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In Vivo-produced Anthrax Toxin

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Specific anthrax antigens were demonstrated in the blood of animals dying from anthrax. These antigens, which appear in the blood at the time when organisms are first detected and whose concentration continues to increase as the number of organisms increases, do not elicit a strong antibody response. The in vivo-produced toxin differs from the in vitro in killing more rapidly and being more difficult to detect. The in vivo toxin exists as an aggregate whose biological and serological activity depends upon its particular composition or configuration, or both.

Little is known about the synthesis of the anthrax toxin molecule or the extent of the differences between the in vivo and in vitro toxin. In fact, only the Porton group has reported purification and characterization of in vivo-produced toxin isolated from terminal blood of guinea pigs dying of anthrax (14, 16, 17). Their attempts were partially successful but resulted in contamination by serum proteins. The aggressin-like properties of anthrax toxin were noted (15), and the effect of certain metabolic analogues on in vivo formation of toxin was studied (19). Sargeant, Stanley, and Smith (12) showed serological identity between purified preparations from in vivo and in vitro toxin.

This paper presents the results of our studies on the kinetics of toxin production, the formation of specific antiserum, differences between in vivo- and in vitro-produced toxins, and studies on the dissociation of the toxin molecule.

MATERIALS AND METHODS

Culture conditions. Spore stocks of *Bacillus anthracis*, strains V1b, 116, 770, and Sterne, were prepared by growth in N-Z-Amine-A medium for 24 hr. Spores, collected by centrifugation, were washed with water until negative for catalase and free from sporangia by light microscopic examination. They were then heat-shocked at 60 C for 30 min and stored in the presence of 0.1% phenol at 4 C until used.

Vegetative cells were grown in casein acid digest-yeast medium. A 50-ml amount of medium in a 250-ml Erlenmeyer flask was inoculated and placed at 37 C on a reciprocating shaker [99 strokes (2-inch)/min]. Subcultures were prepared by inoculation of 0.5 ml into a second flask, and populations of 10^9 cells/ml were obtained in 24 hr. This subculture was used within 24 to 26 hr with no further treatment.

Anthrax toxin and its components. The method of Fish et al. (4) was used to prepare (i) whole toxin, (ii) protective antigen (PA) after final filtration, (iii) edema factor (EF), 35-fold purified, and (iv) lethal factor (LF), 35-fold purified.

Assay conditions. Serological (antigenic) activity was determined by the Ouchterlony method (20) with the use of antiserum (DH-1-6C) prepared by repeated injections of spores of the Sterne strain of *B. anthracis* into a horse and staining with azocarmine (4). Rat lethality was measured by the method of Haines, Klein, and Lincoln (5), and guinea pig skin edema was measured by the method of Beall, Taylor, and Thorne (1).

Other procedures. Animals were immunized by five injections of protective antigen (PA5), a live vaccine (LV), or a combination of these (PA5 plus LV). The procedure used was that described by Klein et al. (7). Cellulose-acetate electrophoresis (Technical Manual RM-1M-2, p. 22, Beckman Instruments Corp., Palo Alto, Calif.) was used to examine the serum of monkeys that died from anthrax.

RESULTS

Kinetics of in vivo toxin production. Eight guinea pigs (Hartley strain, 300 to 400 g) per bacterial strain were injected intraperitoneally with 1 ml of an unsporulated culture containing 8×10^8 to 16×10^8 cells/ml. The guinea pigs died within 18 to 20 hr after challenge with strains V1b or 116 and within 26 to 42 hr after challenge with strains 770 or Sterne. Ouchterlony plates prepared from the fluid in the thoracic and peritoneal cavities show that every sample contained antigen, as demonstrated by lines of precipitation against antiserum (Fig. 1). In some of the samples, two lines of precipitation were observed; in others, only one line was visible. The number of lines was not related to the strain used to challenge the host.

In a second experiment, 12 guinea pigs were injected intraperitoneally with 10^8 V1b spores in 1 ml. Individual guinea pigs were sacrificed at 2- to 3-hr intervals, and their serum was assayed

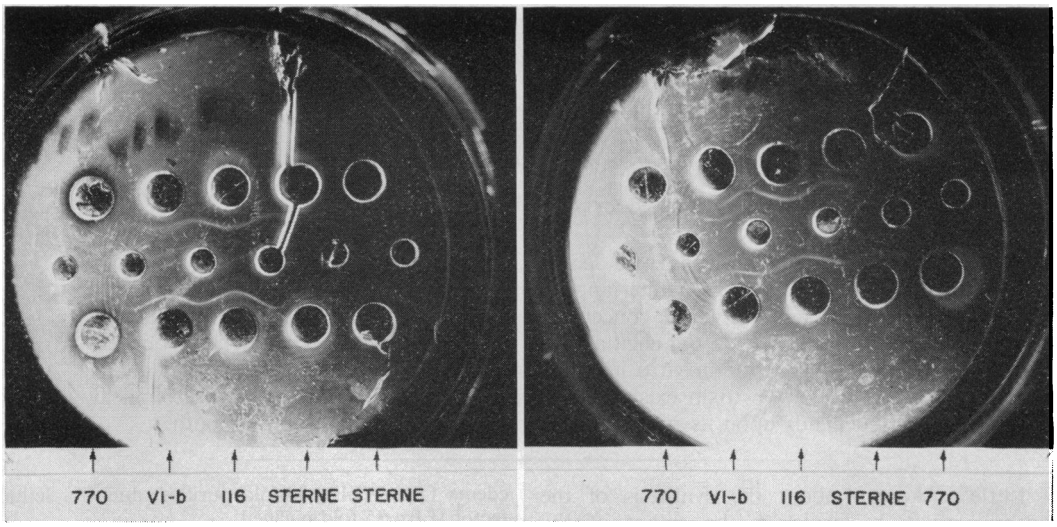

FIG. 1. *Demonstration of the presence of antigens in the thoracic fluid (left) and peritoneal fluid (right) of guinea pigs after challenge with different strains of anthrax.*

for the presence of antigen. Antigen was found in a single animal at 6 hr after challenge. Several negative samples were then found, but by 17 to 22 hr a single, strong line of precipitation was observed. Between 23 and 28 hr, two lines of precipitation were clearly visible, and at death (30 hr) a third line was also visible.

In a parallel experiment, five rhesus monkeys (2 to 2.5 kg) were challenged intramuscularly with 2×10^9 V1b spores. The results (Table 1) indicated that the longer the animal survived, the higher the bacillus count was and the more toxin antigens were present in the blood. Cellulose acetate electrophoresis of serum before challenge and at the time of death revealed an identical protein pattern. Two of the animals had serosanguinous exudate in the peritoneal and thoracic cavity which contained toxin antigens, as indicated by two lines of precipitation by Ouchterlony analysis at a dilution of 1:16 and by lethality to rats after intravenous injection of 1 ml of fluid (124 and 155 min).

In an experiment on the time course of toxin (antigen) production, the jugular vein of a monkey was cannulated (6), and the monkey was challenged by an intradermal injection of 3×10^6 V1b spores. Amounts of 5 ml of blood were withdrawn at 4-hr intervals and assayed for the presence of toxin. The results (Table 2) show that as soon as organisms could be demonstrated in the blood it was possible to demonstrate toxin.

When the serum of a single monkey, injected with 10^7 spores of the Sterne strain, was examined by Ouchterlony analysis, evidence was found for the presence of all three components of the Sterne strain toxin as well as some additional unidentified components.

Formation of specific antiserum. When guinea pigs were immunized with whole toxin as the antigen by the PA5 protocol, antiserum against LF, but not against PA, could be demonstrated. Injection with PA induced the formation of antiserum specific for PA. The titers of these sera were quite low (1:4) compared with the titer of horse serum, which is normally used (1:256).

When rabbits (New Zealand white, 1.5 to 2 kg) were injected with whole toxin or its components by the PA5 protocol, no antibody could be demonstrated in the initial bleedings at 28 days. However, if the protocol was repeated 1 month later, and then again 1 month after that, a moderately titered (1:16) antiserum was produced. In contrast to the results obtained in guinea pigs, whole toxin resulted in the production of antibodies against all three components, PA or EF resulted in the production of antibodies to PA, and LF resulted in production of antibodies to PA and LF components.

The development in rabbits of antibodies to the antigens of the V1b, 116, 770, and Sterne strains was followed, on days 0, 7, 14, 21, 52, and 84, after intraperitoneal injection of 10^5 or 10^9 spores of each of the strains. Establishment of anthrax and death was prevented by the intramuscular administration of 80,000 units of cystalline penicillin and 15 mg of chlortetracycline per kg at 12, 24, and 36 hr postinjection. At 48

TABLE 1. *Time course of organism and toxin content of the serum after spore challenge*

Time to death (hr)	Terminal viable-cell count (organisms/ml)	Rat lethal units/ml	Ouchterlony titer
41	7.7×10^3	0	1:1
44.5	2.1×10^3	0	1:1
45.5	4.4×10^6	48	1:4
48	1.2×10^6	16	1:2
51.5	2.5×10^8	307	1:16

TABLE 2. *Time course of toxin formation in a monkey after spore challenge*

Time (hr)	Viable organisms/ml	Presence of toxin[a]
0	0	0
4	0	0
8	1	0
12	0	0
16	6.6×10^3	+
20	2.3×10^6	+
22[b]	8.4×10^6	+

[a] Toxin was detected by the Ouchterlony analysis.

[b] Taken immediately after death of the animal.

and 72 hr postinjection, 10,000 units of procaine penicillin per kg was injected intramuscularly. At 3 months, the serum from rabbits injected with V1b contained antibodies against all three of the toxin components isolated from the Sterne toxin. The serum from the rabbits that had received 770, 116, and Sterne contained antiserum against PA and LF but not against EF. Continuation of immunization at 120, 150, and 180 days, with no antibiotic therapy, resulted in serum from the animals injected with V1b and Sterne containing antibodies against all three toxin components, but the serum from the rabbits injected with 770 contained antibodies only against EF. Rabbits injected with the 116 strain died of anthrax after the challenge at 120 days. At 360 days, with no antigen administered between 180 and 360 days, none of the animals had a demonstrable antibody titer. When challenged by intraperitoneal injection of 2×10^9 V1b spores, all immunized rabbits survived; however, two nonimmunized controls died at 73 and 88 hr.

Similar experiments were undertaken with monkeys, one monkey each being immunized by the (i) PA, (ii) LF, or (iii) PA plus LF antigens, by means of the PA5 protocol. At 28 days, the antibody titers against whole toxin were, for PA two lines of precipitation at a dilution of 1:1, for LF one line of precipitation at a dilution of 1:16, and for PA plus LF no lines of precipitation at a dilution of 1:1.

Eight monkeys were challenged with 10^8 V1b spores and cured with penicillin. At 28 days after challenge with 10^8 spores, none of the monkeys had died, showing that high immunity had developed. At 42 days, their sera titered 1:2 to 1:16 against PA and 1:1 to 1:8 against LF.

In another experiment, one monkey which 4.5 years earlier had been challenged with V1b and cured was challenged with 10^7 V1b spores. At 1 week, when no indication of anthrax was evident, this animal was challenged with 10^9 spores. At death, 93 hr after the 10^9 challenge, terminal serum showed a PA titer of 1:4 and an antibody titer of 1:16.

Difference in rate of killing between in vivo- and in vitro-produced toxin. We verified the observation of Haines et al. (5) that a minimum of 54 min was required to kill Fisher 344 rats, regardless of the amount of in vitro-produced toxin injected. However, on two occasions we were able to kill rats very rapidly with toxin contained in terminal serum of the monkey. The first monkey had been injected with 10^2 V1b spores and treated with 10^6 units of penicillin 72 and 86 hr later. The animal died 90 hr after challenge. When 1.0 ml of its blood was injected into a rat, the rat died in 10 min. Rats receiving 1.0 ml of normal monkey blood showed no ill effects. An analysis of the serum on Ouchterlony plates (Fig. 2) showed two lines of precipitation visible at a dilution of 1:16. The second plate for component identification showed two and possibly three lines of precipitation. Two of these show some similarity to protective antigen; none shows any similarity to either edema factor or lethal factor.

On the second occasion, the monkey had been challenged with V1b spores and died without further treatment at 40 hr. Within 30 min after death, 1.0 ml of the blood killed a rat in 33 min and 2.0 ml was lethal in 5 min. After storage at 4 C for 24 hr, 0.5 ml killed in 103 min, 1.0 in 44 min, and 2.0 ml in 13 min. Ouchterlony analysis showed one line of identity with protective antigen with a titer of 1:8. The presence or absence of lethal factor could not be determined clearly.

Evidence for dissociation of the toxin molecule. The terminal serum of six monkeys injected with 10^8 V1b spores was collected and assayed. The serum then was frozen and stored at -20 C for 3 days. Upon thawing, it was again assayed. The results (Table 3) show a marked loss in activity after freezing and thawing, as measured by rat lethality and guinea pig skin edema. The Ouchterlony titer did not decrease; in fact, the number of lines increased. These results indicate dissociation of a complex, with concomitant increase in

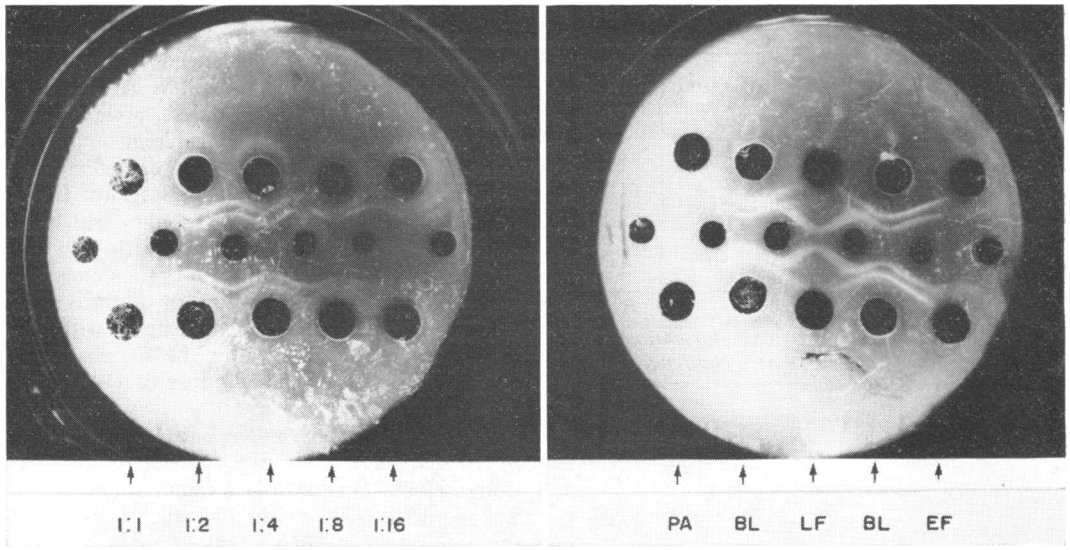

FIG. 2. *Serological examination of monkey blood which killed rats within 10 min. Titers are shown on the left and component identification on the right.*

TABLE 3. *Effect of freezing and thawing on in vivo-produced anthrax toxin*

Animal no.	Fresh serum				Frozen and thawed serum					
	Ouchterlony[a]		Guinea[b] pig	Rat[c] (min)	Ouchterlony[a]				Guinea[b] pig	Rat[c] (min)
	2 lines	1 line			4 lines	3 lines	2 lines	1 line		
1	Lost	Lost	1:80	75	—	1:2	1:16	1:16	NE[d]	1,110
2	—	>1:32	1:80	61	—	—	1:16	1:16	NE	75
3	—	1:16	1:20	50	—	—	—	1:32	NE	73
4	1:4	1:16	1:20	50	1:4	1:8	>1:32	—	NE	113
5	—	1:8	1:80	101	—	1:16	—	—	NE	Survived
6	—	>1:32	1:80	63	—	>1:32	—	—	NE	Not done

[a] Highest titer at which indicated number of lines is visible.
[b] Highest titer that still gives visible edema.
[c] Average time to death of two rats after intravenous injection.
[d] NE = no edema.

serological activity and decrease in biological activity, rather than destruction of a single molecule. Similar results were found with serum from six monkeys that died after challenge with strain 116 spores.

In another type of experiment, the terminal blood was stored at 4 C and assayed periodically. The time to death for rats decreased to a minimum at 6 to 12 hr after death of the host; then it increased. The maximal titer and number of lines by Ouchterlony assay paralleled the rat lethality. These results indicate that molecular changes occur during storage at 4 C.

DISCUSSION

Our observations with both guinea pigs and monkeys support those of Smith et al. (15) that toxin is found in the thoracic and peritoneal fluid at death. The presence of toxin in the lymph and serum of monkeys infected with anthrax has been reported (8). At least two distinct antigens were visible in the thoracic and peritoneal fluid and in the serum of both guinea pigs and monkeys dying of anthrax.

When rats were injected with the serum or thoracic or peritoneal fluid, they died in the same

manner as rats which had received in vitro-produced anthrax toxin. Although Sargeant et al. (12) did find some minor differences in the pattern of lines produced on Ouchterlony plates by toxin isolated from in vitro versus in vivo sources, they concluded that the major components appeared to be the same. Fish et al. (4) reported that they were only able to identify two lines of precipitation in toxin produced in vitro. Therefore, the toxin produced in vivo under these conditions appears to resemble that produced in vitro.

The results reported here also indicate that the same sort of antigens are produced by V1b, 116, and 770 that are produced by the Sterne strain.

A kinetic study of the formation of toxin indicates that it can first be observed about 13 to 15 hr prior to death in the guinea pig and 6 hr prior to death in the monkey. These results, and those in which the correlation between number of organisms and amount of toxin present in the blood was determined, agree with the observations of Smith et al. (14), Tempest and Smith (19), Lincoln et al. (9), and Mahlandt et al. (10).

The toxin in the serum of the guinea pig between 8 and 13 hr prior to death exhibited only one strong line of precipitation by the Ouchterlony technique. Between 2 and 7 hr prior to death, a second line of precipitation became visible, and still later a third line became visible. The appearance of the toxins in the blood stream indicates either that the formation of the different toxin components in vivo is different or that the toxin is synthesized as one multicomponent aggregate which is subsequently broken apart.

Although antisera can be formed by repeated injections of either the partially purified toxin components or spore suspensions, the specificity and extent of the response have not been satisfactory. Sargeant et al. (12) reported that none of the purified preparations they examined was serologically homologous. They especially noted that some preparations of edema factor contained nonimmunogenic but serologically active PA. Our results seem to indicate either that the antigens we used were not homologous or that common antigenic sites existed, because PA and EF led to the formation of antibody against PA; however, LF antigen led to the formation of antibody against both PA and LF.

Both the toxin components and the spore suspensions appear to be poor antigens. Repeated injections were needed before any titer was observed, and the antibody titer soon returned to zero after immunization ceased. The best titer was obtained with the more virulent organisms. It is probable that the animal had a low-grade infection which kept the concentration of antigen higher for a longer period of time. One of the more interesting observations from this work, though, is that often the animals have a fairly high degree of protection with no visible antibody titer.

Both our experiments and those of Belton and Henderson (2) have shown that monkeys are able to build an antibody titer rapidly after rechallenge. We have verified the observation of Ward et al. (21) that immunized animals may die with both toxin and antibody present in their blood.

Contrary to the observations of Watson et al. (22) and Ward et al. (21), we did not find any alteration in the pattern of serum electrophoresis at death.

One of the most interesting observations was that, in several instances, in vivo-produced toxin would kill a rat in an extremely short time. The Ouchterlony titer was not very high (in fact, we could not even detect lethal factor in the serum), yet the rat died. We are tempted to believe that this is the result of a particular configuration of the toxin molecule which is readily altered outside the body. The evidence in the literature that the toxin molecule is not of a stable configuration or composition is extensive (3, 4, 11, 13, 15, 16, 17, 18; Fish et al., J. Infect. Diseases, *in press*). In every case, these workers found that the molecule could dissociate or aggregate to yield a compound with slightly altered properties.

The fact that the Ouchterlony, guinea pig skin edema, and rat lethality titer of monkey serum from animals dying of anthrax changed so markedly upon storage at −20 C is further evidence for the lability of a special toxin configuration. The results from the experiments on the kinetics of toxin formation in the guinea pig during the disease indicate that either (i) the rate of formation or release into the blood stream of the various toxin components is different or (ii) the toxin complex is dissociated into several components in vivo. This dissociation affects and influences the properties of the toxin to a different extent, depending upon the parameter used to measure activity.

The observations reported here are that the toxin not only loses its biological activity after freezing and thawing but yields an increase in the number of lines of precipitation by Ouchterlony analysis with no decrease in the terminal titer. This can most easily be explained by dissociation of a complex molecule or aggregate, rather than by destruction of a single structure. It is important to remember that the results reported here were obtained with crude toxin and that the purified toxin or its components may behave differently.

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