THE NATURE OF THE FIBRINOLYTIC SUBSTANCE OF THE PLAGUE BACILLUS

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 53, No. 4,
pp. 87-91, April, 1962
Original article submitted May 5, 1961

The fibrinolytic properties of the plague bacillus have been attributed by many researchers to the presence of "fibrinolysins" in filtrates of broth cultures [5, 24]. It has recently been shown, however, that the fibrinolytic activity of the plague bacillus is associated with the cell of the microorganism, and is not found to any appreciable extent in the nutrient medium [12].

The object of the present investigation was to study the chemical nature of the fibrinolytic substance of the plague bacillus.

EXPERIMENTAL METHOD

In order to determine the chemical components of the cell with which its fibrinolytic activity was associated, the microorganisms were fractionated by different methods. Avirulent vaccine strains 1, 17, and EV were used for fractionation, because the fibrinolytic activity of the avirulent and virulent strains of the plague bacillus is the same.

Because the lytic action of the plague bacillus on the purified fibrinogens of different species of animals is almost the same [3], in the present research we used fibrinogens from the horse and ox, for there is no difficulty in obtaining the blood of these species of animal. The fibrinogen preparations were obtained by Astrup's method [14]. The fibrinogen concentration in the solution was determined by a modification of Morrison's technique [1].

Thrombin was prepared from ox plasma by E. K. Zabolotnykh's method [13]. The preparation was not purified with acetone, for the thrombin was used only to assess the results of the fibrinogenolysis reaction.

In view of the identical results obtained from determination of the fibrinolytic activity of the plague bacillus by means of the reactions of fibrinolysis and fibrinogenolysis, we used fibrinogen as substrate of the reaction in our experiments. To 0.2 ml of different dilutions of suspensions of microorganisms or their fractions (in the case of water-soluble fractions, their solutions) was added 1 ml of a 0.1% solution of fibrinogen, and the mixture was incubated at 37° for 16-18 hours. To read the results, a drop of thrombin was added to the mixture. A positive reaction was shown by inability of the mixture to gelatinize, while in the absence of a fibrinolytic action a fibril clot was observed to form. The unit of fibrinolytic activity of the preparation was taken to be the smallest amount to give a positive reaction. The activity was expressed in units per 1 mg air-dry weight of the preparation.

It was pointed out above that the fibrinolytic substance of the plague bacillus is associated with the cell of the microorganism and does not pass into the surrounding medium. In order to extract this substance from the bacilli we used one of the most widely used methods of fractionation of the plague bacillus, namely Baker's method [16, 17]. In this method the bacterial mass is treated with a 2.5% solution of common salt at room temperature. Extraction is considerably improved by constant and energetic agitation of the suspension on a shaker. When treated in this way, the capsular substances and toxin pass into the extract along with other substances, and they may be obtained in a purified form by successive precipitation with ammonium sulfate.

EXPERIMENTAL RESULTS

The investigations showed that fractions of the membrane antigen and toxin do not possess fibrinolytic activity. The whole, unpurified extract showed only traces of fibrinolytic activity, whereas the water-insoluble bacterial residue after extraction with salt preserved its initial activity, thus confirming our opinion that fibrinolytic activity is connected with the cell of the microorganism.

Because the attachment of the fibrinolytic substance to the water-insoluble bacterial products interferes with the study of the chemical nature of this substance, we subsequently extracted it by methods breaking down the complexes of the cell contents. In the first place we used phenol for this purpose, for this causes total destruction of the cell at a comparatively low temperature (40-50°) without affecting its chemical components, and at the same time it dissolves proteins and polysaccharides [6, 9].

When treating the bacilli with phenol we used the methods of A. P. Konikov [6] and Westphal, by means of which Davis [19] obtained the lipopolysaccharides of the microorganism of pseudotuberculosis, and we also treated whole bacilli with phenol in accordance with the scheme suggested by V. I. Tovarnitskii [9] for decomposition of an antigen complex.

Irrespective of the method of phenol treatment, the fibrinolytic activity was preserved only in those breakdown products consisting mainly of proteins. This activity, however, was measured in hundredths of the activity of intact bacilli. The decrease in the fibrinolytic activity of the microorganisms in the course of phenol treatment indicates destruction of the fibrinolytic substance and demonstrates that these phenolic methods are unsuitable for the extraction of this substance from the bacilli.

These results show, moreover, that the other fractions obtained by phenol treatment, containing mainly polysaccharides, possessed no fibrinolytic activity. To test this hypothesis, instead of using phenol, we obtained polysaccharides from the microorganisms by means of hydrolysis with dilute acetic acid, as described by Friedman, Morgan, and White [9]. The solution into which the polysaccharides usually pass during hydrolysis of the bacilli does not give a fibrinolytic reaction. It may be assumed from these experiments that fibrinolytic activity is not connected with the polysaccharide breakdown products of plague bacilli.

The same assumption can also be made in respect of the lipids, for the fibrinolytic activity is fully preserved in the microorganisms not only after treatment with acetone and ether, but also after removal of their free and combined lipids by Anderson's method [8], and after prolonged boiling of ether-treated bacilli with alcohol.

Since hydrolysis with dilute acetic acid and treatment of the bacilli with phenol were unsuitable for extraction of the fibrinolytic substance from the plague bacillus, we used other methods. These showed that the fibrinolytic substance may be extracted with urea and potassium thiocyanate.

We carried out extraction with potassium thiocyanate by the method used by Amies [10]. The microorganisms were treated at 37° for 5 hours with a 0.5 M solution of potassium thiocyanate, the pH of which was maintained at the level of 7.6-7.8. The activity of the extract was 100-200 units, whereas the activity of the whole bacteria varied between 200 and 1000 units/mg weight.

The fibrinolytic activity passed into the solution also when the bacilli were treated by Walker's method [29]. The activity of the extracts obtained by extraction with a 15% aqueous solution of urea at 38° for 9 hours was 50-200 units/mg of preparation. This preparation, moreover, was toxic: the dose causing death of 50% of albino mice weighing 16-18 g when inoculated intraperitoneally was 10 μg.

It will be shown below that the coexistence of properties such as fibrinolytic activity and toxicity in the urea extracts is no indication that the substances responsible for these properties are identical in nature. In particular, the absence of fibrinolytic activity in the toxins obtained by Baker's method points to a difference between these substances. This combination does exist however, in other bacteria. For instance, the fibrinolytic activity of staphylococci is evidently associated with staphylococcal toxin, since the method of precipitation of toxin and fibrolysin from the filtrates of broth cultures is the same, and furthermore, the fibrinolytic activity of staphylococci may be neutralized by ordinary antitoxic serum [2].

Reliable information concerning the existence of two different substances responsible for the toxicity and fibrinolytic properties of the plague bacillus were obtained in experiments to separate the crude complex obtained by Walker's method into fractions. By the successive addition of increasing concentrations of ammonium sulfate (20, 35, 50, and 80% saturation) to the crude extract, 4 fractions were obtained. After lyophilization, all were readily soluble in distilled water and physiological saline: the fraction obtained at 20% saturation gave a strongly opalescent solution, and the solutions of the subsequent fractions gave a less intensive opalescence; the fractions obtained at 50 and 80% saturation gave transparent solutions. The fibrinolytic activity was concentrated in the first fraction and amounted to 1000 units/mg of preparation; the activity of the second fraction was 100 units/mg, and the following fractions were completely inactive. The toxicity of the fractions to albino mice weighing 18 g, when injected

intraperitoneally, varied in the opposite manner. The fraction ob tained at 20% saturation was the least toxic (LD_{50} = 100 and 136 μg), and that obtained by saturation to 50% with ammonium sulfate was most highly toxic (LD_{50} = 6.7 and 8 μg). The results of these experiments confirm the view that the fibrinolytic activity of the plague bacillus is unconnected with its toxin.

By analysis of these results we can form some idea of the chemical nature of the fibrinolytic substance of the plague bacillus.

As we have pointed out above, the fibrinolytic activity is associated neither with the lipids nor the carbohydrate breakdown products of the plague bacillus. After treatment with phenol, partial activity is preserved only in those products which are protein in nature. Hydrolysis of the bacilli with a 0.1 N solution of acetic acid at 100° for 1½ hours leads to complete loss of fibrinolytic activity. In this case the fibrinolytic activity is evidently lost as a result of the destruction of a particular complex by acid during heating, and not as a result of thermal denaturation, since the fibrinolytic substance of the plague bacillus is a thermostable compound. The activity of the bacteria or of their individual fractions is preserved when they are heated on a boiling water bath for 1½–2 hours.

The thermal stability of the fibrinolytic substance of the plague bacillus does not exclude the possibility that it is protein in nature, for the degree of stability of proteins, i.e., their power of denaturation, varies within wide limits, and we know of resistant proteins such as insulin and ribonuclease, which can withstand boiling for several minutes at pH 3.0 [26].

On the other hand, the thermal stability of the fibrinolytic substance of the plague bacillus simply means that it can maintain its activity during heating, and it is not proof that denaturation of this substance does not occur. The preservation of the activity of proteins (enzymes) after denaturation has been described in the literature. Such observations have been made in respect of papain [27] and ribonuclease [11], in which the polypeptide bonds can be broken without loss of activity. It has been shown similarly that the partial denaturation of aldolase by the action of sulfhydryl compounds not only is not accompanied by loss of activity, but the activity may actually increase [28].

The fibrinolytic substances of other bacteria, which are protein in nature, are also thermostable. Purified streptokinase, for example, is a simple protein, the fibrinolytic activity of which is only partially destroyed after heating to 100° [7]. There are reports that staphylokinase possesses equally high thermal stability [23].

Proteins may be resistant to all forms of denaturation except alkaline [26]. Indirect proof of the protein nature of the fibrinolytic substance of the plague bacillus is therefore afforded by its destruction by hydrolysis with 0.1 N alkali at 37° for 24 hours, and its stability during hydrolysis with 0.1 N acetic acid under the same conditions.

This association between the fibrinolytic substance and the cell of the microorganism suggests that the fibrinolytic activity is a property of the structural proteins of the bacterial cytoplasm. In this respect the fibrinolytic substance of the plague bacillus has much in common with the tissue plasminogen activator, which is found in animal tissues [12, 25]. Tissue plasminogen activator is a very stable compound found, like the fibrinolytic substance of the plague bacillus, in insoluble tissue proteins. It cannot be transferred into solution by ordinary methods, but may be extracted by potassium thiocyanate solution [13, 15].

This association with the cell of the microorganism is an interesting feature of the fibrinolytic substance of the plague bacillus, distinguishing it from other fibrinolytic substance of bacterial origin, such as strepto- and staphylokinase, which are secreted during the vital activity of these microorganisms into the nutrient medium, and may be obtained by precipitation from the filtrates of broth cultures [7, 18, 20, 21, 22].

SUMMARY

Earlier it was shown by the authors that the fibrinolytic activity of the plague microbe is connected with the microbial cell. By fractionation of bacteria with the aid of various methods it was demonstrated that the fibrinolytic activity is retained in those degraded products which are mainly represented by proteins. The connection of the fibrinolytic substance with the microbial cell enables to suggest that fibrinolytic activity is characteristic of the structural proteins of bacterial cytoplasm. The fibrinolytic substance is extracted from bacteria by urea and potassium thiocyanate solutions. By means of successive precipitation with ammonium sulfate the plague microbe toxin and an active fraction (fibrinolytic ally) may be obtained separately from the crude extracts.

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