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OF SECONDARY CULTURES OBTAINED BY  
THE ACTION OF BACTERIOPHAGE  
ON PLAGUE BACILLUS**

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CHARACTERISTICS OF SECONDARY CULTURES OBTAINED BY THE ACTION  
OF BACTERIOPHAGE ON PLAGUE BACILLUS

Thoughts on the Mutation of Plague Bacillus Into Pseudo-  
Tuberculosis Bacillus

by

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Among the bacterial species the plague bacillus is perhaps the most homogeneous one in respect to the action of bacteriophage. We have never seen a strain which was naturally resistant to lysis by the bacteriophages at our disposal and which are active at a dilution of  $10^{-8}$ . The lysis of the culture when the phage is added after 24 hours, and its inhibition when the addition is contemporaneous with inoculation are total and definite in almost all cases, and this property is utilized for the microbiological diagnosis of plague (M. Advier, G. Girard) and for the control of the purity of the strains (1). Samples maintained over 50 years in the laboratory and deprived of all pathogenic power as well as very virulent strains freshly isolated from the human or animal organism, behave in an identical fashion toward specific bacteriophages. Our experience relates to several hundreds of strains, since we have always subjected all the cultures isolated at Madagascar between 1932 to 1940 to the action of phage and have done the same over the past five years with the strains of other origin which make up our collection in the laboratory. Recently J. Robic has brought us 19 new cultures from Tananarive on which we obtained identical observations. Exceptionally one sees the appearance of secondary cultures in the broth hemocultures or subcultures, particularly if one utilizes the bacteriophagic filtrates maintained without transfers over several years at laboratory temperature and whose virulence has, therefore, become strongly attenuated.

(1) G. Girard, These Annals, 1942, 68, 476.

E. I. Korobkova (2), in a study performed at the Institute of Microbiology in Saratov dealing with the variants or mutants obtained in the aforementioned manner has stressed the great morphological diversity of their colonies and of the elements of which the latter are constituted, describing, in particular, giant shapes with nuclei (3). She noted a dissociation into clear-cut types R and S, the loss of virulence of the mutants, their more or less marked resistance to secondary lysis by a more active phage as well as the possession, in a latent stage, of a lysogenic capacity among certain elements which had become phagoresistant. She has also observed certain modifications of the biochemical properties the most important of which, fermentation of rhamnose with the production of acid, particularly deserves attention. It is known, in fact, that this carbohydrate is always attacked by the bacillus of the pseudotuberculosis of rodents, but never by the plague bacillus, and that the differentiation of the two germs, so closely related to each other in many of their characteristics, is in part based on this reaction.

The researches which we have carried out at Tananarive and Paris and which are far from being as extensive as those of Korobkova, nevertheless confirm her conclusions regarding the physiognomy of the secondary cultures. In the same tube one notes the two variants R and S, but the macroscopic part S, which is present in excess in the case of the plague bacillus, does not contain in all secondary smooth (lisses) cultures all the properties inherent in this type such as are known, for example, in the case of the Salmonella group. Thus in the case of the plague bacillus the presence of an antigenic carbohydrate-lipid complex is never detected (Pirowsky, Girard). As for the colonies specifically denoted as "R" by Korobkova, some of them definitely have an appearance which is practically never encountered in the case of "ultrapure" cultures (using the term "ultrapure" according to the definition of d'Herelle) of the bacilli of Yersin; they are dry, greyish or translucent, with indented edges, very difficult to preserve, producing on broth very delicate cultures which in stirring look very much like a suspension of very fine and shiny sand.

The first secondary cultures which we observed came from a very virulent strain of pulmonary plague whose inocula on nutritive broths had been treated in some tubes by a phage isolated from the feces of rats (*R. rattus*) and in other tubes by a phage found in fleas (*X. cheopis*) taken from the same rats in an endemic-epidemic plague center in Madagascar. The tubes remained clear for 12 days with the first phage, 33 days with the second phage, after which time there developed a culture having the appearance of a plague culture. Transferred to gelatin, the two variants described by the Russian author were clearly visible, but we failed to keep the colonies of R alive. Examination

(2) E.I. Korobkova, Rev. Epid. Microb. Parasit. Saratov, 1937, 16, 15.

(3) E.I. Korobkova, ibid., p 4.

following the staining of the constituent elements of these original colonies revealed a wide morphological variety: only a few coccobacillar elements, but many small or large ring-shaped elements were observed, as well as racket- or biscuit-shaped elements of which some were enormous, poorly stained and evidently in a prelytic state. In the subcultures these abnormal forms tended to disappear in order to make room for the classic forms. Frankly it does not seem to us as if the bacteriophage had provoked microbial structures which we do not already know in cultures of plague bacillus whose morphology undergoes considerable variations according to the conditions under which they develop (temperature, composition of medium, age of culture). These so-called involutinal forms may assume all the above-mentioned shapes even in young cultures, but this polymorphism is much more marked in secondary cultures following the action of bacteriophage.

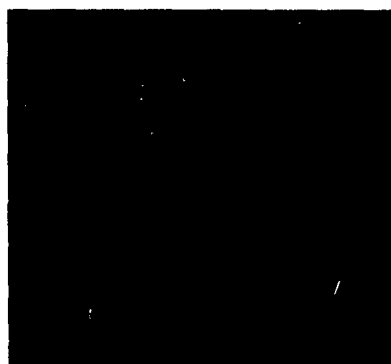


Fig.1. Plague Bacillus, Normal Colonies, Type S.

In these first experiments we have studied, from the point of view of biochemical properties and virulence, three colonies which could be subcultured in series. Their behavior toward carbohydrates, and notably toward glycerol and rhamnose, was the same as that of the original strain. As for virulence, this had considerably diminished, but to a different extent with each sample. Thus, variant B 7 no longer killed either the guinea pig or mouse in doses 1000 times that of the original virulent strain maintained in the same conditions as the secondary cultures. The inoculation, on a shaved and excoriated portion of the skin of guinea pig, of a suspension containing several billions of bacilli per cubic centimeter did not lead to the slightest reaction. The guinea pig inoculated via the cutaneous route treated 15 days later with the pathogenic strain from which this variant derived did not possess any trace of immunity and succumbed to acute plague after the same period of time as did the control guinea pigs; but the mice resisted this exposure which, however, killed the controls in 24 hours. With variant B6, non-virulent both for the guinea pig and mouse, the opposite result was obtained:

guinea pigs resisted the exposure which killed the controls in six days, while mice succumbed in the same period of time as did the controls. Culture B 26 was not found to be deprived of pathogenic power since it caused the guinea pigs to die of chronic plague in 12 days when administered subcutaneously, and on shaved skin it produced an adenitis which was resorbed in 15 days. The subcutaneous inoculation of mice remained without effect, but when inoculated peritoneally, the animals died of acute plague in three days.



Fig.2. Plague Bacilli. Type R Colony Under the Effect of Bacteriophage

Observations of the same order were made in 1942 in Paris on a variant derived from a virulent strain treated with plague bacteriophage (isolated by Advier in Dakar, in 1931, and kept since that time in ampules at room temperature): Resistance of the guinea pig to inoculation of large doses and subsequent immunity; weakened but still marked virulence in the case of mice which die of chronic plague lesions in 6-8 days after the inoculation of between one-half and one billion germs, while the original strain in doses one thousand times less than the latter causes them to die of acute plague in 2-3 days. As was the case above, this variant has not undergone any changes in its fermentative properties toward carbohydrates.

By an analogous procedure we have obtained secondary cultures of strain E.V. (Girard and Robic) used as a live vaccine for the immunization of humans and the preparation of serum-producing horses. The majority of these cultures, true mutants, could only be subcultured in presence of antiphage serum. One of them, studied together with R. Neal, was partly made up of S-type colonies (Strain E.V. 2 S) whose subcultures were easily maintained without antiphage serum; they had the property

inoculation with the greatest of ease in physiological saline solution, forming stable suspensions. Their antigen value and toxicity were below those of the original E.V. strain, but the biochemical properties were identical.

We will add that all the variants whose principal characteristics we have briefly described were lysed by a more virulent bacteriophage than that which has given rise to them.

In a fatal case of bubonic plague treated by bacteriophage and which we followed together with Milliau (4) we made an observation which deserves to be stressed because it was the only time as far as we know that two cultures were isolated -at the beginning of the illness and after death, respectively- of which the second had the characteristics of a true mutant. We did not grasp the full significance of this observation until much later. When one contrasts the lesions of chronic plague produced by the inoculation, in the guinea pig, of material taken from the bubos and liver after death with the customary high virulence of the germ isolated from the bubo prior to treatment, the impossibility of keeping alive the germs isolated by the culturing of these lesions makes us assume at the present time that this anomaly could be attributed to a contamination of the plague bacillus by the bacteriophage. In the course of our long practice in the field of human plague we have not noticed a similar occurrence and our guinea pigs inoculated with plagued material always died with acute or superacute plague lesions, the culture of the specific agent having been easily obtainable and subsequently subculturable, even when this material originated in plague-stricken patients who were receiving bacteriophage as part of their therapy.

#### Discussion

On the basis of the experimental facts which we are reporting it follows that the plague bacilli which develop secondarily in the midst of a virulent culture treated by a bacteriophage of low activity tend to lose all or part of their virulence. This remark naturally applies only to colonies which may be subcultured in series and which exhibit type B. We confirm on this point the conclusions of Korobkova. The only observation which we possess of a mutant obtained in vivo under the action of a specific phage further supports this theory. Let us mention in passing that we cannot subscribe to d'Herelle's opinion who, envisaging the actuality of the appearance of mutants in plague patients treated with bacteriophage which would only act to a partial extent, wrote that "the mutants of the plague bacillus are very virulent (5)." This is a gratuitous statement, not based on any experimental research. The fact that a plague patient who has received bacteriophage succumbs to a germ

(4) H. Girard and M. Milliau, Bull. Soc. Path. Exot., 1935, 23, 880-883.

(5) M. Herelle, The Phenomenon of Cure in Infectious Diseases, Masson, Paris, 1928, 248.

of high virulence, equal to that of the germ in a non-treated patient, does not imply that the former germ is a mutant, but simply that it is a normal bacillus that has not been acted upon by the lytic principle. In all cases where we have studied, under similar conditions, the strains isolated from patients or cadavers, we have found them -with the exception of the sole case referred to above and which invalidates d'Hervelle's thesis- to be very sensitive to lysis by phage even when the latter had been administered during the course of the illness; they proliferate in a normal fashion and are in no way different from classic strains.

As for the changes in biochemical properties of the secondary cultures, indicated by Korebkova but which we ourselves have never observed, the fact is of importance on account of the deductions which are drawn by our colleagues in Saratov and whose significance should not be underestimated. In fact, under the action of the bacteriophage, the two mutants R and S having become avirulent and having acquired the property of fermenting rhamnose, should thus have lost the principal characteristics which distinguished them from pseudotuberculosis bacilli. By analogous reasoning *Pasteurella pseudotuberculosis* could only be *P. pestis* mutated under the influence of the lytic principle. Already in 1928 Zlatogereff and Meghilevskaja tended to consider the bacillus of Malasses and Vignal as a variant R of the bacillus of Yersin and W.F. Harvey (6) considered it, in 1933, as a plague bacillus in symbiosis with the bacteriophage.

This truly appealing concept which the Russian scientists deduced logically from their work would tend to draw even closer the two microorganisms which are already so closely related in many of their properties. Nevertheless the hypothesis engenders serious reservations since the individuality of these two pathogens remains indisputable. To make from the bacillus of Malasses and Vignal a bacillus of Yersin of weakened virulence does not correspond to the facts based on actual observation. We do not here intend to develop this point of view any further and we will limit ourselves to reminding our readers that in human pathology the disease of pseudotuberculosis is a very definite clinical and bacteriological entity; it is a grave and generally fatal illness (7), while there are ambulatory forms of bubonic plague, occurring rather frequently, in which the specific agent does not retain all its classical characteristics. The chronic plague of the guinea pig may quite resemble anatomically a case of pseudotuberculosis, but bacteriologically it remains a case of plague.

In the absence of all apparent intervention of a bacteriophage, Bessonova et al (8) had reported, in 1937, several cases of spontaneous

(6) W.F. Harvey, Trop. Dis. Bull., 1933, 30, 338 and 412.

(7) E. Dujardin-Beaumets, Rev. Path. Comp., 1938, 38, 884.

(8) A. Bessonova, G. Lenskaia, P. Molodtsova and O. Mossolova, Bull. Off. Int. Hyg. Publ., 1937, 29, 2106.

transmutation of plague bacillus into pseudotuberculosis bacillus (5 strains out of the 214 in their collection). In reconciling these observations with those of Korobkova, while we ourselves have never seen anything similar and while none of our plague strains, subjected to regular control in this regard, acidified the glycerol- or rhamnose-containing media, we believe that this divergence may be attributed to a characteristic peculiar to the Russian strains which, in the original form, have the power of fermenting glycerol for the same reason as do all strains of pseudotuberculosis. Glycerinated litmus-treated gelatin cannot serve for differentiating the two microbial species in Russia, but for us it retains the full value assigned to it in 1926 by J. Colas-Belcour. If one examines the problem somewhat more closely, one will remember that all plague-bacillus strains without exception, of which the rat and its fleas are the virus reservoirs, correspond to the type described by Yersin in 1894, and that their passage by man, guinea pig or mouse does not in any way alter their characteristics. On the other hand the acidification of glycerinated media is a property of strains of sylvatic plague such as are found in wild rodents, spermophiles of the Russian steppes, ground squirrels of California and also, sometimes, in jerboas in South Africa. Since they already possess one of the particular characteristics of the bacillus of pseudotuberculosis, these sylvatic-plague bacilli have the tendency to deviate toward the latter type, wither naturally or under the influence of bacteriophage. It would be very instructive in this line of thought to compare the toxicity of original cultures of these two sources: rat-caused plague and sylvatic plague. Extracts or filtrates of plague culture are toxic for the rat and mouse, those of the bacilli of Malassez are not toxic. Let us hope that the reestablishment of normal cultural relations on a global scale will enable us to fill this lacuna by means of exchange of material relevant to this study which at its highest level concerns certain as yet obscure aspects of the epidemiology and prophylaxis of plague.

To sum up and by way of conclusion, we shall say that under the action of bacteriophage one may note the appearance of secondary plague-bacillus cultures whose dominant characteristics are, compared to the strains from which they derive, weakened from the point of view of virulence and antigenic value. The colonies isolated from these secondary cultures take on the most varied shapes in going from type R to type S, the latter being only subculturable in series with or without the addition of antiphage serum. In the case of certain sylvatic-plague strains mutations have been observed, transforming secondary cultures identified at the origin as those of plague into bacilli of pseudotuberculosis. So far this phenomenon has never been observed with murine plague strains originating in areas where sylvatic plague is unknown. But it is important to underline that all the modifications brought about by the bacteriophage-variants R and S, loss of virulence, mutation- may be produced naturally, without having to attribute them to the action of a hypothetical lytic principle, since the strains modified in this way remain, as do those from which derive, sensitive to lysis and are not by themselves lyso-genic. The bacteriophage, however, provokes the onset of the process.

The sole manifestation truly attributable to the bacteriophage is the appearance of colonies of type R the major portion of which does not survive subculturing and whose study can only be undertaken with the aid of an antiphage serum. This study remains to be carried out.