THE BIOLOGIC ACTIVITIES OF BACILLUS ANTHRACIS AND BACILLUS CEREUS CULTURE FILTRATES

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The sole species within the large bacterial family Bacillaceae which is classified as being pathogenic for man and higher animals is *Bacillus anthracis,* the etiologic agent of anthrax. *Bacillus cereus,* however, has been shown to produce fatal infection when injected in large numbers in small laboratory animals.[1] The two species are quite similar in most respects and are difficult to distinguish by routine cultural characteristics, antigenic analysis and biochemical reactions. These similarities are so striking that Smith, Gordon and Clark[2] suggested that *B. anthracis* was a pathogenic variety of *B. cereus.* This conclusion can be considered justifiable since many variants of *B. anthracis* can become avirulent. Under such circumstances, the criterion of pathogenicity as a means of differentiation becomes useless.

The pathogenesis of anthrax remained relatively obscure until Smith and Keppie[3] showed that *B. anthracis* produced a lethal substance during multiplication *in vivo.* In addition, it was found that avirulent strains of *B. anthracis* produced as much "lethal factor" as the fully virulent encapsulated strains. Thorne, Molnar and Strange[4] succeeded in obtaining the anthrax toxin from culture filtrates of the avirulent Sterne strain of *B. anthracis* grown in the modified casamino acids medium described by Belton and Strange.[5]

Recently, Burdon, Wende and Davis[6] reported fatal infections in mice and guinea pigs due to *B. cereus* but concluded that bacterial invasion was very limited and that death was due to a generalized toxemia. In addition, they found that injection of cell-free culture filtrates resulted in death within several hours. The observations of these investigators established the fact that *B. cereus,* as well as *B. anthracis,* was capable of producing a toxic substance during growth which was lethal for laboratory animals.

In view of the marked similarity between *B. cereus* and *B. anthracis,*

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an attempt was made to compare the biologic activities of the lethal substances produced by the respective bacterial species.

Material and Methods

Organisms

The virulent strain of *B. anthracis* (VIB) and the avirulent Sterne strain were obtained from Dr. C. Thorne, Fort Detrick, Maryland. *B. cereus* strains Ba-21 and Ba-25 were obtained from Dr. K. Burdon, Baylor University Medical School; strain ATCC-10987 from the American Type Culture Collection, Washington, D.C., and strains "X," B-47, B-48, and B-49 from the stock culture collection of the Department of Microbiology, College of Medicine, University of Cincinnati. The majority of the experimental work was carried out with the avirulent Sterne strain of *B. anthracis* and *B. cereus* strain "X."

Culture Mediums

Stock cultures were stored at 4° C. on trypticase soy agar slants. Freshly prepared beef infusion broth and the semi-synthetic casamino acids medium of Thorne and coworkers [4] were used in the cultivation of the organisms for toxin production.

Toxin Production

B. anthracis was grown in the casamino acids broth medium in 2 liter Erlenmeyer flasks containing 500 ml. of medium. The cultures were incubated statically at 37° C. for 21 to 26 hours and were then centrifuged at 4° C. The supernatant fluid was decanted, horse serum added to a final volume of 10 per cent and the mixture sterilized through ultrafine porosity fritted glass filters. The serum was required so that the anthrax lethal factor was not adsorbed to the glass during filtration.[4] The filtrates were lyophilized and stored at −70° C.

Toxic culture filtrates of *B. cereus* were obtained by growth in beef infusion broth. Cultures were incubated at 37° C. on a reciprocal shaker for 12 to 15 hours and the filtrates obtained as described for the anthrax cultures, with one modification. It was found that the addition of serum before filtration was not necessary since the toxic substance found in *B. cereus* cultures was not adsorbed to the glass filter. In a few cases, active filtrates of *B. cereus* were obtained from cultures grown in the casamino acids medium used for anthrax toxin production.

Toxin Assays

The toxicity of culture filtrates was determined by inoculation in white mice and albino rats. Several hundred mice and rats were used (Table I). The majority of

Table I

Lethality of *B. cereus* and *B. anthracis* culture filtrates

Organism*	No. mice	Mortality (%)
B. cereus ("X")	300	100
B. cereus (B-47)	50	100
B. cereus (B-48)	50	100
B. cereus (B-49)	50	100
B. cereus (Ba-21)	50	100
B. cereus (Ba-25)	50	100
B. cereus (ATCC-10987)	50	100
B. anthracis (VIB)	50	0
B. anthracis (PM-36)	50	0
B. anthracis (Sterne)	300	1.0

* Organisms grown either in beef infusion broth or in casamino acids medium. Mice given intravenous injections of 0.5 ml. of sterile culture filtrate.

animals were given intravenous injections although other routes of inoculation were employed in a few instances. The doses administered to mice and rats respectively were 0.5 ml. and 1 ml. Dilutions of the toxic filtrates were made in gelatin-phosphate buffer, pH 6.8. Death of the animal was used as the criterion for toxicity of the sterile culture filtrates in all cases.

Phospholipase Activity

The lecitho-vitellin reaction in egg yolk nutrient broth was used for measurement of phospholipase (lecithinase) activity of culture filtrates. Tubes containing 5 ml. of egg yolk broth [7] were incubated at 37° C. with 0.5 ml. filtrate. The opalescence of the medium caused by the action of the phospholipase was measured in a Klett-Summerson colorimeter at 540 mμ. The rate at which the reaction occurred was used as an index of enzyme concentration.

Hemolysin Activity

The hemolytic activity of culture filtrates was determined by observing hemolysis of rabbit erythrocytes. Two-fold dilutions of the filtrates were made in 2 ml. physiologic saline to which was added 0.1 ml. of a 5 per cent suspension of red cells. Tubes were incubated in a 37° C. water bath for 2 hours. Hemolytic titers were expressed as the highest dilution of filtrate showing complete hemolysis.

Pathologic Features

Tissues were obtained immediately after death of the mice or rats. Tissue sections were prepared and stained according to standard histologic procedures. Hematoxylin and eosin was used for staining of the sections in all cases.

Experimental Results

Lethality of B. cereus and B. anthracis Filtrates for Mice and Rats

A preliminary survey of 10 strains showed that there was a striking difference in the response of mice when given injections of culture filtrates of *B. cereus* or *B. anthracis*. Intravenous inoculation of active *B. cereus* filtrates resulted in death within 5 minutes whereas *B. anthracis* culture filtrates were found to be relatively innocuous. The data in Table I show a 100 per cent mortality for mice that received injections of culture filtrates of *B. cereus*. On the other hand, only 3 of the several hundred mice given injections of the anthrax filtrates succumbed. In contrast to the extremely rapid action of the *B. cereus* culture filtrate, these 3 deaths were recorded between 24 to 72 hours after inoculation.

When the albino rat was used as the test animal, it was found that culture filtrates of both bacterial species were lethal. Beall, Taylor and Thorne [8] recently reported that purified preparations of *B. anthracis* lethal factor were lethal for the albino rat. In the present investigation, an inbred strain of Fischer rat was found to respond uniformly when the anthrax filtrates were injected. All rats died within 1 to 3 hours following intravenous injection. As in mice, rats given injections of *B. cereus* culture filtrates died within several minutes after inoculation.

These observations were surprising for two reasons: (1) They showed that the two bacterial species, which are similar in most respects, produced extracellular lethal factors which were quite dissimilar in their biologic properties; and (2) the rat, which is more resistant than the mouse to natural anthrax infection, was much more sensitive to the anthrax lethal factor.

Effects of Anthrax Culture Filtrates on Fischer Rats

Fischer rats weighing approximately 100 gm. were given intravenous injections of 1 ml. of anthrax culture filtrate. These exhibited a syndrome of initial restlessness followed by progressive debility and dyspnea. During the terminal stages of the toxemia the animals showed a marked cyanosis, and breathing became uneven and labored. Death occurred approximately 1 hour following the onset of symptoms. The lethal activity of the anthrax filtrates was not apparent when they were injected intraperitoneally or intramuscularly.

Necropsy examination revealed nothing abnormal except in the lungs. Grossly, these appeared congested and mottled. The pleural cavity contained a considerable quantity of clear fluid. Incision of lung substance showed that the tissue was swollen and edematous.

Microscopic examination of the lung showed a diffuse interstitial edema in both the intra-alveolar and peribronchial spaces (Fig. 1). Beall and co-workers[8] reported similar pathologic findings following the injection of purified anthrax lethal factor in albino rats.

In Vivo Effects of B. cereus Culture Filtrates in Mice

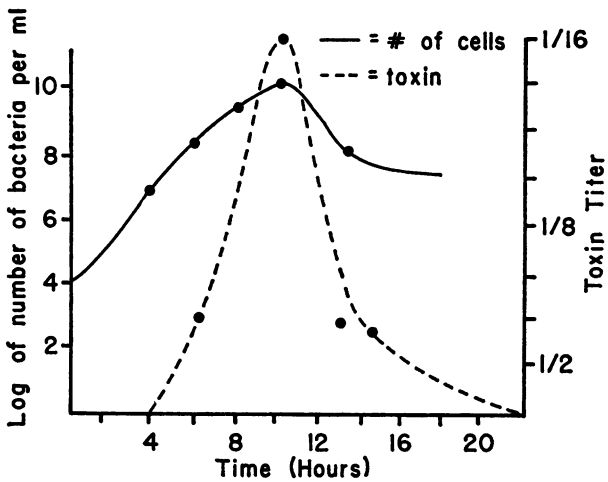
Within seconds after intravenous injection of *B. cereus* toxic filtrate in mice, clinical symptoms were observable. The animals became extremely quiet and arrhythmic breathing developed rapidly. Respiration became increasingly labored and death ensued in a few minutes. The average survival time of mice given injections of 0.5 ml. of the toxic filtrate was less than 2 minutes.

Examination of the lungs at necropsy showed that they were intensely congested. Microscopically, massive thrombi were evident in the blood vessels and capillaries of the lungs (Fig. 2), but there were no pathologic changes in tissues other than the lungs. Identical clinical and histologic effects were produced in rats.

B. cereus filtrates were also active when injected intraperitoneally. There was a lag, however, between the time of injection and the appearance of symptoms and death of the animals. The latent period varied from 15 minutes to several hours. The filtrates were not effective when administered by mouth.

Kinetics of Toxicity in Cultures of B. cereus and B. anthracis

The production of the anthrax lethal factor *in vitro* was found to be dependent upon the length of the incubation period. There was a critical period during which active filtrates could be obtained, but prolonged incubation resulted in a spontaneous inactivation of the lethal factor.[9] In our hands, the toxic culture filtrates were demonstrable only between 21 to 26 hours following inoculation of the casamino acids medium with 3×10^6 anthrax spores. Cultures were incubated statically at 37° C. Samples taken before 21 hours and later than 26 hours were found to be inactive when tested in rats. This inactivation phenomenon was probably due to proteolytic enzymes produced by the organism during growth.



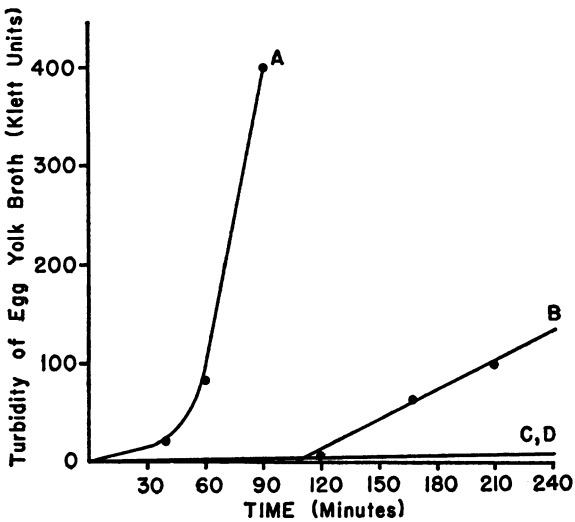
TEXT-FIG. 1. Extracellular toxicity in relation to growth of *B. cereus* in beef infusion broth. Shake cultures were incubated at 37° C. Toxicity is measured by intravenous inoculation of 2-fold dilution of culture filtrates in mice.

The inactivation phenomenon was also demonstrable with toxic culture filtrates of *B. cereus*. The toxic factor was produced and excreted into the culture fluid during the phase of active growth and reached its highest concentration at approximately the same time cell numbers reached a maximum (Text-fig. 1). There was a lag period of approximately 4 hours during which toxicity was not demonstrable by the mouse lethality test. This was followed by a period of accelerated growth during which a sharp rise in the toxicity of culture filtrates was noted. During the stationary growth phase, a rapid decrease in toxicity took place, and after 24 to 27 hours, the filtrates were no longer toxic for mice.

Association of B. cereus Lethal Activity with Lecithinase Activity

Previous studies have shown that strains of *B. cereus* produced potent lecithinases (phospholipases),[10] whereas *B. anthracis* demonstrated a very weak lecithinase activity.[11] The lecithinase activity of the 7 strains of *B. cereus* and the 3 strains of *B. anthracis* listed in Table I was measured in egg yolk broth. The culture filtrates of all strains of *B. cereus* gave a strongly positive egg yolk reaction whereas *B. anthracis* culture filtrates were inactive.

An attempt was made to correlate the lecithinase activity of *B. cereus* with toxicity. *B. cereus* (B-49) was grown in the casamino acids medium and incubated statically at 37° C. Samples were removed periodically and the sterile culture filtrates were tested for lecithinase activity, hemolytic activity and toxicity for mice. The kinetics of the egg yolk reaction



TEXT-FIG. 2. Lecithinase activity of *B. cereus* culture filtrates measured by kinetics of the lecitho-vitellin reaction. Opalescence of egg yolk medium was measured in a Klett-Summerson colorimeter at 540 mμ.
A = 16-hour culture filtrate; toxic for mice; hemolysis titer, 1:128.
B = 24-hour culture filtrate; nontoxic for mice; hemolysis titer, 1:2.
C = 16-hour culture filtrate; heated at 100° C. for 20 minutes; nontoxic for mice; hemolysis titer, 1:2.
D = 48-hour culture filtrate; nontoxic for mice; hemolysis titer, 1:2.

of the various samples are shown in Text-figure 2. The 16-hour culture filtrate which was lethal for mice and demonstrated a hemolysin titer of 1:128 also gave a very strong lecithinase reaction. The heated 16-hour sample, the 24-hour and the 48-hour samples were all nontoxic for mice, and were comparatively without hemolytic capacity. In addition, the

lecithinase activity of the filtrates was very much reduced or completely absent. These observations suggested that the toxic activity of *B. cereus* filtrates was closely associated with the phospholipases produced by the organism during growth. In all cases, toxicity was demonstrable only when the filtrate was hemolytic and showed a relatively high phospholipase activity.

Although *B. cereus* and *B. anthracis* are similar in many respects, the lethal substances produced by these two organisms are quite different in their biologic activities. This was most dramatically seen in the differential response of the mouse when challenged with either of the culture filtrates. Active culture filtrates of *B. cereus* were lethal for mice in all cases, and death occurred within minutes after intravenous challenge. *B. anthracis* culture filtrates, on the other hand, were relatively nontoxic for mice. Only 3 of 400 animals inoculated succumbed when challenged with *B. anthracis* filtrates, and these deaths were recorded between 24 and 72 hours. It is conceivable that the mouse lethality test could be used as a reliable criterion for differentiating *B. cereus* from *B. anthracis*.[12]

The difference in the biologic activity of the materials was also shown by the pathologic lesions produced in mice and rats. Intravenous injection of active *B. cereus* filtrates in both mice and rats resulted in extensive thrombus formation in the pulmonary blood vessels. This in no way resembled the alterations seen in the lungs of rats following intravenous challenge with the anthrax lethal factor. Lung sections of these rats showed an extensive edema with accumulation of fluid in the alveolar and peribronchial spaces (Fig. 1).

Eckert and Bonventre[13] showed that toxic culture filtrates of *B. anthracis* produced severe disturbances in blood chemical values and the distribution of body fluids. The marked hemoconcentration observed during the terminal stages of anthrax toxemia was probably caused by an increased permeability of the pulmonary circulation system with the resulting loss of fluid from the circulation. Whether the anthrax lethal factor acted directly upon the blood vessels to change their permeability or acted at a distant site is still not known.

The mode of action of the toxic material produced by *B. cereus* is still obscure. The thrombus formation in the lungs of mice and rats, however, was not incompatible with the action of potent phospholipases. Mice given injections of snake venom demonstrated lung lesions which were not identical but were similar to the lesions produced by *B. cereus* toxin.[14] This difference might be due to the fact that snake venom contains a variety of toxins other than phospholipases.

The correlation of toxicity with lecithinase and hemolytic activities

was suggestive but did not constitute a definite association between the lethal substance elaborated by *B. cereus* and a lecithinase. Molnar[15] has very recently presented evidence that the toxicity and phospholipase activity might be due to distinct molecular entities.

SUMMARY

The biologic activities of the extracellular, lethal substances produced by *B. cereus* and *B. anthracis* were compared. When challenged intravenously, the mouse was found to be extremely sensitive to *B. cereus* filtrates and insensitive to the *in vitro* filtrates of *B. anthracis*. In view of the difficulties often encountered in differentiating these two bacterial species, it is suggested that the mouse lethality test could be adopted as a diagnostic criterion.

The filtrates of both species were lethal for the albino rat. The mechanisms by which the toxic filtrates acted, however, were not similar. This was seen by difference in the time required to cause death of the animals, and in the pathologic lesions induced. *B. cereus* filtrates, which killed both mice and rats within several minutes, produced massive thrombi in the blood vessels of the lungs. The anthrax lethal factor on the other hand caused an extensive edema of the lungs after a latent period of at least one hour.

It was suggested that the lethal factor of *B. anthracis* acted by altering the permeability of blood vessels in the lung. Evidence was also presented which showed a close relationship between the toxic activity of *B. cereus* filtrates and the potent phospholipases produced by this organism during its multiplication.

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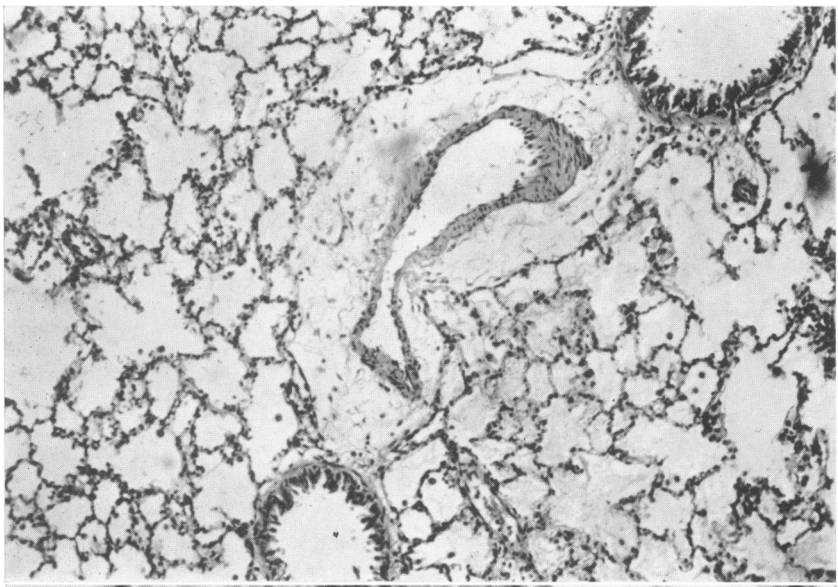
[*Illustrations follow*]

Legends for Figures

FIG. 1. Rat lung after injection with anthrax culture filtrate. Diffuse intra-alveolar and peribronchial edema is manifest. Hematoxylin and eosin stain. × 125.

FIG. 2. Mouse lung after injection with *B. cereus* culture filtrate. A capillary is occluded by a thrombus. Hematoxylin and eosin stain. × 562.



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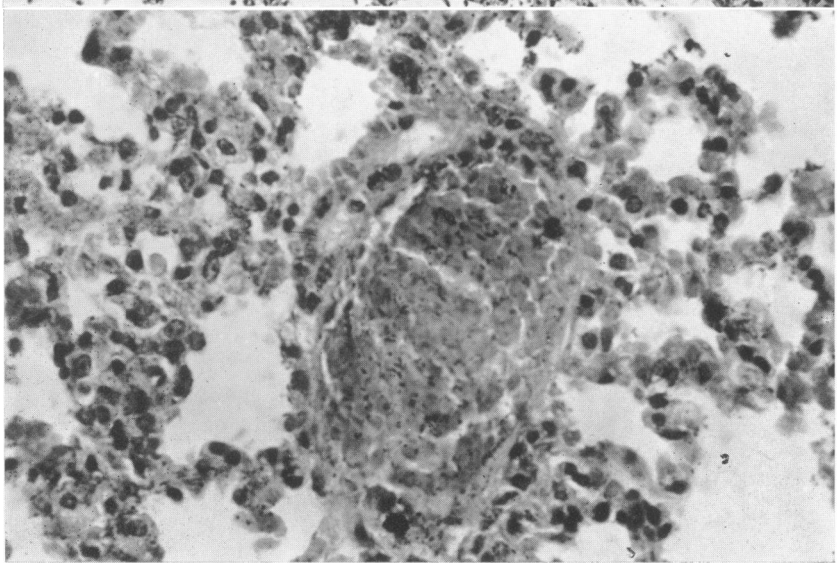

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