IN VITRO SELECTION OF VARIANTS WITH VACCINAL PROPERTIES FROM VIRULENT STRAINS OF P. PESTIS

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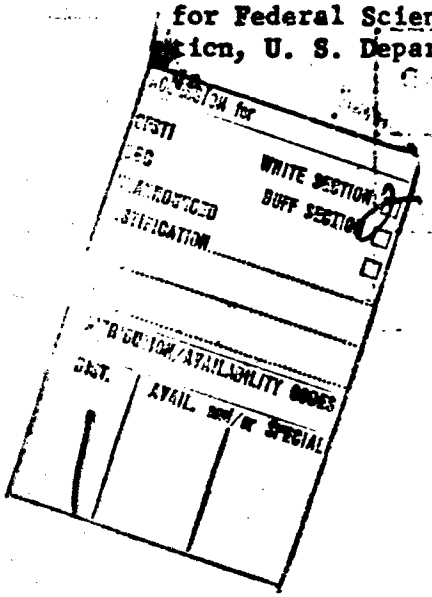

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IN VITRO SELECTION OF VARIANTS WITH VACCINAL PROPERTIES FROM VIRULENT STRAINS OF P. PESTIS

[Following is the translation of an article by V. V. Akimovich, N. I. Nikolayev, L. F. Zykin, N. G. Ponomarev, and A. A. Popov, "Mikrob" All-Union Scientific-Research Antiplague Institute, Saratov, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), No. 6, 1965, pages 64--68. It was submitted on 29 May 1964. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

At the present time the selection of variants of plague bacteria with the properties of vaccine strains is realized based on the results of their behavior in tests on laboratory animals. Such a method of investigation is characterized by a heavy labor expenditure and expensiveness, which sharply limits the number of strains studied and is reflected in the final result of the investigations. Therefore, an attempt to develop a method, with the help of which it would be possible to make an in vitro preliminary selection of the most promising variants before their detailed and conclusive testing on laboratory animals, is completely justified and a necessity. Modern information concerning the virulence and antigenic structure of plague bacteria make it possible to propose that this mission can be resolved successfully.

The heterogeneity of the cellular composition of a population of bacteria is the premise for the attempt to isolate from virulent strains those subcultures which meet the requirements which are set forth for plague vaccine strains.

For selecting variants with an attenuated virulence we used the data of Jackson and Burrows (1956), who point out that plague bacteria, which do not form pigmented colonies on a chemically specific medium with hemin, are weakly virulent for white mice and guinea pigs. This method of selecting avirulent variants is simple and completely practicable.

All told we investigated 6 virulent reference strains of P. pestis (No. 708, 363, 293, 331, 356, 358) and 2 vaccine strains (EV and No. 1).

The proportion of pigmented and nonpigmented colonies in the strains investigated turned out to be very diverse. While the bacteria of both vaccine strains grew solely in the form of colorless colonies, in the remaining strains, which possessed a more or less expressed virulence, there was observed a mixture of pigmentless and dark brown colonies, and the number

of the latter fluctuated in various strains from 3.2 up to 100%. From colonies of both types subcultures were made on beveled agar for accumulating a colony and its subsequent study.

All the pigmentless subcultures turned out to be nonvirulent for mice in doses of from 100 to 10 million microbial cells. On the other hand, pigmented variants caused the death of mice in the same doses as their initial strains, with a certain exception. Analogous investigations were conducted with subcultures of strain No. 708 in tests on guinea pigs. The results turned out to be approximately the same as in the experiments on white mice.

Thus, without resorting to artificial influences on the plague bacteria, it is possible to obtain variants with a naturally changed virulence.

Further it was necessary to clear up to what extent the pigmentless variants preserved a "residual" virulence, that is, the capability to multiply and take root in the organism of an inoculated animal, and did they possess a full-value immunogenic activity.

We attempted to solve the stated problems by means of the further selection of pigmentless variants of plague bacteria on the Higuchi-Smith (1961) medium. These authors showed that magnesium-oxalate agar can be used for the differentiation of virulent and avirulent plague microbes. In one of the works, Akimovich and Ponomarev showed that on one and the same plate of magnesium-oxalate agar during cultivation of the plague bacteria, by changing the length and temperature of incubation it is possible to obtain colonies of three orders: At 37° for 40--42 hours colonies of the I order grew, and by prolonging the incubation up to 70--80 hours at the same temperature, colonies of the II order were detected. In the same seeding with an increase of the periods of cultivation by still 30 hours and incubation at 28°, colonies of the III order were formed. These were formed from cells which are dependent on calcium ions at 37°.

The pigmentless variants of strains No. 293, 708, 331, and 358 which we obtained were subjected to a further selection on Higuchi-Smith medium. The seedings were incubated at 37° for 42 hours, after which colonies of the I order were noted, and part of these were seeded out on beveled agar for further investigation. Colonies of the III order were removed following an 80 hour cultivation at 37° and a subsequent 30 hour incubation at 28°.

Bacteria which are capable of multiplying at 37° on magnesium-oxalate agar (I order) were detected in all the pigmentless and pigmented subcultures. Their number fluctuated from 0.04 to 0.15% depending on the individual peculiarities of the cellular composition of the variants. As a rule, in all the subcultures there were significantly more bacteria which formed colonies of the II order (cells with a potential for the loss of dependency on calcium), and in a number of cases they represented the main mass of the cellular population. The number of bacteria, dependent on calcium ions at 37° (III order) fluctuated in the majority of subcultures from 23 up to 99%.

After 4 months of storage on agar and repeated reseedings, subcultures of the III order were again subjected to investigation on a medium with a deficit of calcium. Approximately in half of the cases the subcultures turned out to be stable, which testified to the absence of multiplication of bacteria on this medium at 37°.

Virulence for white mice was determined in 10 subcultures of the III order of strain No. 708. Each subculture in doses of $5x10^2$, $5x10^3$, $1x10^7$, and $1x10^8$ of bacterial cells was administered to 3 white mice under the skin of the inner surface of the femur. The degree of loss of virulence in various subcultures was expressed differently and this was especially demonstratively detected in the tests with the maximum dose of bacteria. Three subcultures caused the death of all the test animals, 5 others caused a lethal outcome in one mouse in each group, from one subculture 2 mice out of 3 died, and finally one subculture in this dose turned out to be nonvirulent. Out of 10 subcultures investigated, 4 caused the death of singla mice in doses of $5x10^2$ and $5x10^3$ microbial cells. Bacteria were detected with great constancy in the tissues from the site of administration and in the regional lymph node, however, a positive result for the bacteriological investigation was recorded only in 10 mice out of 23 which died following infection. Plague septicemia was observed only in 4 animals. The results of the bacteriological investigation make it possible to consider that the death of a significant part of the mice resulted from specific intoxication or from alien causes.

Subcultures of strains No. 331, and 358 were investigated in a somewhat different arrangement. Along with subcultures of the III order, subcultures of the I order, both those which formed and those which did not form pigmented colonies on the Jackson-Burrows medium, were selected for further analysis. An attempt was made to clear up the behavior of various subcultures in tests on white mice following their joint administration with ferrous sulfate, which, according to the data of Jackson and Burrows (1956a), Avanyan et al. (1963) and others, increases the virulence of plague bacteria which had lost pathogenicity for laboratory animals to a greater or lesser degree. A suspension of ferrous sulfate in sunflower oil (0.1 ml) was administered intraperitoneally 5 and 2 days prior to infection, and then on the day of infection together with an 0.1 ml suspension of bacteria ($1x10^4$ and $1x10^7$). The control animals were infected with the subcultures being investigated ($1x10^7$ bacteria) also intraperitoneally, but without iron.

Under the influence of the preparation of iron used a distinct increase of virulence was observed for subcultures of the III order. This was very demonstratively detected in tests with strain No. 331, the subcultures of which, as a rule, did not cause the death of the control mice. The same subcultures in a dose equal to $1x10^4$ bacteria, introduced jointly with ferrous sulfate, caused a lethal outcome in all the test animals. In individual mice which had died following infection ($1x10^7$) without iron, bacteria were detected primarily in the exudate of the abdominal cavity and considerably less often in the blood. On agar plates a relatively small number of colonies grew. For the test animals an abundant growth of plague bacteria was noted in the seedings of the exudate and the blood.

Subcultures of the I order of both pigmented as well as pigmentless variants of both strains did not cause the death of mice with the doses used ($1x10^6$ and $1x10^7$) either when administered independently or jointly with ferrous sulfate.

The immunogenic properties were studied in certain subcultures of the III and I orders. For immunization, 20-hour agar cultures, incubated at 28°, were used. A suspension containing $5x10^3$ of bacteria was administered to the animals under the skin of the inner surface of the femur. In various periods following infection of the microbes (8--19 days) 9 mice died out of the 59 which were in the test, but their death could not always be connected with plague infection. Guinea pigs turned out to be more resistant: Out of 35 which were in the test, 2 died, and only from one of these was plague bacteria isolated. After 21 days from the onset of immunization all the test and control (not subjected to vaccination) animals were infected with a culture from the virulent plague strain No. 363. The suspension of bacteria in a dose equal to 200 Dcl ($2x10^4$ bacteria) was administered under the skin.

The control mice (10) and guinea pigs (10) died in periods from 3 to 7 days following infection. Subcultures of the I order guaranteed an intensive immunity in 77.5% (in 31 out of 40) mice (see table). Here it was not possible to establish a significant difference in the immunizing capability of the various subcultures, including pigmented and nonpigmented. Out of 40 guinea pigs only 16 survived (40%), and a somewhat more expressed immunological activity was observed in pigmented variants. For subcultures of the III order of all the investigated strains, a characteristic was their ability to immunize both white mice and guinea pigs, however, at the same time that out of 50 mice which were immunized with subcultures of the III order, 43 survived following infection with virulent microbes (86%), and only one out of 33 guinea pigs died.

The results of these investigations made it possible to propose a specific arrangement for the preliminary in vitro selection of subcultures with vaccine properties from virulent strains of plague bacteria. The first phase consists in the selection of variants with a changed virulence based on the feature of the formation of nonpigmented colonies on a medium with hemin. However, by this method it is possible to detect only a weakening of virulence of bacteria, but the level of virulence which is necessary for the development of the vaccinal process cannot be determined. This problem is resolved to a significant degree by means of using magnesium-oxalate agar, on which colonies of the III order grow. These consist of bacteria, dependent on calcium ions at 37°, and characterized (Higuchi and Smith, 1961) by a "latent" virulence, similar to bacteria of the highly immunogenic EV vaccine strain. Following repeated investigations it is possible to exclude those subcultures in which following storage on nutrient media reveal a tendency for growth at 37° on a medium with a deficit of calcium.

A supplementary test for the selection of subcultures with "residual" virulence is their administration to mice simultaneously with ferrous sulfate.

It is possible to consider as full-value only those subcultures which under these conditions prove to be highly virulent for white mice. If the bacteria completely lost their virulence or it turned out to be beyond the limits of a specific threshold, the stated effect is not obtained. The described test is also of value due to the fact that it makes it possible to exclude from further investigations those subcultures in which there is noted a tendency toward restoration of the capability for pigmentation on a medium with hemin and for the restoration of virulence.

Various subcultures of the III order even of the same strain possessed a different immunological activity. In an immunogenic respect those subcultures should be considered as full-value which in a dose of 5000 bacteria guarantee immunity in 80--90% of white mice and guinea pigs following infection with a massive dose (200 Dcl) of virulent plague bacteria.

Conclusions

1. Subcultures with vaccine properties from virulent strains of the plague microbe may be selected according to the following features: On the Jackson-Burrows medium they should grow in the form of pigmentless colonies, on magnesium-oxalate agar of Higuchi--Smith they should form colonies of the III order and should not disclose a tendency for a loss of "residual" virulence; they should be nonvirulent for mice in doses of 1×10^{8} bacteria, and under the influence of ferrous salts their virulence should be raised but without the restoration of the capability for pigment formation and without the restoration of virulence; in a dose of 5000 bacteria they should cause immunity in 80--90% of white mice and guinea pigs in respect to virulent plague bacteria in a dose of 200 Dcl.

2. A final judgement of the selected subcultures is decided following their approval on laboratory animals based on those tests which at the present time are accepted for evaluating vaccine strains of the plague microbe.

Literature

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Results of using virulent plague bacteria for infecting animals which had been vaccinated with subcultures of the I and III orders

Number of strain	Subculture	Species of animals	Number of animals		
			In the test	Survived following immuniza-tion	Survived following infection
708	P-1 III/1	Mice	13	10	9
		Pigs	5	4	4
708	P-2 III/3	Mice	13	12	8
		Pigs	5	5	5
708	P-7 III/1	Mice	13	13	12
		Pigs	5	5	5
331	P-2 III/2	Mice	10	7	7
		Pigs	10	9	9
358	P-1 III/1	Mice	10	[illegible]	7
		Pigs	10	10	9
331	P+1 I/1	Mice	10	10	8
		Pigs	10	10	5
331	P-1 I/2	Mice	10	10	8
		Pigs	10	10	3
358	P+1 I/1	Mice	10	10	7
		Pigs	10	10	5
358	P-1 I/1	Mice	10	10	8
		Pigs	10	10	8

Legend: P- nonpigmented subcultures
P+ pigmented subcultures
Arabic numbers -- number of colonies on Jackson-Burrows and Higuchi-Smith media
Roman numbers -- subcultures of the corresponding order.