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The Spread factor of the Plague Bacillus.

Communication II*. Discovery of hyaluronidase in the plague bacillus.

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In our preceding communication we showed in experiments on animals that the plague bacillus possesses a spread factor that intensifies the permeability of tissues and capillaries. The discovery of the spread factor in plague bacilli throws a different light on the mechanism of their invasive and aggressive properties. The diffusion factor undoubtedly plays a large role in the pathogenesis of plague, the basic and determining feature of which is the mass propagation of the plague bacilli in an organism.

The present work was undertaken for the purpose of determining whether active plague preparations which develop the spread phenomenon in vivo contain the enzyme hyaluronidase. This question is completely uninvestigated and we are the first to touch upon it.

The detection of hyaluronidase in vitro is based upon the fact that because of its action hyaluronic acid loses its ability to form a clot in an acid medium, its viscosity is decreased (depolymerization) and it is hydrolytically decomposed into glucuronic acid and glucosamine. Smirnova and Shtutser, Chistovich, Taratorina, Ogloblina et al. have proposed very simple and successful methods for the production of hyaluronic acid (from umbilical cords); these methods have been completely proved in practice. With a few changes, one of the proposed methods for the preparation of hyaluronic acid was used by us in our experiments.

Before conducting the primary experiment we determined the titer of hyaluronate, i. e., the minimal dose of Wharton-jelly extract that produced a clot upon addition of acetic acid. For the experiment we took double the

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minimal dose. Thus, for example, if the hyaluronate in a dose of 0.1 cubic centimeter (cc) formed a clot in the presence of acetic acid, then the working dose comprised 0.2 cc of the substratum. The specificity of the hyaluronate was checked in a test with a testicular extract (the aforesaid hyaluronidase).

The following preparations of plague cultures were tested for the presence of hyaluronidase: 1) lysates; 2) the liquid centrifuged from a crowded suspension of plague microbes that was produced from a washing of a 48-hour-old agar culture; 3) broth cultures; 4) autolysates from washed agar cultures.

We investigated the hyaluronidase content in preparations made from virulent and avirulent strains of plague bacilli. The first experiments were conducted with a suspension from a 48-hour-old agar culture of the strain EV. The suspension contained 3 billion bacilli per cc. The test proved to be unclear for the reason that the suspension of plague bacilli without a substratum (the control) also produced a sediment upon the addition of acetic acid, whereupon in the experimental test tubes the clot was larger than in the control (hyaluronate - distilled water). Therefore future experiments were conducted with extracts of plague cultures containing insignificant quantities of bacilli: with lysates, and with the fluid centrifuged from a washing of an agar culture.

A test of the lysates of virulent strains No. 100, No. 202 and also strain EV, showed that they contain a principle capable of preventing the formation of a clot from the hyaluronate in the presence of acetic acid. On the basis of the data presented in table 1 it is possible to draw the conclusion that hyaluronidase is contained in the lysates of all three strains - the highly virulent No. 100, the median virulent No. 202, and the practically avirulent EV.

Somewhat more of the hyaluronidase was contained in strain No. 100 - its lysate in an amount of 0.2 cc produces complete dissolution of the hyaluronate.

Further experiments were conducted primarily with the fluid from a washing of a crowded agar culture. The experimental method and the quantitative correlations between the components of the test were the same as in the first experiments. It is possible to produce a more or less clear fluid from a washing of a live agar culture of plague bacilli only after prolonged centrifugation. For the test we used both clear and opalescent fluids. This produced no noticeable effect on the results of the test. But what is an important factor is the concentration of the bacilli in the washed agar culture: one will not detect the hyaluronidase in the liquid suspensions. In table 2 are presented the test results of a fluid centrifuged from a wash of cultural growths of strains No. 100, [illegible] 143 (of median virulence) and EV. The concentration of the bacilli per cc was 100 billion, 105 billion and 102 billion, respectively. The wash was kept on ice for 72 hours prior to the test.

It is evident from table 2 that the hyaluronidase is contained in identical amounts, both in the highly virulent (No. 100) and in the avirulent (EV) strains. Proceeding from this, we decided to determine whether there exists any connection between the virulence of a strain and the presence of hyaluronidase. The enzymatic activities of 15 strains of plague bacilli of different virulence were

simultaneously tested. As an object for the detection of the hyaluronidase we used the fluid from a wash of agar cultures. Table 3, in which the results of this experiment are presented, shows that in general there exists a certain coincidence between a strain's virulence and its hyaluronidase content although a complete parallelism cannot be noted. Practically, the avirulent EV strain gives a positive reaction in almost the same dilutions of the object as does the highly virulent strain No. 100; and the strains No. 286 and No. 202, which retain sufficient virulence for animals, contain a lesser amount of the enzyme than does strain EV. In a more detailed study of the cultural and morphological properties of the latter two strains (Nos. 286 and 202) it was learned that both had begun to dissociate and that they contain S-forms, which represent the completely avirulent growth phase of the plague bacillus. The same applies to strain No. 96. There still remained the unexplained question about the conduct of the avirulent strain EV. In relation to this we conducted an experiment to determine the greatest quantity of hyaluronate that the plague strains of different virulence are capable of protecting from clot formation (in an acid medium). The experiment was conducted with the fluid centrifuged from a wash of 48-hour-old agar cultures of strains No. 100 and EV. The hyaluronate was added in increasing amounts. From the results of this experiment (table 4) it follows that both strains contain almost identical concentrations of hyaluronidase. In experiments on animals, however, it was established that the area of diffusion of an indicator that was injected intracutaneously together with extracts of virulent plague bacilli is significantly greater than when the indicator is injected with an extract from avirulent strains. In line with this, there are observations that some avirulent strains possess invasive properties and have the ability to spread quickly in an organism, producing a bacteremia in like manner to the virulent bacilli. The only difference is in the reaction of the macroorganism and in the weak resistance of the avirulent bacilli: whereas the latter, in comparison, are quickly eliminated from the organism (5-6 days) by the activity of the natural protective functions, the virulent bacilli by destroying all barriers (which are insufficiently complete and have historically been weakly developed for contention with plague infection) and by progressively increasing in number cause the organism's death. Thus, in the plague bacillus the virulence and the ability to spread represent independent functions. The plague bacillus, while losing its virulence, can retain its spread factor. There is every reason to believe that avirulent strains containing hyaluronidase can possess higher immunogenic capabilities than do the strains without it. The noticeably larger area of diffusion of the dye in the experiments with the virulent strains on animals is related to a combination of the spread factor and the virulent bacilli remaining in the fluid after centrifugation. The latter, by propagating in the organism, release new portions of hyaluronidase that also increase the diffusion area of the dye.

We later tested autolysates of plague cultures for their hyaluronidase content. We were unable to detect hyaluronidase in the autolysates of a suspension of 2 billion plague bacilli. could be for this reason all of our searches for hyaluronidase in broth cultures of the plague bacillus proved unsuccessful.

We investigated the hyaluronidase content in active plague preparations after they were subjected to heating at 58°C for one hour and at 100°C for

30 minutes. Using agar-culture washes, which were processed in the manner indicated, we conducted a test for the prevention of clot formation from the hyaluronate. As a result (table 5) it proved that heating at 58°C and even at 100°C does not conclusively inactivate the hyaluronidase - the killed preparations inhibited the clot formation somewhat more weakly than did the unprocessed.

It remained unclear: if the factor preventing the clot formation is identical to the enzyme hyaluronidase, then how to explain its heat-stability; are we dealing with some sort of non-specific agent? In order to answer the question whether we are actually dealing with hyaluronidase we made a series of supplementary investigations.

It is well known that bacterial enzymes demonstrate a significantly greater heat-stability than do the pure chemical enzymes. In our experiments the heat processing of the active plague objects for 30 minutes at 100°C (in a water bath) only weakened the titer of the plague microbe's hyaluronidase, but did not destroy it completely. In a careful check of all the circumstances with which the inactivation of the object was accomplished we noticed that the entire crowded bacterial mass had been subjected to the heating at 58°C and at 100°C and not just the centrifuged clear portion that the test was usually conducted with. As a result of such a processing the intracellular enzymes were not completely destroyed, and it is possible that they were even partially liberated. In relation to this we began to heat only the clear fluid received from centrifugation of a wash of a crowded agar culture. We poured off the clear liquid that was produced from the centrifugation of the bacterial mass and divided it into three portions: the first portion was left unprocessed, the second was heated at 100°C for one hour, and the third was autoclaved at 120°C for 30 minutes. After the processing at 100°C sedimentation occurred, and the fluid processed in the autoclave produced a still larger precipitate. At the same time a portion of the liquid subjected to the autoclave processing, by itself, without hyaluronate, formed a precipitate upon the addition of acetic acid; this precipitate by appearance reminded of a clot from hyaluronate in the presence of acetic acid, therefore for the test we used the autoclaved fluid in a dilution of 1:3 (with distilled water). The test, which was conducted with processed and unprocessed fluids, gave the results that are summarized in table 6.

On the basis of the results of the latter experiments it is possible to draw the conclusion that the enzyme hyaluronidase of the plague bacillus possesses a greater heat-stability than does the pure chemical enzyme inasmuch as the processing at 100°C for one hour still does not inactivate it conclusively. According to physico-chemical properties this enzyme is close to the endotoxin (the heat-stable O-antigen), with which it is possibly connected by common topography.

Further experiments were conducted for the purpose of determining the fission products that are formed from the action of the plague microbe's hyaluronidase on hyaluronate.

The effect of hyaluronidase on hyaluronic acid, as is well known, includes three phases: 1) the prevention of clot formation in an acid medium; 2) the

lessening of viscosity - depolymerization and 3) a more protracted reaction - the hydrolysis of the hyaluronic acid with the release of reducing substances. Together with this it was proved that the hyaluronic acid of the mesenchyme is hydrolyzed only by a specific enzyme - hyaluronidase. In order to define the products of hydrolysis we conducted special experiments that conclusively proved that plague bacilli contain the enzyme hyaluronidase - which specifically breaks down hyaluronic acid.

Hyaluronidase is evidently not a product of the life processes of plague bacilli - we detected it neither in whole broth cultures, young and old, nor in filtrates of these cultures. It is attached to the microbic cell and is released from it after the cell's disintegration as a result of lysis, autolysis, or other processes. The massive propagation of the plague bacillus that occurs in the first hours after infection assures the release of a sufficient amount of hyaluronidase to dissolve the viscous base of the mesenchyme; as a result the permeability of the tissues and capillaries is increased and this itself facilitates the penetration and spread of plague bacilli, toxins and other pathogenetic factors throughout the entire organism.

In the present work we also made an attempt to study the antihyaluronidase in the serum of animals immunized by plague cultures of those strains in which the enzyme hyaluronidase was detected in vitro. We stduied sera received from a horse that had been immunized with strain EV and from two rabbits immunized with strains EV and No. 143 (both strains form hyaluronidase). The immunization cycle consisted of 12 intravenous injections of a live culture with intervals of 4-5 days. Ten days after the last injection the rabbits were exsanguinated.

The antihyaluronidase properties of the antiplague sera was established by determining their ability to neutralize the enzymatic action of active plague preparations. For an unit of the hyaluronidase activity we used the minimal amount of the plague preparation that prevented the formation of a clot of mucin from 0.3 cc of hyaluronate (umbilical cord). We calculated the antihyaluronidase unit as the minimal amount of serum that neutralizes 3 hyaluronidase units. The formation of a clot of mucin was noted in the experimental test tubes containing the serum with the antihyaluronidase; in the control test tubes without the serum no clot was formed. In table 7 are presented the results from the antiplague sera titrated for the presence of antihyaluronidase.

It is evident from table 7 that sera from animals immunized with live cultures containing hyaluronidase possess antihyaluronidase properties. It should be pointed out that the antihyaluronidase sera neutralize the enzyme in more than just the homologous strain, being also active in regards to heterologous strains (Nos. 143 and 100).

Conclusions

1. For the first time, a spread factor (hyaluronidase) has been detected in the plague bacillus in vivo and in vitro.

2. The detection of the spread factor injects an important clarity into the understanding of the mechanism of the invasive and aggressive properties of the plague bacillus. Together with other pathogenetic factors hyaluronidase undoubtedly plays a large role in the pathogeno-immunological process of plague infection. Destroying the viscous base of the connective tissue the hyaluronidase secures a path for the spread of the plague bacilli and their products throughout the entire organism.

3. In the majority of cases the virulent plague strains possess hyaluronidase. However, there is not a complete parallelism between the virulence and the presence of hyaluronidase. Some avirulent strains of the plague microbe contain hyaluronidase in the same quantities as do the virulent bacilli.

4. The presence of the spread factor and the virulence in a plague microbe are frequently dissociated. A loss of virulence does not necessarily cause the loss of hyaluronidase.

5. Hyaluronidase is evidently not secreted by the plague bacilli, but it is released by them in the process of mass destruction - autolysis, lysis and similar phenomena occurring <u>in vivo</u> and <u>in vitro</u> together with the mass propagation of the bacilli.

6. The hyaluronidase of the plague bacillus is relatively heat-stable. Heating of active preparations at 58°C only weakens them, and heating at 100°C for one hour does not completely destroy this enzyme that is attached to the plague bacilli. Objects that have been processed in an autoclave for 30 minutes lose their enzymatic activity.

7. Antiplague sera received from animals that have been immunized with plague cultures of strains containing hyaluronidase neutralize the enzymatic action of active plague preparations.

8. Antihyaluronidase neutralizes the enzyme of heterologous strains as well as that of the homologous strain.

Table 1

The presence of hyaluronidase in plague-culture lysates.

Strain	Amount of lysate (in cc's) 0.5	0.4	0.3	0.2	0.1	0.005	Control
No. 100	++++	++++	++++	++++	-	-	-
No. 202	++++	++++	++++	++++	-	-	-
EV	++++	++++	++++	++++			

Designation: ++++ complete lack of clot; +++ traces of a clot; + clot weaker than in the control; - clot the same as in the control.

Table 2

The detection of hyaluronidase in the fluid centrifuged from a wash of an agar culture.

Strain	Amount of fluid (in cc's) 0.5	0.4	0.3	0.2	0.1	0.005	Control
No 100	++++	++++	++++	++++	++++	-	-
No 143	++++	++++	++++	++++	++++	++	-
EV	++++	++++	++++	++++	++++	-	-

Table 3

The relationship between the virulence of a strain and the presence of hyaluronidase.

Strain	Degree of virulence	Amount of the object (in cc's) 0,5	0.4	0,3	0.2	0,1	0.05	Control
No 100	High	+++	+++	+++	+++	+++	+	-
No 202	Median	+++	+++	+	-	-	-	-
No 286	Median	+++	+++	+++	-	-	-	-
No 14	Median	+++	+++	+++	+++	+++	+	-
No 13	Avirulent	+++	+++	-	-	-	-	-
No 15	Avirulent	+++	-	-	-	-	-	-
No 29	Avirulent	+++	+++	-	-	-	-	-
No 46	Avirulent	+++	-	-	-	-	-	-
No 230	Median	+++	+++	++	-	-	-	-
No 170	Weak	+++	++	-	-	-	-	-
No 66	Weak	+++	+++	+++	-	-	-	-
No 96	Weak	+++	-	-	-	-	-	-
EV	Avirulent	+++	+++	+++	+++	+++	-	-
No 205	Median	+++	-	-	-	-	-	-
No 161	Weak	+++	+++	-	-	-	-	-

Table 4

The effect of constant quantities of the object on increasing quantities of the substratum.

Strain No 100			Strain EV			Strain No 100			Strain EV		
Amount of the object (cc's)	Amount of hyaluronate (cc's)	Results	Amount of the object (cc's)	Amount of hyaluronate (cc's)	Results	Amount of the object (cc's)	Amount of hyaluronate (cc's)	Results	Amount of the object (cc's)	Amount of hyaluronate (cc's)	Results
0.5	0.1	+ + +	0.5	0.1	+ + +	0.4	0.1	+ + +	0.4	0.1	+ + +
0.5	0.2	+ + +	0.5	0.2	+ + +	0.4	0.2	+ + +	0.4	0.2	+ + +
0.5	0.3	+ + +	0.5	0.3	+ + +	0.4	0.3	+ + +	0.4	0.3	+ + +
0.5	0.4	+ + +	0.5	0.4	+ + +	0.4	0.4	+ + +	0.4	0.4	+ + +
0.5	0.5	+ + +	0.5	0.5	+ +	0.4	0.5	+ +	0.4	0.5	+

Table 5

The thermostability of preparations containing hyaluronidase

Identification of the object	Amount of the object strain No. 100 (cc)							Amount of the object strain EV (cc's)						
	0.5	0.4	0.3	0.2	0.1	0.05	Control	0.5	0.4	0.3	0.2	0.1	0.05	Control
Unheated fluid from a wash. . .	+++	+++	+++	+++	+++	+	-	+++	+++	+++	+++	+++	-	-
Heated (at 58°C) fluid from a wash.	+++	+++	+++	++	-	-	-	+++	+++	+++	++	-	-	-
Heated (at 100°C) fluid from a wash.	+++	+++	++	++	-	-	-	+++	+++	+++	++	-	-	-

Table 6

The thermostability of the plague microbe's diffusion factor

Identification of the test object	Amount of the whole object (cc's)						Amount of the object diluted 1:3 (in cc's)						
	0.5	0.4	0.3	0.2	0.1	0.05	0.5	0.4	0.3	0.2	0.1	0.05	Control
Unprocessed fluid.	+++	+++	+++	+++	+++	+	+++	+++	+++	+	-	-	-
Fluid heated at 100°C. . . .	+++	++	-	-	-	-	-	-	-	-	-	-	-
Autoclaved fluid.	0*	0	0	0	0	0	-	-	-	-	-	-	-

* Test not conducted.

Table 7

The antihyaluronidase activity of the tested antiplague sera.

Origin of the serum	Strain used to immunize the animals	Number of antihyaluronidase units
Horse (Series No 859)	EV	40
Rabbit (Series No 276)	EV	25
Rabbit (Series No 277)	No. 143	50