STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

X. Toxin Production by Normal and by Phage-Resistant Shiga Dysentery Bacilli.

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That the administration of bacteriophage during the course of an infectious disease frequently fails to affect the course of the infection is a common finding. One of the principal reasons assigned for this is the development of a race of bacteria resistant to lysis. Since it is well recognized that the resistant bacteria often show marked divergences from the type in many of their biological characteristics, it becomes of importance to know if their ability to produce disease is also different. There have been few studies of this question.

The commonly accepted view is that resistant bacteria are also characterized by an increased virulence. D'Hérelle (1) makes the general statement that bacteria resistant to the bacteriophage show an enhanced virulence, and then extends this, saying (2) "the degree of virulence of a bacterium is strictly in relation with its degree of resistance to the bacteriophage." Later (3), he states that susceptible *B. coli* is harmless, and that when pathogenic, it is the result of its resistance to phage, but he gives no experimental data in support of this contention.

In studying *B. pestis*, d'Hérelle (4) found that while the susceptible organism kills a guinea pig in a dose of 0.1 cc., the resistant strain kills in a dose of 0.0002 cc. Quiroga (5) obtained a resistant strain of *B. pyocyaneus*, of which a loopful injected intravenously killed in 24 hours, while a loopful of the susceptible strain killed in 3 days. Gratia (6) described a resistant strain of *B. coli* that was highly virulent for guinea pigs, not subject to phagocytosis, and that caused septicemia; while the susceptible strain was weakly virulent, subject to phagocytosis, and caused purulent peritonitis. Kauffmann (7) described resistant (*schleimige*) strains of *B. coli* characterized by greatly increased virulence for rabbits and guinea pigs and by resistance to phagocytosis. Wollstein (8) described a resistant strain of Shiga dysentery bacilli that would kill rabbits in 1/5 the dose required for the susceptible strain. Dutton (9), working with streptococci, says that the

outcome of disease depends upon the balance between the virulence of the bacteriophage and the virulence of the bacterium. He, however, considers all streptococci to be mixed with bacteriophage, so that virulence of the bacteria in this case indicates their ability to survive, and not variation in any other sense.

All of the reported studies were made with suspensions of the bacteria and concerned their ability to kill animals; none was related to toxin production. We therefore undertook the study of toxin production by *B. dysenteriæ* Shiga, an organism that causes disease by the elaboration of toxin and not by invasion.

Olitsky and Kligler (10) described the production of two types of toxin by the Shiga dysentery bacillus. The exotoxin acts on the nervous system, determining a paralysis, while the endotoxin acts on the intestinal tract, causing diarrhea and hemorrhages in the intestinal wall. Their methods were followed in our studies, and while a complete separation of the effects of the two toxins was not usually obtained, the predominance of those of one type over the other, depending on the method of preparation, was ordinarily found. In additional experiments an anti-exotoxin was also used for purposes of separation, but the results did not differ from those secured by other methods, so the protocols are not included. An exact quantitative study of toxin production was not attempted, as it sufficed for the object of the experiments to detect whether loss or great increase of this property had occurred. Only the time of death is recorded in the tables, but not only the symptoms but the autopsy findings were characteristic for the type of toxin used. The tables are each typical of experiments that were repeated several times.

EXPERIMENTAL.

B. dysenteriæ Shiga 109 and Laudman phage, active on it to a titer of 10^{-9}, were used.

Resistant bacteria were isolated by continuing incubation after lysis in broth until an overgrowth of resistant bacteria occurred. This was streaked in a Petri dish, and restreaked each day from single colonies for six generations, and then inoculated on an agar slant. The resistant strain thus secured was shown to be resistant to lysis and free from phage before being used.

Exotoxin was prepared by growing the bacteria in broth for 5 days at 37°C. and then filtering through a Berkefeld V candle. This sterile material was injected intravenously into rabbits (Table I).

The filtrates from the normal and from the resistant cultures resulted in similar symptoms and autopsy findings, and the severity of the effects was roughly proportional to the dose administered. The variation in the time of death was within the ordinary limits of individual variation.

TABLE I.

The Production of Exotoxin.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 3–94 1110 gm.	Normal	0.75	Died in 18 hrs.
No. 3–44 1300 gm.	Normal	0.3	Died in 64 hrs.
No. 3–49 1325 gm.	Normal	0.3	Died in 130 hrs.
No. 3–95 1100 gm.	Resistant	0.75	Died in 29 hrs.
No. 3–46 1400 gm.	Resistant	0.3	Died in 52 hrs.
No. 4–07 3125 gm.	Resistant	0.3	Died in 55 hrs.

TABLE II.

The Production of Endotoxin.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 3–43 930 gm.	Normal	0.75	Died in 18 hrs.
No. 3–48 1175 gm.	Normal	0.3	Dead in 40 hrs.
No. 3–93 710 gm.	Resistant	0.75	Dead in 18 hrs.
No. 3–50 1160 gm.	Resistant	0.3	Dead in 93 hrs.

Endotoxin was prepared by the method of Olitsky and Kligler (10). The 24 hour growth of the bacteria in Blake bottles was washed off in saline solution (15 cc. to each bottle) and incubated for 3 days at 37°C., and then filtered through a Berkefeld V candle. The filtrate

thus secured was heated to 80°C. for 1 hour before intravenous injection. As the results show (Table II), there was no appreciable difference in the production of endotoxin by the normal and by the resistant strains.

After the completion of the first series of experiments, it was found that the resistant organism showed a tendency to revert to susceptibility. This was not evidenced by visible lysis, but by the fact that when a diluted phage was incubated with the bacterium, it would increase in titer. Recourse was then had to growing the resistant bacteria in the presence of phage to prevent reversion. Toxin preparations obtained from organisms so treated had the same effects as those preparations of which the results are recorded in the previous tables.

TABLE III.

Examination of the Resistant Strain.

Bacteria inoculated	0	Normal Shiga	Resistant Shiga	Resistant Shiga after 10 daily transfers
Laudman phage diluted 1–10,000	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Titer before incubation	10^{-3}	10^{-3}	10^{-3}	10^{-3}
Incubated 48 hrs. Heated to 56°C. for 50 min. Titrated by Appelmans' method.				
Titer	10^{-3}	10^{-9}	10^{-3}	10^{-3}

The series of experiments was again repeated, with freshly isolated resistants. After isolating them in the usual manner and demonstrating that they were not subject to visible lysis and did not carry phage, they were incubated with diluted phage and shown to be unsuitable for its increase. As a further control, the strain was carried through repeated transfers in broth and tests showed that it had not reverted sufficiently to serve for the increase of phage. The results of these tests are given in Table III. This eliminates any doubt of the resistance of the bacteria used, but as a further control, the tests were repeated on bacteria taken from each flask just prior to filtration.

Exotoxin was prepared in the manner previously described. *Endotoxin* was prepared according to the method of McCartney and Olitsky (11), the method

being varied by using bacteria grown overnight on the agar surface in a Blake bottle. The growth was suspended in 25 cc. of saline for each bottle. 10 cc. of this were centrifuged and washed once in saline and then suspended in 5 cc. of 1 per cent sodium carbonate. This was sealed in an ampoule, heated to 56°C.

TABLE IV.

Exotoxin Production by Bacteria Completely Resistant.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
A 195 1950 gm.	Normal	0.5	Dead in 72 hrs.
A 194 1985 gm.	Normal	1	Dead in 24 hrs.
A 190 1975 gm.	Normal	2	Dead in 48 hrs.
A 193 2050 gm.	Resistant	0.5	Dead in 48 hrs.
A 196 2060 gm.	Resistant	1	Dead in 24 hrs.
A 192 2025 gm.	Resistant	2	Dead in 24 hrs.

TABLE V.

Production of Endotoxin by Bacteria Completely Resistant.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 25 620 gm.	Normal	0.05	Dead in 72 hrs.
No. 29 500 gm.	Normal	0.025	Dead in 96 hrs.
No. 26 610 gm.	Resistant	0.05	Dead in 48 hrs.
No. 27 560 gm.	Resistant	0.025	Dead in 72 hrs.

for 1 hour, and incubated overnight. The resulting fluid was very viscous; it was injected intravenously after cultures had shown it to be sterile (Table V).

The results of these experiments (Tables IV and V), as of the previous ones, show that from the standpoint of toxin production, the normal and the resistant bacteria are identical.

DISCUSSION.

The failure of Shiga dysentery bacilli to show an alteration in toxin production with the development of resistance to the bacteriophage is at variance with the commonly accepted view. While an increased virulence has not always been found in such instances, an alteration has usually been reported. Fejgin (12) described three strains of phage-resistant Shiga dysentery bacilli showing a loss of toxin production to such an extent that 4 to 5 cc. of a 24 hour broth culture failed to produce characteristic symptoms in a rabbit when injected subcutaneously. Blair (13), by the action of bacteriophage, secured two strains of diphtheria bacilli that were atoxic for guinea pigs. Only one of these, however, was resistant to phage. In this laboratory it was found (14, 15) that resistant strains of *B. pestis caviæ* were avirulent, and that virulence returned with the reversion to susceptibility.

The experimental data presented and the review of the literature show that the relation existing between resistance to bacteriophage and toxin production or virulence is by no means a constant one. Furthermore, it is a well recognized fact that bacteria resistant to phage may show quite pronounced differences from the ordinary in other characteristics. The most logical explanation of these facts seems to be that the properties of resistance to phage, and of toxin production are two independent manifestations of bacterial variation or dissociation. If this is correct, it should be possible, by examining a sufficiently large number of strains, to secure races showing all possible combinations in the degree of these two properties.

SUMMARY.

1. The production of exotoxin and of endotoxin by normal Shiga dysentery bacilli and by strains resistant to Laudman phage was found to be the same.

2. The presence of phage did not alter toxin production by the resistant organism.

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