

Inactivation of Endotoxin by Polymyxin B

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The limulus gelation assay was utilized to investigate endotoxin inactivation by a number of antibiotics in vitro. Endotoxin activity was sharply reduced by polymyxin B and sodium colistimethate. The effect of the polymyxin was not significantly inhibited by 0.001 M calcium or 90% serum. Crude endotoxins from a variety of aerobic gram-negative bacteria, including several not previously studied, could be inactivated 1 or more logs by as little as 1 μ g of polymyxin B per ml, whereas *Bacteroides fragilis* endotoxin was poorly detoxified. A 10,000-fold range in the relative susceptibility of different endotoxins to inactivation by polymyxin B was found. The endotoxin most susceptible to polymyxin B was derived from an organism resistant to polymyxin B by disk sensitivity testing, suggesting that the bacteriocidal and endotoxin detoxifying properties of polymyxin need not be directly related.

Recent studies have demonstrated the capacity of polymyxin B to protect animals from a variety of toxic effects of endotoxin, including the generalized Schwartzman reaction (5, 23), diffuse intravascular coagulation (6, 7), renal cortical necrosis (6, 7), leukopenia (6, 7), and death (6, 12, 21, 23). The limulus gelation test, developed by Levin and others (13, 14), has provided a useful in vitro means to assess an endotoxin activity which corresponds with in vivo indices of toxicity (3, 15, 24). We have previously found this assay to be useful in evaluating endotoxin inactivation processes (4). The present study was designed to evaluate the effect of polymyxin B and other antibiotics upon endotoxin, the conditions of the reaction, and the relative polymyxin susceptibility of endotoxins from a variety of gram-negative bacteria.

MATERIALS AND METHODS

Limulus assay. A lysate of *Limulus polyphemus* amoebocytes (horseshoe crab blood cells) was prepared by the method of Yin et al. (27). In preliminary experiments, it was found that maximal sensitivity and reproducibility of the assay was achieved by diluting initial lysate preparations 1:8 in 0.15 M tris(hydroxymethyl)aminomethane buffer before use. Equal volumes of lysate and test solution were incubated together for 4 h at 37 C and observed for readily visible changes in viscosity. The limulus lysate used in the majority of these studies consistently developed a viscous gel when incubated with 0.1 ng of a standard

endotoxin (*Escherichia coli* O26:B6 Boivin preparation, Difco Laboratories, Detroit, Mich.) per ml. With this assay, diluents, reagents, and media were determined to be free of detectable endotoxin at the concentrations used. The diluent used for all reagents was pyrogen-free water for injection (Travenol Laboratories, Inc., Deerfield, Ill.).

Antibiotic screening. A number of antibiotics were incubated for 20 min with 5 ng of *E. coli* O26:B6 endotoxin per ml and tested with the limulus assay as described above; a positive test was taken to indicate failure of the antibiotic to significantly inactivate endotoxin.

Effect of calcium. Serial 10-fold dilutions of *E. coli* O26:B6 endotoxin were incubated for 20 min with 1 μ g of polymyxin B (Chas. Pfizer & Co., New York) per ml or 1 μ g of sodium colistimethate (Warner-Chilcott Laboratories, Morris Plains, N. J.) per ml in the presence of 0.001 M calcium chloride and were compared by limulus assay with identically treated calcium-free controls. Sodium colistimethate was previously hydrolyzed by incubation at 37 C overnight to develop its full activity (1).

Effect of serum. Serial 10-fold dilutions of *E. coli* O26:B6 endotoxin were incubated overnight at 37 C in 1-ml volumes containing 8 μ g of polymyxin B per ml and 90% serum obtained from a normal adult donor. This material was then treated by a minor modification of the method of Levin et al. (15) to recover residual endotoxin; the mixtures were emulsified with 1 ml of chloroform in stoppered tubes (10 by 75 mm) on a reciprocating shaker (no. 75-683, Eberbach Corp., Ann Arbor, Mich.) at 630 2.5-cm strokes per min for 6 h and then centrifuged at $2,400 \times g$ for 9 min. A portion of the cloudy aqueous phase from each tube was then tested by limulus assay and compared with identically treated polymyxin-free controls.

Effect of polymyxin B upon endotoxins. Culture supernatants of a variety of gram-negative bacteria

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isolated from clinical sources were prepared in serial 10-fold dilutions in water and heated at 100 C for 15 min. The Boivin-endotoxin of *E. coli* O26:B6 and the spinal fluid of an infant with *E. coli* meningitis were similarly prepared. High dilutions of these crude endotoxins were incubated at 37 C for 20 min with polymyxin B at a concentration of 0, 1, and 10 $\mu\text{g/ml}$ and then tested by limulus assay. All samples were tested in triplicate, and the dilution giving 50% end point of limulus reactivity was estimated for each endotoxin preparation by the method of Reed and Muench (19).

RESULTS

E. coli O26:B6 endotoxin (5 ng/ml) was not demonstrably altered by chloramphenicol (1,000 $\mu\text{g/ml}$), gentamicin (50 $\mu\text{g/ml}$), streptomycin (100 $\mu\text{g/ml}$), tetracycline (25 $\mu\text{g/ml}$), ampicillin (100 $\mu\text{g/ml}$), carbenicillin (100 $\mu\text{g/ml}$), or sulfisoxazole (400 $\mu\text{g/ml}$), although it was neutralized by polymyxin B (50 $\mu\text{g/ml}$). Polymyxin B (1 $\mu\text{g/ml}$) deactivated 100 ng of this endotoxin per ml by at least 2 log despite the presence of 0.001 M CaCl_2 ; hydrolyzed sodium colistimethate also sharply inhibited endotoxin activity, although this effect may have been somewhat reduced in the presence of 0.001 M CaCl_2 (Table 1). Greater than 1-log inactivation could also be demonstrated in the presence of 90% normal human serum (Table 2).

In further experiments, polymyxin B was observed to induce a marked decline in the activity of endotoxin preparations from five aerobic gram-negative organisms, whereas there was relatively minimal decline in the activity of polymyxin-treated *Bacteroides fragilis* endotoxin (Fig. 1). Susceptibility of the various endotoxins to polymyxin B varied over a 10,000-fold range. The endotoxin most sensitive to polymyxin B was derived from a strain of *Proteus mirabilis* which had been demonstrated to be resistant to the antibacterial action of polymyxin B by routine disk-sensitivity testing.

TABLE 2. *Inactivation of endotoxin by polymyxin B in human serum*^a

Initial endotoxin (ng/ml)	Limulus reactivity after incubation with serum and:	
	No antibiotic	Polymyxin B (8 $\mu\text{g/ml}$)
1.0	+	0
0.1	±	0
0	0	0

^a Endotoxin and polymyxin B were incubated overnight in 90% normal human serum at the concentrations noted; after chloroform treatment, the mixture was tested with the limulus assay. Symbols are as defined in footnote to Table 1.

DISCUSSION

Polymyxin B, a cationic cyclic decapeptide containing both lipophilic and lipophobic groups, is known to act upon gram-negative bacterial cell walls (26). The present study adds further evidence of this by demonstrating an interaction between polymyxin and the Boivin-endotoxin of *E. coli* O26:B6. Recent studies have clearly demonstrated that endotoxin toxicity is attributable to cell wall lipid A (16). Endotoxin neutralization by polymyxin is thus likely to occur at this site.

Previous investigations have shown in vivo protection by polymyxin against *E. coli*, *Aerobacter aerogenes*, and *Serratia marcescens* endotoxins (5-7, 12, 20-23); the current study extends these observations to include inactivation of *Proteus*, *Klebsiella*, and *Neisseria* endotoxins as well. However, a 10,000-fold variation in susceptibility of the various endotoxins was found. Rifkind (21) has clearly demonstrated some degree of interdependence between parent-bacterial susceptibility and susceptibility of the derived endotoxin to polymyxin B. Nonetheless, it is evident that these

TABLE 1. *Inactivation of endotoxin by polymyxin B and colistimethate in the presence of 0.001 M calcium chloride*^a

Initial endotoxin (ng/ml)	Limulus test reactivity after incubation with:				
	No antibiotic	Polymyxin B (1 $\mu\text{g/ml}$)	Polymyxin B (1 $\mu\text{g/ml}$) in 0.001 M CaCl_2	Colistimethate (1 $\mu\text{g/ml}$)	Colistimethate (1 $\mu\text{g/ml}$) in 0.001 M CaCl_2
100	+	0	0	0	±
10	+	0	0	0	0
1	±	0	0	0	0
0	0	0	0	0	0

^a Endotoxin, antibiotic, and calcium were incubated together at the concentrations noted and then tested by limulus assay. +, Positive; ±, weakly positive; 0, negative test for endotoxin.

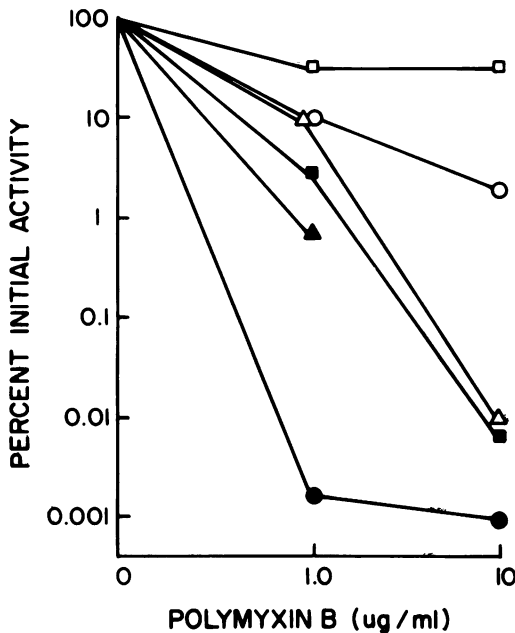


FIG. 1. Effect of polymyxin B upon the activity of endotoxins from a variety of gram-negative organisms. Symbols: □, *Bacteroides fragilis*, wound culture supernatant; ○, *Klebsiella pneumoniae* type 18, cholecystitis culture supernatant; △, *Neisseria meningitidis*, spinal fluid culture supernatant; ■, *E. coli* O18a,c:K77:H7, neonatal meningitis spinal fluid; ▲, *E. coli* O26:B6 Boivin endotoxin (Difco; Detroit, Mich.); ●, *Proteus mirabilis*, polymyxin B resistant, urinary tract infection culture supernatant.

two properties may at times be sharply divergent, since *Proteus mirabilis*, an organism resistant to the bactericidal effect of polymyxin, produced an endotoxin which was highly sensitive (Fig. 1).

Bactericidal activity of the polymyxins may be blocked by low concentrations of calcium or serum (9, 18). However, endotoxin detoxification by polymyxin B was not inhibited by such treatment in the present study.

The finding that a variety of gram-negative bacterial endotoxins can be inactivated in vitro by polymyxin B, together with previous in vivo animal protection studies (5-7, 12, 20-23), suggests that it might well be possible to decrease endotoxin activity in man with polymyxin B therapy. Serum levels of polymyxin well above 1 $\mu\text{g/ml}$ can be readily achieved (25). Levels of endotoxin which can be inactivated by this means (Table 1) appear to be in excess of those observed in the plasma of adult (2) and pediatric patients (M. S. Cooperstock, unpublished data). However, the clinical role of endotoxin-detoxifying agents can be defined only with

clarification of the role of endotoxin in human disease. While circulating endotoxin-like materials have been demonstrated in clinical populations, the findings are divided as to whether endotoxemia can (8, 10, 15) or cannot (10, 16, 24) be correlated with clinical outcome. Since quantitative studies of human endotoxemia are few, toxic levels under various clinical conditions remain undefined. One such study found that levels of circulating endotoxin-linked antigens correlate well with mortality in meningococemia (10). This finding and the present demonstration that *Neisseria meningitidis* endotoxin is sensitive to polymyxin merit further evaluation in view of possible clinical usefulness if confirmed.

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