INFECTION AND IMMUNITY, Nov. 1971, p. 550–555

Vol. 4, No. 5
Printed in U.S.A.

Enhanced Toxicity for Mice of Combinations of Antibiotics with *Escherichia coli* Cells or *Salmonella typhosa* Endotoxin

W. C. ROSE AND S. G. BRADLEY

Department of Microbiology, Virginia Commonwealth University, Richmond, Virginia 23219

Received for publication 12 August 1971

Enhanced lethality for BALB/c mice has been observed after the administration of *Salmonella typhosa* endotoxin with either actinomycin D, cycloheximide, or nogalamycin. The dose of actinomycin D required to kill half of the mice (LD_{50}) was 0.8 mg/kg in normal animals, 0.35 mg/kg in mice administered 0.08 mg of endotoxin per kg, and 0.28 mg/kg in mice administered 0.2 mg of endotoxin per kg. The LD_{50} of endotoxin in normal mice was 12 mg/kg and in mice given 0.4 mg of actinomycin D per kg was 0.067 mg/kg. The LD_{50} of actinomycin D in mice administered 1.8×10^8 live *Escherichia coli* cells per kg or 1.8×10^9 heat-killed *E. coli* cells per kg was reduced to 0.4 mg/kg. The LD_{50} of cycloheximide was 181 mg/kg in normal animals and 28 mg/kg in mice administered 4 mg of endotoxin per kg. The LD_{50} of endotoxin in mice given 120 mg of cycloheximide per kg was 0.02 mg/kg. Enhanced lethality due to various combinations of cycloheximide and endotoxin was abolished by pretreatment of mice with endotoxin. The LD_{50} of nogalamycin was 21 mg/kg in normal mice and 13 mg/kg in mice receiving 1 mg of endotoxin per kg.

Bacterial endotoxin potentiates the responses of animals to a number of biological and nonbiological agents, including various antibiotics (4, 8, 11). Berry (1), for example, observed an enhanced lethal effect in mice receiving both endotoxin and actinomycin D.

Pieroni et al. (7) and Dowling and Feldman (3) have used this potentiation as the basis for an extremely sensitive bioassay for the detection of submicrogram quantities of endotoxin. In addition, Karp and Bradley (5) have observed a synergistic toxicity for mice of combinations of endotoxin with either sparsomycin or pactamycin. Subsequently, Bradley and Rose (Fed. Proc. **29:** 682, 1970) noted an increased rate of death in mice given combinations of daunomycin and endotoxin.

As a first step in determining the potential clinical significance of these interactions, we conducted a survey for enhanced toxicities involving bacterial endotoxin and selected chemotherapeutic agents. Enhanced death occurred in mice administered endotoxin in combination with cycloheximide or nogalamycin. In addition, enhanced mouse lethality was demonstrated in animals receiving gram-negative bacterial cells and actinomycin D.

MATERIALS AND METHODS

BALB/c male mice weighing 22 to 27 g were used. Drug solutions were prepared such that the required amount could be administered intraperitoneally (ip) in 0.01 ml per g of mouse. Actinomycin D (Calbiochem, Los Angeles, Calif.), nogalamycin (Upjohn Co., Kalamazoo, Mich.), cycloheximide (Nutritional Biochemicals Corp., Cleveland, Ohio), and *Salmonella typhosa* 0901 W lipopolysaccharide (Difco) were dissolved or suspended in sterile 0.15 M NaCl and adjusted to *p*H 7.0. When endotoxin or bacterial cells were given to mice with a drug, the injections were administered simultaneously, i.e., within 15 seconds, in a random sequence.

Stock cultures of a strain of *Escherichia coli* obtained from the collection of the Department of Microbiology were maintained on Penassay agar (Difco). Cultures grown in peptone-yeast extract (0.5 and 0.3%, respectively) broth for 18 hr at 37 C were harvested by centrifugation and suspended in sterile saline to give an absorbance of 0.25 in a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) at 420 nm. The number of viable organisms in this suspension was determined as colony-forming units after 24 hr of incubation at 37 C on Penassay agar. The logarithmic mean and standard error of duplicate determinations for 10 suspensions was $8.7 \times 10^7 \pm 2.1 \times 10^7$ cells/ml. The LD_{50} for mice 7 days after ip inoculation was 5.5×10^9 cells/kg.

The bacterial cells were subjected to two different heat treatments. Suspensions were heated in a water bath at 58 C for either 20 min or 1 hr. Heating for 20 min reduced the viable count of the suspension of *E. coli* having an A_{420} of 0.25 to 2.2 $\times$ 10^5 $\pm$ 0.85 $\times$ 10^5 cells per ml. Heating for 1 hr reduced the viable count of this same suspension to less than 10^3 viable cells per ml.

Synergy was determined by analyzing isobolograms (10) and by using the method of Treffers and Muschel (12). The amount of drug that killed 50% of the treated animals (LD_{50}) was determined by the method of Reed and Muench (9) or, where applicable, by interpolation from probit plots (6). Potency ratios and tests for parallelism were determined by the method of Litchfield and Wilcoxon (6). The acceptable level of significance was $P < 0.05$.

Pretreatment of mice with *S. typhosa* endotoxin involved the following regimen: 2 mg of endotoxin per kg on day -5, 4 mg of endotoxin per kg on days -4 and -3, and 8 mg of endotoxin per kg on day -2. The mice were challenged on day 0.

RESULTS

The LD_{50} of actinomycin D for BALB mice 3 days postinjection was 0.82 mg of actinomycin D per kg (Fig. 1). During this series of experiments, the LD_{50} of *S. typhosa* endotoxin was 12.2 mg/kg by Reed-Muench determination and 13.8 mg/kg by probit analysis (Fig. 2). Mice simultaneously given 0.4 mg of actinomycin D per kg and endotoxin, and scored on day 3, gave an LD_{50} with respect to endotoxin of 0.067 mg/kg (Fig. 2). Lesser reductions in endotoxin lethality were elicited by the simultaneous administration of 0.3 or 0.2 mg of actinomycin D per kg with endotoxin (Fig. 3). Analysis of the probit plot of endotoxicity, with and without the simultaneous administration of 0.4 mg of actinomycin D per kg, showed a potentiation of endotoxin lethality of greater than 200-fold (Fig. 2). The LD_{50} of actinomycin D was decreased to 0.35 mg/kg when administered to mice in combination with 0.08 mg of endotoxin per kg and to 0.28 mg/kg with 0.20 mg of endotoxin per kg (Fig. 1). This represents potentiations of 2.3- and 2.9-fold, respectively. The lethality of various combinations of endotoxin and actinomycin D was greater than the additive effects of the corresponding drug doses (Fig. 3).

Potentiation of actinomycin D lethality was also observed after simultaneously administering nonlethal doses of live or heat-killed *E. coli* cells to mice (Table 1). As few as 1.8 $\times$ 10^8 viable cells per kg given to mice in combination with 0.4 mg

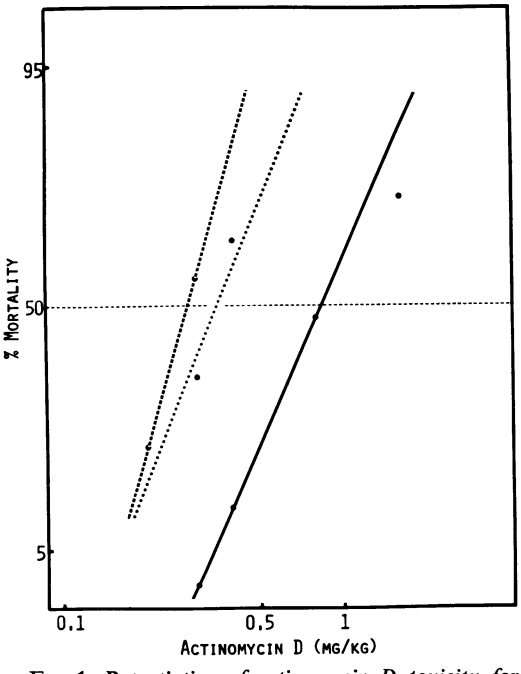

FIG. 1. *Potentiation of actinomycin D toxicity for mice by Salmonella typhosa endotoxin. The lethal responses of BALB mice to various doses of actinomycin D with no endotoxin (—), 0.08 mg of endotoxin per kg (···), or 0.2 mg of endotoxin per kg (– – –) are plotted on a probit scale.*

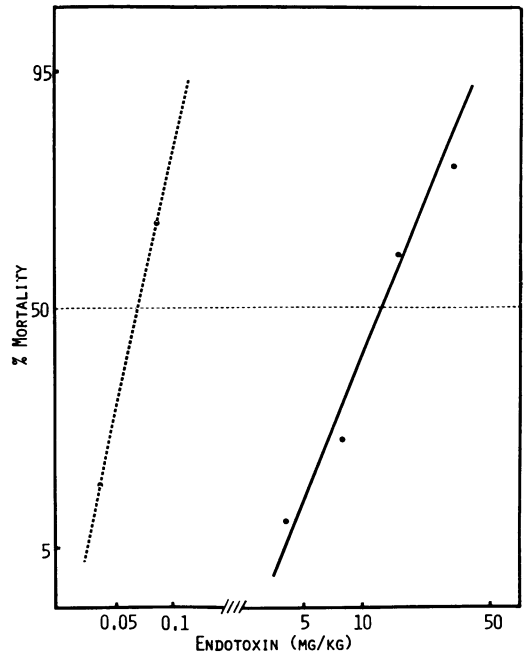

FIG. 2. *Potentiation of S. typhosa endotoxin lethality for mice by actinomycin D. The lethal responses of BALB mice to various doses of endotoxin with no actinomycin D (—) or 0.4 mg of actinomycin D per kg (– – –) are plotted on a probit scale.*

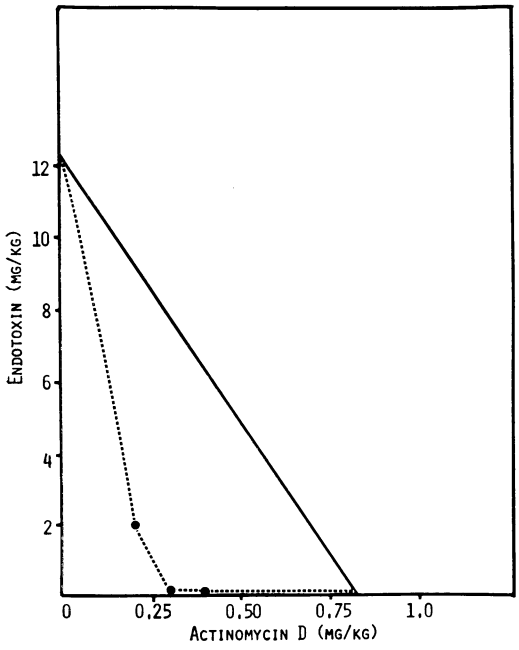

FIG. 3. *Synergistic toxicity for mice of combinations of actinomycin D with S. typhosa endotoxin. The dashed line connects LD_{50} values for various combinations; the solid line indicates the responses expected for additive effects.*

of actinomycin D/kg resulted in 55% death within 3 days. The administration of 1.8×10^9 heat-killed cells per kg, obtained by heating $8.7 \times 10^7 \pm 2.1 \times 10^7$ cells per ml at 58 C for 20 min or 1 hr, with 0.4 mg of actinomycin D per kg, resulted in 63 and 50% lethality, respectively, within 3 days after inoculation. The LD_{50} for actinomycin D changed from 0.82 mg/kg to 0.38 mg/kg when the drug was given in combination with either 1.8×10^9 heat-killed cells (from the 20 min, 58 C heat treatment) or 1.8×10^8 viable cells per kg. This corresponds to a greater than twofold potentiation of actinomycin D lethality.

The LD_{50} of cycloheximide 3 days after challenge was 181 mg of cycloheximide per kg by the Reed-Muench method and 185 mg of cycloheximide per kg by probit analysis (Fig. 4); 120 mg of cycloheximide per kg was nonlethal. The LD_{50} of endotoxin for mice during this period of experimentation was 20.5 mg/kg by Reed-Muench determination and 18.5 mg/kg by probit analysis (Fig. 5). When administered with 120 mg of cycloheximide per kg, the LD_{50} of endotoxin after 3 days was decreased to 0.02 mg/kg, representing a 925-fold potentiation (Fig. 5). Other combinations of endotoxin and cycloheximide also resulted in synergistic potentiation of mouse lethality; 20 mg of cycloheximide per kg with 8 mg of endotoxin per kg, 40 mg of cycloheximide per kg with 4 mg of endotoxin per kg, and 120 mg of cycloheximide per kg with 0.2 mg of endotoxin per kg resulted in 50, 90, and 100% lethality, respectively, within 3 days. The administration of these same drug-endotoxin combinations to mice which were previously treated with endotoxin proved nonlethal.

The LD_{50} on day 3 of cycloheximide decreased from 185 mg/kg to 28 mg/kg when the drug was given to mice with 4 mg of endotoxin per kg (Fig. 4). Mice receiving 4 mg of endotoxin per kg did not die. This reduction in the LD_{50} of cycloheximide in the presence of 4 mg of endotoxin per kg represents a 6.6-fold drug potentiation. The lethality after 3 days due to various combinations of cycloheximide and endotoxin was greater than the additive effects of the corresponding drug doses (Fig. 6).

The LD_{50} values for nogalamycin, as determined by the Reed-Muench method for days 3 and 7, were 22.9 and 21.5 mg of nogalamycin per kg, respectively. By probit analysis, the 7-day mortality data gave an LD_{50} of 21 mg of nogalamycin per kg (Fig. 7). The LD_{50} of endotoxin for mice dur-

TABLE 1. *Enhanced toxicity for mice of actinomycin D with Escherichia coli cells*

Actinomycin D (mg/kg)	*E. coli* cells/kg	No. of mice	Cumulative per cent death after	
			3 days	7 days
0.4	None	68	9	15
0.4	9.0×10^7	22	27	36
0.4	1.8×10^8	22	55	68
0.4	1.8×10^9	6	100	100
0.4	3.6×10^8 [a]	10	10	20
0.4	9.0×10^8 [a]	16	56	69
0.4	1.8×10^9 [a]	16	63	63
0.4	1.8×10^9 [b]	10	50	60
0.3	1.8×10^8	12	8	17
0.3	3.6×10^8	12	8	25
0.3	9.0×10^8	12	42	42
0.3	9.0×10^8 [a]	12	0	0
0.3	1.8×10^9 [a]	18	11	17
0.2	3.6×10^8	12	0	0
0.2	9.0×10^8	24	33	42
0.2	1.8×10^9	6	100	100
0.2	9.0×10^8 [a]	12	0	0
0.2	1.8×10^9 [a]	18	11	22
0.0	1.8×10^9	18	0	0
0.0	1.8×10^9 [a]	18	0	0

[a] *E. coli* cells were heated at 58 C for 20 min. This resulted in a 400-fold decrease in viability (e.g., heat treatment of an A_{420} of 0.25 left $2.2 \times 10^5 \pm 0.85 \times 10^5$ viable cells per ml).

[b] *E. coli* cells were heated at 58 C for 1 hr. The viable cell count of the treated suspension (A_{420} of 0.25) was less than 10^3 cells per ml.

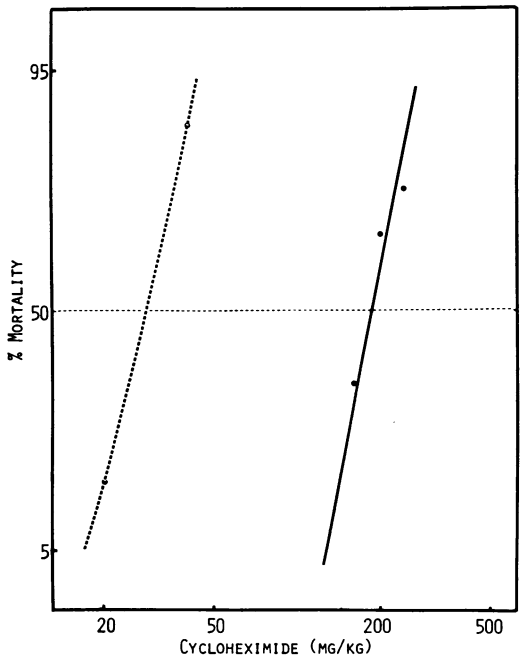

FIG. 4. *Potentiation of cycloheximide toxicity for mice by S. typhosa endotoxin. The lethal responses of BALB mice to various doses of cycloheximide with no endotoxin (—) or 4 mg of endotoxin per kg (——) are plotted on a probit scale.*

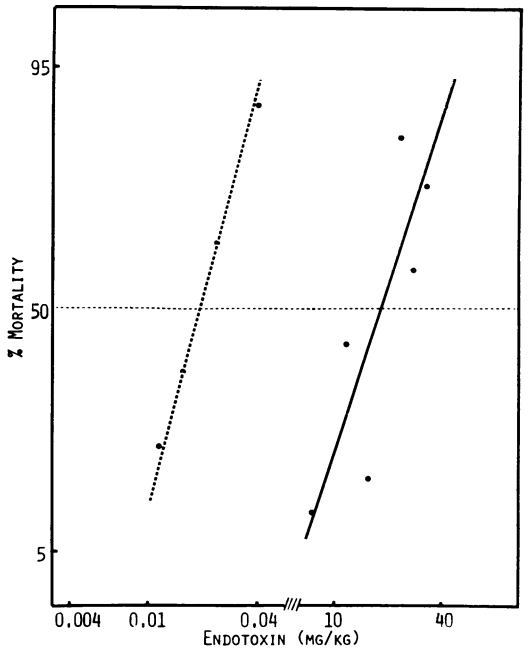

FIG. 5. *Potentiation of S. typhosa endotoxin lethality for mice by cycloheximide. The lethal responses of BALB mice to various doses of endotoxin with no cycloheximide (—) or 120 mg of cycloheximide per kg (——) are plotted on a probit scale.*

ing this period of experimentation was 17.9 mg/kg as calculated by the Reed-Muench method and 17.5 mg/kg by probit analysis (Fig. 8). The LD_{50} of endotoxin, scored on day 3, was reduced to 0.2 mg/kg when mice were simultaneously administered 20 mg of nogalamycin per kg, thus representing an 88-fold potentiation (Fig. 8). It should be noted that, when given alone, 20 mg of nogalamycin per kg resulted in only 3% lethality. Other nogalamycin-endotoxin combinations likewise resulted in enhanced lethalities. The 7 day LD_{50} of nogalamycin was reduced from 21 mg/kg to 13.2 mg/kg when administered to mice in combination with 1 mg/kg of endotoxin (Fig. 7), thus representing a 1.6-fold potentiation. The lethality due to various combinations of nogalamycin and endotoxin was greater after 3 days than the additive effects of the corresponding drug doses (Fig. 9).

DISCUSSION

The previously reported potentiation of mouse lethality due to simultaneous administration of combinations of actinomycin D and endotoxin has been confirmed (1, 3, 7). The smaller potentiation of endotoxicity observed by us may be due to any number of factors including, strain and sex of mice, source and dose of antibiotic,

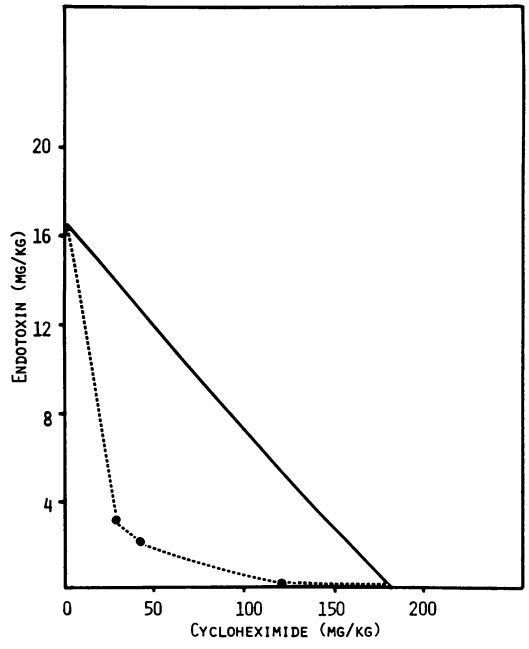

FIG. 6. *Synergistic toxicity for mice of combinations of cycloheximide with S. typhosa endotoxin. The dashed line connects LD_{50} values for various combinations; the solid line indicates the responses expected for additive effects.*

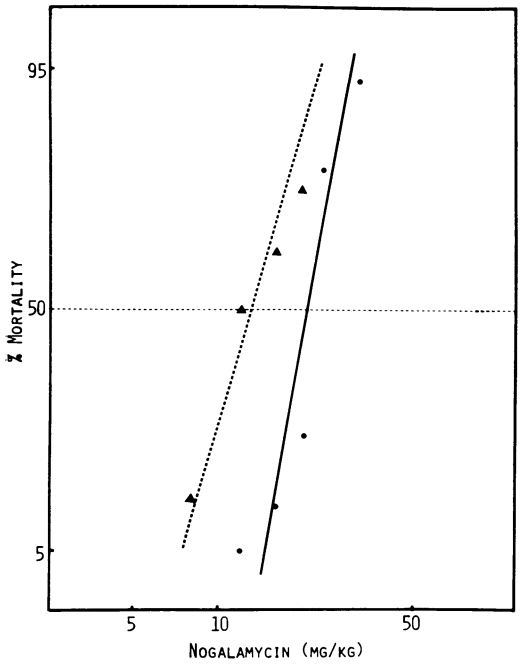

FIG. 7. *Potentiation of nogalamycin toxicity for mice by S. typhosa endotoxin. The lethal response of BALB mice to various doses of nogalamycin with no endotoxin (—) or 1 mg of endotoxin per kg (——) are plotted on a probit scale.*

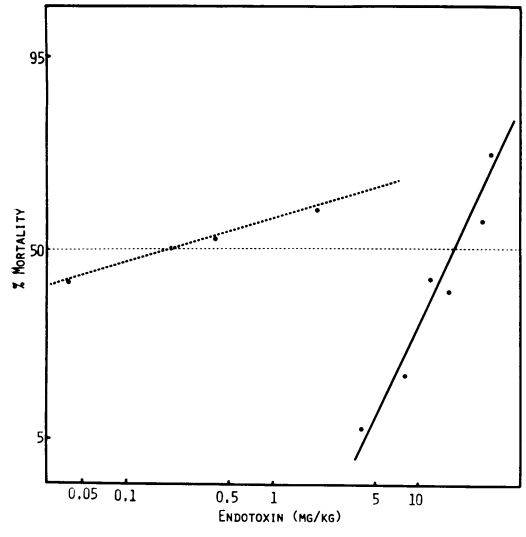

FIG. 8. *Potentiation of S. typhosa endotoxin lethality for mice by nogalamycin. The lethal responses of BALB mice to various doses of endotoxin with no nogalamycin (—) or 20 mg of nogalamycin per kg (——) are plotted on a probit scale.*

and day of LD_{50} determination. The slopes of the dose-response curves for mice given endotoxin and actinomycin D are parallel to the responses to endotoxin alone and to antibiotic alone. In addition, the isobologram for the 3-day LD_{50} data depicts a synergistic interaction. Accordingly, the observation that both actinomycin D and endotoxin significantly potentiate each other in a parallel manner indicates that the underlying mechanism of the synergy may involve a dualistic enhancement of each agent's mode of toxic behavior.

The approximately twofold potentiation of actinomycin D lethality by either live or heat-treated *E. coli* cells, although not parallel to the lethal response to actinomycin D alone, nevertheless demonstrates that endotoxin per se need not be administered to elicit a synergistic reaction. The 10-fold differential between the number of live cells and the number of heat-killed cells required to elicit a similar actinomycin D potentiation might be due to multiplication of the live cell inoculum. The potentiation of actinomycin D lethality by 0.08 mg of endotoxin per kg (2.3-fold) and 1.8×10^9 heat-killed cells per kg (2.2-fold) is approximately the same. Assuming 10

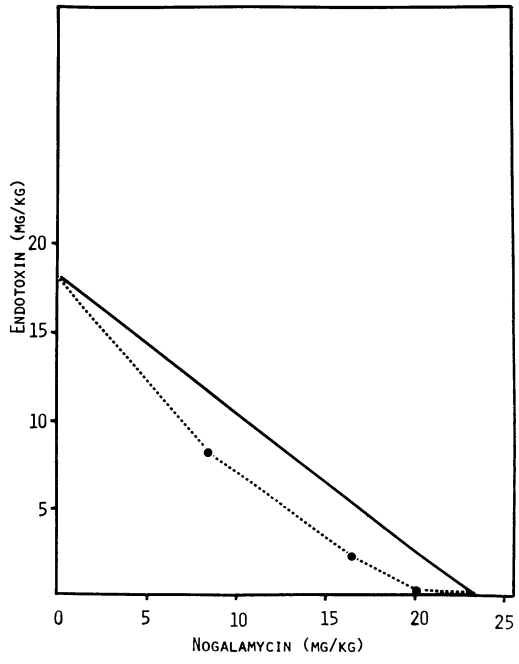

FIG. 9. *Synergistic toxicity for mice of combinations of nogalamycin with S. typhosa endotoxin. The dashed line connects LD_{50} values for various combinations; the solid line indicates the response expected for additive effects.*

μg of endotoxin per 10^9 gram-negative cells (2), the effect of the *E. coli* cells can be reasonably attributed to their endotoxin composition. Because the biological effect resulting from the simultaneous administration of 0.4 mg of actinomycin D per kg with 1.8×10^9 heat-killed cells per kg, derived from either the 20-min or 1-hr heat-treatment of the same number of viable cells, is approximately equal, the endotoxin content of the cells, and not the presence of viable organisms, is probably responsible for the drug potentiation.

The lethal responses to combinations of cycloheximide with endotoxin are parallel to the corresponding responses of each agent administered separately. This complementary interaction is consistent with the symmetrical nature of the isobologram for the cycloheximide-endotoxin data based upon 3-day LD_{50} values. The successful elimination of the cycloheximide-endotoxin-enhanced lethality by pretreatment of the mice with endotoxin is consistent wtih similar results previously reported involving combinations of sparsomycin with endotoxin (5) and daunomycin with endotoxin (Bradley and Rose, Fed. Proc. 29:682, 1970).

The lethal responses to combinations of nogalamycin and endotoxin are not parallel to the lethal responses for endotoxin alone but are parallel ($P < 0.05$) to the lethal responses for the antibiotic alone. Moreover, deaths due to endotoxin occur during the first 48 hr after administration, whereas deaths due to nogalamycin and combinations of nogalamycin and endotoxin occur up to 7 days after injection. These data indicate that the nogalamycin-endotoxin interactions resulting in enhanced lethality are mainly due to a potentiation of the antibiotic's mode of injury.

The questions remains: does the antibiotic retard the clearance or detoxification of endotoxin, or both? Conversely, endotoxin may retard the clearance or detoxification of the antibiotic. The data presented suggest that both processes may be involved, depending upon the synergy being studied.

ACKNOWLEDGMENTS

The research upon which this publication is based was performed pursuant to Public Health Service contract no. NIH-69-2266 from the National Institutes of Health. W. C. Rose was the recipient of an A. D. Williams Fellowship.

LITERATURE CITED

1. Berry, L. J. 1964. Effect of endotoxins on the level of selected enzymes metabolites, p. 151–159. *In* M. Landy and W. Braun (ed.), Bacterial endotoxins. Rutgers University Press, New Brunswick, N.J.
2. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. 1968. Microbiology, p. 119. Harper and Row, New York.
3. Dowling, J. N., and H. A. Feldman. 1970. Quantitative biological assay of bacterial endotoxins. Proc. Soc. Exp. Biol. Med. 134:861–864.
4. Huang, K-Y., and M. E. Landay. 1969. Enhancement of the lethal effects of endotoxins by interferon inducers. J. Bacteriol. 100:1110–1111.
5. Karp, R. D., and S. G. Bradley. 1968. Synergistic toxicity of endotoxin with pactamycin or sparsomycin. Proc. Soc. Exp. Biol. Med. 128:1075–1080.
6. Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99–113.
7. Pieroni, R. E., E. J. Broderick, A. Bundeally, and L. Levine. 1970. A simple method for the quantitation of submicrogram amounts of bacterial endotoxin. Proc. Soc. Exp. Biol. Med. 133:790–794.
8. Pieroni, R. E., and L. Levine. 1964. Enhancing effect of insulin on endotoxin lethality. Experientia 25:507.
9. Reed, L. J., and H. Muench. 1938. A simple method for estimating fifty per cent end points. Amer. J. Hyg. 27:493–497.
10. Sabath, L. D. 1968. Synergy of antibacterial substances by apparently known mechanisms. Antimicrob. Ag. Chemother. 1967, p. 210–217.
11. Selye, H., B. Tuchweber, and L. Bertok. 1966. Effect of lead acetate on the susceptibility of rats to bacterial endotoxins. J. Bacteriol. 91:884–890.
12. Treffers, H. P., and L. H. Muschel. 1954. The combined actions of chloramphenicol and of bactericidal antibody plus complement on *Salmonella typhosa*. J. Exp. Med. 96:155–165.