STUDIES ON THE TWORT-D'HERELLE PHENOMENON.

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Since the first publications of Twort (1) and d'Hérelle (2), and the important contributions of Bordet and Ciuca (3) and of Kabeshima (4), experimental studies of the Twort-d'Hérelle phenomenon have been numerous, but there is still no generally accepted interpretation of it. However, as time passes the hypothesis of d'Hérelle of the existence of an ultra parasite seems to find less favor. The bibliography of the subject is already quite extensive. For a complete review we need only refer to the monograph of d'Hérelle, the critical articles of Maitland (5) and Davison (6), and the works of Seiffert (7) and Kuttner (8).

The present studies may be divided into two series. In the first of these we have repeated some observations on the phenomenon in general and have given special attention to certain peculiarities of the lytic principle which have aroused our interest. In the second series the antigenic properties of the filtrates of lysed cultures[1] have been studied.

Origin and Characteristics of the Lytic Principle Studied.

We have employed in these experiments a lytic principle isolated from the feces of a normal rabbit. We have not made a systematic study on the frequency of bacteriophage in the intestinal tract of normal rabbits, but frequent examinations indicate that bacteriophage is constantly present in the feces of caged laboratory rabbits. To

[1] During the preparation of this report there has appeared a paper on the same subject by Weiss and Lloyd (Weiss, E., and Lloyd, A., *J. Infect. Dis.*, 1924, xxxiv, 317). Their results are in partial accord with ours.

obtain the lytic principle we have employed the technique described by d'Hérelle.[2]

Some particles of fecal matter are ground up with a little bouillon in a sterile mortar. This coarse suspension is added to 70 or 80 cc. of sterile bouillon in a small Erlenmeyer flask and allowed to incubate at 37°C. overnight. Next day it is filtered first through paper and then through a Mandler filter. It is then distributed into sterile tubes and each tube is inoculated with a culture of the bacteria to be tested. After incubation for 24 hours the contents of each tube are again filtered separately. A small quantity (0.5 to 1.0 cc.) of this second filtrate is added to the young bouillon culture of the same organism, and incubated at 37°C. After 2 hours 2 or 3 drops of this mixture are flowed over the surface of a sterile agar slant. After incubation overnight the extent of lysis is indicated by the number of clear spaces ("plages" of d'Hérelle) which appear in the bacterial growth on the slant.

The following strains of bacteria were employed; *B. dysenteriæ* Shiga (two strains), *B. dysenteriæ* Flexner (two strains), *B. typhosus*, *B. paratyphosus* A and B, *B. choleræ suis*, *B. coli communis*, *B. coli communior*, and *B. lactis aerogenes*, all stock strains found in this laboratory. Strain Shiga 73 was isolated in 1919 from a fatal case of dysentery; it has been maintained on artificial media and has never shown spontaneous lysis. The typhoid strain (Rawlings) was one used in many laboratories of the United States for the preparation of antityphoid vaccine.

At the beginning of these studies we obtained lytic filtrates for one or many of the bacteria tested. The most active filtrate was selected for further experiment. The first series of titrations gave the following results. The lytic principle was active in a dilution of 1:1,000,000 for Strains Shiga 73 and Flexner 57, 1:10,000 for the other Flexner strain, and 1:1,000 for the typhoid. It was inactive for the other strains. I started immediately to increase the activity of the active principle by successive inoculation and filtration of cultures. For these experiments the attempt was limited to two strains, Shiga 73, which was the most sensitive to lysis, and the Rawlings typhoid strain, which was the least sensitive. After twenty successive passages a titration of the ultimate filtrates gave the following results. The Shiga filtrate was active for its homologous strain in a dilution of about 1:10,000,000,000 but no longer possessed any lytic activity for the typhoid strain. The typhoid filtrate was similarly active in a dilution of 1:10,000,000,000 for the typhoid strain but was only feebly

[2] d'Hérelle (2), p. 24.

active for the Shiga strain (in a dilution of about 1:1,000). With these two filtrates animals were immunized and the experiments to be described were carried out.

Filtration Experiments with Collodion Membranes.

Maisin (9) and Wollman (10) have studied the filterability of bacteriophage through collodion membranes. We have repeated the experiments, following about the same technique.

A collodion sac is mounted on a glass tube of about 6 mm. diameter. It is filled with bouillon and half immersed in a larger test-tube containing some of the same bouillon. The small tube attached to the collodion sac was allowed to pass up through the cotton plug of the test-tube and to project 4 or 5 cm. The projecting end of the small tube was also plugged with cotton. The assembled apparatus was sterilized in the autoclave at 120°C. After sterilization the contents of the collodion sac were removed by means of a bulb pipette and replaced by active filtrate. The bouillon outside the collodion sac was inoculated with the sensitive organism. After incubation for 6 hours a few drops of the culture in the outer tube were inoculated onto the surface of an agar slant. This process was repeated after 18 hours and at the same time an agar slant was inoculated with some of the contents of the collodion sac to test its sterility. If the bacteriophage had passed through the membrane the cultures made with material from the outer tube should have shown the characteristic plages on the agar slants, if not, the culture was regarded as normal. If the contents of the collodion sac were found not to be sterile the experiment was disregarded, as it was considered that the membrane had perforated.

This experiment was carried out many times and with membranes of different thickness. The passage of the lytic principle through collodion membranes was never observed. Experiments with sacs made with collodion of less than 4.5 per cent were not satisfactory because these sacs were not resistant to autoclaving or handling. Only sacs made with collodion solutions of 4.5 to 5.0 per cent were able to withstand these procedures.[3]

Attempts to Inactivate the Active Principle by Suspensions of Cholesterol, Lecithin, or Killed Bacteria.

Otto, Munter, and Winkler (11, 12) and Seiffert (7) have published studies relative to the ability of certain suspensions to absorb the

[3] The collodion used in these experiments was known as Parlodion, obtained from Arthur H. Thomas Company of Philadelphia.

lytic principle. For this purpose they have employed animal charcoal, bolus alba, and Kieselgur. We have made comparative experiments with suspensions of cholesterol, lecithin, and suspensions of typhoid and dysentery bacilli killed by heat.

The suspensions of cholesterol and lecithin in distilled water were prepared from acetone (for cholesterol) or alcohol (for lecithin) solutions. The acetone or alcoholic solutions were titrated, and after the addition of distilled water the acetone or alcohol was evaporated off on the water bath. The aqueous suspensions contained 0.25 mg. of cholesterol or 1.0 mg. of lecithin per cc. The bacterial suspensions were made quite thick, being composed of the growth from ten agar slants washed off in physiological salt solution, centrifuged, and resuspended in 5 cc. of salt solution. 0.1 cc. of the lytic filtrate was added to 5 cc. of the suspension of cholesterol, lecithin, or killed bacteria. The mixtures were incubated at 37°C. for 3 hours and then refrigerated 24 or 48 hours, after which they were centrifuged and the lytic action of the supernatant fluid titrated by dilution in the usual manner. No appreciable diminution of the activity of the filtrates was observed after exposure to cholesterol or lecithin. The bacterial suspensions, however, had considerable neutralizing action on the active principle. After contact with a suspension of killed typhoid bacilli for 48 hours the antityphoid filtrate was 30 to 35 times less active than the original filtrate.

The Formol Titration of Lysed Cultures.

To determine whether or not the bacteriophage was able to produce a true proteolysis, a number of formol titrations were made of cultures before and after exposure to the lytic principle. For these titrations we have employed the method of formol titration described by Brown (13) for the titration of culture media. These were made with cultures after exposure to lytic principle for 8, 24, and 48 hours. The lysed cultures did not show any increase in formol titration as compared with normal control cultures of the same age kept under similar conditions. It is concluded from these titrations that the bacteriolysis caused by the bacteriophage is not a true proteolysis, but rather a simple plasmolysis of the bacterial cell without splitting of the protein molecule. This conclusion is supported by experiments now to be reported showing the persistence of antigenic properties in the filtrates of lysed cultures.

Immunization against the Lytic Principle with Filtrates of Lysed Cultures.

With the two filtrates (twentieth passage) employed in the preceding experiments, the activity and specificity of which have been described above, rabbits were immunized.

For these experiments twelve rabbits were employed. Six of them received anti-Shiga principle and six received antityphoid principle. During the course of immunization five rabbits died, three that had been injected with anti-Shiga filtrate and two injected many times with antityphoid filtrate. Others besides ourselves have noted the great toxicity of lysed culture filtrates. The Shiga filtrates were much more toxic than the typhoid filtrates. 0.1 cc. of filtrate from a lysed Shiga culture injected intravenously killed a rabbit of 1,500 to 2,000 gm. in 3 or 4 days. The autopsy picture was the same as that obtained by the injection of dysentery toxin. Filtrates of lysed typhoid cultures were much less toxic for rabbits, 2 or 3 cc. injected intravenously being generally tolerated. The same quantity of the typhoid filtrate injected subcutaneously into guinea pigs of 350 to 400 gm. was generally fatal in 6 to 8 days. The rabbits were given various quantities of the filtrates. Those receiving lysed typhoid filtrates were given five to eight intravenous injections at 5 day intervals. The total quantity of filtrate injected into each rabbit was 16 to 32 cc. Those rabbits receiving lysed Shiga filtrates were immunized in the same way and at the same intervals but the doses were much smaller, starting generally with 0.05 cc. and increasing progressively to 2.5 or 4.5 cc. for each injection, the total amount given being about 9 or 10 cc.

The sera of the rabbits were studied after five, seven, and eight injections. 8 days after the last injection the rabbits were bled to death from the carotid artery. Each serum was tested for its neutralizing action against both homologous and heterologous lytic principle. Its ability to fix complement with either filtrate as antigen was also titrated. The agglutinins for both Shiga and typhoid bacilli were titrated. The lytic properties of the washed and unwashed leucocytes of the immune animals were also studied. The presence of lytic principle in filtrates of the intestinal contents of the same animal was also investigated.

To study the lytic properties of the leucocytes, in conformity with the experiments of Lisbonne, Boulet, and Carrère (14), a leucocytic exudate was procured by the intrapleural injection of sterile bouillon into rabbits 16 hours before they were bled to death. This exudate was collected immediately after the death of the animal and divided into two parts. One portion was washed two or three times by centrifugation in physiological salt solution; the other was used without washing. The leucocytes were suspended in equal parts of salt solution and bouillon, distributed into many tubes, and inoculated with a small quantity of a young culture of typhoid or Shiga 73 dysentery bacilli; incubated at 37°C. for 48 hours; filtration done; and the lytic properties of the filtrates examined.

After bleeding the animals the intestinal contents were collected from three portions of the intestine, the duodenum, ileum, and cecum. As described above, the bacteriophage was sought for in the filtrates after incubation of the fecal suspensions.

Using the leucocytes of these immune animals we have never obtained active filtrates by the method of Lisbonne, Boulet, and Carrère. All attempts to find the bacteriophage in the intestinal contents of immunized rabbits were likewise negative. It would appear, from these experiments, that the immunization of rabbits with the lytic principle results in the disappearance of the bacteriophage from their intestines.

A study of the sera of the immunized rabbits yielded the following results.

Agglutinins.—All the sera from rabbits immunized with anti-typhoid filtrate agglutinated typhoid bacilli in rather high dilution (1:4,000 to 1:9,000). They never showed any non-specific agglutinins for the Shiga strain or the paratyphoid strains. The sera from rabbits immunized with anti-Shiga filtrate agglutinated the Shiga bacilli but only in lower dilutions (1:300 to 1:800). The agglutination tests were read after 1 hour in the water bath at 55°C. and 12 hours in the refrigerator.

Fixation of Complement.—All the immune sera fixed complement intensely in the presence of the filtrate which had served as antigen for immunization. Complement fixation occurred also in the presence of the heterologous antigen but to a much less degree. If, however, the immune sera were sufficiently diluted complement fixation occurred only in the presence of the specific antigen. These facts are easily explained as due to the antigenic complexity of filtrates of lysed cultures. It is possible that all these filtrates may possess a fraction common to the different strains of bacteria employed and that these common fractions are the cause of the apparently non-specific complement fixation in moderate dilutions of serum. All of the sera fixed complement strongly in dilutions of 1:50, 1:80, or 1:100 in the presence of the homologous antigen, whereas five or six times the amount of serum was required to fix complement in the presence of the heterologous antigen.

Antilysin.—Increasing quantities (0.1 to 1.0 cc.) of immune serum

were mixed with a constant quantity (0.1 cc.) of the homologous lytic filtrate in sterile tubes. The contents of each tube were made up to 2.0 cc. by the addition of physiological salt solution. The mixtures were incubated at 37° C. for 2 to 2½ hours. At the end of this period 0.75 cc. of a young (3 or 4 hour) bouillon culture of the homologous organism was added to each tube and after another period of 2 hours in the incubator a few drops from each tube were inoculated onto agar slants. In this manner the degree to which the lytic principle had been neutralized by the serum could be determined quite accurately. All of the immune sera were found to be more or less antilytic, although only one of the sera was found to have a titer sufficiently high to produce complete neutralization. This serum neutralized not only its homologous (antityphoid) bacteriophage but to a considerable extent neutralized also the anti-Shiga principle. However, to effect complete neutralization of the latter it was necessary to use five or six times as much serum as was required for neutralization of the homologous principle. Though there were quantitative differences all of the sera exhibited the same phenomena. In no case was a strictly specific antilysin obtained.

SUMMARY AND CONCLUSIONS.

1. The experiments here reported were carried out with a lytic principle isolated from the feces of normal rabbits.

2. It seems that bacteriophage is constantly present in the feces of caged laboratory rabbits.

3. A lytic filtrate active for Strain Shiga 73 (in a dilution of 1:1,000,000) and for the typhoid bacillus (in a dilution of 1:1,000), after twenty successive passages with Shiga bacillus was active, for its homologous strain, in a dilution of about 1:10,000,000,000 but no longer possessed any lytic activity for the typhoid strain. The same filtrate, after twenty successive passages with typhoid bacillus, was similarly active in a dilution of 1:10,000,000,000 for the typhoid strain but was only feebly active for the Shiga strain.

4. The passage of the lytic principle through collodion membranes was never observed.

5. No appreciable diminution in the activity of the filtrates was observed after exposure to cholesterol or lecithin. Killed bacterial

suspensions, however, were found to exert a considerable neutralizing effect on the active principle.

6. Formol titrations of lysed cultures lead to the conclusion that the bacteriolysis caused by the bacteriophage is not a true proteolysis but rather a simple plasmolysis of the bacterial cell and not a splitting effect of the protein molecule.

7. We have never obtained active filtrates with the leucocytes of immune animals by the method of Lisbonne, Boulet, and Carrère (14).

8. It would appear, from the experiments, that the immunization of rabbits with the lytic principle results in the disappearance of the bacteriophage from their intestines.

9. All the sera from rabbits immunized with antityphoid or anti-Shiga filtrates agglutinated respectively the typhoid or Shiga bacilli.

10. Similarly, these sera fixed complement strongly in the presence of the homologous antigen. Five or six times the amount of serum was required to fix complement in the presence of heterologous antigen.

11. All of the immune sera were found to be more or less antilytic. In no case was a strictly specific antilysin obtained.

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