A STUDY ON THE DISSOCIATION OF THE DIPHTHERIA BACILLUS

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Comparatively little work has been done on the dissociation of the diphtheria bacillus into smooth and rough forms, despite the fact that virulent and non-virulent strains of the diphtheria bacillus have been described as long ago as Roux and Yersin's work in 1890. In 1897 Corbett and Phillips actually showed that a pure line culture of the diphtheria bacillus yielded two types of colonies, one virulent and the other non-virulent. In 1898 Slawyk and Maincatide may well have been describing smooth and rough colonies when they stated that among 30 strains of the diphtheria bacillus they found one kind of colony which was convex and glistening and another kind which was flat and dull. It was not, however, until nearly thirty years later that it was shown that virulence was associated with the smooth type of colony, and non-virulence with the rough type. Cowan in 1927 showed that a stock culture of the Park 8 strain split into a smooth, virulent and a rough, non-virulent form.

In the present study an attempt has been made to answer the following questions:

1. Is virulence always associated with the smooth form of the diphtheria bacillus and non-virulence with the rough form?
2. Is toxin formation related to the morphological difference in the colonies?
3. Is it possible to transform at will the smooth form into the rough form, and *vice versa?* If so, under what conditions does this transformation take place?

4. Which forms are found in the acute stage, the convalescent stage and the carrier stage of diphtheria?

5. Do the rough forms possess any immunizing value?

In addition, it was kept in mind that the objection might be raised that rough, non-virulent bacilli found in throat cultures, although they might resemble diphtheria bacilli under the microscope and even in fermentation reactions, might not be true diphtheria bacilli. This objection is difficult to meet conclusively, but if it were possible to show that rough forms could be derived in the test tube from a pure, smooth culture, and that these rough forms were similar in every respect to those found in throat cultures, it would be very strong evidence that the rough forms occurring in the throat were genuine diphtheria bacilli.

MATERIAL

With the above problems in mind, the study was commenced by securing throat cultures from diphtheria cases at different stages of the illness.

The surface growth on the Loeffler slant was washed off with about 5 cc. of hormone broth and gently emulsified. A loopful of the emulsion was then streaked on each of two blood agar plates. The blood agar plate was used because it was easy to pick out the colonies of diphtheria bacilli from those of streptococci and pneumococci. The plates were carefully examined the next morning, after about twenty hours' incubation, under the stereoscopic binocular microscope. Diphtheria-like colonies were picked out and replated on blood agar plates. After the second plating, colonies—whether rough or smooth—which showed characteristic diphtheria bacilli were transferred to Loeffler's slants, and the fermentation reactions and virulence tested.

In the acute stage, 47 throat cultures were studied, and all showed smooth colonies only.

In the convalescent stage, among 79 cultures 16 were found with both rough and smooth colonies.

In the carrier stage, the 3 cases that were investigated showed both types of colonies.

Thirty-one cases of clinical diphtheria were cultured system-

atically up to the time of their release. In the acute stage, no rough forms were found, but in 5 cases the cultures for release showed a few rough colonies.

Lastly, a culture of the Park-William 8 strain that was being used for toxin production was studied carefully. Colonies were found which appeared to be intermediate in type. They had a granular surface, but were not so rough as the rough forms found in throat cultures.

Examination of material

The various strains that had been obtained from these sources were examined for their morphological and cultural characteristics, their fermentation reactions, their virulence, their toxin production, and their immunizing properties.

Morphology and cultural characteristics. The smooth forms are generally thinner, longer and more striated than the rough forms, which are apt to be somewhat short, plump, and to stain uniformly. The smooth colonies are glistening and whitish in colour, while the rough colonies are smaller, denser, more granular and greyish in colour. The smooth type forms a thick pellicle on broth with little growth at the bottom of the tube, while the rough form, when freshly isolated, forms no pellicle at all and tends to grow at the bottom of the tube. On passage in broth a thin pellicle is ultimately formed. The surface growth on a Loeffler slant of the smooth organism can be emulsified into a uniformly turbid suspension, but the rough form, similarly treated, gives a granular suspension.

Fermentation reactions. There has been considerable discussion as to the reliability of fermentation in differentiating the diphtheria bacillus from the diphtheroids. However, many reliable workers, including A. Knapp (1904), Hans Zinsser (1907), J. G. Fitzgerald (1924), C. C. Okell and Baxter (1924), working with a great number of strains of diphtheria bacilli as well as with diphtheroids, have come to the conclusion that the fermentation test does differentiate the Klebs-Loeffler bacillus from the other organisms. Two-hundred and sixteen cultures of diphtheria bacilli were studied, of which 164 were smooth forms and 52 were

rough. These, as well as cultures of *B. hoffmanni* and *B. xerosis*, were tested in serum water media containing glucose, dextrin and sucrose. The tubes were read seven days after inoculation. All the diphtheria bacilli formed acid in glucose and dextrin serum

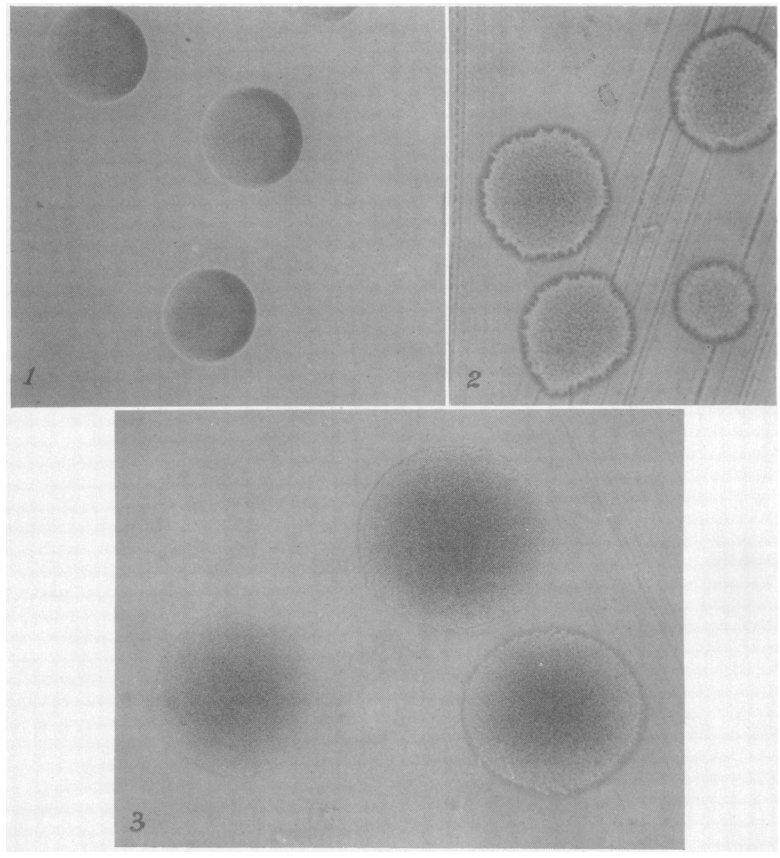

FIG. 1. SMOOTH TYPE COLONIES
FIG. 2. ROUGH TYPE COLONIES FROM THROAT CULTURE
FIG. 3. INTERMEDIATE TYPE COLONIES FROM PARK 8 STRAIN

water, but not in sucrose. The rough forms gave the same fermentation reactions as the smooth forms.

Virulence. In testing for virulence, the various strains were grown for twenty-four hours on Loeffler's medium. The growth

on the slant was washed off in 10 cc. of saline and 0.1 cc. of this emulsion was injected intradermally into each of 2 guinea pigs, one normal and the other protected with 500 units of antitoxin. In general, 5 strains were tested on each pair of guinea pigs. Two-hundred and sixteen strains were tested in this way with the following result: 164 strains were smooth and 146 of these were virulent and 18 avirulent; the 18 avirulent smooth forms were all obtained from convalescents or carriers; 52 strains were rough and all were avirulent.

Toxin production. In testing for the production of toxin, the procedure was as follows: Cultures were transplanted daily for three days in broth, and then a piece of pellicle was inoculated into 40 cc. of special broth contained in a 300 cc. flask. The special broth was prepared according to Harrison's formula, from the United States Hygienic Laboratory. The initial pH of the broth was 7.3. The inoculated broth was incubated for five days at 36°C., the reaction of the broth being checked daily. At the end of the incubation, the culture was centrifuged, the supernatant fluid filtered through a Berkefeld "N" filter, and the filtrate tested for toxicity by guinea pig injection. Twelve rough strains and 5 avirulent, smooth strains were found to be completely atoxic. The cultures remained acid during the incubation period, and 5 cc. of the filtrates had no effect whatever on guinea pigs, when injected subcutaneously. Five smooth, virulent strains were tested. Two out of the 5 produced a toxin with a M.L.D. of 0.004 cc. The remaining 3 strains, although virulent, produced a toxin with a M.L.D. of 1.0 cc. It is interesting that these 3 strains were isolated from the throats of 3 different carriers. The toxicity of the cultures of the 2 types of colonies isolated from the Park 8 strain was tested intracutaneously on guinea pigs. The filtrates were first diluted 1:500 and then titrated (table 1).

Table 1 shows that the smooth colony type of Park 8 strain produces a toxin at least 5 times stronger than the toxin produced by the intermediate colony type.

Immunizing properties. Guinea pigs were injected both subcutaneously and intraperitoneally with filtrates of cultures of the

rough colony organisms. Three weeks after the last injection of rough filtrate, the guinea pigs were tested with 1 M.L.D. of toxin. All the animals died within six days. The rough filtrates, then, produce no antitoxic immunity whatever.

The experiment was repeated, using the filtrates of smooth avirulent organisms. Again no immunity resulted.

As was to be expected, the injection of living, rough bacilli did not confer any immunity on guinea pigs, the subsequent injection of living, smooth, virulent organisms being fatal to these animals.

TABLE 1

AMOUNT OF FILTRATE	1:500 FILTRATE FROM PARK 8 SMOOTH COLONY	1:500 FILTRATE FROM PARK 8 INTERMEDIATE COLONY
cc.		
0.125	++++	++++
0.08	++++	++++
0.04	++++	+++
0.01	++++	++
0.005	++++	0
0.0025	+++	0
0.0015	++	0
0.001	0	0

IN VITRO EXPERIMENTS ON THE DISSOCIATION OF THE DIPHTHERIA BACILLUS

1. Attempt to dissociate the "S," or smooth form, into the "R," or rough form, by means of incubation at a temperature of 45°

Hewlett and Knight (1897) reported that virulent diphtheria bacilli were changed into avirulent vacilli by seventeen hours' exposure at 45°C. Accordingly, 5 smooth, virulent toxic strains were incubated at 45° for seventeen hours for 20 passages, plating out on blood agar plates and incubating the plates at 37°C. between each 45° cultivation. Examination of the plates usually showed only a few scattered colonies, but these were as smooth and glistening after the twentieth passage as were the original colonies. A Loeffler slant at 36°C. after the tenth passage, however, was completely avirulent for guinea pigs. Examination of all the strains at the end of the experiment showed that they were

all atoxic as well as avirulent. Hewlett and Knight's work was thus confirmed, but no dissociation into the "R" form had taken place under these conditions.

2. Attempt to dissociate the "S" form into the "R" form by means of cultivation in antitoxic serum

Observations on the transformation of toxic and virulent strains of diphtheria bacilli into atoxic and avirulent strains have been made by Bernhardt (1916), Levinthal (1926), Becker (1927), and recently by Jungeblut (1928). Jungeblut, after cultivating 2 strains of diphtheria bacilli in broth containing normal horse serum on the one hand and antitoxic serum on the other, found that the cultures in normal horse serum broth remained unchanged, but that the cultures in the antitoxic serum broth lost their capacity to produce toxin after 6 passages in this medium, while retaining their virulence of guinea pigs.

Five strains of smooth, virulent, toxic bacilli were cultivated in 3 different media.

a. 1.0 cc. normal horse serum + 4.0 cc. of broth.

b. 1.0 cc. of concentrated antitoxic serum (10,000 units per cubic centimeter) + 4.0 cc. of broth.

c. 1.0 cc. of unconcentrated antitoxic serum (50 units per cubic centimeter) + 4.0 cc. of broth.

After 10 passages, none of the strains in any of the 3 mediums showed any colony difference; so they were then cultivated in undiluted normal horse serum and undiluted antitoxic serum for another 10 passages. Still the appearance of the colonies was unchanged, being perfectly smooth and glistening, while the morphology of the bacteria also remained normal. Cultures from both the normal serum and the antitoxic serum were tested at the end of the experiment for virulence and toxin production. Both sets of cultures were virulent, and the M.L.D. of the toxin produced was about 0.02 cc.

No dissociation into the "R" form occurs, then, if "S" strains are cultivated in antitoxic serum.

3. Attempt to dissociate the "S" form into the "R" form by means of cultivation in anti-bacterial serum

The method used for the production of anti-bacterial serum was similar to that used by Fitzgerald and Doyle (1923) in making agglutinating serum for *B. diphtheriae*, except that the cultures were washed 3 times instead of 14 times, as in Fitzgerald and Doyle's technique. A suspension of the washed bacilli of 10 strains of smooth virulent organisms was made up and injected into rabbits in the following way:

a. 1.0 cc. of the suspension was heated to 60°C. and injected subcutaneously.

b. One week later, 2.0 cc. of the heated suspension was injected subcutaneously.

c. One week later, 2.0 cc. of the unheated suspension was injected intravenously.

d. One week later, 5.00 cc. of the unheated suspension was injected intravenously.

e. One week later, 10.0 cc. of the unheated suspension was injected intravenously.

Rabbits were bled seven days after the last injection. The sera so obtained usually agglutinated in a concentration of 1:3000. They also contained a small amount of antitoxin—about 2.0 units per cubic centimeter.

The serum of one such rabbit was distributed into small tubes, each tube containing 0.5 cc. of the undiluted serum. A similar set of tubes, each containing 0.5 cc. of normal rabbit serum was prepared at the same time. Five strains of smooth, virulent toxin-producing diphtheria bacilli, together with a strain of *B. xerosis*, were cultivated in the anti-bacterial serum and in the normal serum. Sub-cultures were made every other day and plates streaked at the same time. On the tenth passage a few rough colonies were observed in 2 out of the 5 strains. These were replated and found to produce rough colonies only. The other 3 strains were then transferred to the serum of another immunized rabbit and cultivated as before. After the sixth passage a few rough colonies were seen in one strain, and the number of rough

colonies increased with the number of passages until, at the twentieth passage, there were an equal number of rough and smooth colonies. The remaining 2 strains and the culture of *B. xerosis* underwent no change.

The rough colonies were tested and found to conform in every way to those found in the throats of convalescents. The morphology of the bacilli and the appearance of the colonies were indistinguishable from those of the rough forms described earlier in this paper, the fermentation reactions were typical, the strains were totally avirulent and atoxic and they produced no immunity. No explanation was found to account for the fact that 2 of the strains cultivated under these conditions remaining unchanged during 30 passages.

4. Attempt to change the "R" form back into the "S" form by animal inoculation

One rough strain, transformed from the smooth form by means of cultivation in anti-bacterial serum, and the Park 8 intermediate form were used. The organisms were grown separately in 100 cc. of broth, the cultures were then centrifuged and the sedimented bacilli resuspended in 2 cc. of saline. Each suspension was injected into a guinea pig subcutaneously. With the rough organisms an abscess formed at the site of injection, but cultures of the pus showed pure cultures of the "R" form. The guinea pig injected with the Park 8 strain died and cultures showed intermediate forms only.

5. Attempt to change the "R" form back into the "S" form by cultivation in "R" antibacterial serum

For this experiment serum was prepared in the same way as with the "S" anti-bacterial serum, but rough bacilli were used instead of the smooth, virulent organisms, and the suspensions were not heated for any of the injections. Two rough strains were cutivated in this "R" anti-bacterial serum and showed no change after 15 passages.

DISCUSSION

In the light of the investigations and experiments that have been recorded above, the questions that were posed in the beginning of this paper can now be discussed.

Is virulence always associated with the smooth form of the diphtheria bacillus and non-virulence with the rough form? The answer to the first part of the question is in the negative, as many typically smooth forms were found that lacked virulence, although it is significant that these smooth, avirulent forms were only found in the convalescent stage and not in the acute stage of the disease. Whether they are, or are not intermediate forms between the smooth and the rough forms is not known at present. The answer to the second part of the question is in the affirmative, as was of course to be expected. All the rough forms tested were completely avirulent. This is in conformity with the most important characteristic of the "R" forms of the majority of pathogenic organisms.

Is toxin formation related to the morphological difference in the colonies? The answer to this question is closely parallel to what has been said about virulence. The rough forms are always atoxic, but some of the smooth forms produce no toxin. It is interesting that in the throats of carriers, smooth forms were found which, although virulent by the usual test, formed only a very feeble toxin. Attention has been called to the fact that the Park 8 strain produces a powerful toxin and yet is not very virulent, so that the reverse picture described here is additional evidence that there is another factor in virulence beside the ability to produce a strong toxin.

An intermediate form was found in studying a culture of the Park 8 strain. This was not a glistening, smooth colony, nor was it a typical rough. It produced a toxin which, although fairly strong, was yet very definitely weaker than that produced by the smooth Park 8 colony.

Is it possible to transform at will the "S" form into the "R" form and *vice versa*? If so, under what conditions does this transformation take place? The loss of virulence or the capacity

to produce toxin has been reported many times in the literature, and in some cases this has been brought about by subjecting the virulent, toxic bacilli to an abnormal cultural environment. The experiments described in this paper, however, show that this loss in biological activity is not accompanied by any change in morphology or colony formation. Apparently the only condition which results in the transformation into the "R" form is the presence of specific anti-bacterial serum in the culture medium and even strong, undiluted antitoxic serum does not bring about this change. "R" forms occur in the throats of convalescent cases, and it would thus seem probable that the *in vivo* transformation is due to the anti-bacterial antibodies in the patient's tissues and not to the antitoxin antibodies. Carriers have been treated by the injection of diphtheria bacilli vaccines in order to rid the throat of the organisms, so far with very little success, but this treatment should have raised the anti-bacterial content in tissues and it is at least possible that the bacilli which persisted after the treatment were rough forms and therefore innocuous.

Experimentally it has been proved that the rough, avirulent, atoxic diphtheria bacillus is a variant of the smooth, virulent, toxic organism, but it has not been possible either *in vitro* or *in vivo* to change the "R" form back into the "S." Although this reversion cannot be demonstrated, there is always the possibility that it may take place in the human body under conditions of which we are at present ignorant.

Which forms are found in the various stages of the disease? In the acute stages only the "S" forms are found. The "R" forms begin to make their appearance during convalescence and this is presumably the result of the elaboration of anti-bacterial antibodies in the patient. Avirulent "S" forms also occur later in the disease and it is possible that this change may be brought about by the antitoxic antibodies.

Do the rough forms possess any immunizing value? As was to be expected, the "R" forms have no immunizing value whatever.

The "R" forms, which were derived from pure cultures of the "S" forms *in vitro* resembled in every particular the "R" forms isolated from convalescent diphtheria cases and carriers, and this

constitutes very strong evidence that the latter forms were genuine diphtheria bacilli and not diphtheroids.

SUMMARY

1. Forty-seven cultures of diphtheria bacilli isolated from patients suffering from diphtheria showed smooth, virulent forms only. Of 79 throat cultures from diphtheria patients during the convalescent stage, sixteen cultures showed the presence of both smooth and rough forms of diphtheria bacilli.

2. Thirty-one cases of clinical diphtheria were cultured at different stages, from the onset of illness up to release. Not a single rough form was found during the acute stage, but 5 cases showed the presence of rough types of diphtheria bacilli from cultures for release.

3. In 3 cases of carriers which were studied, the rough forms of diphtheria bacilli formed the predominating type.

4. One culture of Park 8 diphtheria bacilli was dissociated into smooth and intermediate types by repeated plating on blood agar plates, and while both forms produced toxin, the smooth type produced a much more potent toxin than did the intermediate type.

5. Sugar fermentations give reliable data for the recognition of diphtheria bacilli, but do not differentiate the smooth from the rough type, nor the virulent bacilli from the avirulent.

6. The virulence of 216 strains of diphtheria bacilli was tested. Fifty-two were rough forms and were all found to be totally avirulent and atoxic. One hundred and sixty-four were smooth in cultural characteristics but while 146 were virulent, 18 of them were avirulent. All the avirulent forms were isolated from patients during the convalescent stage or from carriers. These 18 cultures demonstrate that while loss of toxicity is usually associated with the cultural characteristics spoken of as "rough," a diphtheria culture may retain its smooth form and still have lost its toxic properties.

7. Toxicity tests were made on 12 strains of rough types of diphtheria bacilli and 6 strains of rough types transformed from smooth types. They were totally atoxic. Avirulent, smooth

forms were also found to be atoxic, while 3 strains of virulent, smooth forms isolated from carriers were toxic, although the toxin produced by them was weak.

8. Eighteen guinea pigs immunized with filtrates of the rough cultures of diphtheria showed no protection against the filtrates of toxic, smooth types of diphtheria bacilli.

9. Eleven guinea pigs immunized with cultures of the rough type of diphtheria bacilli showed no protection against the smooth types of virulent diphtheria bacilli.

10. By means of repeated exposure of virulent cultures to a temperature of 45°C. for seventeen hours the virulence can be reduced, but this treatment has no effect upon the appearance of the colonies.

11. Smooth, virulent diphtheria bacilli inoculated into diphtheria antitoxin-broth mixtures and then into pure antitoxin showed no change after 20 passages, while 3 out of 5 strains of diphtheria bacilli tested showed a change of smooth type to rough type after subculturing in anti-bacterial serum.

12. Various attempts were made to transform the rough type of diphtheria bacilli into the smooth type again by means of growing the rough type in the toxin-broth mixture, animal inoculation and subculturing in antibacterial serum of rough diphtheria bacilli. By none of the above mentioned methods was it found possible to convert the rough type back to the smooth type.

CONCLUSIONS

1. Smooth, virulent and toxic diphtheria bacilli are transformed to non-virulent, "R" forms in the throats of patients during convalescence.

2. The transformation may pass through an intermediate stage in which diminished toxin formation is not associated with morphological change.

3. *In vitro* experiments indicate that the dissociation "S" → "R" is governed by contact with anti-bacterial rather than with antitoxic serum.

4. The vaccination of carriers of "S" diphtheria bacilli with "S" vaccine is thus rendered logical.

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