THE RATE OF FORMATION OF PHAGE RESISTANT MUTANTS IN THE CELL POPULATION OF VIRULENT STRAINS OF PLAGUE BACILLI

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The results of a study of mutation rates of phage resistant plague bacilli of varying virulence have been discussed in a previous communication [2]. In connection with these data, mutations from phage sensitive to phage resistant have been observed in studying both the virulent and the avirulent strains. It has not been possible to establish the dependence of mutation rate on the nature of applied phage, and in all cases the rate varied from 2×10^8 to 6×10^8.

The study of the isolated phage resistant mutants demonstrated that they were not identical, since some of them are related in their properties to the activators of pseudotuberculosis (R variants), while others (S) have the properties of inducers of phage and of pseudotuberculosis. Because of the appearance of mutants in the population of virulent strains, which had to be more homogeneous in all respects, including virulence, than the avirulent population, as well as the breakdown of the phage resistant mutants isolated from the S-type virulent strain into rough (R) and smooth (S) forms, we decided to carry out additional studies of the rate of formation of phage resistant mutants in the population of phague bacteria and of stability of their properties.

EXPERIMENTAL METHODS

Four virulent strains of plague bacteria (No. 231, 352, 356, and 363) having typical properties of continental variety have been used in the study, together with the strain No. 293, a glycerin nonfermenter and belonging to the oceanic variety.

Isolation of the phage resistant mutants and determination of the rate of their formation have been carried out according to the previously suggested methods [8, 9, 10]; and the procedure used for studying the plague organisms [1, 5, 7] has been employed for establishing their characteristics. The details of the methods and procedure have been described previously [2].

For isolation of the phage resistant variants by replica plating, the primary petri dishes were inoculated with 20, 100, and 1000 bacteria.

EXPERIMENTAL RESULTS

As a rule, the colonies on the primary plates appeared as rough variants of the plague organism, with slight morphologic deviations in the direction of not significant smoothness, loss of lacy border and formation of edging (Fig. 1). Other colonies had a sharply defined edge and a depressed center; the third type retained a raised center but did not show a lacy zone.

Distinguishing features of primary colonies of the virulent strains are their sensitivity to phages and very rapid (after one day) appearance in the area of lysis of stable smooth colonies, resistant to all phages used in the experiments, including the pseudotuberculosis phage (Fig. 2).

According to the analysis of replica plates, the phage resistant mutants have been obtained in studying strains No. 231 and 352 (after observing 42,000 microorganisms). One phage resistant colony has been isolated from

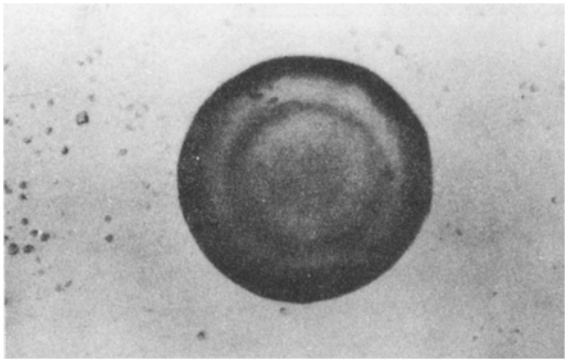

Fig. 1. Original colony of strain No. 352 (14).

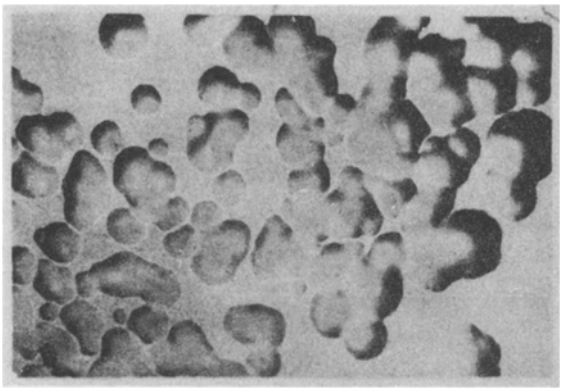

Fig. 2. Development of phage resistant colonies in the lysed area of the original.

bacteria of strain No. 231 (on the replica plate with phage (1-17), and four colonies have been found in the inocula of strain No. 352 bacteria, of which two developed in plates with the phage EV (No. 2 and 14) and two in plates with phages 1-17, EV and D'Herelle (No. 1 and 3). The colonies of phage resistant mutants could be considered, morphologically, to belong phenotypically to the plague bacillus: clear, smooth, round colonies, slightly elevated, with a flat center and a smooth edge (Fig. 3). The obtained mutants have not been isolated using any of the plague and pseudotuberculosis phages.

The rate of formation of phage resistant mutants in the population of the virulent strains is independent of the phage used, but is somewhat dependent upon the virulence of the investigated strains. In the population of virulent bacteria the mutation frequency is 1×10^{-9} and in the avirulent bacteria it is 2 to 6×10^{-8}.

The study of phage resistance of the primary colonies demonstrated that they are not completely resistant to phages, although, as is indicated above, their replicates on plates with phage are resistant.

The study of other properties of the primary cultures and of the isolated phage resistant mutants have been carried out according to the generally accepted scheme for investigating etiologic agents of plague.

It can be seen from Table 1 that organisms in the original colonies having the properties of plague bacteria, became avirulent to guinea pigs. Bacteria from the resistant colonies are differentiated by decreasing biochemical activity, inability to grow on selective diagnostic media and fermentation activity on mannitol and occasionally on glycerin. Mutants $352/EV_2\text{-}R_2$ and 231/1-17-R, isolated from livers of sacrificed guinea pigs infected with the S-mutants of these strains, have been the exceptions. These mutants have been characterized by greater activity than the bacteria from the original colonies and were similar in activity to the causative agents of pseudotuberculosis.

As it has been already indicated, in the experiments carried out on avirulent and virulent strains, there has been observed a breakdown of a smooth (S) phage resistant mutant into R and S variants. In the opinion of investigators studying variations in the plague bacillus [4, 6], this points to the intraspecies dissociation of this activator while at the same time the appearance of the organisms with properties of the pseudotuberculosis agent represents a true mutation. Stable variants capable of dissociation with formation of true mutants, belong to the pre-mutational phase [5]. In order to verify stability of properties of phage resistant mutants of the virulent strains, their colonies have been kept on MPA with phage for 12 days. The transfer of these mutants to broth, with subsequent inoculation on agar medium demonstrated that two of them ($352/EV_2$ and 352/EV-14) formed R and S variants. The rough variants of both strains resembled in their properties the R form of the plague bacillus, have been lysed by plague phage and killed guinea pigs in 3-4 days. The smooth variants did not lead to death of guinea pigs and were not lysed by the plague and pseudotuberculosis phages. Passage of the varient $352/EV_2$ previously subjected to phage in a guinea pig led to the action of plague phages, but sensitive to pseudotuberculosis phage (Fig. 4).

Mutant 231/1-17, smooth in appearance and subjected to previous action of phage and after passage in a guinea pig, also produced R forms, analogous in properties to R form $352/EV_2$ (both cultures have been isolated from guinea pig liver, sacrificed on the 25th day after injection with S mutants).

The obtained phage resistant mutants have been characterized by labile properties and ability to dissociate with formation of variants having properties of another species (pseudotuberculosis).

The Properties of the Original Colonies and of the Phage Resistant Mutants, Isolated from the Population of Virulent Strains

Strain	Glucose	Mannitol	Maltose	Glycerin	Rhamnose	Urea	Kol-Bel'Kur medium	Acid free agar	Peptone-free agar	Otten's medium	Nitrification	Denitrification	Reduction of methylene blue	Phage EV	Phage 1-17	D'Herelle's phage	Phage	Motility	Broth	HS
									Original											
B. pestis 352_1	5+	3+	3+	3+	–	–	2b	–	1p	red	+	+	±	+s.g.	+s.g.	+s.g.	+s.g.	–	Somewhat cloudy, flaky sediment	–
" " 352_2	3+	3+	3+	3+	–	–	2b	–	"	ditto	+	+	±	+s.g.	+s.g.	+s.g.	+s.g.	–	Ditto	–
" " 352_3	3+	3+	3+	3+	–	–	2b	–	"	"	+	+	±	+s.g.	+s.g.	+s.g.	+s.g.	–	"	–
" " 352_{14}	3+	3+	3+	3+	–	–	2b	–	"	"	+	+	±	+s.g.	+s.g.	+s.g.	+s.g.	–	"	–
" " 231	3+	3+	3+	3+	–	–	2b	–	"	"	+	+	±	+s.g.	+s.g.	+s.g.	+s.g.	–	"	–
									Resistant											
	3+	3+	3+	3+	+2d	+3d	++++2d	poor growth	8p	blue	+	–	incomplete reduction	–	–	–	+	+	agglutination, growth	–
B. pestis $352/EV_2$-R_2	2+	–	2+	–	–	–	–	–	1p	red	–	–	–	–	–	–	–	–	cloudy	–
" " 352/EV-S																				
" " $352/D_1$-S	2+	–	2+	+5d	–	–	± 5d	–	"	ditto	–	–	–	–	–	–	–	–	"	–
" " $352/1\text{-}17_1$-S	2+	–	2+	+5d	–	–	± 5d	–	"	"	–	–	–	–	–	–	–	–	"	–
" " $352/EV_1$-S		–	2+	–	–	–	–	–	"	"	–	–	–	–	–	–	–	–	"	–
" " $352/EV_{14}$-S		–	2+	–	–	–	–	–	"	"	–	–	–	–	–	–	–	–	"	–
" " 231/1-17-R		4+	4+	+2d	+2d	+2d	++++2d	poor growth	8p	blue	–	–	–	–	–	–	+	+	agglutination, growth	–

Legend: – no reaction; + weak rose coloration; 2+ definite rose coloration; 3+ raspberry coloration; 4+ intensive raspberry coloration; +s.g. lysis and secondary growth; ± initial activity; +2d, +3d, +5d time, in days, to reaction; 8c, 1c – 8th clearing; 1st clearing.

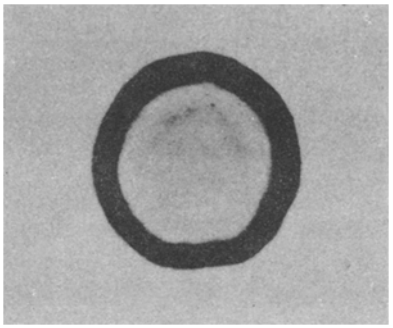

Fig. 3. Resistant colony of strain No. 231.

Fig. 4. Growth of a rough mutant of strain 352/EV_2-R_2, isolated from a sacrificed guinea pig.

In summarizing the studies of the frequency of formation of phage resistant mutants in populations of cells of virulent strains and their isolation in the absence of phage, to which they are resistant (replica plating technique), one can make the following conclusions. The frequency of formation of phage resistant mutants in the population of virulent strains of plague bacteria is lower than the frequency determined by analysis of the population of avirulent strains. The properties of phage resistant mutants are unstable. The data, pointing to the sensitivity of the original colonies to phage action with formation of stable variants, as well as to the appearance of mutants as a result of phage action and passage in a guinea pig, contradict the hypothesis according to which phage resistant mutants are not adaptive in origin but develop as a result of selective mutations [8].

SUMMARY

The rate of formation of phage resistant mutants in the cell population of virulent strains of plaque bacilli.

A continued study of the frequency of formation of bacteriophage resistant mutants in the cell populations of virulent strains, as well as an analysis of colonies, isolated with the aid of the replica plating technique in the absence of the destructive factor (bacteriophage) demonstrated mutations of individual cells to have an adaptive nature. The frequency of bacteriophage resistant mutant formation in the virulent plague strains constitutes $1 \cdot 10^{-9}$.

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