A Simple Method for the Quantitation of Submicrogram Amounts of Bacterial Endotoxin[1,2] (34565)

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(Introduced by G. Edsall)

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A variety of procedures have been described which are capable of increasing the susceptibility of experimental animals to the lethal effect of bacterial endotoxin. These include adrenalectomy (1), injection of pertussis vaccine (2), BCG inoculation (3), treatment with insulin (4), and the use of various agents, such as Thorotrast (5), zymosan (3), and lead acetate (6). Several of these procedures, as well as others (7–9), have been utilized in an attempt to detect and quantitate small amounts of bacterial endotoxin. To date, however, no standardized procedure is available for such quantitation.

The literature contains several reports of synergistic toxicity between bacterial endotoxins and certain antimetabolic drugs. Braude reported that mytomycin C is capable of enhancing the sensitivity of mice to endotoxin (10). Karp and Bradley found that sublethal doses of the antitumor antibiotics, sparsomycin and pactamycin, act synergistically with endotoxin to produce ocular hemorrhage and death (11). Cooney *et al.* (12) found a potentiation of toxicity between actinomycin D and pertussis vaccine. Berry and co-workers, in a series of reports (13, 14), have shown that actinomycin D can increase the lethality of bacterial endotoxin. Their investigations were conducted primarily to elucidate the mechanisms of endotoxin action.

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In most of these studies, relatively large amounts of endotoxin have been used and the phenomenon was not investigated with a view towards developing a practical test for detecting and quantitating endotoxin. During our investigation, however, we found that when mice were injected with actinomycin D, they could be rendered over 100,000 times more susceptible to endotoxin than normal mice. The purpose of this paper is to describe a method for the detection and quantitation of submicrogram amounts of bacterial endotoxin which offers advantages over other procedures because of its simplicity, reproducibility, and high sensitivity. Some possible applications of this method are discussed.

Materials. Female CFW mice, weighing 15 to 25 g (obtained from Carworth Farms, New City, N. Y.) were used in these studies. Mice of the same approximate weight were used in individual experiments. The bacterial endotoxins were commerically obtained Westphal extracts of *Salmonella typhosa* 0901 and *Escherichia coli* 026:B6 (Difco Laboratories, Detroit, Michigan). Dilutions were prepared in isotonic saline. Actinomycin D (Cosmogen), lot nos. 1039K and L588, were purchased from Merck, Sharp and Dohme, West Point, Pa. Each ampoule, containing 0.5 mg of the antimetabolite, was dissolved in isotonic saline immediately prior to use. Tetanus and diphtheria toxins were prepared in these Laboratories.

Results. In a preliminary experiment we injected groups of mice subcutaneously with either saline or 25 μg of actinomycin D, lot no. 1039K, and intraperitoneally with dilutions of *S. typhosa* 0901 lipopolysaccharide. Deaths were tabulated in 2 days and the

TABLE I. Effect of Actinomycin-D (AM-D) on the Lethality in CFW Mice of *S. typhosa* Endotoxin (ET).

AM-D (μg) Lot 1039K	Route	ET (μg)	Route	D/T[a]	LD_{50} (μg)
—	—	125	ip	0/8	
—	—	250	ip	2/8	
—	—	500	ip	4/8	425.
—	—	1000	ip	8/8	
25	sc	0.0001	ip	1/8	
25	sc	0.001	ip	1/8	0.0034
25	sc	0.01	ip	5/8	
25	sc	0.1	ip	8/8	
25	sc	—	—	0/8	
25 (mixed with)		0.0001	ip	0/5	
25 (mixed with)		0.001	ip	1/5	
25 (mixed with)		0.01	ip	5/5	0.0024
25 (mixed with)		0.1	ip	5/5	
25 (mixed with)		1.0	ip	5/5	
25		—	ip	0/5	

[a] Dead to total.

LD_{50} was calculated by the Reed and Muench method. As shown in the first two sections of Table I, the LD_{50} of the typhoid endotoxin in control mice was approximately 425 μg. In mice treated with actinomycin D, however, the LD_{50} was reduced to approximately 0.0034 μg. Actinomycin D alone was nonlethal. The indications were, therefore, that treatment with the antimetabolite could enhance sensitivity to endotoxin approximately 125,000-fold. This test was repeated several times with similar results.

In order to facilitate the inoculation procedure, we attempted to determine the effect of mixing actinomycin D with the endotoxin preparations immediately prior to testing. Groups of five mice were injected intraperitoneally with mixtures of from 0.0001 to 1 μg of typhoid endotoxin and a constant dose of 25 μg of actinomycin D. Deaths were tabulated at 2 days. Results shown in the last section of Table I indicate that the LD_{50} of the endotoxin–actinomycin D mixture was approximately 0.0024 μg. Injection of actinomycin D alone resulted in no deaths. The experiment was repeated a second time with similar results. Because of the ease of inoculation and high sensitivity obtained, in all subsequent experiments the drug was mixed with test preparations immediately before testing and a single intraperitoneal injection was administered.

In order to gain some knowledge of the possible specificity of this synergistic action, we considered it of interest to determine the effect of the drug on the sensitivity of mice to other bacterial toxins. A constant amount of 12.5 μg of actinomycin D was added to dilutions of tetanus toxin ranging from 0.125 to 1.0 mouse MLD. Controls received similar doses of the toxin and deaths were tabulated in 1 week. Results shown in Table II indicate that actinomycin D did not augment the lethality of tetanus toxin in mice. A similar experiment was performed using diphtheria toxin as the challenge agent. Again there was no significant difference in mortality between actinomycin-treated and control mice receiving bacterial exotoxin (Table III).

It is well accepted that bacterial endotoxin is rapidly eliminated from an animal's circulation. Much of this work has been done in rabbits using sublethal doses of radioactively labeled endotoxin (15, 16). In order to determine if the present procedure could detect circulating endotoxin, we injected 1 mg of *S. typhosa* endotoxin into the marginal ear vein of a rabbit. Blood was drawn immediately

TABLE II. Effect of Actinomycin-D (AM-D) on Tetanus Toxin Lethality in CFW Mice.[a]

AM-D (μg) Lot 1039K	Tetanus toxin (mouse MLD)	D/T[b]
—	0.125	0/6
—	0.25	0/6
—	0.50	4/6
—	1.0	6/6
12.5	0.125	0/6
12.5	0.25	0/6
12.5	0.5	1/6
12.5	1.0	5/6
12.5	—	0/6

[a] AM-D was mixed with toxin prior to ip injection.

[b] Dead to total.

before the injection and again 60 min after injection. The blood was then diluted, as shown in Table IV, and 12.5 or 25 μg of actinomycin D were added to the samples which were then injected ip into mice. Actinomycin-treated blood samples obtained before the endotoxin injections were uniformly nonlethal for the mice. Samples obtained 1 hr after inoculation with typhoid endotoxin, however, were capable of inducing a high mortality, with dose–response regression.

E. coli *endotoxin.* The assay method was tried with a different source of endotoxin as follows: Four groups of 10 mice were injected ip with mixtures of from 10^{-5} to 10^{-2} μg of *E. coli* endotoxin and a constant dose of 12.5 μg of actinomycin D. Four additional groups of 10 mice received *E. coli* endotoxin only (125-1000 μg), and a fifth group was injected with 12.5 μg actinomycin D. Deaths were tabulated at 2 days. Results shown in Table V indicated that the LD_{50} of the *E. coli* endotoxin was approximately 224 μg. When actinomycin D was mixed with dilutions of endotoxin immediately prior to challenge, however, the LD_{50} was decreased to approximately 0.001 μg. Actinomycin D injected alone resulted in no deaths within the 48-hr observation period. This experiment, which was repeated with similar results, indicated that the antimetabolite could increase the lethality of *E. coli* endotoxin about 225,000-fold. It should be noted that the batch of actinomycin D used in this experiment (lot no. L588) was different from that used in the previous experiments described (lot no. 1039K). Subsequent tests have confirmed the fact that L588 possessed a greater capacity to increase the lethality of endotoxins than 1039K. A more detailed examination of the characteristics of particular batches of actinomycin D, as well as those obtained from different sources of supply, is currently under way.

TABLE III. Effect of Actinomycin-D (AM-D) on Diphtheria Toxin Lethality in CFW Mice.[a]

AM-D (μg) Lot 1039K	Diphtheria toxin (mouse MLD)	D/T[b]
—	0.01	0/8
—	0.1	0/8
—	1.0	8/8
—	10.0	8/8
12.5	0.01	0/8
12.5	0.1	1/8
12.5	1.0	8/8
12.5	10.0	8/8
12.5	—	0/8

[a] AM-D was mixed with toxin prior to ip injection.

[b] Dead to total.

TABLE IV. Test for Endotoxin (ET) in Rabbit Blood before, and 60 min after, Injection of a Rabbit with 1 mg of *S. typhosa* ET, Using CFW Mice Injected ip with Mixtures of Blood and Actinomycin-D (AM-D).

AM-D (μg) Lot 1039K	Blood (ml)	D/T[b]
25	0.0005	0/5
25	0.005	1/5
25	0.05	5/5
12.5	0.001	3/8
12.5	0.01	4/8
12.5	0.1	8/8
25	0.05[a]	0/5
12.5	0.1[a]	0/8

[a] These control samples were taken from the rabbit prior to ET injection; other test samples, 60 min after ET injection.

[b] Dead to total.

Discussion. Bacterial endotoxins exert a wide variety of effects on the physiological

TABLE V. Effect of Actinomycin-D (AM-D) on Lethality in CFW Mice of *E. coli* Endotoxin (ET).[a]

AM-D (μg) Lot L588	ET (μg)	D/T[b]	LD_{50} (μg)
—	125	1/10	
—	250	6/10	224
—	500	9/10	
—	1000	10/10	
12.5	0.00001	2/10	
12.5	0.0001	3/10	
12.5	0.001	5/10	0.001
12.5	0.01	5/10	
12.5	—	0/10	

[a] AM-D was mixed with endotoxin prior to ip injection.

[b] Dead to total.

responses of experimental animals, and are believed to play an important role in several human pathological conditions, including fatal shock states. Although many tests have been devised to quantitate endotoxin, none to date has been entirely satisfactory. Numerous investigators have alluded to the need for a simple and sensitive test for the accurate determination of very small quantities of endotoxin. The procedure, which we have described, based on the capacity of the antimetabolite, actinomycin D, to potentiate markedly the lethality of bacterial endotoxin, may answer this need.

The test itself is easy to perform. A constant dose of actinomycin D is mixed with dilutions of the endotoxin-containing preparation, and the mixtures are injected into the peritoneal cavities of groups of mice. In 48 hr the number of deaths in each mouse group is tabulated and the LD_{50} calculated. This LD_{50} value reflects the endotoxin content of the starting preparation.

Variables that affect the assay. The toxicity for mice of actinomycin D depends on several factors including the strain, sex, age, and weight of the test mice being used. There also appear to be variations between batches of the antimetabolite supplied by the same manufacturer. For this reason, the largest dose of a specific lot of actinomycin D, which can be tolerated by the particular mice being used for testing, should be determined prior to performing the actual endotoxin assay. In the present experiments a smaller dose of the drug, 12.5 μg, was administered whenever young mice were used in order to avoid toxic deaths from the antimetabolite itself.

A further variable is the period of observation of death rates of mice following challenge with test mixtures. When these mixtures containing graded doses of endotoxin straddle the LD_{50}, most of the mice die on the day of injection, some the day after, and some deaths are often delayed beyond 48 hr. We selected the 48-hr cutoff period because we obtained reproducible results using it.

The strain of mouse may be an important variable. In earlier reports of lethal synergism between antimetabolites and endotoxin (10–14), effects have been obtained using a variety of different antimetabolites, bacterial endotoxins, and mouse strains. In none of these studies, however, has the degree of potentiation of endotoxin lethality approached that presently reported using actinomycin D in mice of the CFW strain. We are currently investigating the response of other mouse strains to these mixtures.

Finally, the antimetabolite was found incapable of potentiating the lethality of other bacterial toxins such as tetanus and diphtheria exotoxins (Tables II and III). This finding, of course, does not prove that the potentiating effect is specific with respect to bacterial endotoxin. However, it should be noted that the symptomatology and mode of death of endotoxin-treated mice is pathognomonic of endotoxin shock. The ocular hemorrhaging, weight loss, and other pathological symptoms of endotoxin poisoning have been referred to elsewhere (11, 17). These symptoms were present in mice treated with our actinomycin–endotoxin mixtures and hence identified the mouse deaths as endotoxic in nature. Mice receiving antimetabolite-treated blood from an endotoxin-injected rabbit (Table IV) also displayed these characteristic symptoms.

Possible uses. We have successfully used this procedure with endotoxins derived from

Neisseria meningiditis, Pseudomonas aeruginosa, and *Shigella dysenteriae,* in addition to the two sources described here in detail. We have also used it to monitor the removal of endotoxin during the fractionation of pertussis vaccine (18). The method thus appears to have a general applicability for the lipopolysaccharide endotoxins of gram-negative bacteria. We are currently investigating the feasibility of using this procedure as an alternative to the more expensive and relatively imprecise rabbit pyrogenicity test on preparations designed for parenteral use in man. A further application is suggested by our successful assay of circulating endotoxin in rabbit blood, namely, to detect endotoxin in the blood of patients suffering from gram-negative septicemia and endotoxin shock. A collaborative study on this is now in process.

Summary. The antimetabolite, actinomycin D, is capable of enhancing the lethality of bacterial endotoxin in CFW mice over 100,000-fold. This procedure appears to offer a simple, reproducible, and highly sensitive test for the detection and quantitation of submicrogram amounts of bacterial endotoxin. The lethality in CFW mice of tetanus and diphtheria exotoxins was not similarly augmented by the antimetabolite. The procedure was capable of detecting small quantities of circulating endotoxin in rabbit blood 60 min after injection of 1 mg of *S. typhosa* endotoxin. Some characteristics and possible applications of this test are discussed.

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