THE USE OF HIGUCHI-SMITH MEDIUM FOR IMPROVING THE IMMUNOGENIC PROPERTIES OF PLAGUE VACCINE STRAINS

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One of the deficiencies of live plague vaccines is the lowering of their immunogenic activity during the process of storage and reseeding on nutrient media. Of the great number of strains which have been proposed as vaccine strains, only a few have passed the test of time. In connection with this the problem of searching for methods of increasing the immunogenic properties of vaccine strains requires constant attention.

In previous investigations (Akimovich and Ponomarev, 1964) we showed that on the Higuchi-Smith medium (1961), depending on the time of incubation and the temperature conditions, plague bacteria form colonies of the I, II, and III order. Colonies of the I order consist of bacteria which are not wanting in calcium ions for their development at 37°, colonies of the II order are bacteria with a potential for losing the dependency on calcium, and colonies of the III order are formed due to bacteria which are capable of multiplying at 28° on a medium which is deficient in calcium.

Ist order subcultures of the EV vaccine strain turned out being capable of immunizing mainly white mice, but not guinea pigs, but on the other hand, subcultures of the III order caused a specific immunity both in mice and in pigs.

In the EV strain the number of colonies of the I order did not exceed 0.03%, while their number in strain I * reached 64.6%. Apparently this circumstance was the reason for the loss by this strain of the ability to immunize guinea pigs and served as the grounds for removing it from the stock of substances with a specific prophylaxis for plague.

The results of these observations made it possible for us to use the peculiarities in the behavior of plague bacteria on the Higuchi-Smith medium for resolving two problems: 1) by means of selection on a medium with a deficit of calcium to isolate subcultures of the EV vaccine strain which possess the greatest immunizing capability, and 2) with the help of the same method to improve the immunological activity of the I vaccine strain.

* A component of the 1-17 antiplague vaccine (Altareva et al.,1958).

For selecting the most stable variants of the III order of the strains being investigated we used the following method: 50 subcultures of the III order of the EV and No 1 strains were reseeded on a Hottinger agar slant and incubated at 28° for 48 hours. The cultures which grew were placed in a dryer and preserved at room temperature (16--18°). In 15, 30 and 45 days the subcultures were sown on a medium with a deficit of calcium and the colonies of the I, II and III order which grew were calculated.

It developed that subcultures of the III order, regardless of strain, have a different tendency for losing the dependency on calcium ions. Together with subcultures in which a large number of colonies of the I and II order (up to 0.95%) were observed, there were subcultures in whose populations bacteria which grew on magnesium-oxylate medium at 37° were detected in the minimum amount. From the latter we selected for further study subculture 9 of the EV strain and subculture 18 of the I strain. The populations of these proved to be the most stable; 0.073 and 0.027% of colonies of the I and II order respectively were detected.

In tests on guinea pigs we studied the ability to "take" of subcultures 9 and 18, and also the periods for the formation and the intensity of the immunity which they caused in the animals. The magnitude of the minimum immunizing dose of the subcultures was determined simultaneously in 2 species of animals -- guinea pigs and white mice.

For evaluating and comparing the properties of the subcultures selected by us, we included in the experiments the reference EV vaccine strain and the vaccine strain I from which these variants were isolated.

The experiments for determining the ability to "take" were conducted on 108 guinea pigs which were divided into 6 groups. The suspension of the culture was administered subcutaneously in quantities of 5×10^8 and 3×10^9 microbial cells. On the 4, 7, 10, 12, and 21st day following the injection of bacteria, 3 guinea pigs each were sacrificed. Tissue from the site of administration of the bacteria, the regional lymph node, liver, spleen, lungs, blood and marrow were seeded in dishes on a medium with gentian violet and sulfite and blood agar.

Positive results of bacteriological investigations following infection with the reference EV strain and subculture 18 were noted up to 12 days inclusively (table 1). Bacteria of subculture 9 were detected only up to 10 days following infection. Regardless of the strain and the subculture, it was possible to isolate bacteria as a rule only from the tissues from the site of infection and the regional lymph nodes. As is apparent from the data cited, no significant differences were noted in the periods for the ability to "take" and the invasion ability of the plague microbe subcultures under study in the tissues and organs of guinea pigs.

For the purpose of determining the minimum immunizing dose guinea pigs and white mice were immunized with subcultures9 and 18; a 2-day agar culture was introduced under the skin of the left groin in quantities of 5×10^2 and

$5x10^3$ microbial cells, suspended in 0.2 ml of physiological solution. The initial strain I and its subculture 18 were additionally investigated in a dose of $2x10^4$ bacteria. Each dose of bacteria was tested on 10 animals of each species. After 21 days following immunization the test animals were infected subcutaneously in the area of the groin with the virulent P. pestis strain No 363 in a dose of $2x10^4$ of bacteria (200 Dcl).

As a control we had 10 nonimmunized guinea pigs and 10 white mice which were infected subcutaneouly with the same strain in the same dose.

All the control guinea pigs died in 7--9, and the control mice -- in 4--7 days following infection (table 2). In the group of test animals the guinea pigs died in 6--15 days, and the white mice in 5--12 days following administration of the virulent strain.

Following the administration of $5x10^3$ of bacteria, the reference EV strain protected 8 out of 10 of the test guinea pigs from death, that is, based on immunizing activity it satisfies the requirements which are set forth for vaccine strains of the plague microbe. Based on the ability to immunize guinea pigs, subculture 9 of the EV strain somewhat exceeded the reference strain; out of 10 animals vaccinated with $5x10^3$ bacteria, 9 survived.

As it should have been expected, the initial vaccine strain I turned out to be little effective: When guinea pigs were immunized with doses of $5x10^3$ and $2x10^4$ approximately 1/3 of the animals used in the test survived following subsequent infection with virulent plague microbes. On the other hand, subculture 18 of this strain in a dose of $5x10^3$ bacteria turned out to be somewhat more effective than the reference EV strain.

In the tests on the immunization of white mice the EV strain and its subculture in a dose of $5x10^3$ microbial cells prevented the death of around half of the animals used in the test following their infection with a virulent strain of plague bacteria. Approximately the same results were obtained with the I strain and its subculture.

In order to determine the periods for the formation of immunity in guinea pigs which had been vaccinated with subcultures 9 and 18, the animals were given a single immunization under the skin of the left groin with a 12-day agar culture in a dose of $5x10^6$ microbial cells. There were 5 guinea pigs in each group. After 5, 6, 8, and 10 days following the onset of immunization the test animals were infected with virulent strain No 363 in a dose of 200 Dcl (20,000 microbial cells).

The death of the immunized animals was observed in a period from 3 up to 22 days from the time of infection with the virulent bacteria (table 3). In pigs which were immunized with the reference EV strain an immunity, capable of protecting the animals from infection, was developed by the 6th day following immunization. Bacteria of subcultures 9 and 18 stimulated the creation of an intense immunity in guinea pigs on the 8th day following immunization. It protected 4 out of the 5 animals which were in the test.

From similar animals, sacrificed in 30 days, it was not possible in one case to isolate plague bacteria.

In tests on 30 guinea pigs the intensity of immunity was determined following immunization with a reference strain and two subcultures. The animals received a 2-day agar culture in a quantity of $1x10^6$ microbial cells suspended in 0.2 ml of physiological solution subcutaneously in the area of the groin. After 21 days following the onset of immunization all the animals were infected with the virulent P. pestis strain No 363 in a dose of 50,000 Dcl ($5x10^6$).

As it follows from the data obtained, based on its intensity the immunity in guinea pigs turned out to be high in all the animals, regardless of the culture which was used for immunization (table 4).

Subculture 18 of plague microbe strain No 1, which was preserved in a dry state for more than 2 years (period of observation) without reseeding, stably preserved its immunogenic properties.

Conclusions

1. By means of selection on the Higuchi-Smith medium ~~it is possible to obtain~~ P. pestis variants which possess a high immunogenic activity.

2. When using the stated method, variants with a somewhat more expressed immunological effectiveness are obtained from the approved EV vaccine strain.

3. By the method of selection on a medium with a deficit of calcium ~~it was possible to isolate~~ from a degraded vaccine strain a subculture with an immunological activity which was not subordinate to the activity of the industrial EV strain.

4. By means of the selection of the appropriate subcultures on the Higuchi-Smith medium ~~it is possible to maintain~~ the immunization activity of commercial vaccine strains at the proper level.

Literature

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Table 1

Ability to "take" by bacteria of subcultures 9 and 18 in the organs of guinea pigs

Strain and subculture	Infection dose	Frequency of isolation in various days following infection					
		4th	7th	10th	12th	16th	21st
EV reference	5×10^8	2	3	2	1	0	0
	3×10^9	2	2	0	1	0	0
Subculture 9	5×10^8	2	3	1	0	0	0
	3×10^9	3	3	0	0	0	0
Subculture 18	5×10^8	2	2	1	1	0	0
	3×10^9	3	2	1	2	0	0

Table 2

Determination of the minimum immunizing doses of subcultures 9 and 18

Strain and subculture	Results of infection with a virulent strain (200 Dc1)					
	Guinea pigs, vaccinated with bacteria in doses of			White mice, vaccinated with bacteria in doses of		
	5×10^2	5×10^3	2×10^4	5×10^2	5×10^3	2×10^4
EV reference	10/5	10/2	-	10/6	10/5	-
Subculture 9	10/3	10/1	-	10/8	9/5	-
I initial	9/9	10/7	8/5	10/4	8/3	9/4
Subcul. 18	10/4	8/0	8/0	10/8	8/5	9/0

Note: Numerator - number of animals in test; denominator - number of animals which died.

Table 3

Periods for the development of immunity in guinea pigs, vaccinated with subcultures 9 and 18

Strain and subculture	Dose of vaccine	Number of animals which died in various periods after infection w/ virulent plague bacteria (days)			
		5th	6th	8th	10th
EV reference	5×10^6	5/2	5/0	5/1	5/0
Subculture 9	5×10^6	5/3	5/2	5/1	5/0
Subculture 18	5×10^6	5/4	5/2	5/1	5/0

Legend: Same as table 2.

Table 4

Intensity of immunity in guinea pigs, immunized with subcultures 9 and 18

Strain and subculture	Immunization dose	Number of guinea pigs	Number of pigs died from plague
EV Reference	1×10^6	10	0
Subculture 9	1×10^6	10	0
Subculture 18	1×10^6	8	1