THE INCREASE OF BACTERIOPHAGE IN VIVO DURING EXPERIMENTAL INFECTIONS WITH SHIGELLA PARADYSENTERIAE, FLEXNER, IN MICE

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Received for publication September 23, 1944

The results obtained by Morton and Engley (1944) on the protective action of dysentery-phage against experimentally induced infections in white mice with Shigella paradysenteriae, variety Flexner, demonstrated that the experimental animals were protected when the ratio of dysentery-phage particles to virulent dysentery bacilli was of the order of 1 to 8. If the protective action of dysentery-phage in vivo is due to lysis of the dysentery bacilli by the dysentery-phage, the protective action is difficult to explain unless the amount of dysentery-phage within the animal's body increases during the course of the infection. Our results show the latter does occur.

During the course of our studies two reports appeared which corroborated our findings. Rakieten and Rakieten (1943) reported that the survival of chick embryos infected with Shigella paradysenteriae, Flexner, was associated with an increase in the amount of dysentery-phage in the embryos. Dubos, Straus, and Pierce (1943), working with Shigella dysenteriae infections in mice, demonstrated an increase in the amount of dysentery-phage in the infected mice.

Krueger and Scribner (1941) interpreted a portion of the results reported by Nungester and Watrous (1934) as indicating that 4 hours after the injection of bacteriophage into the animal body only 0.04 per cent remained in the circulating blood. Only 1 out of 4 rats showed the presence of bacteriophage in its blood, and Krueger and Scribner took one-fourth of the titer of the one rat for the basis of their statement on the rapid elimination of bacteriophage from the animal body. Such a general statement cannot be made on the basis of so few experimental animals.

The purposes of our investigations were to determine (1) how rapidly our strain of dysentery-phage was eliminated from the circulating blood of white mice and (2) if there was an increase in the amount of dysentery-phage in the blood of the experimental animals during the course of infection with dysentery bacilli. We found that the dysentery-phage gradually disappears from the circulating blood within a period of about 1 week. During the course of infection with phage-susceptible dysentery bacilli, the titer of dysentery-phage markedly increases in the blood of the experimental animals.

EXPERIMENTAL

In the experiments to be described cultures of Shigella paradysenteriae, varieties Flexner X-S45 and Newcastle, were used to produce the experimental infections.
Young white Swiss mice weighing 17 to 20 grams were used as experimental animals. The intraperitoneal injection into mice of the organisms suspended in 5 per cent gastric mucin produces in a few hours a bacteremia which terminates fatally in 24 to 48 hours. When suspended in 5 per cent gastric mucin, 4 bacilli constitute a minimum lethal dose.

The bacteriophage preparation used was propagated for several generations against cultures of the X-S45 organism. Filtrates were obtained as follows: About 150 ml of tryptone glucose yeast-extract broth were placed in a 250-ml centrifuge bottle and inoculated with 2 ml of an overnight culture of the X-S45 organism. The bottle was then placed in a 37°C water bath for 2 hours, air being bubbled constantly through the growing culture. After 2 hours of incubation the culture was removed from the water bath and inoculated with 2 ml of the stock homologous bacteriophage. The culture was then replaced in the water bath and kept there for 5 hours, air being bubbled through it constantly. The bottle was then removed from the bath and centrifuged at about 3,500 rpm for 20 minutes. The supernatant was filtered through fritted pyrex glass filters and the filtrate aseptically dispensed into bottles and placed immediately in the refrigerator. The titer of each lyaste was determined 24 to 48 hours after preparation by plaque enumeration on 2 per cent extract agar plates. The plaques were counted after overnight incubation of the plates at 37°C. The filtrates used in the experiments were: LP X-S45 no. 6 (5 x 10^10 particles per ml), LP X-S45 no. 7 (1.4 x 10^9 particles per ml), and LP X-S45 no. 8 (4.8 x 10^9 particles per ml).

The blood level of the bacteriophage in normal and infected animals was determined by bleeding from the heart, using a 1-inch, no. 23 or 24 gauge needles and 1-ml (tuberculin) syringes. The syringes and needles were sterilized in boiling water and were allowed to dry before use.

Either 0.1 or 0.2 ml of blood were withdrawn from the heart, and initial dilution of 1/10 was prepared in a young broth culture of the "X" organism. Appropriate dilutions were made and 0.2 ml of each dilution were placed on the surface of an agar plate and spread with a sterile, bent glass rod. (Dilutions were made with 1-ml pipettes, a different pipette being used for each dilution.) The plates were incubated overnight and examined the following day or placed in the refrigerator after incubation and examined within the next 48 hours. When very small amounts of phage were to be expected, sometimes as much as 0.5 ml of each dilution was spread on the surface of the agar plate.

Experiment 1. A group of 18 mice received intraperitoneally 1 ml of a culture of the X-S45 organism diluted in gastric mucin. The actual amount injected was 0.2 ml of a 10^-7 dilution of the culture suspended in 0.8 ml of mucin. Simultaneously the mice were given 1 ml of a homologous phage (5 x 10^18 particles) intraperitoneally. At the same time an equal number of mice received the injection of dysentery-phage only. (All of the 3 normal mice which received the injection of organisms only were dead within 24 hours.)

An equal number (usually 3) of both the infected and the control mice were bled at intervals of 3, 9, 24, 48, 72, and 96 hours after receiving the injections.
The blood level was determined and the average for each group was obtained. The results are tabulated in Table 1.

The results of this preliminary experiment, shown in Table 1, give only slight indication of multiplication of bacteriophage in vivo among the infected animals. There is some indication, however, that the phage increased in amount and was retained for a longer period of time in the blood of the infected animals. The appearance at the 3-hour interval of less dysentery-phage in the circulating blood of the infected mice as compared with the normal mice suggested that the dysentery-phage may have been fixed by the dysentery bacilli in the peritoneal cavity. The following experiment was devised to determine if this occurred.

**Experiment 2.** Forty-eight white mice, weighing 17 to 20 grams each, were given $1 \times 10^9$ particles of dysentery-phage intraperitoneally. Another 6 mice were kept as normals. The titer of the dysentery-phage in the blood was determined (as previously outlined) 24, 48, 72, and 96 hours after the injection of dysentery-phage. At the 96-hour interval 9 mice were injected intraperitoneally each with 127 M.L.D. of the X-S45 organism in 5 per cent mucin; 9 mice received mucin only, and 9 mice received 446 M.L.D. of a Newcastle strain of *Shigella paradysenteriae* not lysed by the dysentery-phage in vitro.

Three of the normal mice received 127 M.L.D. of the X-S45 organism and the remaining 3 received 446 M.L.D. of the Newcastle strain. These served as controls for virulence of the organisms. All 6 mice were dead within 24 hours. The results of this experiment are shown in Table 2.

As may be seen from the data in Table 2, the injection of the homologous organisms at a time when the dysentery-phage level in the blood had decreased to 200 particles per ml produced a rise in the blood level to an average of 16,000-000 particles per ml in 24 hours. This means that the number of particles had increased 80,000 times during this period. Seventy-two hours after the injection of the bacteria, the dysentery-phage level was still high, 5,300 particles per ml of blood.

On the other hand, in the group of mice which had received dysentery-phage but subsequently did not receive any organisms, the dysentery-phage level in
the blood decreased from the time of injection until it could no longer be detected with the dilutions employed after 144 hours, being less than 50 particles per ml.

The mice injected with mucin alone (96 hours after having received the injection of dysentery-phage) showed no increase in the number of particles in the blood. The average number of particles for each of the different groups of mice which received only mucin could not be determined since 5 of the total number of 9 mice showed less than 50 particles per ml of blood.

<table>
<thead>
<tr>
<th>Bleeding time after injection of dysentery-phage</th>
<th>Number of dysentery-phage particles per ml of blood in mice injected with dysentery-phage only</th>
<th>Number of dysentery-phage particles per ml of blood in mice injected with X-845 organisms, 96 hours after injection of phage</th>
<th>Number of dysentery-phage particles per ml of blood in mice injected with Newcastle organisms, 96 hours after injection of phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>28,000,000 phage particles/ml blood</td>
<td>16,000,000</td>
<td>800 (1 mouse)</td>
</tr>
<tr>
<td>24</td>
<td>(no results; wrong dilutions used)</td>
<td>180</td>
<td>&lt;50 (2 mice)</td>
</tr>
<tr>
<td>48</td>
<td>940</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>570</td>
<td>all mice dead</td>
<td>150 (1 mouse)</td>
</tr>
<tr>
<td>120</td>
<td>50</td>
<td></td>
<td>&lt;50 (2 mice)</td>
</tr>
<tr>
<td>144</td>
<td>&lt;50</td>
<td></td>
<td>100 (2 mice)</td>
</tr>
<tr>
<td>168</td>
<td></td>
<td></td>
<td>&lt;50 (1 mouse)</td>
</tr>
</tbody>
</table>

In the group of mice infected with the Newcastle strain only 2 mice remained alive 28 hours after the injection of organisms. The dysentery-phage level in the blood of these 2 mice did not show any increase in titer when compared with the titers of the control mice bled at the same time. This was in marked contrast to the rise in titer in the mice which had been injected with phage-susceptible organisms (the X-845 strain), as is illustrated in figure 1.

Experiment 3. This experiment was a repetition of experiment no. 2 but with slight alterations. Before the experiment was started, 2 white mice were injected with 1 ml of a 10⁻² dilution of a culture of the X-845 organism in mucin. The culture in this dilution gave a count of 292 organisms per ml or about 73
M.L.D. The mice were bled from the clipped tail at intervals of 15, 30, 60, 90, and 180 minutes after inoculation. A drop of blood from each was diluted in 0.5 ml of broth and plated on MacConkey’s agar. The organisms were shown to be present in the blood stream as early as 1 hour after inoculation.

In order to find out how long it took for the dysentery-phage to increase in the blood after the injection of organisms, the intervals of time were altered in this experiment. The mice receiving dysentery-phage alone were bled 3, 12, 24, 72, 96, 120, 144, and 168 hours after the injection of the dysentery-phage. The group of mice which received organisms 96 hours after the injection of the dysentery-phage were bled 3, 12, 24, 48, and 72 hours after injecting the organisms. Thus, there was a parallel series from the 3- to the 72-hour intervals.

![Graph]

**Fig. 1. The Average Number of Dysentery-Phage Particles per ml of Blood in White Mice as Given in Table 2**

--- normal mice; —— mice injected after 96 hours with phage-susceptible dysentery bacilli, X-S45 strain; ······· mice injected after 96 hours with phage-resistant dysentery bacilli, Newcastle strain.

The mice inoculated with the homologous organism (X-S45) received only 66 M.L.D. in this experiment, which might serve as an explanation for the proportionally smaller rise in the blood level of bacteriophage in this experiment as compared with that in experiment no. 2.

The mice inoculated with the Newcastle strain (which was not susceptible in vitro to the bacteriophage used) received an estimated 166 M.L.D. of the organism. The titer of the bacteriophage was $4.8 \times 10^8$ particles per ml.

Organisms recovered from the blood of the mice which received both the injection of the X-S45 organism and the homologous bacteriophage, employing the technique of Kliger, Oleinik, and Caaskes (1943), gave the biochemical reac-
TABLE 3

Blood level of bacteriophage in control mice, in mice injected with phage-susceptible dysentery bacilli (X-345) and in mice injected with dysentery bacilli not susceptible to the lytic action of the dysentery-phage (Newcastle strain)

<table>
<thead>
<tr>
<th>BLEEDING TIME AFTER INJECTION OF DYSENTERY-PHAGE</th>
<th>NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH DYSENTERY-PHAGE ONLY</th>
<th>NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH X-345 ORGANISMS, 96 HOURS AFTER INJECTION OF PHAGE</th>
<th>NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH NEWCASTLE ORGANISMS, 96 HOURS AFTER INJECTION OF PHAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>120,000,000</td>
<td>no plaques seen</td>
<td>not tested</td>
</tr>
<tr>
<td>12</td>
<td>80,000,000</td>
<td>9,800</td>
<td>150 (1 mouse)</td>
</tr>
<tr>
<td>24</td>
<td>17,000,000</td>
<td>130,000</td>
<td>1,000 (1 mouse)</td>
</tr>
<tr>
<td>72</td>
<td>1,000</td>
<td>300</td>
<td>&lt;200 (2 mice)</td>
</tr>
<tr>
<td>96</td>
<td>560</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>460</td>
<td></td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Log of the number of phage particles per ml of blood

FIG. 2. THE AVERAGE NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN WHITE MICE AS GIVEN IN TABLE 3

— normal mice; — — — mice injected after 96 hours with phage-susceptible dysentery bacilli, X-345 strain; — — mice injected after 96 hours with phage-resistant dysentery bacilli, Newcastle strain.

The average number of phage particles per ml of blood in normal mice was 120,000,000. In mice injected with phage-susceptible dysentery bacilli, X-345 strain, the number decreased to 9,800 after 96 hours. In mice injected with phage-resistant dysentery bacilli, Newcastle strain, the number was not tested. The number of phage particles per ml of blood in normal mice was not tested.
for injecting the mice. The results of this experiment are shown in table 3 and in figure 2.

Feces from phage-infected mice were found to contain dysentery-phage, whereas feces from mice not infected with dysentery-phage did not contain a bacteriophage active against the strain of dysentery bacilli employed in the experiment.

**Summary**

When a dysentery-phage, prepared against *Shigella paradysenteriae*, Flexner X-S45, is injected intraperitoneally into normal white Swiss mice, it rapidly enters the circulating blood.

The concentration of the dysentery-phage in the blood stream remains high for about 24 hours; however, it diminishes constantly, more or less rapidly at first then rather slowly, until it is barely detectable after 5 to 7 days. It is not eliminated as rapidly as Krueger and Scribner (1941) lead one to believe in their review.

Lytic action *in vitro* is accompanied by protective action *in vivo*; no lytic action *in vitro*, no protective action *in vivo*.

The injection into mice of dysentery bacilli susceptible to the action of the dysentery-phage, after the level of the dysentery-phage in the blood stream is fairly low, causes a temporary (3-hour) decrease in the amount of dysentery-phage in the circulating blood. This decrease is followed by a rapid rise in the amount of dysentery-phage in the circulating blood until a maximum titer is reached in about 12 hours after the injection of the organisms. The rise appears to be roughly proportional to the number of susceptible organisms injected (80,000-fold increase following the injection of 127 M.L.D.). After the maximum amount of dysentery-phage is reached in the blood stream, the titer decreases rapidly within the next 24 hours, thereafter more slowly.

The temporary decrease in the amount of dysentery-phage in the circulating blood, within 3 hours after the injection of phage-susceptible organisms, suggests a concentration of the dysentery-phage at the site of the large number of organisms injected into the peritoneal cavity. The reappearance of dysentery-phage in the circulating blood may come about through one or both of two possible ways. The dysentery-phage liberated in the peritoneal cavity through the lysis of the dysentery bacilli which were injected may enter the blood stream, as happens after dysentery-phage is injected into the peritoneal cavity of normal mice. Following the intraperitoneal injection of virulent dysentery bacilli into normal mice or phage-infected mice, the dysentery bacilli gain entrance to the blood stream. The bacilli are first detected in small numbers 1 hour after injection and are present in greater numbers after 3 hours. Another possible explanation for the increase in the amount of dysentery-phage in the circulating blood between the third and twelfth hours after the intraperitoneal injection of phage-susceptible dysentery bacilli is that those bacilli in the circulating blood undergo lysis in the blood stream. It is reasonable to expect that if the mice are protected against several lethal doses of dysentery bacilli by dysentery-phage, the
bacilli are destroyed wherever phage particles are able to combine with the bacilli. Three hours after the injection of dysentery bacilli into phage-infected mice, both dysentery-phage particles and dysentery bacilli are in the circulating blood.

Regardless of where bacteriophagy takes place in vivo, the amount of dysentery-phage in the circulating blood increases during the course of infection with phage-susceptible dysentery bacilli.

Dysentery-phage is an unique antibiotic in that as it protects animals from fatal infections with dysentery bacilli more dysentery-phage is produced.

REFERENCES


