BACILLUS DIPHTHERIAE IN ITS RELATIONSHIP TO BACTERIOPHAGE

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Among the more thoroughly studied bacterial species must be included the diphtheria bacillus, and yet, if we judge from contemporary evidence, its behavior, both as a biological entity and as an agent of infectious disease, is but imperfectly understood. With each new report on its attributes, data are presented bearing on mutation phenomena, dissociation, reversion processes, cyclogeny, and like changes, which all point toward the conclusion that fixity in type and in properties involves a concept no longer tenable.

The profound effect of external environmental conditions upon such characters as morphology, virulence, and toxigenesis is clearly shown by the work of Gins and Jermoljewa (1928–9), to mention but a single instance. That other, and perhaps more subtle, features may equally influence the organism and its behavior is apparent from the results herein to be recorded, bearing as they do upon the relation of bacteriophage to this organism. Study of the relation of *B. diphtheriae* to bacteriophage has been limited (Blair, 1924, Fejgin, 1925), and the observations on record offer nothing of great importance beyond demonstrating the fact that with this organism also the phenomenon of bacteriophagy may occur. It is obvious that, could well-defined bacteriophagy be demonstrated with *B. diphtheriae*, this organism would offer possibilities for study not presented by the majority of bacterial species. Questions of pleomorphism, of fluctuations in virulence, of modifications in toxigenic power, and on the constitution of toxin and, in particular, on the nature of the toxin-antitoxin reaction, might be approached through new avenues.

With some of these possibilities in mind, sporadic attempts have been made in this laboratory, since 1921, to discover or derive a race of bacteriophage active with respect to *B. diphtheriae*, but until this past year no clear-cut results had been obtained. The problem has always been complicated, since there was no way other than mere chance of bringing into contact a potent bacteriophage filtrate and a susceptible bacterial strain. Both of the essential components were, in effect, unknown quantities, and with no assurance that the bacterial strain under examination was sensitive, it was always impossible to affirm that a filtrate did or did not contain bacteriophage. Recently, however, renewed attempts made in connection with other studies upon the diphtheria group of organisms have yielded results of some interest.

The susceptible strain of *B. diphtheriae* which has served throughout the experiments here reported was derived under the following circumstances.

A boy, aged four and one-half years, entered the New Haven Hospital on September 17, 1929, with an admission diagnosis of mild scarlet fever, although at this time there were no throat symptoms. The only point worthy of mention in connection with the previous history is that the child had received smallpox vaccination on the 6th of September, 1919, and on the 15th a cutaneous eruption developed which spread somewhat during the 16th and 17th. The temperature was but slightly elevated. After admission to the Hospital, the rash disappeared and by the 20th of September had completely faded. No further symptoms of any kind developed until September 29, when a swelling of the cervical glands was noted. This was not associated with a temperature reaction or with pain. On the 7th of October the temperature suddenly became elevated, the glands became still further enlarged, and the throat was definitely inflamed. On the 11th a membrane developed and throat cultures revealed diphtheroid-like organisms in direct smears. Later reports on the virulence of the cultures taken at this time were "virulence questionable." The child received antitoxin, and suffered no further complications, although additional cultures which were taken and with which repeated virulence tests were made were recorded as being positive and virulent. After the requisite period of negative cultures the boy was discharged from the Hospital on the 4th of November.

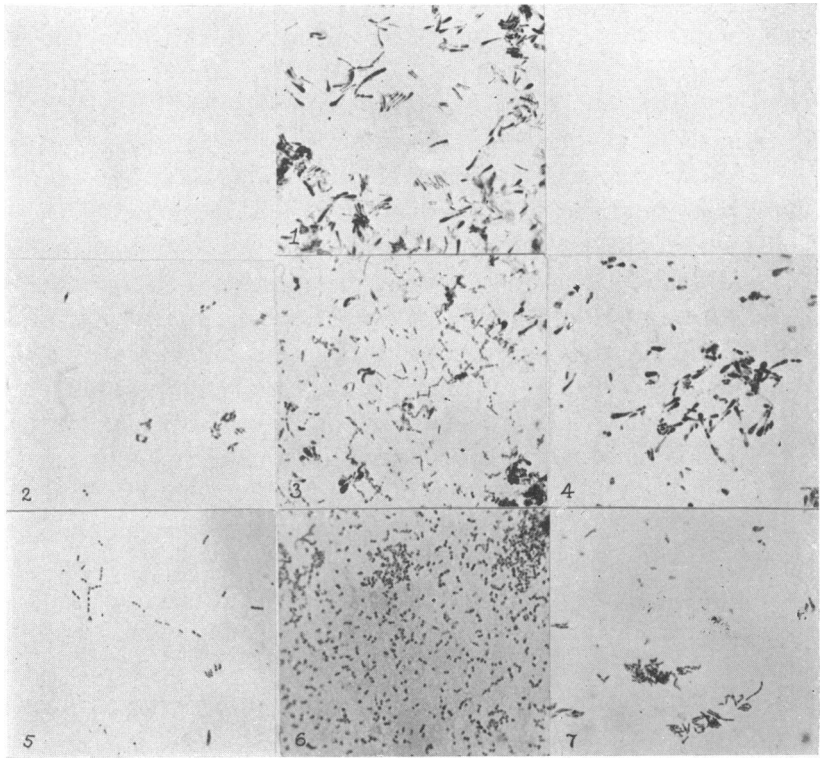

FIG. 1. THE ORIGINAL WRIGHT CULTURE AS FIRST ISOLATED FROM THE PATIENT

FIG. 2. RESISTANT ORGANISMS OF THE LARGE COLONY COMPONENT OF THE WRIGHT CULTURE

FIG. 3. SUSCEPTIBLE ORGANISMS OF THE SMALL COLONY COMPONENT OF THE WRIGHT CULTURE, AS THEY APPEARED AT THE TIME OF ISOLATION

FIG. 4. SUSCEPTIBLE ORGANISMS, WITH ADDED BACTERIOPHAGE, JUST PRIOR TO THE BEGINNING OF LYSIS

FIG. 5. THE SUSCEPTIBLE STRAIN AS IT APPEARS SEVEN MONTHS AFTER ISOLATION

FIG. 6. THE SUSCEPTIBLE STRAIN ON BESREDKA'S EGG MEDIUM

FIG. 7. THE SUSCEPTIBLE STRAIN TAKEN FROM BESREDKA'S EGG MEDIUM AND GROWN UPON SERUM-AGAR

The culture which has served for the experiments herein recorded was a virulent organism derived from this case, and in its original form, is designated as the "Wright" strain. When obtained it possessed no distinctive characters and was of the Wesbrook C type (fig. 1). The alterations which it has subsequently undergone will be discussed later.

The bacteriophage which served in the initial experiment to effect bacteriophagy with this Wright strain was obtained from a sewage filtrate. The raw sewage in question had previously served as a source of supply for bacteriophage races showing a variety of potencies. For present purposes this sewage, after filtration, was added to nutrient broth in the proportion of nine parts of sewage filtrate to one part of a ten-fold concentrated broth. This material, in 50 cc. quantities, was inoculated with 0.2 cc. of broth cultures of each of nine freshly isolated strains of diphtheria bacilli. Four such preparations were made. The object, of course, in such multiple inoculations was to offer any bacteriophage that might be present in the filtrate an opportunity to become enriched if any of the nine strains contained susceptible organisms. Of the four bottles inoculated, only one exhibited, after incubation, any inhibition of growth, and here it was incomplete.

The material from this bottle was filtered (in all filtration experiments Chamberland L3 or L5 filters were employed), and, after demonstration that the filtrate was sterile, it was distributed throughout a series of tubes, and these were in turn each inoculated with one of the nine cultures previously employed, in order to ascertain with which of the nine strains the filtrate was most active.

This titration showed that if the inhibition previously observed was bacteriophagy, culture Wright was the organism most susceptible, and it was upon the basis of this examination that this culture was selected for all further studies. That the result obtained with this filtrate and culture Wright was in effect bacteriophagy was proved by subsequent tests in which it was shown that serial transmission of the phenomenon could be effected indefinitely with the titer of the bacteriophage maintained at a dilution of

10^{-6}, and also by the demonstration of plaque production upon solid media.

In the course of the initial studies upon plaque formation it was noted that colonies of two types were frequently present in cultures of this Wright strain which had grown for periods of forty-eight hours or longer. The first of these was a large, rough, freely-growing colony, resembling in many respects diphtheria cultures which have been for a long time under artificial cultivation. Morphologically, the bacteria composing colonies of this sort were of the Wesbrook C type, but presented a marked tendency toward extreme pleomorphism with the occurrence of a variety of somewhat bizarre "involution forms" (fig. 2).

The second variety of colony was small and fine, presenting no distinctive features other than its minute size. Morphologically the organisms were of the C and C^2 types. They were beaded and very definitely presented a polar distribution of the deeply-staining areas (fig. 3). Subsequent study has indicated that these two types of colony and the morphologically distinct organisms composing them bear definite relationships to various factors entering into bacteriophage phenomena with this organism.

When this apparent dissociation was observed the two types of colony were separated and the cultures thus derived were tested for their susceptibility to the bacteriophage filtrate. It was then found, and this has subsequently been confirmed many times, that the large colony derived from this Wright strain is not susceptible to bacteriophagy. The small colony, on the contrary, manifests, just so long as it maintains its own distinctive features, a complete susceptibility to lysis.

For the studies in mind it was desired to have available several cultures of diphtheria bacilli of diverse derivation, all susceptible to bacteriophage, and, consequently attempts were made to adapt this bacteriophage to other diphtheria strains. To this end repeated contacts between filtrates of known potency for the Wright strain and the other cultures were made. In most instances no evidence whatever of such an adaptation could be disclosed, while in others results of a questionable nature were obtained. In no instance, with this series of cultures, did a complete adaptation occur. Failing, therefore, to adapt this race of bac-

teriophage to other cultures it seemed reasonable to resort to the alternative expedient and seek other races of bacteriophage.

At that time the question of the homogeneity or heterogeneity of diphtheria bacilli as regards bacteriophage was an open one. As sources for such races recourse was had to a variety of materials. Two other sewage filtrates were tested, stool filtrates from a diphtheria patient at different periods during the disease, stool filtrates from a convalescent diphtheria case, blood from a patient convalescent from a streptococcus infection in which the blood culture showed organisms of the diphtheroid type; stool, urine, and throat washings from a diphtheria carrier; stool filtrates from an untreated, fatal case of diphtheria; throat washings from normal persons as well as stool filtrates from normal individuals, and culture filtrates of the first isolation of the Wright strain.

Without entering into details as to the success obtained with these various materials, since the question of distribution of diphtheria bacteriophage merits consideration elsewhere, it may be stated that races of bacteriophage exhibiting a greater or less degree of potency were recovered from many of the above sources. In all, 11 races of bacteriophage were secured. During the meantime additional strains of *B. diphtheriae* had been isolated directly from field cultures received from the Connecticut State Department of Health, and with these, 36 in number, contacts were made with each of the 11 filtrates.

It was felt that such a titration might serve to yield additional susceptible races of *B. diphtheriae* and might also aid in resolving the question of homogeneity. For reasons which will appear below, these tests were largely unsatisfactory, but they did serve to demonstrate that among the 36 cultures under test no other example of a completely susceptible strain was present. It may be added that although several hundred cultures have since been examined we have yet to find a strain comparable in susceptibility to the Wright culture described above.[1] Thus, all of the work

[1] Since this manuscript was submitted for publication a second susceptible strain, isolated from a field culture, has been obtained. For a period after its isolation this culture behaved in every respect like the Wright culture. It also developed a resistance as did the Wright strain, but unlike the latter it has thus far failed to yield susceptible derivatives.

which has been done has been conducted with this susceptible derivative of the original Wright strain, and the very fact that in all of the tests only one susceptible organism has been employed militates against drawing broad conclusions from the data here offered. Nevertheless, under the limitations imposed by these conditions, the results which have been obtained are of such clear-cut character and are so suggestive that this preliminary report may be justified.

In presenting this material two aspects of the question may be considered, since each is of importance, although from different points of view.

First, the peculiar attributes and behavior of this susceptible Wright strain merit attention since the potentialities of this individual culture may be of significance to an understanding of the cyclogeny of *B. diphtheriae*. The second feature worthy of comment involves a consideration of the properties of field cultures of diphtheria bacilli in their relationship to this particular Wright strain and to the various bacteriophage preparations active upon it.

The Wright strain of *B. diphtheriae* has now been under daily observation for a period of over eight months and during this time on various occasions it has exhibited rather striking variations in some of its attributes. It will be recalled that this strain was derived from what might be termed a mixed culture and that it represents the small, fine colony component of that culture. On several occasions throughout the continued study of this culture it exhibited marked changes, not only with reference to its susceptibility to the bacteriophage, but in morphology as well. Because of the readiness with which this small colony derivative of the Wright strain grew in fluid media, during the early studies broth cultures were used throughout, without intervening transfers to solid media (fig. 4). After a series of such transfers, during which time the organisms had retained their susceptibility to bacteriophage, a gradually increasing resistance became apparent. This increased resistance was evidenced not so much by a developing refractoriness to lysis as by the fact that after an apparently complete lysis the period intervening before the development

of a secondary culture progressively diminished. At the end of about 15 serial transfers from broth to broth it became obvious that the culture had become transformed, although morphological study of the strain at this time failed to disclose profound differences as compared with the strain used in the initial tests. The tendency toward bipolar staining was possibly more in evidence and it seemed that that portion of the rod lying between the deeply-staining granules tended to take the stain less intensely. Cursory examination of the culture in stained preparation at this time gave the impression of a streptococcus rather than of the diphtheria bacillus (fig. 5). This tendency progressed, but at no time did a culture ever mature without solid-staining rods, unmistakably diphtheria, being present with the cocci. Even when the single minute colonies have been fished from blood plates with the aid of the dissecting microscope an admixture of both types has always been encountered.

Reverting to the original Wright culture, a second isolation was made and the strain thus obtained was apparently identical with the first, stained preparations showing the small, bipolar staining rods, completely susceptible to lysis without subsequent secondary growth. Repeated serial passages with this derivative were effected and at the third passage evidence of secondary growth was apparent, and in successive passages this became more marked.

In studying this transformation it was noted that when secondary cultures followed lysis, platings from the control tubes invariably revealed colonies of the two types, comparable in all respects to the two types originally derived from the Wright strain. Platings of the secondary growth which appeared yielded colonies of but one type and these were identical with the colonies of the large, rough type as derived from the control tube.

It was obvious that the original Wright culture represented a strain tending toward "spontaneous" dissociation and this dissociative phenomenon was accelerated in the fluid medium. Examination of a serum-agar slant inoculated with the culture when the isolation was made and which showed only the almost invisible transparent growth typical of the substrain now revealed

many white, vigorously-growing colonies superimposed upon the original culture. These colonies and the bacteria composing them corresponded in every respect with the large, rough colonies. Fishings from the small, fine colonies of this culture, as well as from the original have been made periodically since this observation was made and they have been carried along through successive cultures simultaneously, both in broth and on agar.

The serial broth cultures do one of two things. They may become resistant to the bacteriophage, and when plated show, usually after a prolonged incubation, the presence of large, daughter colonies resembling in every particular the growth of the resistant strain, or they may exhibit a tendency toward spontaneous disappearance.

Filtrates made from such "suicide cultures" contain bacteriophage, and agar slants made from the culture when it first shows evidence of a failure to develop will show plaques. It may be stated again that serum-agar slants inoculated with the freshly isolated, susceptible derivative never show plaques or daughter colonies, and these cultures, when transferred to broth, grow, at first, very slowly so that incubation for 48 hours at least is necessary for obtaining growth from the initial transfers. These broth cultures are always more or less susceptible to bacteriophage. When a susceptible culture of this type is transferred to Besredka egg medium the morphological aspect of the organism becomes very distinctive. At the end of twenty-four hours smears reveal cocci only (fig. 6). These coccus forms are large, in diplo-arrangement, and seem characteristic of growth on this medium. That they represent a true phase of the development of this culture seems certain, since if smear preparations are made at short intervals during the period of growth upon this medium the transitional stages between the bacillary morphology and the diplococcus arrangement may be readily followed. These cocci are apparently associated with the accumulation of deeply-staining material at either end of the rod. The portion of the rod connecting these bipolar zones gradually loses its staining property and in some cases seems to disappear entirely.

In older cultures single cocci are occasionally found, but the

most common arrangement is in the diplococcus form with pairs of diplococci forming either tetrads or short chains. Cells of this coccus type are not to be confused with typical cocci, for at no time are these apparent cocci perfectly round in contour nor do they exhibit uniformity in size. They could not possibly be confused with staphylococci. When the cultures on the Besredka medium are allowed to stand for ten days or more before subculturing, the agar slant in almost every case will show at least half of the organisms to be typical diphtheria bacilli, belonging to the barred or solid-staining types (fig. 7). The colonies on plates are all alike and most of them contain both the coccoid and solid-staining forms. It must be admitted, however, that up to the present time a complete reversion from this coccus form to the original bacillary form has not been effected. Incidentally it may be added that broth cultures grown from such a culture on Besredka's medium resist bacteriophage.

The behavior of the susceptible Wright strain, that is, its tendency to give rise to the resistant type of colony or to "commit suicide," introduced the question as to whether the organism as we have studied it was not really a secondary culture. To study this problem filtrates from broth cultures which had been made some three weeks previously were prepared and 0.5 cc. of each filtrate was added to a tube of broth. Two drops of a broth culture of the susceptible diphtheria organism were added to each tube. Of the 36 filtrates thus tested 13 exerted no inhibitory action upon the test organism, another 13 showed a slight inhibitory effect, and the remaining 10 caused a complete inhibition of growth in the first passage. These cultures were again filtered and a second passage was made, using in each case one drop of the filtrate. In four instances no inhibition of growth followed, and in six but a slight clouding of the medium was obtained, while with 26 of the 36 strains lysis was complete, although readings made shortly after the inoculation showed that the susceptible strain underwent growth in all tubes. In the third passage with these culture-filtrates complete lysis was obtained in all except three and these exhibited such a slight turbidity that

they were read as partial lysis. All of the control tubes showed the absence of lysis and the controls containing known bacteriophage gave complete lysis. This third passage of the filtrates was prepared in duplicate and after the twenty-four-hour readings one set was returned to the incubator, and by the end of the fourth day secondary growth had developed in all except one. The duplicate set of tubes was held at room temperature and developed secondary growths in about a third of the tubes. In no instance, however, did the intensity of growth equal that obtained in the control tube.

While the tests above described were in progress, additional strains of diphtheria bacilli were being accumulated as rapidly as possible, the cultures being derived largely from field cultures supplied us by the Connecticut State Department of Health Laboratories and from clinical cases present in the New Haven Hospital. When an additional 40 such strains had been obtained they were tested in the same way and without exception the results were of the character above described. In no instance did a culture filtrate cause complete lysis after the first passage, although in about one-half of the cultures the filtrates caused more or less inhibition of growth. With the second passage, however, a complete absence of growth or lysis occurred in all but four cases, and these showed an inhibition. The twenty-four-hour reading of the tubes prepared from the third passage showed complete absence of growth in all tubes save the appropriate controls. At the end of four days about half of these tubes had yielded secondary cultures. Later a third set of ten cultures was prepared and these were filtered after they had grown in broth for three days. Nine of these failed to show evidence of lysis until the third passage. Upon the fourth passage all filtrates inhibited growth. Thus, while all of the cultures examined exhibited constancy in behavior as regards the ultimate result, there seemed to be differences of a quantitative nature indicating that if these field cultures really represent lysogenic strains, the readiness with which they yield demonstrable bacteriophage varies.

With the idea of determining whether this variability could be

referred merely to quantitative factors or whether quality also influences the result, ten cultures from among those previously examined were selected at random. Filtrates were prepared and these filtrates were subjected to ten passages with the susceptible diphtheria strain. After the tenth passage the filtrates were subjected to serial dilution for testing the relative potency of the bacteriophage races present. In 5 cases a complete bacteriophagy was obtained in a dilution of 1:500,000, in four it was complete at 1:3,500, and in one at 1:5,000. Obviously these races of bacteriophage are not of high potency, a fact which may be significant, but of even greater importance than this is the demonstration of the fact that such a difference actually exists. This indicates, of course, that the incitant for bacteriophagy was to be found in the filtrates derived from the diverse strains rather than in a splitting-out of bacteriophage from the suceptible strain used in all of the tests. It should, perhaps, be emphasized that this conclusion is merely indicated and is not proved by this experiment.

In addition to the large number of cultures subjected to examination as described above, another group of cultures was tested. These cultures were those described in a prior publication and were the strains which had been subjected to adverse environmental conditions and had yielded filter-passing organisms.

Cultures of the latter were made in broth and, after a period of incubation, filtrates were prepared and these were tested against the susceptible Wright strain. All of these filtrates contained lytic principles, but they were invariably of low potency, since, even after seven successive passages, nine of them failed to cause a complete and permanent lysis. That bacteriophage was present, however, could readily be seen from the second passage on throughout the series.

The nature of the Wright suceptible strain, fluctuating as it does, apparently on the basis of dissociative changes and selection, between resistance and suceptibility, inevitably introduces an element of uncertainty in matters of interpretation. Nevertheless, it appears certain that this strain possesses certain attributes which tend to set it apart from the other cultures which we have

studied. In the first place, it is the only culture from which a completely susceptible derivative has been recovered. Many of the other strains have exhibited a partial susceptibility, but a complete dissociation and segregation through colony types has not been attained. It is equally certain that this Wright culture, even in its susceptible form, is in reality, or through unknown causes may become, a lysogenic strain as is evidenced by its "suicide" behavior under certain circumstances. The facility with which it passes from the susceptible to the resistant state, with the accompanying morphological changes, appears to be peculiar to it, and while continued growth in broth accelerates the "susceptible-to-resistant" transformation, the true nature of the incitant is not known. While in the resistant form this culture is unquestionably lysogenic and must certainly carry the lytic component through its suceptible stage, it is equally true that many filtrates made of the culture during its sensitive periods have proved to be devoid of action on the organism itself. And yet, bacteriophage must have been present. This observation may very well serve as an example of the very insecure nature of the apparent stability of many cultures and to emphasize again the extreme subtility of those forces competent to disturb equilibrium.

The intimate relationships between the bacteriophage and the elementary diphtheria organism are obscure, and the fact that all strains of diphtheria bacilli thus far isolated from field cultures yield lytic filtrates suggests that the diphtheria "bacillus" as we know it may be a most complex being. The coccoid forms occurring in the transformation of the susceptible derivative and the coccus stage appearing in the development of the bacillary form from the filter-passing stage may well be worthy of consideration. At the moment, however, it would seem that the most urgent problem is that associated with deriving additional strains susceptible to bacteriophagy. Until this is accomplished the studies here recorded must be regarded as but an isolated observation, and while suggestive, they do not justify the conclusion that the diphtheria bacillus as we know it is always a lysogenic culture, that is, a being in which the bacterium is

uniformly found in association with bacteriophage, possibly an obligate symbiosis.

As for diphtheria bacteriophage, it would seem to be fully as common as is bacteriophage for *B. coli* or for pyogenic cocci. Its demonstration in sewage, in patients, in convalescents, in field cultures, and under certain circumstances in dust and air, suggests that it may play a significant rôle under many circumstances, and offers to the epidemiologist an additional point of attack in the study of those conditions governing the behavior of communicable disease.

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