LYSIS OF HUMAN AND ANIMAL FIBRINS BY THE PLAGUE MICROBE

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The incompleteness of our knowledge concerning the mechanisms allowing the unobstructed multiplication and spread of the plague agent in the host organism has forced the present authors to turn their attention to the study of fibrinolysins of the plague microbe, which to the present time have attracted the attention of only a few investigators. The first investigation of the fibrinolytic properties of the plague microbe was undertaken by Madison [16], who utilized the serum-free human and animal fibrin. In accordance with his data, the most sensitive to the action of the plague microbe fibrinolysin factors was rat fibrin, approximately six times less sensitive were human and guinea pig fibrins, even less sensitive were the fibrins of the ground squirrel and the cat, rabbit, cow, and finally, monkey. Horse, ram, and pig fibrins, according to Madison, were not lysed by the plague microbe.

Data concerning the presence of fibrinolytic properties in the plague microbe have been confirmed by a number of works [5, 6, 7, 11].

The research method for fibrinolytic properties, utilized by the authors of the mentioned studies, was disadvantageously different from Madison's method; the substrates for the action of the fibrinolytic factor were plasma clots rather than purified fibrin. The source of fibrinolytic activity, as in Madison's work, was a broth culture of the plague microbe.

By utilizing the mentioned method, A.S. Kvashina [5] has studied 20 cultures and found out that not all of the plague microbe cultures could dissolve human and animal plasma clots. Part of the cultures (6 out of 20) did not possess these properties. Without regard to the presence of fibrinolytic properties in the avirulent culture EV, the author arrived at the conclusion that the "avirulent cultures of Bact. pestis do not dissolve blood fibrin" and that the ability to produce fibrinolysin was resident in virulent cultures.

The data of A.S. Kvashina concerning the intensity of plasma fibrin dissolution from different animal types did not agree fully with Madison's results; Rat plasma fibrin appeared less sensitive to the fibrinolytic factor of the plague microbe in comparison to human and guinea pig fibrins. On the other hand, blood plasma clots from animals such as the horse and the ram, in a way similar to Madison's data, were not lysed even by the most active cultures. This permitted the author to compare the degree of animal susceptibility to plague infection with the susceptibility of blood fibrin to the fibrinolytic factor of the plague microbe. Here A.S. Kvashina referred to Hadfield's data[14]concerning the point that "the stability of blood fibrin is an indicator of unreceptivity of the animal to the corresponding infection".

Sera of animals immunized with cultures of Bact. pestis neutralize the action of the fibrinolytic factor from the plague microbe. This action of immune sera [6] is related to the presence of antifibrinolysins, "accumulation of which takes place gradually in a way similar to the formation of antibodies". However, these data do not solve the question concerning the nature of antifibrinolytic action of the immune sera, since immunization of the animals was conducted with whole microbial cells and not with a purified plague microbe fibrinolysin.

To the plague microbe fibrinolytic factor there is relegated a specific role in the pathogenesis of plague sickness. On the basis of literature data, and also on the basis of his own investigations, V.N. Lobanov [9] indicated that during plague sickness the inflammation foci contained no fibrin, or it was present in only small quantities. The author assumed that in the complex process of fibrinolysis, the fibrinolytic factor was not the only cause; however, its presence was without doubt.

The fibrinolytic factor dissolved the exudate fibrin from the inflammation focus, and thanks to this, the plague microbe spreads easily from the locus of penetration and from primary foci [4]. The opinion has been expressed that the value of fibrinolysins as pathogenic factors is exaggerated [3].

The nature and the mechanism of action of the plague microbe fibrinolytic factor have not been studied. Besides, the variability of results obtained by various authors dictated the necessity for a comparative investigation of the susceptibility of serum-free fibrin and plasma fibrin to the plague microbe fibrinolytic factor.

In the present investigation plasma and purified fibrins were utilized as substrates for demonstrating the fibrinolytic action of the plague microbe.

EXPERIMENTAL METHODS

In order to prepare blood plasma, the blood was mixed at bleeding with an equal volume of 1% solution of sodium citrate, or to 9 volumes of blood there was added 1 volume of 2.5% solution of potassium oxalate. The oxalated or citrated bloods were diluted with a physiological solution at the ratio of 1:5 and centrifuged. The plasma was stored at 5°.

Whole oxalated plasma generally served as the source of fibrinogen. The exception was human plasma, which was obtained from stabilized (stabilizer No. 7) donor blood.

Dry fibrinogen preparations were prepared by a single-stage precipitation with a saturated solution of sodium chloride and subsequent dialysis of the moist precipitate against a 0.5% solution of sodium citrate [8]. The fibrinogen that passed over into the solution was poured into ampoules and dried from the frozen state. The dry fibrinogen was also stored at 5°. Immediately prior to the experiment, the dry preparation was dissolved in a phosphate buffer (M 1/15, pH 7.4) and following fibrinogen determination, there was prepared a 0.1% solution.

The amount of fibrinogen was determined by Weinberg's method [2], as modified by the authors.

Cleaner fibrinogen preparations, relatively free from albumin admixtures, were prepared by Astrup's method * [12]. After a preliminary removal of plasma prothrombin (adsorption with trisubstituted calcium phosphate), fibrinogen was twice precipitated with ammonium sulfate. The precipitate was dissolved in veronal buffer (pH 7.8, ionic strength 0.05), and a preparation containing 5-10 mg of fibrinogen per ml was obtained. Such fibrinogen solutions were stable for two weeks of refrigerator storage.

Fibrinogen was coagulated by thrombin. The latter was obtained by activating the prothrombin with thromboplastin. A 5% suspension of fresh human brain in a phenolphysiological solution [17] served as the source of the thromboplastin factor. Purified prothrombin preparations were prepared from oxalated plasma by adsorbing it onto barium sulfate with subsequent washing of the adsorbent with 0.02 M acetate buffer and eluting it with 5% solution of sodium citrate. After dialysis against a physiological solution for 48-56 hours at 1-5° the eluent comprised a prothrombin preparation [10].

In the present investigation all experiments were carried out with a vaccination culture of Bact. pestis 17. In preliminary experiments it was ascertained that the fibrinolytic activity of this culture was sufficiently high.

The B. pestis 17 culture was grown at 28° for 48 or 20-24 hours on fluid and agar nutritional media. Agar media were prepared on Hottinger's broth. From among the fluid nutritional media there were utilized broths from digests of Hottinger and Martin, meat-peptone broth, and a phosphate broth according to Konikov [8], which was utilized by him for culturing of hemolytic streptococci with the idea of obtaining purified streptokinase preparations. There were conducted separate experiments with aerated broth cultures.

For the purpose of the fibrinolytic reactions, to various dilutions of the culture with the volume of 0.2 ml there were added 0.1 ml of 5% calcium chloride solution or thrombin, this depending on the substrate utilized (plasma or a 0.1% fibrinogen solution). After addition of 0.1 ml of the substrate, the contents of the test tubes were mixed rapidly and after clot formation they were placed in a thermostat at 37°. During the arrangement of the fibrinogenolytic reaction, the coagulant was added to the fibrinolysin-substrate system only after coagulation. Results of the fibrino-and fibrinogenolytic reactions were ascertained after 16 hours.

EXPERIMENTAL RESULTS

In the first experiments there were reproduced some data obtained by A.S. Kvashina [5]. The authors observed lysis of clots from plasmas of the human, the guinea pig, and the rabbit in the presence of centrifuged day-old broth cultures. Clots from plasma of the pig, ram, horse, and the bull were not lysed by even whole broth cultures. The highest fibrinolysin titres were given by poorly precipitable broth cultures; on the contrary, in those cases where the supernatant fluid was clear, lysis was limited by the first dilutions (1:2-1:4). The presence of bacteria has a direct influence on the duration of the reaction; this was completely contrary to A.S. Kvashina's opinion [5]. Further, the microbial cells were precipitated from the broth culture, and a suspension of them was prepared in a physiological solution. The results were completely clear: Microbe suspension produced lysis, and the microbe-free supernatant fluid did not possess fibrinolytic properties. It should be pointed out that in some cases plasma clots from human, guinea pig, and rabbit bloods could be partially and even completely lysed by some sterile broths, at the same time that plasma clots from other animals were always stable. This, evidently, could explain the contradictory data of A.S Kvashina concerning the filterability of the fibrinolytic factor. In the first communication A.S. Kvashina [5] noticed the ability of fibrinolysin to penetrate through biological filters; in her subsequent works she established that "after transfer through Chamberlaine [3] or Zeitz filters the fibrinolytic titre dropped sharply (from 1:32 - 1:64 to 1:1)" [6]. In the present experiments broth cul-

* The purity of fibrinogen preparations was checked by paper electrophoresis.

ture filtrates, as well as the supernatant fluid, did not produce lysis, whereas a bacterial cell suspension gave reproducible results. Fibrinolytic properties of microbes precipitated from broth cultures or grown on agar were the same. Three-fold washing of the bacteria with a physiological solution was not reflected in their fibrinolytic activity; this attested to the continuous connection of the fibrinolytic factor with the microbial cell. Fibrinolytic factor of the plague microbe was preserved after bacterial lyophilization.

The thermostability of the plague microbe fibrinolytic factor was high: Boiling in a water bath for 15-20 minutes did not lower its activity. In this respect, plague microbe fibrinolytic factor disclosed similarity to streptokinase, activity of which was lowered to one half by half hour heating at 65°; however, after further heating, even up to 100°, it was nearly unchanged [8]. Results of determinations of the thermoresistance of the plague fibrinolysins by A.S. Kvashina were completely contrary to present data: In her experiments heating at 60° for 10 minutes resulted in complete inactivation of the fibrinolytic factor [5].

Investigation of the susceptibility of the serum free fibrin and plasma fibrin to the plague microbe fibrinolytic factor exposed basic differences in the degree of lysis of their substrates.

Human and animal blood fibrins, obtained by coagulation of purified fibrinogen by homologous thrombin, was lysed by the plague microbe without regard to particular species. Plague microbe fibrinolytic factor lysed not only fibrin but also fibrinogen, which as a result of the lysis was transformed into a protein not coagulable by thrombin.

The susceptibility of unpurified human and animal plasma fibrin to the plague microbe fibrinolytic factor was different. Unpurified human, guinea pig, and rabbit plasma fibrin was lysed relatively easily by the plague microbe, whereas lysis of the unpurified pig and ram plasma fibrin could take place only in the presence of large numbers of bacterial cells, reaching several billions.

Stability against lysis during the action of the plague microbe was evidently related to the presence in animal blood serum of fibrin-protective factors, which were inhibitors of the blood fibrinolytic enzyme and are known in the literature as antifibrinolysins [1]. Concentration of fibrin-protective factors in blood of different animal types and the human differed. Thus, for example if in 1 ml of normal human plasma there were 79 antifibrinolysin units [13], then in 1 ml of bovine plasma there were 222 units [15]. In accordance with present data, the most active fibrin-protective factors were in pig and ram sera, less active in horse and bull sera. Addition of pig or ram serum to fibrin from any source made it more stable to fibrinolysin. Lability of human, rabbit, and guinea pig plasmas was possibly evidenced in a small content of fibrin-protective factors. Plasma antifibrinolytic properties, which were not observed by A.S. Kvashina and other authors, masked the true susceptibility of blood fibrin from animals unsusceptible to plague infection.

The present data let one state that there does not exist a connection between the susceptibility of various animal types to plague infection and the action of plague bacillus fibrinolysin on blood fibrin.

SUMMARY

Bact. pestis is able to lysate purified fibrin not only of human blood, but also that of animals investigated by the author (pig, sheep, horse, bull, dog, guinea pig, and rabbit). Antifibrinolysins, i.e., fibrin-protective factors, contained in the blood sera of various animals, depress fibrinolysis provoked by B. pestis. The fibrinolytic factor of B. pestis is connected with the microbial cell and does not pass into the nutritive medium.

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