RAPID LETHAL EFFECT IN RATS OF A THIRD COMPONENT FOUND UPON FRACTIONATING THE TOXIN OF *BACILLUS ANTHRACIS*

FRANCIS A. BEALL, MARTHA J. TAYLOR, AND CURTIS B. THORNE[1]

U.S. Army Chemical Corps, Fort Detrick, Frederick, Maryland

Received for publication December 22, 1961

ABSTRACT

BEALL, FRANCIS A. (U.S. Army Chemical Corps, Frederick, Md.), MARTHA J. TAYLOR, AND CURTIS B. THORNE. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. **83**:1274–1280. 1962.—Rats were found to be more susceptible to the lethal effect of toxin produced by *Bacillus anthracis* in vitro than were several species considered less resistant to anthrax. Rats were killed much faster by less toxin per gram of body weight than were mice. Guinea pigs survived doses of toxin that killed rats. Intravenous injection of Fischer 344 rats is a rapid test for lethal activity, which facilitates the demonstration of two components, different from protective antigen, in toxin. One of these, a lethal factor, was separated from the other component, which causes cutaneous edema in the guinea pig. The latter component was not necessary for lethal effect. Neither of these factors was active unless combined with protective antigen. Although the guinea pig skin reaction has been used routinely to assay the toxicity of samples, the present results show that this test does not assay the lethal component.

Two essential factors have been demonstrated in the toxin produced by *Bacillus anthracis*. Smith et al. (1956) showed that toxin in plasma from guinea pigs dying of anthrax could be separated into two components by ultracentrifugation. Toxin produced by *B. anthracis* in vitro was found to contain the same components (Harris-Smith, Smith, and Keppie, 1958). Toxin produced in a medium without added protein also has been separated into two fractions by passing culture supernatant liquid through a fritted-glass filter (Thorne, Molnar, and Strange, 1960). One fraction, designated as filter factor, was eluted from the filter. The culture filtrate contained another component, evidently the protective antigen previously purified by Strange and Thorne (1958). Neither fraction was toxic alone; together they killed mice and produced cutaneous edema in the guinea pig. The present report shows that anthrax toxin is composed of at least three components with distinct biological activities. Two components, different from protective antigen, are adsorbed by fritted-glass filters. When combined with protective antigen one of them kills mice and rats but does not produce cutaneous edema in the guinea pig. With fritted-glass filters it was possible to obtain this lethal factor free from the other component which, along with protective antigen, is necessary for the formation of edema in the guinea pig skin but is not essential for lethal effect. Neither component was active without protective antigen. Throughout previous studies it was observed that the lethal effect of toxin was concomitant with the ability to produce edematous lesions when injected into the skin of rabbits or guinea pigs. Titration of this latter capacity has been used routinely to assay the toxicity of various preparations. However, the present results show that this assay does not indicate lethal capacity.

The investigators cited above have demonstrated the lethal effect of toxin with mice and guinea pigs. We found that the Fischer 344 rat was more susceptible to the lethal action of toxin than was either of these species. Intravenous injection of this rat with anthrax toxin is evidently the most sensitive test for lethal effect now available.

MATERIALS AND METHODS

Preparation of toxin. The methods for preparing anthrax toxin were essentially those described by Thorne et al. (1960) for large-scale preparation of filter factor. Casamino acids medium, 500 ml in each of ten Povitsky diphtheria-toxin bottles (5-liter capacity), was in-

[1] Present address: Department of Microbiology, Oregon State University, Corvallis, Ore.

oculated with 2.5×10^6 spores of the avirulent Sterne (Weybridge) strain of *B. anthracis*. Cultures were incubated statically at 37 C for 24 hr; all subsequent processing was carried out at 4 C. After centrifugation, the culture supernatant liquid (4 to 4.5 liters) was filtered through a fritted-glass filter (fine porosity, 90-mm diam, Corning no. 36060). The filter was eluted with 50 ml of alkaline buffer (0.3 M $NaHCO_3$-Na_2CO_3 buffer, pH 9.7). The eluate (filter factor) and the culture filtrate were sterilized by filtration through fritted-glass filters of ultrafine porosity (60-mm diam, Corning no. 36060, or $\frac{5}{8}$ in. by $4\frac{3}{4}$ in. tubular type Corning no. 35000). Samples of culture supernatant fluid were also sterilized in the same way after the addition of horse serum (10% v/v). The presence of serum allowed toxin to pass through fritted glass without adsorption (Thorne et al., 1960). Eluates were stored at 4 C; culture supernatant liquid and culture filtrates were kept frozen until just before use.

Assay by skin reaction in guinea pigs. The titer of a sample is expressed, in units per milliliter, by the reciprocal of the highest dilution that caused a palpable edematous lesion when 0.2 ml was injected intradermally (Smith et al., 1956). Each sample was titrated, within 48 hr of its preparation, in two female guinea pigs weighing about 400 g each. Culture supernatant liquid, culture filtrates, and eluates were serially diluted in a culture filtrate containing 8 units of protective antigen per ml. When a component is said to produce cutaneous edema in the guinea pig, this means that it had this capacity when mixed with protective antigen. The alkaline buffer used to elute filters caused necrosis unless diluted 1:10 or more before injection. This prevented the detection of less than 10 units per ml in an eluate; the titer of an eluate reported as zero, therefore, means that it contained 0 to 9 units per ml.

Assay of protective antigen. An agar diffusion method standardized by Thorne and Belton (1957) was employed with certain modifications. Plates were kept at 30 C from the time antiserum was added to the center wells until the final reading. Antigen was placed in the outer wells 6 hr after the center wells were filled. Eluates, as well as culture filtrates and culture supernatant fluid, were examined by this procedure. Two lots of partially purified protective antigen were used as standards for identifying the precipitation line previously associated with the immunizing activity of culture filtrates (Thorne and Belton, 1957). Both lots of partially purfied protective antigen had been prepared by the procedures given by Strange and Thorne (1958) for fraction II-C, lyophilized, and stored at −15 C for several years. These were reconstituted in 10% horse serum at concentrations of 128 units or less per ml. At these concentrations, this material formed a single line of precipitation with antiserum in the agar diffusion assay. The antiserum used in these experiments was prepared in a horse by injecting spores of the Sterne (Weybridge) strain of *B. anthracis*.

Determination of lethal effect. Lethality of various preparations was determined by intravenous injection into several species of animals. Test material was injected into Balb/c AnNLi mice (weighing 11 to 21 g) via the tail vein, and into Hartley guinea pigs (weighing 290 to 375 g) via a superficial vein on the lateral side of the hind foot. The mice were from Ralph Lincoln's colony and the guinea pigs from the Animal Farm, both at Fort Detrick, Md. With rats, the dose was injected into the dorsal vein of the penis. Male Fischer 344 rats, 2 to 6 months old and weighing 200 to 400 g, were used in most experiments. These were from the colony described by Taylor, Kennedy, and Blundell (1961). A small number of germ-free Fischer 344 rats, obtained from Lobund Germfree Life Research Laboratories, was also tested. In other experiments, Sprague-Dawley, Osborne-Mendel, and NIH Black rats (developed from Long-Evans stock at the National Institutes of Health) were used. Test animals were usually observed for 2 weeks, but in some cases survivors were killed after 5 days for microscopic examination of tissues.

Eluates were tested for lethal effect by diluting them in solutions of partially purified protective antigen or in culture filtrates. The routine test was whether or not 2.0 ml of a mixture consisting of 1 volume of eluate plus 4 volumes of culture filtrate killed rats. The unqualified statement that an eluate had lethal effect means that it killed rats under these conditions. Eluates were also tested for lethal activity in the absence of protective antigen. Animals were given 2.0 ml of a mixture of 1 volume of eluate and 4 volumes of gel-phosphate solution [0.02 M phosphate buffer, pH 8.0, with 0.5% (w/v) gelatin].

Culture filtrates, solutions of partially purified protective antigen, or culture supernatant fluid were injected alone and in mixtures consisting of 4 volumes of the test material plus 1 volume of alkaline buffer.

RESULTS

Fractionation and assay of toxin. Upon testing batches of toxin, prepared and fractionated as described in Materials and Methods, we found that the fraction eluted from the glass filter did not have lethal action unless mixed with the culture filtrate. However, some culture filtrates, when tested alone, killed rats. The factor necessary for lethal effect could be removed from these culture filtrates by additional filtration and was eluted from filters with the alkaline buffer. Biological assays of various eluates showed that some, in combination with protective antigen, caused cutaneous edema in the guinea pig and had lethal effect, but that others exhibited only the latter capacity.

Fractionation of three batches of toxin, designated 1, 2, and 3, is described below; results from assay of various fractions are given in Table 1. The first eluate of each batch was obtained by the procedure described in Materials and Methods for preparation of filter factor. The culture filtrate from this procedure (i.e., the first culture filtrate of each batch) had lethal activity. One such culture filtrate, first culture filtrate (3), is listed in Table 1; its lethal effect, when given intravenously to mice and rats, is shown in Table 2. First culture filtrates of batches 1 and 2 were refiltered by passing each through two additional fine-porosity filters in succession. Eluates from these two filters were pooled and designated intermediate eluate. The resultant culture filtrate, which still had lethal effect, was finally filtered in small amounts (150 to 300 ml) through filters of ultrafine porosity. These filters were eluted successively with the same portion of alkaline buffer and this eluate was called final eluate. The final culture filtrate from this procedure did not kill rats. No intermediate eluate was prepared from the first culture filtrate of batch 3; it was subjected only to the final filtration procedure described above.

All the eluates listed in Table 1, except first eluate (3), killed rats. The first eluates caused cutaneous edema in the guinea pig; the final eluates did not. The intermediate eluate of batch 1 also had this capacity, indicating that, with this batch, all the factor necessary for the guinea pig skin reaction had not been adsorbed on the initial filter. No eluate exhibited the activities mentioned above unless combined with protective antigen furnished either by the partially purified preparation or by the final culture filtrate. Neither

TABLE 1. *Fractions prepared from anthrax toxin*

Preparation*	Protective antigen	Guinea pig skin test	Test for lethality in rats†
	units per ml	*units per ml*	
Culture supernatant fluid (1)	16	128	+
First eluate (1)	0	3,200	+
Intermediate eluate (1)	0	1,600	+
Final eluate (1)	0	0	+
Final culture filtrate (1)	16	0	−
Culture supernatant fluid (2)	16	64	+
First eluate (2)	0	1,600	+
Intermediate eluate (2)	0	0	+
Final eluate (2)	0	0	+
Final culture filtrate (2)	8	0	−
First eluate (3)	0	1,600	−
First culture filtrate (3)	16	0	+
Final eluate (3)	0	0	+
Final culture filtrate (3)	8		−

* Numbers 1, 2, and 3 indicate batch numbers.

† See Materials and Methods. None of the eluates killed rats unless given in combination with final culture filtrate or partially purified protective antigen.

TABLE 2. *Lethal effect of first culture filtrate from batch 3 for rats and mice*

Dose	Units in dose		No. killed/no. injected	Time to death
	Guinea pig skin test	Protective antigen		
ml				*hr*
2.0	0	26–32*	33/33 (rats)	1–2
1.0	0	16	2/2 (rats)	1.5–2
1.0	0	16–21†	14/14 (mice)	12–62
0.5	0	8	1/5 (mice)	113

* Variation due to two ways of testing (see Materials and Methods).

† Some doses augmented with partially purified protective antigen.

of these, when tested alone, caused cutaneous edema in the guinea pig or had lethal effect. Final culture filtrates had no apparent effect in 48 rats given 2.0 to 4.0 ml or in 10 mice given 1.0 ml.

Results of the following experiments with various fractions of toxin indicate that the component necessary for formation of edema in the guinea pig skin is not essential for lethal effect. (i) The eluates of batch 1 were assayed for lethal capacity by serially diluting each of them in the homologous final culture filtrate and injecting 2.0-ml doses into rats divided into groups of comparable age and weight. The results of this titration are given in Table 3. These data reveal no correlation between the capacities of an eluate to kill rats and to produce edema in the guinea pig skin. (ii) Four of the eluates listed in Table 1 had a titer of zero in the guinea pig skin test but each had lethal activity. One of these, final eluate (1), shown to be lethal for rats when mixed with the final culture filtrate (line 3, Table 3), was tested in mice also. Five mice given this mixture and sufficient partially purified protective antigen to raise the protective antigen content of the 1.0-ml dose to 20 units died in 17 to 48 hr. Final eluate (3) killed rats rapidly (Table 4). In contrast, the first eluate of batch 3, with a titer of 1,600 units per ml in the guinea pig skin assay, had no apparent effect on rats when tested under the same conditions. (iii) As previously mentioned, first culture filtrate (3) killed mice and rats (Table 2), but did not elicit cutaneous edema in the guinea pig. (iv) Mice given 1.0 ml of culture supernatant fluid (1) or first culture filtrate (3) were killed as rapidly by either material, despite the fact that the former contained 128 units by guinea pig skin assay and the latter had none.

Additional evidence that the lethal factor is different from the factor necessary for cutaneous edema in the guinea pig was obtained by testing four other eluates previously prepared by one of us (C. B. T.). These eluates, which did not produce cutaneous edema in the guinea pig, had been dialyzed against 40% ethanol. The resultant precipitates were then dissolved in 0.05 M phosphate buffer (pH 7.0) and the solutions stored at −15 C. We found that this material alone did not have lethal effect but, in combination with partially purified protective antigen or final culture filtrate, killed rats and mice. The dose for mice contained 24 units of protective antigen.

TABLE 3. *Titration of eluates for lethal effect in rats*

Eluate	Guinea pig skin test	Dilution of eluate in final culture filtrate (1)						
		5*	10	20	40	80	160	320
	units/ml							
First eluate (1)	3,200	4/4†	4/4	0/4				
Intermediate eluate (1)	1,600	4/4		1/1		4/4	4/4	0/4
Final eluate (1)	0	4/4		4/4	4/4	0/4		

* Reciprocals of dilutions.

† No. of rats killed by 2.0-ml dose/no. of rats tested.

TABLE 4. *Lethal effect of final eluate from batch 3 in rats*

Dose (2.0 ml)	No. killed/ no. injected	Time to death
		hr
Final eluate (3)* + gel-phosphate diluent	0/5	
partially purified protective antigen	2/2	1.5
Final culture filtrate (3)†	27/27	1–2.5

* Eluate diluted 1:5.

† Eluate diluted from 1:5 to 1:20.

Examination of eluates by agar-gel diffusion technique. The lines of precipitation, formed when eluates were diffused against anthrax spore antiserum, also indicated the existence of two distinct components different from protective antigen. Eluates formed from one to three lines of precipitation on gel diffusion plates. All of these were different from the line formed by protective antigen placed in adjacent wells. When eluates forming several lines were compared, it was found that the predominant line (i.e., the line continuing to the end-point dilution) of an eluate with a high titer in the guinea pig skin test was different from the predominant line formed by an eluate without detectable titer in that assay. This latter line of precipitation was the only one formed by serial twofold dilutions of final eluate (3) and continued through 1:32, the end point. Moreover, this same line was among those formed by all eluates that, in combination with protective antigen, killed rats. The highest dilution of first, intermediate, and final

eluates of batch 1 forming this line was 1:8, 1:256, and 1:32, respectively. There is good correlation between these end points and the respective lethal capacities of the eluates (Table 3). A subsequent experiment with these same eluates indicated, however, that although the titer of this line of precipitation remained the same considerable lethal activity had been lost upon storage.

Relative susceptibility of different species to lethal effect of toxin. We observed repeatedly that rats are more susceptible to the lethal effect of anthrax toxin than are mice or guinea pigs. For example, 75 mice and 20 guinea pigs survived intravenous injection of material that killed rats. In no case was a mouse or guinea pig killed by the same amount of toxin per gram of body weight that was lethal for rats. Not only did mice and guinea pigs survive equivalent doses without apparent signs of illness, but in some cases they survived greater doses than did rats. The data in Table 2 are typical of those indicating that proportionately larger doses are required to kill mice, and that mice are killed less rapidly than rats. The equivalent doses per gram of body weight for the mice in this experiment would be 0.07 and 0.13 ml, but four of five mice survived a dose of 0.5 ml. All rats given 1.0 ml of culture supernatant fluid (2) died within 3 hr, but this dose killed only two of five mice and 0.5 ml caused no deaths in five mice. The mixture of eluate and culture filtrate that killed all of 27 rats given 2.0 ml (Table 4) was not lethal for two guinea pigs given 4.0 ml and 5.0 ml, respectively. Rats given 0.5 ml of culture supernatant fluid (1) died within 3 hr; mice receiving 1.0 ml died in 23 to 39 hr; a guinea pig given 8.0 ml was sacrificed 7 days after injection.

Similar results were also obtained upon testing three batches of toxin furnished us by M. Puziss. Rats were killed within 2 hr by less toxin per gram of body weight than that required to kill mice. Death in mice occurred from 23 to 96 hr after injection. Doses of toxin that killed rats were without apparent effect when given intravenously to guinea pigs of similar weight.

Response of rats to lethal doses of toxin. We have observed approximately 400 rats given lethal doses of anthrax toxin. After injection, all of these appeared normal for a period of time that varied with the time to death. For example, rats dying in 80 to 100 min did not appear to be sick during the first 50 to 60 min after injection. After this time respiratory difficulty was obvious and eventually cyanosis was evident. The rats died with a frothy liquid emerging from their nostrils. Pulmonary edema and hydrothorax were found at necropsy. From 1 to 3 ml of clear fluid could be aspirated from the thoracic cavity of rats killed by toxin, and their lungs weighed about 2.5 times more than normal rat lungs. Death occurred from 45 min to 7 hr after injection of various doses. The time of death in Fischer 344 rats varied in a uniform manner with the amount of lethal material injected.

TABLE 5. *Neutralization of lethal effect in rats by specific antiserum*

Dose*	No. killed/ no. injected
Toxin +	
antiserum (*B. anthracis*)	0/12
horse serum	15/15
antiserum (*B. cereus*)	4/4
rabbit serum	5/5

* Toxin (1.5 ml) and antiserum or serum (0.5 ml).

Tests for lethal effect in other rats. The preceding data were obtained using Fischer 344 rats. To find out whether these rats were peculiarly sensitive to anthrax toxin, the following tests were made. Doses of toxin or appropriate control material were given to 14 Sprague-Dawley, 21 Osborne-Mendel, 8 germ-free Fischer 344 rats, and 5 NIH Black rats. Although the response to toxin was not as uniform as that observed with our Fischer 344 rats, rapid deaths along with the characteristic symptoms previously described were observed with all strains except the NIH Black. The five NIH Black rats were apparently unaffected by doses of toxin that killed Sprague-Dawley and Osborne-Mendel rats in the same experiment. The materials used for these tests and selected tissues from germ-free rats killed by toxin were found to be free of bacteria when cultured aerobically on fresh blood agar.

Neutralization of lethal effect by specific antiserum. To demonstrate that the rapid death in rats was caused by the specific toxin of *B. anthracis*, the experiments summarized in Table 5 were carried out. Fischer 344 rats were apparently unaffected by mixtures of otherwise lethal doses of toxin and hyperimmune horse serum prepared

against the Sterne (Weybridge) strain of *B. anthracis*. In some experiments, this antiserum was mixed with culture supernatant fluid and in others with a combination of final eluate and final culture filtrate. Lethal activity of anthrax toxin was not neutralized by normal horse serum, normal rabbit serum, or by hyperimmune serum from a rabbit injected with spores of *B. cereus*, a closely related organism that also produces a toxin.

DISCUSSION

Smith, Keppie, and Stanley (1955) concluded that cutaneous edema in the guinea pig and lethal activity were caused by the same substance. Our results show, however, that it is possible to separate a factor that, in combination with protective antigen, has lethal activity but does not elicit cutaneous edema in the guinea pig.

Our experience with filtration of culture supernatant liquid indicates that the factor necessary for cutaneous edema in the guinea pig is preferentially adsorbed on fritted-glass filters. Therefore, it was possible to remove all of this component by filtration while some of the component necessary for lethal effect remained unadsorbed. Eluate from subsequent filters then contained only the latter component. The filter factor of Thorne et al. (1960) evidently contained both factors. Our preparation of first eluate (3), which was a fraction causing cutaneous edema in the guinea pig but lacking sufficient lethal factor to kill rats under the test conditions, was fortuitous. In fractionating culture fluid by filtration, the procedure to be followed is dictated by the results from assay of the starting material and each subsequent fraction for biological activity. The sensitivity of the Fischer 344 rat to the lethal factor greatly facilitated its detection and separation and may prove useful in the development of a less tedious and more effecient fractionation procedure.

Guinea pigs and many of the mice surviving doses of toxin lethal for rats probably received less than the amount of protective antigen required for lethal effect in these species. Optimal proportions of components and exact requirements for lethal effect have not been determined for any species. It remains to be ascertained to what extent the precipitation line associated with the lethal capacity of eluates will be useful in the investigation of these problems. Sargeant, Stanley, and Smith (1960) reported that anthrax toxin contained at least three major serological components. One of these they associated with the factor causing cutaneous edema and another with protective antigen. The third component could be the factor responsible for lethal effect.

The rat is generally considered more resistant to anthrax than the mouse or guinea pig. The LD_{50} (*B. anthracis* spores) for the rat has been shown to be considerably greater than the LD_{50} for these other species (Young, Zelle, and Lincoln, 1946). The adult Fischer 344 rat is not killed by the intradermal injection of more than 10^8 spores (Taylor et al., 1961) but is highly susceptible to lethal action of anthrax toxin given intravenously. This suggests that low levels of toxin, in vivo, may be lethal for species usually considered resistant to anthrax.

ADDENDUM

During the final preparation of this manuscript, a paper by Stanley and Smith appeared in which evidence for a third factor of the anthrax toxin is described. (Stanley, J. L., and H. Smith. 1961. Purification of Factor I and recognition of a third factor of the anthrax toxin. J. Gen. Microbiol. **26**:49-66.) Although these authors used a different method for separating the toxin components, the results indicate that their third factor is the same as the lethal component we describe.

ACKNOWLEDGMENT

We are indebted to M. Puziss and F. Klein for some of the material used in these studies.

LITERATURE CITED

HARRIS-SMITH, P. W., H. SMITH, AND J. KEPPIE. 1958. Production *in vitro* of the toxin of *Bacillus anthracis* previously recognized *in vivo*. J. Gen. Microbiol. **19**:91–103.

SARGEANT, K., J. L. STANLEY, AND H. SMITH. 1960. The serological relationship between purified preparations of factors I and II of the anthrax toxin produced *in vivo* and *in vitro*. J. Gen. Microbiol. **22**:219–228.

SMITH, H., J. KEPPIE, AND J. L. STANLEY. 1955. The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis in vivo*. Brit. J. Exptl. Pathol. **36**:460–472.

SMITH, H., D. W. TEMPEST, J. L. STANLEY, P. W.

HARRIS-SMITH, AND R. C. GALLOP. 1956. The chemical basis of the virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin: their relation to known immunizing aggressins. Brit. J. Exptl. Pathol. **37**:263–271.

STRANGE, R. E., AND C. B. THORNE. 1958. Further purification studies on the protective antigen of *Bacillus anthracis* produced *in vitro*. J. Bacteriol. **76**:192–202.

TAYLOR, M. J., G. H. KENNEDY, AND G. P. BLUNDELL. 1961. Experimental anthrax in the rat. I. The rapid increase of natural resistance observed in young hosts. Am. J. Pathol. **38**:469–480.

THORNE, C. B., AND F. C. BELTON. 1957. An agar-diffusion method for titrating *Bacillus anthracis* immunizing antigen and its application to a study of antigen production. J. Gen. Microbiol. **17**:505–516.

THORNE, C. B., D. M. MOLNAR, AND R. E. STRANGE. 1960. Production of toxin *in vitro* by *Bacillus anthracis* and its separation into two components. J. Bacteriol. **79**:450–455.

YOUNG, G. A., JR., M. R. ZELLE, AND R. E. LINCOLN. 1946. Respiratory pathogenicity of *Bacillus anthracis* spores. I. Methods of study and observations on pathogenesis. J. Infectious Diseases **79**:233–246.