Quantitative Biological Assay of Bacterial Endotoxins[1] (34899)

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That antimetabolic drugs can enhance the effects of bacterial endotoxins on animals was reported by Braude (1) who found that mitomycin C reduced the LD_{50} of endotoxin fivefold. Karp and Bradley (2) stated that pactamycin or sparsomycin given together with endotoxin produced higher rates of ocular hemorrhage and death in mice than either of the drugs or endotoxin alone. Berry (3) and Berry and Smythe (4) demonstrated that actinomycin D (AM-D) significantly potentiated endotoxin lethality but were primarily concerned with the metabolic effects of endotoxin. Recently, Pieroni *et al.* (5, 6) utilized the synergistic toxicity of AM-D to quantitate the effects of submicrogram amounts of typhoid and coli lipopolysaccharides (LPS). They found that when small doses of AM-D were administered with either typhoid or coli LPS that their lethal effects on mice were increased 100–200,000 times. While the same system (7) permitted them to detect minute amounts of endotoxin in pertussis vaccines, it had no detectable effect on either tetanus or diphtheria toxins (6).

We have repeated and extended these observations of Pieroni[3] on the combined action of *Salmonella typhosa* LPS and AM-D in mice. After confirming the synergistic lethality of the two, the method was used to quantitate the biological activity of meningococcal LPS.

Materials and Methods. The AM-D used was Merck, Sharp and Dohme Cosmegen from various lots; each vial contains 0.5 mg of AM-D and 20 mg of mannitol. LPS extracted by both the Boivin (B) and Westphal (W) methods from *S. typhosa* 0901 were purchased from Difco Laboratories. Purified meningococcal LPS (8) were prepared in our laboratory by Dr. E. Hackenthal. Their bacteria-free, supernatant fractions were used in some experiments. Cell bodies (dried sediments from these cultures) also were studied. Group A meningococcal polysaccharide preparations were kindly supplied by both Drs. E. C. Gotschlich[4] and M. Artenstein.[5] Materials to be injected were diluted with sterile distilled water to appropriate concentrations. All injections were made intraperitoneally in 1-ml volumes with disposable syringes. Female CFW mice, weighing approximately 20 g, were used in all experiments. Dilutions were tested in groups of five or six mice depending upon the amount of material available. Deaths were recorded daily and the LD_{50}'s were calculated by the Spearman-Karber (9) method at 2 and 7 days.

Results. In two preliminary trials, AM-D alone was found to have an LD_{50} of 28.1 μg after 7 days. In another experiment, 50 mice

[1] Conducted under the sponsorship of the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board and supported by the United States Army Research and Development Command, Department of the Army, under a Research Contract (DA-49-193-MD).

[2] During part of this study J.N.D. was a trainee under Grant 1 T1-AI239, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. During the remainder of his time, he was supported by the Army Research and Development Command research contract.

[3] We are indebted to this worker for prepublication copies of his reports.

[4] Lot III F made by E.C.G. at The Rockefeller University.

[5] Batch A-5 produced by Squibb Institute for Medical Research and provided by the Department of Bacterial Diseases, Walter Reed Army Institute of Research.

received 12.5 μg and 50 others, 25 μg of AM-D. The 12.5 μg dose killed 2 mice (4%) within 48 hr and 4 mice (8%) within 7 days; following the larger dose, 8 (16%) died by the second day and 42 (84%) in 7 days. The 12.5 μg dose was selected for all subsequent experiments. In 18 experiments, 87 control mice received only this dose; none succumbed within 2 days but 8 (9.2%) died within 3 to 7 days. (A single experiment in which all five control animals died was discarded. Deaths among control groups fitted almost perfectly a binomial distribution; the deaths of three or more control mice out of five was to be expected fewer than six times in 1000.)

In each of two experiments with *S. typhosa* (B) LPS, eight groups of six mice were injected with various doses of the LPS alone while five groups of six mice received, in addition, 12.5 μg of AM-D (Table I, 1 and 2). LD_{50}'s were calculated at 2 and 7 days. In both experiments all deaths among mice who received endotoxin alone occurred within 48 hr. Animals receiving AM-D and endotoxin succumbed throughout the 7 days. Consequently, their lethality ratios were greater at 7 than at 2 days. The 7-day LD_{50}'s of LPS alone were reasonably consistent in the two experiments but varied almost sixfold when AM-D was added. Thus, in one experiment the simultaneous administration of AM-D led to a 4000-fold increase in the 7-day lethality of the endotoxin, while in the other the difference was only 552-fold.

When *S. typhosa* (W) LPS was used, LPS alone again tended to exert its effect during the first 2 days while deaths from endotoxin combined with AM-D occurred more evenly over 1 week's time (Table I, 3). In comparison with the previous experiments, the Westphal preparation appears to be somewhat more active than the Boivin, with and without AM-D. When the two LPS were titrated simultaneously (Table I, 4), they were equally potent alone, but in combination with AM-D, the Westphal preparation again was seemingly more lethal. The smaller than fourfold difference between the two in the 7-day LD_{50}'s with AM-D was well within this biological system's limits of error.

The effects of a larger AM-D dose were studied in a single experiment in which *S. typhosa* (W) LPS was administered, alone and with 25 μg of AM-D (Table I, 5). AM-D produced decreases in the LD_{50} of 100,000 and 300,000-fold in 24 and 48 hr, respectively, while none of the five control animals succumbed during the first 2 days. LD_{50}'s were not calculated at a later time since the first control animal had died by day 3 and after day 4 no animals receiving AM-D survived the lowest dose of LPS (0.0001 μg).

Minute amounts of four purified meningo-

TABLE I. Effect of AM-D on the LD_{50}'s of *S. typhosa* LPS in Mice.

Exp.	LPS	Day	LD_{50} (μg); AM-D (μg)			Fold increase
			0	12.5	25	
1	Boivin	2	743	1.00	—	743
		7	743	0.18	—	4180
2	Boivin	2	552	3.16	—	175
		7	552	1.00	—	552
3	Westphal	2	240	0.126	—	1900
		7	162	0.008	—	20,400
4	Boivin	2	442	0.501	—	881
		7	361	0.079	—	4540
	Westphal	2	470	0.501	—	938
		7	384	0.020	—	19,300
5	Westphal	1	530	—	0.0050	110,000
		2	377	—	0.0013	300,000

TABLE II. Seven-Day LD_{50}'s of Purified Meningococcal LPS Administered with 12.5 μg of AM-D /Mouse.

LPS	Exp. 1		Exp. 2	
	LD_{50} (μg)	Fold increase[a]	LD_{50} (μg)	Fold increase[a]
A_4	0.0501	> 200	0.0079	>1266
B_7	0.5012	> 20	0.0050	>2000
B_8	0.0079	>1266	0.0050	>2000
C_4	0.0316	> 317	0.0316	> 317

[a] None was lethal when 10 μg were administered without AM-D. The fold increases represent the minimum resulting from the addition of AM-D.

coccal LPS preparations (8) were available and were injected with AM-D. One was of serogroup A, two were of B, and one, a C. Their 7-day LD_{50}'s are presented in Table II. The first experiment with B_7 was a poor one, but it is included for completeness. Because of the limited quantities available, these materials could not be titrated without AM-D but none of five mice succumbed when 10 μg of each preparation were injected alone. Since the LD_{50}'s of these LPS exceeds 10 μg, the true increase in lethality due to the additon of AM-D is greater than the minimal values shown in Table II. With the possible exception of the one trial with B_7 LPS, the potentiating effects of AM-D were large and consistent with those observed with typhoid LPS.

The supernatant fluids of four large volume broth cultures grown to provide material for LPS purification were titrated by the AM-D system. Supernatant fluids from serogroups A, C, X, and Y were studied (Table III). Because the concentrations by weight of LPS contained in these fluids were not known, the results are stated in terms of their dilutions. These cultures were treated with formalin, so each experiment included mice which received uninoculated broth containing the same amount of formalin. No control animal died. Unfortunately, two of the five mice given undiluted Group A supernatant fluid without AM-D succumbed. This was not true for Group C nor for fivefold concentrates (by drying) by X and Y supernatant fractions. Thus, AM-D potentiated the lethal effects of these three at least 800 to 2400 times.

Meningococcal Group Y cells prepared from a lyophilized culture, failed to kill mice injected with 0.005 to 10 mg doses. When administered with 12.5 μg of AM-D/mouse, 0.5 mg killed four of five, and 0.005 mg, three of five mice. Thus, if 5 μg is assumed to approximate the LD_{50} of meningococcal cells injected with AM-D and if Group Y LPS has effects similar to those of Groups A, B, and C (Table II), then these purified LPS preparations are 158 to 1000 times more potent, by weight, than are the bacterial cells.

Purified meningococcal Group A polysaccharide (10) received from Drs. Gotschlich and Artenstein were titrated with AM-D. The two polysaccharides had been made by the same method but Dr. Artenstein's was negative in rabbit pyrogenicity tests. The other, not so tested, was found to have an LD_{50} of about 20 μg with AM-D in each of two trials. The Artenstein preparation appeared to be free of endotoxin. Neither was lethal when 0.5 mg was given alone to each of five mice.

Discussion. The simultaneous administration of AM-D and bacterial endotoxins resulted in markedly increased lethal effects in mice. In experiments with *S. typhosa* LPS, where direct comparisons were possible, a 12.5 μg dose of AM-D enhanced its lethality from 550 to >20,000-fold. Pieroni *et al.* (5, 6) have reported a potentiating factor of about 100,000 in the 2-day LD_{50} when *S.*

TABLE III. Seven-Day LD_{50}'s of Meningococcal Supernatant Fluids Administered with 12.5 μg of AM-D/Mouse.

Group	LD_{50} (dilution)	Fold increase[a]
A	1:398	> 398 (?)
C	1:813	> 813
X	1:389	>1945
Y	1:479	>2395

[a] 2/5 mice died after injection with undiluted Group A supernatant fluid without AM-D. Similar injections of the other three supernatant fluids were not lethal including fivefold concentrates of X and Y.

typhosa (W) LPS is given with 25 μg of AM-D/mouse. Since we found 25 μg to be lethal in a significant proportion of our mice, we chose to use 12.5 μg. In the one experiment where the higher dose was employed, AM-D seemingly reduced the 2-day LD_{50} of LPS almost 300,000 times.

The apparent increase in sensitivity when 25 μg of AM-D were combined with the endotoxin is somewhat artifactual. If no animals die at the zero dose of endotoxin, then deaths up to the LD_{50} are the specific results of the administered doses. Since the administration of 25 μg of AM-D alone to 50 mice resulted in 16% deaths within 2 days, then an expected 16% of animals receiving this amount of AM-D and endotoxin would die from the former alone. Only the remaining 84% would be at an additional risk of death with respect to the administered endotoxin. Under these conditions the endotoxin dose which kills 50 of 100 mice who also have received AM-D is not the specific LD_{50} of endotoxin. With 16 animals dead as the result of AM-D alone, then only 34 endotoxin-specific deaths should occur in the 84 animals at risk. The specific death rate then is 34/84 or 40.5%. Thus, part of the increase in lethality noted with high doses of AM-D reflects the comparison of a specific $LD_{40.5}$ with a specific LD_{50}. In addition, the calculations of LD_{50} estimates by the Spearman-Karber or Reed-Muench methods rest on the assumption of the symmetry of the dose–response curves on either side of the LD_{50} (9). The substantial death rate at the zero-endotoxin dose produces a strong negative skew which tends to produce computational underestimates of the LD_{50} thereby compounding the bias already present because of the lethal effects of AM-D.

These two types of error were reduced, but certainly not eliminated by employing 12.5 μg of AM-D. This dose alone still killed 8 to 9% of mice within 7 days so that the calculated "LD_{50}'s" are biased to that extent. Thus, caution is required when interpreting comparisons of the LD_{50} of endotoxin administered with AM-D with that of endotoxin alone. Comparisons between the LD_{50}'s of two materials each given simultaneously with the same dose of AM-D should not be affected.

Despite these limitations, this biological system would seem to be useful in determining endotoxin activity as is reported herein in purified meningococcal LPS, in large volume culture supernatant fluids and in cell bodies. In the quantities available, these materials themselves were not lethal for mice, but when administered with AM-D, minute amounts of endotoxin were detected. Similar results were obtained with one purified Group A polysaccharide preparation, while no mouse deaths were observed with another which had passed the rabbit test.

Summary. The administration of AM-D simultaneously with typhoid endotoxin to mice reduced the 7-day LD_{50} of the latter approximately 550 to 20,000-fold. This system was used to quantitate the endotoxin contents of minute amounts of purified meningococcal LPS as well as broth culture supernates and cell bodies of these organisms. Small quantities of endotoxin were detected in one meningococcal polysaccharide preparation but not in another which was nonpyrogenic in rabbits.

The authors are indebted to Miss Patricia Newton for technical assistance and to Dr. Paul R. Sheehe for his constructive criticism.

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Received Mar. 16, 1970. P.S.E.B.M., 1970. Vol. 134.