Alterations in the Biological Activity of Protective Antigen of *Bacillus anthracis* Toxin.* (28656)

DOROTHY M. MOLNAR AND ROBERT A. ALTENBERN
(Introduced by R. D. Housewright)
United States Army Biological Laboratories, Fort Detrick, Frederick, Md.

The toxin of *Bacillus anthracis* contains at least 3 known components — protective antigen (PA), edema factor (EF), and lethal factor (LF) — that can be separated by glass filtration alone(1) or in combination with column chromatography on DEAE cellulose (2). None of the components is toxic when tested alone, but combination of PA with either EF or LF produces the edema reaction or lethality, respectively.

Strange and Thorne(3) purified the protective antigen and described its chemical and physical properties, which indicate a simple protein molecule. Several preparations of partially purified PA made in this laboratory according to their directions failed to kill rats when combined with LF; however, these preparations were serologically indistinguishable from samples of active PA. Recently this loss in lethal activity has been observed *in vivo* along with a transient conversion of PA to an inhibitor of toxin lethality.

Materials and methods. The Sterne strain of *B. anthracis* was grown under the conditions described previously for toxin production(4) except that $NaHCO_3$ concentration was increased to 1.2% and was added after 4 hours' incubation, which frequently increased antigen yields. The toxin was separated into its components by passing culture supernatant fluid through large fritted-glass filters(4,1).

The components were assayed by the agar diffusion technique of Thorne and Belton(5) using an antiserum from a horse injected with spores of the Sterne strain of *B. anthracis*. Toxic activity was determined by the edema reaction in guinea pigs(4) or by the rat lethality test(1). Beall *et al*(1), reported that the time to death in Fischer 344 rats varied in a uniform manner with the amount of lethal material injected. In our experience this relationship exists only over the range of lethal material killing in approximately 80-240 minutes.

Preparations of PA, partially purified by the method of Strange and Thorne(3), formed one strong precipitation line and one weaker line in the agar diffusion assay. The stronger line merged with the line produced by preparations of standard PA obtained from Thorne and was visible at a dilution of 1-5,000 to 1-10,000 (5,000-10,000 agar diffusion units).

Preparations of EF gave 2 lines in the agar diffusion assay, the weaker one of which merged with the LF line. The stronger line had a titer of 128, and both lines crossed the line formed by PA. Preparations of LF formed only 1 line in agar diffusion plates and had a titer of 32-64. No edema activity was demonstrable on addition of PA to these preparations of LF. In the following presentation, "whole toxin" and a "toxic mixture of PA + LF" are used interchangeably.

Results and discussion. Correlation between clearance of toxin components from the bloodstream of rats and loss in lethality: When injected singly into rats, a large dose of PA or LF is nonlethal but when injected in combination at appropriate much smaller concentrations, rats are killed and the time-to-death response of Fischer 344 rats is proportional to concentration of material injected. Varying the order of timed injections of PA and LF resulted in significant changes in the time-to-death response.

Injection of PA first, followed by injections of either LF or PA + LF (toxic mixture) at the time intervals indicated in Table I, resulted in both a gradual loss in ability of PA to form a lethal mixture with LF and

* The technical assistance of Jack Schweitzer, Samuel Hamilton and Harold Barnard is gratefully appreciated. LCDR Andrew J. Gaspar's kindness in bringing to our attention the effect of the order of timed injections of toxin components is also gratefully acknowledged.

TABLE I. Correlation Between Clearance of Toxin Components from Bloodstream and Loss in Lethality.

Injection		Assay time (min after initial injection)						
Initial	At time of assay	0-30	60	90	120	180	240	20-24 hr
				Agar diffusion assay				
PA	none	+	+	±	—	—	—	—
		+	+	+	±	—	—	—
LF	"	+	+	+	+	+	+	—
		+	+	+	+	+	+	—
PA + LF	"	+	+	(Died, 72 min)				
		+	+	+	(Died, 95 min)			
				Lethality assay*				
PA	LF	69	107		S	S	S	S
		71	101		S	S	S	S
"	(PA + LF)	65	78		87	148	S	66
		68			87	151	S	68
LF	PA	75	75			70	77	S
		75	84			77	81	S
"	(PA + LF)		59				63	71
			63				73	71
None	(PA + LF)	58			70		66	75
		72			69		66	76

* PA and LF were mixed and injected into Fischer rats at 0, 2, 4 and 24 hr intervals and time to death in min of each rat noted. Simultaneously, the same PA and LF preparations were injected separately followed by injections of either the other component or a toxic mixture of PA + LF at time intervals indicated. S = survived.

an even slower appearance (about 1 hour later) of inhibition toward the toxic mixture. This inhibition is manifested as a considerable extension of time to death following injection of the toxic mixture. After 20-24 hours, the inhibition was no longer observed as noted by a return of the time-to-death response to that of the control. PA was no longer detectable in the bloodstream by the agar diffusion assay at about the time it no longer formed a lethal mixture with subsequently injected LF.

On the other hand, when LF was injected first followed by injections of PA or PA + LF, over a 4-hour period there was no significant loss in ability of LF to combine with PA to kill rats, no inhibition of toxin lethality, and no disappearance of LF from the bloodstream. After 20-24 hours, LF was no longer demonstrable in the bloodstream, did not form a lethal mixture with PA, and still did not inhibit toxin lethality. In rats injected with lethal mixtures of PA + LF, the LF precipitin line was demonstrable in the blood up to the time the rats died whereas the PA precipitin line disappeared after about 1 hour.

In vitro *alterations in PA activity:* As previously mentioned, several crude preparations of PA obtained as the 28-50% $(NH_4)_2SO_4$ fraction did not form toxic mixtures when recombined with EF or LF. The activities of 4 crude preparations are presented in Table II. All 4 preparations formed similar lines on agar diffusion against spore antiserum but only PA #4 and #1 evoked a toxic response upon combination with EF and LF. They were also noninhibitory to toxin lethality. In contrast, PA #3 failed to form active toxin when combined with either EF or LF and it protected the rat from the known killing effect of a toxic mixture. PA #5 also failed to form a lethal mixture with LF but had some edema activity in combination with EF. It was not tested for ability to inhibit toxin lethality. Attempts to alter PA #4 and #1 *in vitro* so that they would exhibit the activities described for PA #3 have so far failed. Complete loss of lethal activity resulted from incubation for one hour

TABLE II. Biological Properties of Several Preparations of Protective Antigen.

PA preparation	Activity: Agar diffusion (units/ml)	Activity: Edema* +(EF) (units/ml)	Activity: Lethality† + (LF)	Activity: Lethality† + (PA + LF)
3	1280	<16	S S	S S
5	640	80	S S	
4	1280	2048	62 65	68 81
1	2048	2048	71 69	65 68
None				69 68

* To measure edema activity, EF was added to serial dilutions of the PA preparation.

† Eighty agar diffusion units of the PA preparation indicated were mixed with either LF alone or a toxic mixture of PA + LF. Numbers indicate time to death in min after injection of these mixtures. An agar diffusion unit of PA is the smallest amount of PA which will form a visible line of precipitate against a high titer antiserum in an Ouchterlony plate.

S = survived.

at 37°C with trypsin, with rat blood or with 0.05 M phosphate, pH 7.0. Evidently milder treatment is required to convert active PA to the inhibitory state.

Ability of PA to inhibit previously injected toxin: Since PA inhibits lethality of subsequently injected toxic mixtures, it was of interest to determine if PA could inhibit previously injected toxin. The results in Table III show that, depending on the concentration of toxin, administration of large doses of PA up to 5 minutes after toxin injection significantly delayed or prevented death. The significance of the delay in death observed at 15-30 minutes is questionable with only one test animal. However, administration of PA at 30 and 60 minutes after toxin injection had no effect on toxin lethality as judged by the time to death following toxin injection.

TABLE III. Ability of PA to Inhibit Previously Injected Toxin.

PA administered: Units	PA administered: Time after toxin (min)	Time (min) to death after injection of*: 16 units toxin	Time (min) to death after injection of*: 32 units toxin
None	—	100	69
160	60		68
160	30	100	84
160	15		95
160	5	survived	210
160	0†		174

* Each value is the result of a single injection into 1 Fischer 344 rat. Reproducibility of the rat assay using 2 animals per dose can be seen in Tables I and II.

† Added to toxin *in vitro* immediately before injection.

Effect of ratio of PA/LF on lethality: PA and LF were mixed in various ratios based on their precipitin titers and injected intravenously into Fischer rats at the levels indicated in Table IV. A ratio of PA/LF of 5/4 killed in the shortest time at all levels of LF tested. Increasing the ratio to 5/1-10/1 delayed or prevented death, depending on the level of LF. At a concentration of LF of 4 units/ml none of the rats died regardless of concentration of PA. Similarly, when the concentration of PA injected was only 5 units, the rats survived or death was markedly delayed. When serial 2-fold dilutions of a mixture of PA + LF were injected, the response was comparable to that observed with the corresponding mixtures. Again, levels of 5 and 4 units of PA and LF, respectively, were too low to kill.

TABLE IV. Effect of Ratio of PA/LF on Rat Lethality.

Ratio PA/LF (agar units/ml)	Time to death (min)*: Concentration of LF (units/ml) 32	16	8	4
10/1	S	S	>360	S
5/1		134	>180	S
5/2	81	89	161	
5/4	73	85	104	S
5/8		114	>240	
5/16		>180		
5/32	S	S		
Serial 2-fold dilutions of a mixture at ratio of 5/4	72	85	122	S

* Each of 2 rats was injected with 1 ml of a given mixture of PA + LF.

Values reported represent the mean of from 1-5 mixtures of each ratio using different preparations of LF with PA #4 (Table II).

These results permit several general conclusions regarding the *in vivo* fate of injected toxin or its components. Apparently both toxic mixtures (PA + LF) and PA alone

rapidly disappear from the bloodstream of rats and may be fixed at specific tissue sites. If so, absorbed PA not only cannot react with subsequently injected LF to produce the lethal effect but also undergoes some change that inhibits the lethality of toxic mixtures. The exact nature of this change or changes is unknown but probably involves subtle physicochemical changes rather than extensive splitting of the molecule.

The observation that lethality of toxic mixtures can be inhibited by large doses of PA suggest that both are fixed at the same tissue site and that toxic mixtures are fixed through their PA component. The toxin continuously released during anthrax infection might well be antagonized by repeated injections of PA to occupy all the available tissue sites. Since it is generally recognized from the data of Smith *et al*(6) that toxemia is the primary cause of death in anthrax infected animals, these observations may be useful in exploring a rational therapy in anthrax.

Summary. Evidence is presented to show that protective antigen undergoes subtle alterations that affect its biological activity. For one hour following intravenous injection of protective antigen (PA) into the rat, subsequent inoculation of lethal factor leads to death of the rat as expected. Two hours after injection of protective antigen, injection of lethal factor does not kill the rat although challenge with whole toxin produces the classical lethal response. At this time, PA is no longer demonstrable in the bloodstream by immunological methods. In contrast, 4 hours following an injection of protective antigen, rats not only do not die when injected with lethal factor but also are protected from the lethal effect of whole toxin. Some preparations of protective antigen can detoxify *in vitro* whole toxin of known potency. These facts are discussed in relation to the clearance from the bloodstream of injected whole toxin or its components and a possible role for PA in anthrax therapy is suggested.

1. Beall, F. A., Taylor, M. J., Thorne, C. B., *J. Bacteriol.*, 1962, v83, 1274.

2. Stanley, J. L., Smith, H., *J. Gen. Microbiol.*, 1961, v26, 49.

3. Strange, R. E., Thorne, C. B., *J. Bacteriol.*, 1958, v76, 192.

4. Thorne, C. B., Molnar, D. M., Strange, R. E., *ibid.*, 1960, v79, 450.

5. Thorne, C. B., Belton, F. C., *J. Gen. Microbiol.*, 1957, v17, 505.

6. Smith, H., Keppie, J., Stanley, J. L., *Brit. J. Exp. Path.*, 1955, v36, 460.

Received May 21, 1963. P.S.E.B.M., 1963, v114.

Plasma Protein Thyroid Hormone Complexes: Separation by Continuous Flow Paper Electrophoresis. (28657)

EFRAIN TORO-GOYCO AND MARTA CANCIO (Introduced by C. F. Asenjo)

Radioisotope and General Medical Research Laboratories, Veterans Administration Hospital and Department of Biochemistry and Nutrition, University of Puerto Rico School of Medicine, San Juan

The transport of the thyroid hormones thyroxine and triiodothyronine by plasma proteins has received much attention in the last few years(1). It has been shown that their transport is accomplished by several plasma proteins, but there seems to be no general agreement as to which protein plays the main physiological role. The differences in results obtained by the use of various experimental methods are manifest(2). The considerable amount of work performed using single- and 2-dimensional gel and paper electrophoretic techniques, although very useful in identification of thyroid hormone-plasma protein complexes does not allow a determination of the chemical composition of these complexes (3,4). We are reporting our attempt to isolate these complexes by means of continuous flow electrophoretic fractionation. This method enables us to separate the protein-bound thy-