proactivator with streptokinase was placed on standard plates to prove activity of the proactivator preparation.

8. Examination of the pH-resistence of the activator. The euglobulin precipitate was dissolved in twice the original volume of oxalated barbital buffer pH 7,2. 2 ml euglobulin

solution were pipetted into each of 10 tubes. The pH was adjusted to 1.0—10.0 respectively by the addition of N, 0.1 N HCl or N, 0.1 N NaOH. Each sample was divided in 4 equal parts. Each series of samples were incubated at 37, 50, 70 or 100° C respectively. After 30 minutes incubation they were rapidly cooled and neutralized with the necessary amounts of N, 0.1 N NaOH or N, 0.1 N HCl respectively. Enough barbital buffer pH 7.2 was added to make equal volumes. The samples were tested on standard fibrin plates immediately.

9. Casein digestion method was performed in some cases as described by Elsner (9).

10. Fibrinolysis of the human plasma clot: The plasma was used undiluted and clotted with thrombin. The time was observed which was necessary for complete lysis of the clot.

Results

24 different plasmas which were proved to have a high fibrinolytic activity by the preliminary testing with the thrombelastograph were used for this study.

1. The fibrinolytic activity on standard fibrin plates was very marked in all plasmas except two which were moderately active (Fig. 1).

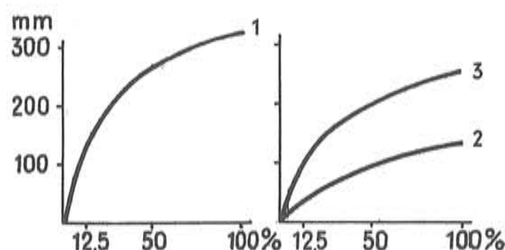

Fig. 1: Comparison of the lytic activity on standard fibrin plates (left) and on heated fibrin plates (right). The increase of the lysed area on the heated fibrin plate after addition of bovine plasminogen is an estimate of the activity of activator. 1 Euglobulin on standard fibrin plate. 2 Euglobulin solution on heated fibrin plate. 3 Euglobulin solution + bovine plasminogen on heated plate. Abscissa: Concentration of euglobulin solution. Ordinate: Lysed area in mm^2.

2. All plasmas showed distinct lytic activity on heated fibrin plates. The lysed areas, however, were much smaller than on the standard plates (Fig. 1). This proves the presence of plasmin and suggests that a great deal of the activity on the standard plates was due to the presence of an activator. At the same time the potency of some samples to split casein and to digest the clot which formed with the patient's own plasma, was tested. The amount of casein digested was relatively low corresponding to the smaller areas on the heated fibrin plates, because both methods are sensitive only to free plasmin. The plasma clot was lysed completely in a short time. This method does not differentiate between plasmin and activators.

3. Presence of *plasminogen*. The addition of streptokinase to the euglobulin solution before testing on the heated fibrin plate induced a very distinct increase

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of the lysed area (Fig. 2). This suggests that plasminogen was present in addition to plasmin. Only in 4 cases were the results negative. This could have been due to a deficiency of proactivator or of plasminogen. However, in a second test, the proactivator was found to be present, and the lysed area on the heated fibrin plates was rather great in these cases. Therefore it may be concluded, that in these 4 cases the total amount of plasminogen was transformed to plasmin. There was generally a correlation between the amount of activated plasmin and residual plasminogen.

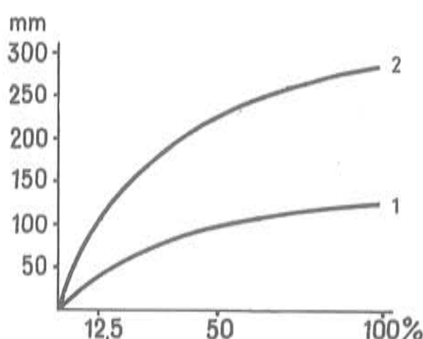


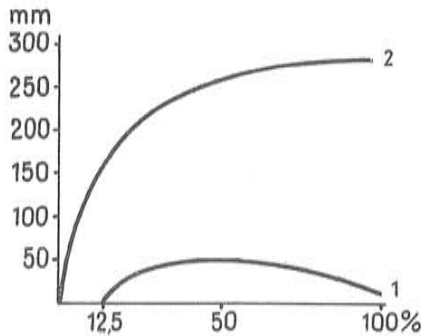
Fig. 2: Determination of plasminogen. Increase of the lysed area on heated fibrin plates after addition of streptokinase. 1 Euglobulin solution. 2 Euglobulin solution + streptokinase.

4. The increase of lysed areas on the heated fibrin plates after addition of bovine plasminogen to the euglobulin solution proved the presence of an *activator* and confirmed the results obtained with the standard fibrin plates and by lysis of the plasma-clot (Fig. 1).

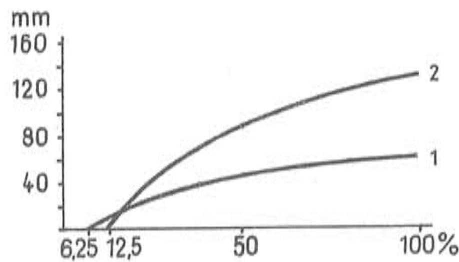
5. An further increase of the size of the lysed areas on heated fibrin plates after addition of streptokinase to the euglobulin fraction with bovine plasminogen added corresponds to the presence of *proactivator* (Fig. 3).

6. The presence of a *fibrinolysokinase* in the euglobulin fraction was tested on heated fibrin plates by addition of proactivator and plasminogen (Fig. 4). This activity was significant only in 5 cases and insignificant in two. In these cases we found only some activator activity and no proactivator activity. In all the other cases this test was negative.

7. To obtain some information on the properties of the activator, its resistance to the change of pH and of temperature was tested on standard fibrin plates. The activity remained unchanged after 30 minutes incubation at 37 and 50° C in the acid pH-range, and some increase of the activity was found at neutral reaction. At 70° it was stable at acid pH, and at 100° only a little



F i g . 3 : Determination of proactivator. Increase of lysed area on heated fibrin plates after addition of streptokinase and bovine plasminogen. 1 Euglobulin solution + bovine plasminogen. 2 Euglobulin solution + bovine plasminogen + streptokinase.



F i g . 4 : Determination of fibrinolysokinase. Increase of lysed area on heated fibrin plates after addition of proactivator and bovine plasminogen. 1 Euglobulin solution + bovine plasminogen. 2 Euglobulin solution + bovine plasminogen + human proactivator.

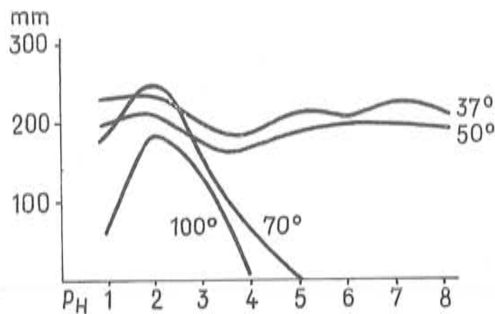


Fig. 5

activity was lost at acid pH. At neutral pH the activity was destroyed at 70 and at 100° C (Fig. 5 and 6).

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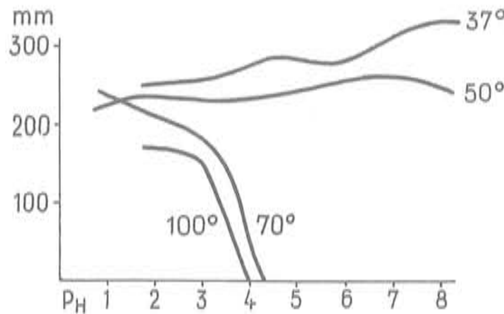

Fig. 6

Fig. 5 and 6: pH resistence of the activator at different temperatures (37, 50, 70 and 100° C) incubated for 30 minutes. Abscissa: pH. Ordinate: Lysed area in mm^2.

Discussion

A certain amount of plasmin was present in all samples included in this study. When the plasmin level was high, only a small amount of plasminogen was left. It was totally consumed only in one case. A potent activator, acting directly on plasminogen was found in all samples except five. In these cases a fibrinolysokinase was found.

There are different types of activators of the fibrinolytic system known which differ in their stability at different temperatures and pH-values. The activator which may be obtained in plasma after incubation with an excess of streptokinase is very labile especially at acid reaction, and even at neutral reaction it is destroyed at 50° C (19, 20). The proactivator in plasma is stable at pH 2—10 at 50° and only a small amount is destroyed at acid pH at 70 and 100° C (18, 20). Astrup and Sterndorff (4) found an activator in tissues which was stable at 37 and 50° at pH-values from 2 to 10, and was not destroyed at acid pH at 70 and 100° C. In spontaneous lytic blood the activator behaved differently. It showed maximal stability at neutral pH after incubation at 37 and 50°, but some activity remained at acid pH after incubation at 70 and 100° C. The activity obtained in human plasma after addition of small amounts of streptokinase behaved like spontaneous lytic blood (20), whereas, the activator in menstrual blood behaved like tissue activator (1).

In our experiments the activator activity remained stable during incubation at 37 and 50° C from pH 2.0 to pH 8.0, The activity was insignificantly higher at neutral pH and after incubation at 37° C (Fig. 6). At 70° and pH 2.0 it was as stable as at 50°. At neutral pH it was destroyed. At 100° C it lost some

activity at acid reaction and was destroyed at pH higher than 4.0 (Fig. 5). This behaviour is very similar to that of the activator in menstrual blood described by Albrechtsen (1). The properties of our activator are comparable with those described for the stable fibrinokinase. The slight elevation of activity at neutral reaction after incubation at 37° C may be caused by the presence of a small amount of a labile type of activator.

These results suggest, that two different ways of activation of the plasminogen may be induced by injection of bacterial pyrogens. The most common way is an activator of the type of a stable fibrinokinase which activates plasminogen directly. In the other type a fibrinolysokinase activates the proactivator of the plasma, which activates the plasminogen.

Our results are in good agreement with the observations of Hörder (12) who found plasmin in blood samples after the injection of pyrogens, and with von Kaulla (13, 14) who observed the presence of an activator. The properties of the activator after pyrogen injection have not been studied until now as far as we know. However, the activator in spontaneous active blood after anoxia and electrical shock seemed to behave a little differently (20).

As far as the source of the activators is concerned, nothing has been known up until now. In the first hour after the injection of a bacterial pyrogen a leucopenia of moderate degree developes (11, 12). The other effects cannot been observed earlier than after a lag period of 60 to 90 minutes. In the most cases fibrinolysis starts earlier than the temperature rises (10, 13, 14, 23). In this time, the "extrinsic" pyrogens are converted to the so called "intrinsic" ones. Cranston and coworkers (5) found, that the lag period may be shortened considerably if the pyrogens are incubated for several hours with blood before being injected. This effect is not observed when cell free serum is used. The authors conclude that the formation of intrinsic pyrogens may also be possible in vitro. To study whether a similar mechanism is involved in the development or fibrinolysis we incubated pyrexal with citrated blood under the same conditions as Cranston (5) and tested the fibrinolytic activity at intervals. Free plasmin could be detected on heated fibrin plates after 2 to 3 hours (Table). The incubation of serum with pyrexal did not induce any activity. These results suggest that a similar mechanism in which cells, probably leucocytes, are involved, may be responsible for the activation of fibrinolysis and for the conversion of extrinsic to intrinsic pyrogens. For this reason, fibrinolysis begins earlier after the injection of extrinsic pyrogens than the temperature rise, whereas, both begin at the same time after injection of intrinsic pyrogens as Hörder (12a) observed. The leucocytes may be the source of the activators as was already suggested by Eichenberger (6, 8).

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The results of our in vitro experiments differ from those of Eichenberger (7) and von Kaulla (14) who could not induce fibrinolysis by incubation of blood and pyrogen in vitro. However, our dosage was, like that of Cranston (5), many times higher, which may be the reason for the difference.

Development of fibrinolytic activity in vitro

Type of material incubated	Type of Plate tested	Incubation Time in Hours								
		0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4
		lysed area in mm^2								
Exp. 1 citrated blood	heated	0	0	0	0	68	58	36	33	32
	standard	0				130				
Exp. 2 citrated blood	heated	0	0	0	0	0	0	60	30	33
	standard	0						85,5		
Exp. 3 Serum	heated	0	0	0	0	0	0	0	0	0
	standard	0				0		0		

The mechanism of activation of fibrinolysis by pyrogens differs from that induced by peptone which acts on serum without interaction of cells (20a).

Summary

1. The mechanism of the activation of plasmin in blood after intravenous injection of bacterial pyrogens was studied.

2. There was plasmin and an activator present in the spontaneous lytic blood samples. In cases with a high level of plasmin a low amount of plasminogen was found and vice versa.

3. There seem to be two mechanisms of activation. In the most cases the activator has the properties of a fibrinokinase. In the other type a fibrinolysokinase was found.

4. The resistence of the activator against changes in pH and influence of temperature was studied. It was found that the activator was very resistent at acid pH, and, therefore, it shoud be classified as a stable type activator. In addition to this a small amount of a labile type activator seems to be present.

5. Some fibrinolytic activity develops in vitro if the pyrogens are incubated with cell-containing plasma or blood. It is supposed that the leucocytes are involved in the activation mechanism.

Résumé

1. Etude du mode d'activation de la plasmine après injection intraveineuse de pyrogène d'origine bactérielle.

2. Les échantillons de sang ayant une action lytique spontanée contiennent de la plasmine et l'activateur. Une haute concentration de plasmine est accompagnée d'un taux abaissé de plasminogène et viceversa.

3. Il y aurait deux mécanismes d'activation: dans la majorité des cas l'activateur a les propriétés d'une fibrinokinase. Dans d'autres cas il s'agit d'une fibrinolysokinase.

4. L'étude des variations de température et du pH sur l'action de l'activateur a révélé que cette enzyme est très résistante au pH acides et doit, en conséquence, être classifiée comme un activateur du type stable. On peut démontrer en plus la présence d'une faible quantité d'activateur du type labile.

5. Une certaine action fibrinolytique se dévelope in vitro après incubation de pyrogène avec du plasma ou du sang contenant des éléments figurés. Il est possible que les leucocytes sont impliqués dans le mécanisme d'activation.

Zusammenfassung

1. Es wurde der Mechanismus der Entstehung der fibrinolytischen Aktivität im Blut nach der Injektion von bakteriellen Pyrogenen untersucht.

2. In den spontan lytischen Proben fand sich neben Fibrinolysin ein Aktivator. Eine hohe fibrinolytische Aktivität ging mit einer geringen Menge Profibrinolysin einher und umgekehrt.

3. Es wurden zwei Mechanismen der Aktivierung gefunden. Bei dem einen war ein Aktivator vom Typ der Fibrinokinase, bei dem anderen, selteneren, ein solcher vom Typ der Fibrinolysokinase nachweisbar.

4. Die Untersuchung der Resistenz des Aktivators gegen Änderung der Wasserstoffionenkonzentration und verschiedene Temperatureinflüsse zeigte, daß es sich um einen stabilen Aktivator handelt. Neben diesem dürfte aber auch eine geringe Menge des labilen Aktivators vorhanden sein.

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5. Auch bei der Inkubation von zellhaltigem Plasma oder Blut mit dem Pyrogen in vitro entsteht fibrinolytische Aktivität. Es wird vermutet, daß die Leukozyten bei der Entstehung dieser fibrinolytischen Aktivität eine Rolle spielen.

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