DETECTION OF THE CAPSULAR ANTIGEN OF *Pasteurella pestis* IN A DIALYZATE

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Much research has been devoted to the study of the properties of the capsular material of *Pasteurella pestis* [1-4, 7, and others]. At the present time the technique most commonly used to obtain and purify the capsular material is that suggested by Baker and co-workers [7]. In this method a saline extract is first prepared from *P. pestis* cells killed with cold acetone, and the substance is then salted out at certain levels. These workers gave the name "fraction 1" of *P. pestis* to the preparation after reprecipitation several times, and this term is in established usage in the literature. It has been shown that fraction 1 is a substance of protein nature, and that part of this protein is bound indissolubly to a polysaccharide.

The preparation of a stable diagnostic agent for the passive hemagglutination and antibody neutralization reactions has opened the way to the immunological study of fraction 1 by means of highly sensitive methods [5]. Quantitative immunological analysis has shown that, in addition to large molecular components of the fraction 1 type, other substances of lower molecular weight must exist, having similar antigenic activity.

In the present paper we describe direct evidence in support of the existence of such a substance in the cells of *P. pestis* when killed with acetone.

EXPERIMENTAL METHOD

In order to study the ability of fraction 1 antigen to pass through cellophane filters, two types were prepared. In some experiments the cellophane was sterilized by immersion in boiling water for 30 min, while in others it was not sterilized. Dialysis was carried out at 4° against physiological saline.

A suspension of bacterial cells (*P. pestis* cells killed with acetone) or fraction 1 solution, in a volume of 5 ml, was placed in a cellophane bag; the total number of minimal neutralizing doses reached 5,000,000. The volume of physiological saline (dialyzate) surrounding the cellophane bag was 25 or 50 ml. The cellophane bag and the surrounding fluid were placed in a glass vessel, sterilized in some experiments by autoclaving.

Specific antigen of fraction 1 of *P. pestis* was detected and titrated by the antibody neutralization reaction [5], the complement fixation reaction, and the precipitation reactions in jelly and in fluids. For the antibody neutralization reaction (ANR) formalinized sheep's red cells sensitized with fraction 1A (red cells of series 87) and plague agglutinating serum from the Saratov "Mikrob" Institute (series 69) were used. For the complement fixation reaction (CFR) the serum of a rabbit immunized repeatedly with fraction 1 of strain EV No. 76, was used. This serum was used to titrate the activity of the antigens in the CFR in a dilution corresponding to 4 serum units. The precipitation reaction in jelly was performed in the usual manner in agar plates, using 0.2 ml of each component. The precipitation reaction in liquids was carried out in agglutination tubes. To 0.5 ml of plague agglutinating serum from the Saratov "Mikrob" Institute, diluted 1:10, 0.5 ml of the test material was added, and the result of the reaction was read from the intensity of the protein precipitate.

At the same time the ability of the antigens to sensitize formalinized and tanninized sheep's red cells was tested, and the activity of the sensitized red cells was tested by means of the serum of a rabbit immunized with fraction 1A of strain EV No. 76.

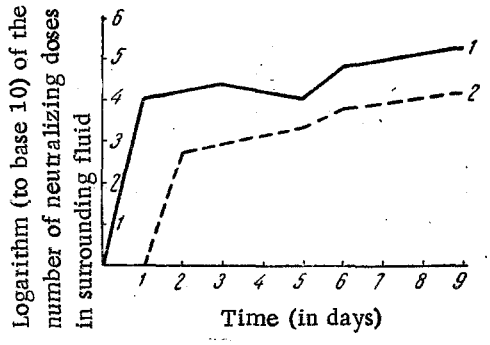
The adsorption of antigen on to aluminum hydroxide suspensions at 4° was studied by titrating the antigens in the ANR before adsorption and the supernatant fluid after adsorption. One milliliter of the suspension contained 2.5 mg $Al_2(OH)_3$.

The antigenic properties of the dialyzate were studied by adsorption on to aluminum hydroxide; 0.5 ml of suspension was injected intramuscularly into an albino mouse, and one week later the same antigen, but not adsorbed on to aluminum hydroxide, was injected subcutaneously; after a further 3 weeks the animals were sacrificed and their serum tested by means of the passive hemagglutination reaction to determine the titer of antibodies to fraction 1.

The presence of protein and its decomposition products was detected by the biuret reaction and ninhydrin test, and polysaccharides were detected by the Molisch reaction. The action of trypsin on fraction 1 antigen was tested by pooling equal volumes of 0.25% crystalline trypsin solution and antigen solution, and the activity of the antigen was determined by the ANR after a suitable period of exposure.

EXPERIMENTAL RESULTS

In a few experiments we showed that the specific fraction 1 antigen of P. pestis passed through the pores of the cellophane, and it was detected in the dialyzate by means of the ANR. It should be noted that the duration of dialysis and the variety of cellophane significantly affected the titer of antigen detected in the surrounding liquid (see fig.).



Changes in the passage of capsular antigen into the surrounding fluid. 1) First variety of cellophane; 2) second variety of cellophane.

In the experiment illustrated in the figure, the bag contained a suspension of bacterial cells of strain No. 1213, equivalent to approximately 1 million minimal neutralizing doses. For control purposes a solution of fraction 1 of strain No. 1213 (approximately 700,000 minimal neutralizing doses) was poured into cellophane bags, but no neutralizing activity was detected in the dialyzate.

During prolonged dialysis even at 4° there was a gross increase in contamination of all the samples, so that we began to sterilize the vessel and the cellophane. The results of an experiment, in which a variety of cellophane (allowing more of the fraction 1 antigen to pass through) was used, are given in Table 1. Into each cellophane bag was poured a suspension of bacterial cells (0.2 g) or solution of fraction 1 (5 mg), and the bag was immersed in 50 ml of physiological saline. Identical volumes of the materials were allowed to stand at 4° in test tubes. The distribution of antigen was investigated 5 days later by means of the ANR and CFR.

The number of minimal neutralizing and complement-fixing doses detected in the tubes was consistently greater than in the cellophane bags, largely as a result of loss of antigenic material, which could not be extracted completely from the bag.

The ANR and CFR revealed a certain amount of fraction 1 antigen in the physiological saline surrounding the cellophane bag, displaying activity in these reactions. As might have been expected, the ANR was much more sensitive than the CFR.

Investigation of the properties of the dialyzates showed that protein was absent both from the dialyzate and from corresponding dilutions (as regards neutralizing activity) of the dialyzed fluid. As a rule the ninhydrin reaction revealed the presence of nitrogen compounds in the dialyzate. The Molisch reaction was positive only with fraction 1 solution. Specific antigen of fraction 1, demonstrable in the dialyzate, was adsorbed on to aluminum hydroxide floccules; no neutralizing activity could be detected in the supernatant fluid by means of the ANR. No antigen was detected in the dialyzate by means of the precipitation reactions in agar jelly and in liquid, which was quite understandable because its concentration was too low. A positive precipitation reaction in jelly and liquid could be observed only in concentrations of fraction 1 corresponding to 5000-20,000 minimal neutralizing doses in 1 ml.

Solutions of fraction 1 or suspensions of bacterial cells sensitized formalinized sheep's red cells in concentrations of several tens of thousands of minimal neutralizing doses in 1 ml. The dialyzate naturally did not sensitize the red cells, for its content of antigen was low. We tested 12 samples of dialyzate by evaporating them down to one-tenth their original volume, and these concentrations were then used to sensitize red cells. In two cases in which the number of minimal neutralizing doses was greatest, the red cells were sensitized, and the serum of a rabbit immunized with fraction 1 agglutinated red cells down to a dilution of 1:5120.

TABLE 1. Study of the Ability of Fraction 1 Antigen to Pass through Cellophane Pores during Dialysis

Character of material and No. of strain	Quantitative distribution of antigenic activity during dialysis				Antigenic activity in control test tubes	
	No. of minimal neutralizing doses		No. of minimal complement-fixing doses		No. of minimal neutralizing doses	No. of minimal complement-fixing doses
	inside the bag	in the dialyzate	inside the bag	in the dialyzate		
Fraction 1, strain No. 1213	1/0 800	0		0	320 000	80 000
Fraction 1, strain No. 1213	153 600	0	38 400	0		
Bacterial cells, strain EV No. 76						
Bacterial cells, strain EV No. 76	6 0 0 000	11 264	64 000	224	1 600 000	320 000
	3 584 000	22 528	89 600	1 760		
Bacterial cells, strain No. 1260	1 920 000	708	192 000	0	3 200 000	640 000
Bacterial cells, strain No. 1260	1 216 000	736	486 400	0		
Bacterial cells, strain No. 1252	3 584 000	5 376	89 600	420	1 600 000	320 000
Bacterial cells, strain No. 1252	1 664 000	22 016	166 400	430		
Bacterial cells, strain No. 1204	1 536 000	720	76 800	0	1 600 000	160 000
Bacterial cells, strain No. 1204	1 792 000	701	44 800	0		
Bacterial cells, strain No. 496, agent of pseudotuberculosis	0	0	0	0	0	0
Phsyiological saline	0	0	0	0	0	0

TABLE 2. Action of Trypsin Solutions (0.12%) on the Neutralizing Activity of the Dialyzate

Capsular antigen of strains	Antigen titer in ANR			
	pH 7.0		pH 8.0	
	trypsin	physiological saline	trypsin	physiological saline
EV No. 76	1:32	1:64	1:64	1:32
EV No. 76	1:64	1:64	1:64	1:64
No. 1252	1:32	1:32	1:32	1:32
Fraction 2, strain No. 1217 (100 μg/ml)	1:1024	–	1:512	–
Physiological saline	0	0	0	0

Ten samples of dialyzate were injected into albino mice (each dialyzate was given to 3 animals), and the four mice tested with dialyzate showing greatest activity in the ANR were found to have antibodies to fraction 1 in the passive hemagglutination reaction in titers of 1:40 - 1:320. In our experiments trypsin did not diminish the neutralizing activity of the dialyzate.

In this experiment mixtures of equal volumes of trypsin solution and dialyzate were kept for 1 h at 37° and for 2 h at 25°, after which they were tested in the ANR. Solutions of fractions 1 and 2 were also used in the experiment. Our results confirmed the findings of Ajl and co-workers [6], who were unable to destroy plague toxin by treating it with trypsin. In our experiment the value of LD_{50} of a mixture of the fraction 2 solution with physiological saline, when injected intraperitoneally into albino mice, was 21 μg, and the corresponding value for a mixture of a solution of fraction 2 with a 0.12% trypsin solution was 12 μg.

The fact that the dialyzate showed antigenic activity proved the existence of substances of low molecular weight, and evidently nonprotein in nature, in the bacterial cell of P. pestis. Since the antigen present in the dialyzate neutralized plague agglutinating serum, which did not thereafter agglutinate red cells sensitized with fraction 1 of P. pestis; there is reason to suppose that the antigenic activity of fraction 1 was, in fact, associated with this substance of low molecular weight. In all probability this substance was present in the dialyzate in a free state, while fraction 1 consists of a complex of protein with this low molecular compound. We consider that the term "capsular antigen" be reserved for this substance of low molecular weight and not applied to fraction 1. Capsular antigen was active not only in the ANR, but also in the CSF, it was adsorbed on to aluminum hydroxide, it sensitized formalinized sheep's red cells, it was not destroyed by trypsin, and it was capable of causing the accumulation of antibodies in the serum of immunized albino mice.

SUMMARY

Quantitative immunological analysis indicates that, along with the fraction 1, there should exist a substance, with a similar antigenic activity, but with a more simple structure. The composition of bacterial mass of acetone-killed Past. pestis contains a substance capable of passing through cellophane pores during the dialysis of bacterial mass against physiological saline. This substance, as distinct from fraction I, was called the capsular antigen; it possessed marked antigenic activity in the complement fixation and in the antibody neutralization reactions. Accumulation of antibodies in the serum and in the fraction 1 was induced by immunization of albino mice with the capsular antigen.

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