

STUDIES ON A BACTERICIDAL AGENT EXTRACTED FROM A SOIL BACILLUS

II. PROTECTIVE EFFECT OF THE BACTERICIDAL AGENT AGAINST EXPERIMENTAL PNEUMOCOCCUS INFECTIONS IN MICE

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In the preceding paper a description was given of the preparation and properties of a soluble agent, extracted from cultures of an unidentified soil bacillus, which exerts a bactericidal effect on Gram-positive microorganisms (1). The bactericidal effect *in vitro* is not inhibited by the presence of serum or ascitic fluid; in fact, the bactericidal agent is also effective *in vivo*. Its protective action against experimental pneumococcus infections in mice is described in the present paper.

EXPERIMENTAL

Cultures.—Virulent pneumococci of Types I, II, III, V, VIII, and virulent Friedländer bacilli type B were grown in blood broth and passed through mice often enough to maintain a degree of virulence such that 0.000,000,01 cc. of an 8 hour culture would regularly kill 20 gm. mice within 72 hours. In all cases, the infective dose, diluted in 0.5 cc. of buffer pH 7.4, was injected by the intra-abdominal route.

Bactericidal Agent.—All the protection experiments reported in the present paper were carried out with the same preparation of the bactericidal agent (NS7) which was used in the experiments described in the preceding paper. The agent was injected by the intra-abdominal route in the form of a solution in phosphate buffer at pH 7.4. The dilutions were so arranged that the desired amount of agent was administered in 0.5 cc. of buffer solution.

Protective Effect of a Single Dose of the Bactericidal Agent.—Mice were infected with varying dilutions of cultures of virulent pneumococci (Type I and Type III) and were subsequently treated within 10 minutes with 2 mg. of the bactericidal agent. No further treatment was given (Table I).

The results presented in Table I show that one single treatment with 2 mg. of the bactericidal substance was sufficient to protect a significant number of mice against infection with 0.0001 and 0.00001 cc. of culture of virulent pneumococci. Although most of the mice infected with larger amounts of culture died within the 6 day observation periods, they sur-

vived longer than the untreated controls. The next experiment aimed at determining whether a higher degree of protection could be obtained by administering the bactericidal extract repeatedly at 24 hour intervals.

Protective Effect of Repeated Doses of the Bactericidal Agent.—Mice were inoculated with 0.1 or 0.01 cc. of cultures of virulent pneumococci Types I, II, III, V, VIII. They were treated on 3 consecutive days; 2 mg. of the bactericidal agent was given 10 minutes after injection of the infective inoculum, 1 mg. 24 hours later, and 1 mg. 48 hours later (Table II.)

TABLE I
Protective Effect of a Single Dose of Bactericidal Substance

Infective dose of pneumococcus		Treat- ment	Number of mice	Result*						
	cc.	mg.								
Type I	0.01	2	6	D 48	D 72	D 72	D 72	D 72	S	
"	0.001	2	6	D 72	D 72	D 96	S	S	S	
"	0.000,1	2	6	D 72	S	S	S	S	S	
"	0.000,01	2	6	S	S	S	S	S	S	
"	0.000,001	0	1	D 44						
"	0.000,000,1	0	1	D 46						
"	0.000,000,01	0	1	D 72						
Type III	0.01	2	6	D 48	D 48	D 72	D 72	D 72	D 72	
"	0.001	2	6	D 72	D 72	D 72	D 96	D 96	D 96	
"	0.000,1	2	6	D 72	D 96	D 96	S	S	S	
"	0.000,01	2	6	D 72	D 96	S	S	S	S	
"	0.000,001	0	1	D 46						
"	0.000,000,1	0	1	D 44						
"	0.000,000,01	0	1	D 44						

*S = survival of the animal (6 day observation periods).

D = death of the animal; the numeral indicates number of hours before death.

The results presented in Table II show that, with 3 consecutive treatments at 24 hour intervals of time and comprising in all 4 mg. of agent, it was possible to protect many mice against 0.1 and 0.01 cc. of cultures of pneumococci of maximum virulence; in all cases the untreated control mice, inoculated with 0.000,000,01 cc. of culture or with larger infective doses, died in less than 72 hours.

In order to study the comparative effectiveness of the bactericidal agent against the different types of pneumococci, an effort was made in the following experiment to determine the minimal amount of agent that would protect mice against the same infective dose of pneumococci of different types.

Titration of the Bactericidal Agent against Different Types of Pneumococci.—Groups of 4 mice each were inoculated with 0.001 cc. of 8 hour cultures of pneumococci of Types I, II, III, V, VIII. Within 10 minutes after inoculation each group was treated with 1 mg., 0.3 mg., or 0.1 mg. respectively of the bactericidal agent. A second treatment with the same amount of extract (1 mg., 0.3 mg., and 0.1 mg. respectively) was again given 24 hours later and the same dose repeated 48 hours after inoculation (Table III).

TABLE II
Protective Effect of Repeated Treatments with the Bactericidal Agent

Infective dose of pneumococcus	Treatment on 3 consecutive days	Num-ber of mice	Results										
			cc.	mg.	mg.	mg.							
Type I	0.1	2	1	1	6	D 22	S	S	S	S	S	S	S
"	0.01	2	1	1	6	D 22	S	S	S	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 40							
Type II	0.1	2	1	1	6	D 26	S	S	S	S	S	S	S
"	0.01	2	1	1	6	D 22	S	S	S	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 72							
Type III	0.1	2	1	1	6	D 72	D 72	D 72	D 72	S	S		
"	0.01	2	1	1	6	D 72	D 72	S	S	S	S		
"	0.000,000,01	0	0	0	1*	D 60							
Type V	0.1	2	1	1	6	D 72	S	S	S	S	S		
"	0.01	2	1	1	6	S	S	S	S	S	S		
"	0.000,000,01	0	0	0	1*	D 72							
Type VIII	0.1	2	1	1	6	D 22	D 22	D 40	D 48	S	S		
"	0.01	2	1	1	6	D 22	S	S	S	S	S		
"	0.000,000,01	0	0	0	1*	D 40							

* The untreated control animals inoculated with 0.000,000,1 and 0.000,001 cc. of culture died within 44 hours.

It is apparent from the results presented in Table III that 1 mg. of extract administered daily on 3 consecutive days was sufficient to protect mice against infection with 0.001 cc. of culture of pneumococci Types I, II, III, V, VIII. When the amount of extract was reduced to 0.3 mg. or 0.1 mg. daily, most animals died within the 6 day observation period, although they survived longer than the controls. One may conclude therefore, that the protective effect of the bactericidal agent is approximately the same against all virulent pneumococci so far tested, irrespective of specific type.

In the three experiments which have just been described, the first dose

of bactericidal agent was administered within 10 to 15 minutes after inoculation of the experimental animals with the infecting organism. In the following experiment an attempt was made to determine the curative effect of the bactericidal agent when administered several hours after injection of the infecting inoculum.

TABLE III
Titration of the Bactericidal Agent against Pneumococci of Different Types

Infective dose of pneumococcus		Treatment			Number of mice	Results			
cc.	mg. of agent per day								
Type I	0.001	1.0	1.0	1.0	4	S	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 120	D 96
"	"	0.1	0.1	0.1	4	D 72	D 72	D 96	D 96
"	"	0	0	0	4	D 24	D 24	D 48	D 48
Type II	0.001	1.0	1.0	1.0	4	S	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 96	D 120
"	"	0.1	0.1	0.1	4	D 72	D 72	D 96	D 96
"	"	0	0	0	4	D 24	D 24	D 48	D 48
Type III	0.001	1.0	1.0	1.0	4	D 120	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 96	D 96
"	"	0.1	0.1	0.1	4	D 48	D 72	D 72	D 72
"	"	0	0	0	4	D 24	D 24	D 48	D 48
Type V	0.001	1.0	1.0	1.0	4	S	S	S	S
"	"	0.3	0.3	0.3	4	D 96	D 120	D 120	S
"	"	0.1	0.1	0.1	4	D 72	D 72	D 96	D 96
"	"	0	0	0	4	D 48	D 48	D 48	D 48
Type VIII	0.001	1.0	1.0	1.0	4	D 120	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 96	D 96
"	"	0.1	0.1	0.1	4	D 48	D 48	D 72	D 72
"	"	0	0	0	4	D 24	D 24	D 24	D 24

The Curative Effect of the Bactericidal Agent.—Mice were inoculated with 0.000,01 cc. of culture of Type I or Type III. They were divided into three groups which were treated with 2 mg. of bactericidal agent respectively 2 hours, 5 hours, and 17 hours after infection. A second and a third dose of 1 mg. each, were given 24 and 48 hours after the first treatment (Table IV).

The results presented in Table III show that mice inoculated with 1000 fatal doses of pneumococci Type I or Type III can be protected even when treatment with the bactericidal agent is delayed for several hours after

infection. In fact, 3 out of 6 mice inoculated with Type I organisms survived, although treatment had been delayed for 17 hours; the 6 mice inoculated with Type III pneumococci and treated 17 hours later all died, but 4 of them survived much longer than the untreated controls.

The Effect of the Bactericidal Agent upon Infection with Klebsiella pneumoniae.—It has been shown in the preceding paper that the bactericidal agent is ineffective *in vitro* against all the Gram-negative bacilli so far tested. It is also ineffective upon experimental infection of mice with

TABLE IV
Curative Effect of the Bactericidal Agent

Infective dose of pneumococcus		Time between infection and first treatment			No treatment
		2 hrs.	5 hrs.	17 hrs.	
Type I	cc. 0.000,01	S	S	D 34	D 30
"	"	S	S	D 72	D 34
"	"	S	S	D 96	D 40
"	"	S	S	S	—
"	"	S	S	S	—
"	0.000,000,01	—	—	—	D 72
Type III	0.000,01	D 96	D 72	D 24	D 18
"	"	S	D 96	D 30	D 30
"	"	S	S	D 48	D 34
"	"	S	S	D 60	—
"	"	S	S	D 72	—
"	"	S	S	D 96	—
"	0.000,000,01	—	—	—	D 48

Klebsiella pneumoniae (Friedländer bacillus), a Gram-negative rod, as appears from the following experiment.

Mice were inoculated with dilutions of a 6 hour culture of Friedländer bacilli type B. They were treated within 5 minutes with 2 mg. of preparation NS7 (Table V).

DISCUSSION

It is clear that the bactericidal agent described in the preceding paper is effective *in vivo* as well as *in vitro* and protects mice against infection with virulent pneumococci. In fact, the protective action is observed not only when the agent is injected simultaneously with the infective dose, but also when it is administered several hours later.

Protection has been obtained against cultures of the five different types of pneumococci (I, II, III, V, VIII) which have been used; these cultures were of maximum virulence since 0.000,000,01 cc. was invariably fatal to mice within 72 hours. It is permissible to assume, therefore, that the agent will be found effective against experimental infection of mice with pneumococci of other types.

It is of special interest that the amount of bactericidal agent required to protect mice against a given amount of virulent culture is approximately the same irrespective of the type of pneumococcus used as infective agent. It is likely, therefore, that the action of the agent is directed against a structure or a function which is qualitatively and quantitatively similar in all pneumococci. The same conclusion had been derived from a study of

TABLE V
Effect of the Bactericidal Agent upon Experimental Infection of Mice with Klebsiella pneumoniae

Infective dose of <i>Klebsiella pneumoniae</i> type B	Treatment	Number of mice	Result
cc.	mg.		
0.000,1	2	4	D 17 D 17 D 17 D 17
0.000,01	2	4	D 17 D 17 D 17 D 72
0.000,001	2	4	D 17 D 17 D 17 D 48
0.000,000,1	0	2	D 48 D 72
0.000,000,01	0	2	D 72 D 72

the action of the agent on pneumococci *in vitro*. As will be shown in a later publication, experiments carried out in collaboration with Dr. R. C. Lancefield have demonstrated that the agent also exerts a protective effect on experimental infection of mice with hemolytic streptococci of group A and C; on the contrary, it does not protect mice against *Klebsiella pneumoniae*, a Gram-negative organism, even when very small infective doses are used. This, again, is in agreement with the results of experiments *in vitro* in which a bactericidal effect was recognized against all the Gram-positive microorganisms so far tested, whereas the Gram-negative bacilli remained unaffected. There is little doubt, therefore, that the protective effect *in vivo* depends upon the same mechanism by which the bactericidal agent causes the death of the Gram-positive cells *in vitro*. It is interesting to contrast this direct bactericidal effect with the mechanism of the protection induced by a bacterial enzyme that hydrolyzes the capsular polysaccharide

of Type III Pneumococcus (2, 3). As described in earlier studies, this polysaccharidase does not in any way affect the viability of pneumococci; by decomposing the capsular substance of Type III organisms, however, it renders these bacterial cells susceptible to destruction by phagocytosis. The polysaccharidase does not attack the specific polysaccharides of other types of pneumococci, and consequently it protects only against infection with Type III organisms. On the contrary, the bactericidal agent considered in the present paper inhibits the growth of all Gram-positive organisms so far tested, and exerts on them a direct bactericidal action *in vitro* and *in vivo*.

SUMMARY

In the first paper of this series, a description was given of a cell-free extract, obtained from autolysates of a particular strain of a soil bacillus, which selectively inhibits the growth of all the Gram-positive microorganisms so far tested, and exerts on them a bactericidal effect *in vitro*.

In the present study it is shown that the same agent protects white mice against infection with large numbers of virulent pneumococci. It also exerts a curative effect when administered to mice several hours after injection of the infecting organisms.

The degree of protection afforded, and the minimal effective dose of bactericidal agent, are approximately the same for all virulent pneumococci, irrespective of type specificity.

The bactericidal agent is entirely ineffective against infection with virulent Friedländer bacilli (type B). This agrees with the fact that the agent does not affect Gram-negative bacilli *in vitro*.

The protective action exerted by the bactericidal agent against experimental pneumococcus infection depends upon the same mechanism which determines its bactericidal effect *in vitro*.

See Addendum to Paper I.

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