THE MULTIPLICATION OF BACTERIOPHAGE IN VIVO AND ITS PROTECTIVE EFFECT AGAINST AN EXPERIMENTAL INFECTION WITH SHIGELLA DYSENTERIAE*

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The knowledge that bacteriophage lysis is inhibited by blood, serum, bile, white cells, tissue debris, etc., has caused many bacteriologists to doubt that the agent can retain its activity *in vivo* (1). Indeed, most investigators feel that the beneficial effects which in some cases follow bacteriophage therapy, may not be due to the lytic or bactericidal effect of the agent, but rather to a number of other mechanisms such as the production of specific antibacterial immunity, or the stimulation of non-specific phagocytic activity by the bacterial and broth components present in the lysate (2, 3). There are, however, a few reports which seem to establish that bacteriophage *per se* can exert a favorable influence on certain experimental infections (4–6).

It would seem that the evaluation of the potential therapeutic efficacy of bacteriophage will be facilitated by a knowledge of the fate of this agent following its introduction into normal and infected animals. In order to study this problem we have made use of the experimental disease which results from the intracerebral injection of *Shigella dysenteriae* (Shiga) into white mice. This infection takes the form of a meningitis which is usually fatal in 3 to 10 days, and in which extensive multiplication of the dysentery bacillus takes place in the brain in the absence of generalized septicemia. It will be shown in the present paper that when anti-Shiga bacteriophage is injected into the general circulation of animals so infected, the agent multiplies very rapidly at the site of bacterial multiplication (in the brain), and may, under the proper experimental conditions, protect the animals against an otherwise fatal infection.

EXPERIMENTAL

Experimental Infection.—The culture used was a smooth variant of *Shigella dysenteriae* (Shiga 2308), obtained through the kindness of Dr. M. Coleman of the De-

* Preliminary experiments were carried out in the laboratories of the Hospital of The Rockefeller Institute for Medical Research.

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partment of Laboratories of the New York State Department of Public Health, Albany, New York. The culture was grown in meat extract peptone broth for 24 to 48 hours; appropriate dilutions (in warm broth or saline) were injected intracerebrally into anesthetized white mice weighing 18 to 25 gm., the final volume of the inoculum being 0.03 cc. The few deaths occurring within 3 hours were considered as due to trauma and have not been included in the experimental results.

The L D_{50} of this culture injected by the intracerebral route is approximately 0.00005 cc.; the injection of 0.005 cc. results in the death of 95 per cent of the infected animals within 2 to 5 days. The infective organisms can be recovered in large numbers from the brain of the infected animals at autopsy; peritoneal washings, on the contrary, always remain sterile and the heart blood gives only an occasionally positive culture.

Bacteriophage Preparations.—The bacteriophage used in these experiments was obtained from New York City sewage water in 1942, and has since been propagated in cultures of *Shigella dysenteriae* 2308 (smooth phase). No attempt has been made to isolate a pure line bacteriophage from it, and in fact we have observed that our preparation forms plaques of at least two different sizes.

The bacteriophage filtrates were obtained by the following technique. Flasks containing 250 cc. of broth were inoculated with 2.5 cc. of an overnight culture of *Shigella dysenteriae* 2308. After 3 hours' incubation at 37 C. the culture containing approximately 10^9 organisms per cc., was placed in the ice box overnight (only as a matter of convenience), and inoculated the following morning with 0.000025 cc. of bacteriophage. After 4 hours' incubation at 37 C. the culture was filtered through a Selas porcelain candle (porosity 03), giving a filtrate which contained from 7×10^9 to 2×10^{10} bacteriophage particles[1] per cc. The final titre was apparently independent of whether or not the culture had undergone lysis at the time of filtration.

The titre of the bacteriophage preparation was determined by plaque enumeration on 2 per cent agar plates, 24 to 48 hours old. The bacteriophage filtrate was diluted far enough to give isolated plaques, the final dilution being made in a broth culture of *Shigella dysenteriae* 2308. The final dilutions were immediately spread on a quadrant of the nutrient agar with a calibrated loop (1/60 cc.), conditions being arranged to obtain confluent bacterial growth. The plaques were counted after 24 hours.

The bacteriophage level in the blood was established by diluting the blood obtained from the clipped tail in a red cell counting pipette. When low bacteriophage numbers were expected, as for instance in the blood of uninfected animals, 0.25 cc. of the heart blood was diluted with 4 cc. of culture, and 0.15 cc. of the mixture spread over an agar plate with a bent glass rod; it will be seen that under these conditions the smallest blood level that could be measured corresponded to approximately 120 bacteriophage particles per cubic centimeter of blood.

To determine the bacteriophage level in the brain, the cerebral hemispheres and cerebellum were ground in an agate mortar with 2 or 4 cc. of saline; the material

[1] The assumption is made in the presentation of the data that each plaque obtained on the agar plates corresponded to one bacteriophage particle in the bacteriophage dilution.

filtered through paper was then diluted and plated as described above in the case of the blood.

TABLE I

Bacteriophage Level in the Brain of Normal and Infected Mice at Different Intervals after Intraperitoneal Injection of 10^5 Bacteriophage Particles

Time after infection	Mouse No.	Normal mice	Mouse No.	Infected mice
hrs.				
1	1	$<1.2 \times 10^{2}$*	17	1.2×10^{2}*
	2	" "	18	6.6×10^{5}
2	3	1.2×10^{2}	19	1.2×10^{2}
	4	" "	20	$<1.2 \times 10^{2}$
4	5	$<1.2 \times 10^{2}$	21	1.2×10^{3}
	6	1.2×10^{2}	22	$<1.2 \times 10^{2}$
8	7	$<1.2 \times 10^{2}$	23	9.9×10^{8}
	8	" "	24	1.0×10^{7}
16	9	" "	25	1.9×10^{9}
	10	" "	26	9.2×10^{8}
26	11	Not tested	27	4.0×10^{6}
	12	" "	28	2.0×10^{9}
54	13	$<1.2 \times 10^{2}$	29	4.6×10^{8}
	14	" "	30	6.6×10^{6}
75	15	Not tested	31	6.6×10^{5}
	16	" "	32	$<6.6 \times 10^{5}$

* The figures refer to the number of bacteriophage particles per gram of brain material.
< indicates that the number of bacteriophage particles was smaller than the smallest amount that could be revealed with the dilutions employed.

The Fate of Bacteriophage Injected into Normal and Infected Mice

Experiment 1.—A group of mice were infected intracerebrally with 0.005 cc. of *Shigella dysenteriae*. Other mice were kept as uninfected controls. All animals received simultaneously with the infection 10^5 bacteriophage particles by the intraperitoneal route. The animals were sacrificed after different intervals of time (1, 2, 4, 8, 16, 26, 54, and 75 hours after infection); and the bacteriophage content of the brain was determined by the method outlined above (Table I).

The results presented in Table I show that bacteriophage injected intraperitoneally into normal mice reaches its maximum titre in the brain within a very short time (2 hours), then rapidly disappears. In infected animals, on the other

TABLE II

*Blood and Brain Levels of Bacteriophage in Normal and Infected Mice**

Time after injection of bacteriophage	Mouse No.	Normal mice		Mouse No.	Mice infected 24 hrs. before	
		Blood level No. of particles per cc.	Brain level No. of particles per gm.		Blood level No. of particles per cc.	Brain level No. of particles per gm.
hrs.						
18	1	6.0×10^4	$<1.2 \times 10^3$	25	3.3×10^7	8.4×10^8
	2	7.2×10^6	2.9×10^4	26	6.0×10^7	3.6×10^7
	3	3.0×10^7	1.2×10^5	27	1.4×10^7	7.8×10^7
	4	$<6.0 \times 10^4$	$<1.2 \times 10^3$	28	9.0×10^7	2.4×10^7
42	5	5.3×10^3	$<1.2 \times 10^2$	29	8.0×10^4	6.0×10^5
	6	2.6×10^3	5.2×10^2	30	2.7×10^4	9.0×10^6
	7	7.9×10^3	2.6×10^2	31	4.9×10^4	1.5×10^6
	8	2.6×10^3	3.9×10^2	32	6.1×10^4	3.2×10^7
66	9	1.2×10^2	$<1.2 \times 10^2$	33	1.2×10^3	1.2×10^8
	10	" "	" "	34	1.9×10^3	6.3×10^7
	11	2.2×10^2	1.2×10^2	35	1.3×10^3	1.2×10^8
	12	$<1.2 \times 10^2$	" "	36	5.3×10^2	5.6×10^7
90	13	$<1.2 \times 10^2$	$<1.2 \times 10^2$	37	9.3×10^2	7.6×10^8
	14	1.2×10^2	" "	38	6.1×10^3	3.0×10^8
	15	" "	" "	39	5.3×10^3	1.5×10^9
	16	1.7×10^2	" "	40	$<1.2 \times 10^2$	$<6.6 \times 10^3$
114	17	$<1.2 \times 10^2$	" "	41	$<1.2 \times 10^2$	$<1.2 \times 10^2$
	18	" "	" "	42	1.0×10^5	5.8×10^9
	19	" "	" "	43	$<1.2 \times 10^2$	1.2×10^2
	20	" "	" "	44	1.0×10^4	9.0×10^8
138	21	3.9×10^1	" "	45	$<1.2 \times 10^2$	$<1.2 \times 10^2$
	22	1.0×10^2	" "	46	" "	" "
	23	$<1.2 \times 10^2$	" "	47	" "	" "
	24	" "	" "	48	Died	Died

* 10^9 bacteriophage particles injected intraperitoneally. Mice infected intracerebrally with 0.005 cc. *Shigella dysenteriae* 24 hours before determination of bacteriophage level.

< indicates that the number of bacteriophage particles was smaller than the smallest amount that could be revealed with the dilutions employed.

hand, the bacteriophage multiplies in the brain to reach in 12 to 18 hours maximum levels far higher than the total amount originally injected, indicating, therefore, multiplication of the agent *in vivo*.

Experiment 2.—A group of mice received 10^9 bacteriophage corpuscles by the intraperitoneal route. Some of the animals were sacrificed after 18, 42, 66, 90, 114, and 138 hours, in order to determine the level of bacteriophage in the blood and brain.

After the same intervals of time similar groups of animals were infected by the intracerebral route with 0.005 cc. of *Shigella dysenteriae*, and sacrificed 24 hours later. This procedure permitted a comparison of the bacteriophage levels in the blood and brain of infected and uninfected mice at various times after the administration of bacteriophage (Table II).

It is clear from the results presented in Table II that, in uninfected mice, the bacteriophage in the blood rapidly reaches a titre compatible with the numbers that could be expected if the total number of particles injected were simply diluted in the total fluid volume of the mouse. The brain level remains at all times below that of the blood, perhaps due to the small blood content of the brain. Both the blood and brain levels begin to fall a few hours after injection of the bacteriophage and, in fact, after 4 days the bacteriophage level in some of the mice was too low to be measured by the titration method employed.

In infected animals one observed immediately a very great increase in the number of bacteriophage particles, the increase being much more pronounced in the brain than in the blood; this situation which is in marked contrast with that which obtained in the uninfected animals (where brain levels were much lower than blood levels) is probably due to the fact that the experimental infection remains localized in the brain and that the bacteriophage can multiply only at the site of bacterial multiplication. It appears therefore that the blood level is only a reflection of the events occurring in the brain.

Protective Effect of Bacteriophage against Infection

Experiment 3.—Twenty-four mice were infected intracerebrally with 0.005 cc. of *Shigella dysenteriae*; 8 of them were treated simultaneously by the intraperitoneal injection of 10^9 bacteriophage particles; 8 other mice were treated with only 10^5 bacteriophage particles, and the other 8 animals were kept as untreated controls.

The 8 control mice died within 2 to 4 days; only 2 of the animals which were treated with 10^5 particles survived, whereas 6 of the 8 having received the largest amount of bacteriophage (10^9 particles) did so.

It is of interest to point out that all these animals comprised part of a larger test already mentioned under Experiment 1. It can be seen from Table I that some of the infected animals which had received 10^5 particles of bacteriophage exhibited immediate multiplication of the agent in the brain, whereas in other animals multiplication of the bacteriophage was delayed. It may not be too far fetched to assume that the two infected animals which survived following treatment with 10^5 particles may have belonged to the group exhibiting immediate multiplication of the agent in the brain.

In the course of many experiments, not to be described in detail, a number of mice were infected with smooth cultures of *Shigella dysenteriae* and treated with active Shiga bacteriophage introduced by the intraperitoneal route. As controls, other infected mice were left untreated, or received by the intraperitoneal

TABLE III

Protective Effect of Bacteriophage against Infection of Mice with Shigella dysenteriae

Experiment	No. of mice	Dose in cc. and nature of material injected intraperitoneally	Dose of 24 hr. culture of smooth Shiga intracerebrally	No. of mice surviving at end of observation period	Remarks All intraperitoneal treatments given within 10 min. of intracerebral injection of *Shigella dysenteriae*.
			cc.		
A	8	—	0.003	1/8	Virulence controls (no trea-t ment)
B	8	—	0.005	0/8	
C	6	—	0.005	0/6	
D	8	—	0.005	0/8	
E	6	—	0.005	0/6	
F	8	—	0.020	0/8	
	44			1/44	
B	8	0.1 cc. phage heated at 60°C. for 10 min.	0.005	1/8	Control mice (treated with material containing no active bacteriophage). All doses made up to 0.5 cc. with saline
B	8	0.1 cc. 1 per cent tryptone broth	0.005	0/8	
B	8	0.1 cc. filtrate of 24 hr. culture of smooth Shiga in tryptone broth	0.005	0/8	
B	8	0.1 cc. filtrate of 24 hr. culture of rough Shiga in tryptone broth	0.005	0/8	
B	8	0.5 cc. 0.85 per cent saline	0.005	0/8	
	40			2/40	
	84			3/84	Mice receiving no active phage, 3.6 per cent survivals
A	8	0.02 cc. active phage	0.003	7/8	Mice treated with active bacteriophage. Preparation gave between 10^9 and 10^{10} plaques per cc. No control of bacterial count injected other than age of culture (24 hr.)
A	8	0.10 " " "	0.003	7/8	
A	8	0.50 " " "	0.003	6/8	
C	6	0.01 " " "	0.005	5/6	
C	6	0.10 " " "	0.005	4/6	
D	7	0.10 " " "	0.005	3/7	
B	8	0.10 " " "	0.005	7/8	
C	5	0.50 " " "	0.005	4/5	
F	8	0.10 " " "	0.020	3/8	
	64			46/64	Mice receiving active phage, 72 per cent survivals

route, saline, broth filtrates of cultures of rough and smooth *Shigella dysenteriae*, or bacteriophage inactivated by heat. The results of a number of such protection experiments are summarized in Table III. These results reveal that of the control mice (untreated, or treated with inactive material) infected with 0.005 cc. *Shigella dysenteriae*, only 3.6 per cent survived; whereas 72 per cent of the infected animals treated with active bacteriophage were saved.

In other experiments, effective protection was also obtained when bacteriophage was administered intravenously or subcutaneously instead of intraperitoneally. Administration of the agent *per os* (by stomach tube or mixed with drinking water) did not influence the course of the infection.

DISCUSSION

Anti-Shiga bacteriophage introduced intraperitoneally into normal white mice can be detected in the brain within 1 hour after injection, but the number of bacteriophage particles per gram of brain (brain level) always remains much smaller than the numbers present per cubic centimeter of blood (blood level). In mice infected intracerebrally with *Shigella dysenteriae*, on the contrary, the intraperitoneal injection of bacteriophage rapidly results in a brain level considerably higher than the blood level. This high brain level cannot be due to a simple selective fixation of the circulating bacteriophage by the susceptible bacteria multiplying in the brain, but implies a considerable multiplication of the lytic agent *in vivo*.

It has also been shown that the intraperitoneal injection of sufficient amounts of active bacteriophage can protect mice against intracerebral infection with *Shigella dysenteriae;* no protection was obtained when bacteriophage inactivated by heat, or culture autolysates free of bacteriophage, were used instead of the active agent. Before concluding, however, that protection was the result of *multiplication* of the agent *in vivo*, it must be noted that a number of animals died even though considerable multiplication of bacteriophage had undoubtedly taken place; this was the case, for instance, in the mice treated with the smaller amount of the agent (10^5 particles) in Experiment 3. It is likely that, with inadequate treatment, the bacteria multiply extensively before the bacteriophage reaches a bacteriostatic or bactericidal level, so that enough toxin and sufficient irreversible pathological changes are produced to cause death irrespective of final bacteriophage level. On the other hand, animals treated with large amounts of bacteriophage (10^9 particles, for instance), survived even though there was no definite evidence of *multiplication* of the agent *in vivo*.

It is difficult to evaluate the importance of the different factors which may play a part in the protection induced by bacteriophage. In certain experiments not reported in this paper, it was found, for instance, that the injection of heat-killed bacteriophage several days prior to the infection was sufficient to

afford some protection. This protective effect may be due to the antibacterial immunity resulting from the injection of the bacterial products present in the lysate, and also to non-specific stimulation of phagocytic activity caused by broth components. The results described in the present report, however, establish the fact that one can produce an experimental situation in which survival of the infected animal depends upon the presence of a sufficiently high concentration of bacteriophage in the tissues at the beginning of the bacterial disease. Whether the protective effect results from an ability of the bacteriophage to prevent further bacterial growth, to cause lysis of the infective cells, to induce variation to a non-virulent form, or simply to sensitize them for phagocytosis, cannot be decided on the basis of available data.

CONCLUSIONS

1. Anti-Shiga bacteriophage injected into the general circulation can multiply in the brain of mice infected intracerebrally with *Shigella dysenteriae*.

2. Under proper conditions, the injection of active bacteriophage into the general circulation can protect mice against an otherwise fatal intracerebral infection with *Shigella dysenteriae*.

3. The protection so induced appears to depend upon the early establishment of a high bacteriophage level in the infected animal.

Addendum.—Shortly after the completion of this manuscript, there appeared in the *Journal of Bacteriology* a paper establishing the protective effect of a bacteriophage preparation against infection of the chick embryo with a Flexner strain of *Shigella dysenteriae* (7).

BIBLIOGRAPHY

1. Evans, A. C., *Pub. Health Rep., U. S. P. H. S.*, 1933, **48**, 411.
2. Eaton, M. P., and Bayne-Jones, S., *J. Am. Med. Assn.*, 1934, **103**, 1769, 1847, 1934.
3. Krueger, A. P., and Scribner, E. J., *J. Am. Med. Assn.*, 1941, **116**, 2160, 2269.
4. Asheshov, I. N., Wilson, J., and Topley, W. W. C., *Lancet*, 1937, **1**, 319.
5. Fisk, R. T., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 659.
6. Yen, C. H., and Stephen Chang, K. P., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 243.
7. Rakieten, T. L., and Rakieten, M. L., *J. Bact.*, 1943, **45**, 477.