Protective Effect of Subinhibitory Polymyxin B Alone and in Combination with Ampicillin for Overwhelming *Haemophilus influenzae* Type B Infection in the Infant Rat: Evidence for *in Vivo* and *in Vitro* Release of Free Endotoxin after Ampicillin Treatment

JUAN W. WALTERSPIEL, SHELDON L. KAPLAN, AND EDWARD O. MASON, JR.

Myers-Black Section of Pediatric Infectious Disease, Department of Pediatrics, Baylor College of Medicine,
Charles T. Parker Memorial Laboratory, Texas Children's Hospital, Houston, Texas 77030

ABSTRACT. The potential endotoxin modifying effects of subinhibitory doses of polymyxin B were evaluated in an animal model of overwhelming septicemia. Five to six day old Sprague-Dawley rats were infected intraperitoneally with 10^6 – 10^7 cfu of *Haemophilus influenzae* type b. At 12 h after infection, at which time mortality was 18%, subinhibitory doses of polymyxin b (0.0125 mg/kg $\times$ 3 q 3 h) either alone or in combination with 500 mg/kg ampicillin significantly increased survival at 17 and 20 h ($p = 0.009$, 0.01 and $p = 0.003$, 0.01) compared to animals treated with 0.5 mg/kg of ampicillin alone. Prolonged survival at 36 h ($p = 0.009$) was seen in animals receiving both ampicillin and low dose polymyxin compared to either ampicillin dose alone. Ampicillin significantly reduced the number of bacteria in blood of survivors ($p < 0.023$ at 30 min) compared to untreated animals but increased the activity of free endotoxin at 30 min compared to controls ($p = 0.006$). *In vitro* endotoxin release from *H. influenzae* type b increased 5-fold after addition of 100 μ g/ml of ampicillin, whereas a six-fold reduction in endotoxin activity was measured after the addition of 7 μ g/ml of polymyxin B. Subinhibitory doses of polymyxin B modulate the ethal effects of overwhelming *H. influenzae* type b infection in infant rats and might be beneficial as adjunct treatment in gram-negative septicemia. (*Pediatr Res* 20: 237–241, 1986)

Abbreviations

HibE, *Haemophilus influenzae* type b Strain Eagan
BHIS, brain heart infusion broth—supplemented
PBS, phosphate buffered saline
LPS, lipopolysaccharide
HIB, *Haemophilus influenzae* type b
cfu, colony forming units
ip, intraperitoneal

Endotoxins or lipopolysaccharides from gram-negative bacteria are potent inducers of a wide variety of biological alterations in humans and experimental animals. LPS consists of a hydro-

philic polysaccharide, a core, and a hydrophobic lipid component, the lipid A. The polysaccharide determines the immunological specificity of the given LPS whereas lipid A, also endowed with antigenic properties, is responsible for the biologic activities of LPS (1). The role of endotoxin in the pathogenesis of shock associated with gram-negative bacteria in humans is not clear; however, the fact that antibodies directed against the core region of LPS significantly decrease mortality in humans with gram-negative infections supports the probability of some contribution of endotoxin to the physiologic effects seen in septic shock (2).

Shenep *et al.* (3, 4) have demonstrated the release of endotoxin from *Escherichia coli* by antimicrobial agents in a rabbit model. Some children with *Hib* meningitis, who initially have normal peripheral perfusion, develop septic shock within 1 to 2 h after receiving their first dose of antibiotics. The LPS of *Hib* is similar in biological action to LPS from other gram-negative bacteria (5). Further indirect evidence that LPS contributes to the virulence of *Hib* includes the observation that antibodies against the related *E. coli* J5 LPS core antigen protects six to 8-wk-old mice against death due to *Hib* infection (6).

Polymyxin B is a cationic, cyclic polypeptide antibiotic that specifically binds to the lipid A-core region of LPS, disrupting the outer membrane. This action neutralizes endotoxin effects *in vitro* and *in vivo* (7–16). However, the neutralizing effect was only seen if polymyxin B was administered in advance or in conjunction with artificially purified endotoxin. This finding was thought to preclude the usefulness of polymyxin B as a neutralizing agent in clinical situations. The recent demonstration of endotoxin release by the action of commonly used antimicrobial agents *in vivo* suggests that the coadministration of polymyxin B with an endotoxin releasing antibiotic might be advantageous (3, 4).

This study investigates the release of endotoxin from *Hib* by antibiotics *in vitro* and *in vivo* and the protective effect of low dose polymyxin B in an infant rat model of overwhelming *Hib* infection.

MATERIALS AND METHODS

In vitro release of endotoxin by antibiotics. *HibE*, kindly provided by Dr. T. J. Inzana, was employed in all experiments. Aliquots were frozen at -70° C in buffered glycerol-tryptic soy broth and were thawed and refrozen once. *HibE* was grown in brain heart infusion broth, supplemented with nicotinamide

adenine dinucleotide and hemin (BHIS) (10 µg/ml each, Sigma Chemical Co., St. Louis, MO). One milliliter of an overnight broth culture was added to 9 ml fresh medium in a 25-ml Erlenmeyer flask and incubated for 2 h at 35° C on a rotary shaker at 150 rpm. A baseline colony count was performed and antibiotics were added to achieve final concentrations approximating achievable peak serum concentrations in humans:

Ampicillin (Bristol Laboratories, Syracuse, NY) 100 µg/ml, chloramphenicol (Parke Davis, Morris Plains, NJ) 25 µg/ml, polymyxin B (Aerosporin, Burroughs Wellcome, Research Triangle Park, NC) 7 µg/ml, and colistinmethanate sodium (Warner/Chilcott, Morris Plains, NJ) 5 µg/ml. Two samples containing no antibiotic served as controls. One control flask was held at 4° C to prevent further replication; one control and all samples were incubated at 35° C after the addition of the antibiotics. Colony counts were performed at 2, 6, and 22 h. Similarly, polymyxin B at 0.1, 0.01, and 0.001 µg/l and a combination of ampicillin 100 µg/ml and polymyxin B at 0.1 µg/ml *versus* ampicillin alone were evaluated at 2 and 6 h after their addition to the cultures.

Cell free endotoxin activity was determined by removing 1 ml aliquots of bacterial suspension for colony quantitation after centrifugation at 12,000 × *g* for 10 min. The supernates were filtered through 0.22-µ filters (Millipore Co., Bedford, MA) and sterility verified by subculture to chocolate agar. Serial 10-fold dilutions of the filtered supernates were prepared with pyrogen free water and assayed for their endotoxin activity.

Determination of endotoxin activity in vitro and in vivo. Endotoxin activity was quantified by comparison to activity of *E. coli* 0 111:B4 standard (LPS) endotoxin in pyrogen-free water. Glassware and equipment were pyrogen free or rendered pyrogen free by baking at 160° C overnight. The chromogenic substrate S 2423 (AB Kabi Stockholm, Sweden) was used as an indicator for the limulus amoebocyte lysate reaction with endotoxin (QCL 1000, Whittaker, MA Bioproducts, Walkersville, MD) as described by Friberger *et al.* (17). The reaction was stopped by addition of 50% acetic acid and the optical density was determined at 405 nm using a MR530 MicroElisa Reader (Dynatech Laboratories, Inc., Alexandria, VA). This standard curve is linear over endotoxin concentrations between 0.01–0.1 ng/ml (0.1–1.0 endotoxin U/ml). Final endotoxin activity of the sample was calculated by multiplying the concentrations obtained from the standard curve by the dilution factor necessary to obtain an OD reading within the standard curve. We found that the addition of polymyxin B at 7 µg/ml to the chromogenic substrate does not interfere with this assay. The activity of LPS derived from *HibE* obtained by the phenol-water method (kindly supplied by Dr. T. J. Inzana) was 100 times less active on a weight basis than the standard endotoxin in this assay (18).

To measure free endotoxin activity in whole blood, 0.01 ml of blood was diluted in 3 ml of pyrogen free normal saline and gently filtered through a 0.45-µ filter unit (Millex HA, Millipore CO.) to remove bacteria. Red blood cells were lysed by the filtration process but the dilution was sufficient to avoid nonspecific light absorption from hemoglobin. The filtrate was immediately frozen at –20° C until assayed. Several healthy animals did not demonstrate any blood endotoxin activity using this method.

Experimental infection. Pathogen-free, outbred albino Sprague Dawley infant rats purchased with their mothers from Harlan Sprague Dawley, Inc., Houston, TX were used throughout the experiments. Litters contained eight to 14 animals and were mixed randomly before the experiments were initiated. Food and water were freely available and the animals were housed under standard conditions. All animals were weighed and infected when 5 to 6 days old.

One milliliter of BHIS overnight broth culture of strain Eagan was added to 9 ml of fresh BHIS and incubated on a rotary shaker at 150 rpm for 2½ h at 35° C. The culture was centrifuged at 12,000 × *g* for 10 minutes, washed once with phosphate

buffered saline containing 0.1% gelatin (Difco Laboratories, Detroit, MI) and was resuspended in PBS containing 0.1% gelatin to a concentration of 10⁸ cfu/ml. The minimal inhibitory concentrations of ampicillin and polymyxin B for *HibE* were 0.1 and 0.003 µg/ml, respectively. Ampicillin and polymyxin B were not synergistic against *HibE* *in vitro*.

Animals were injected intraperitoneally with 0.1 ml of a 10-fold dilution (10⁶ cfu) on the left side of the abdomen. To minimize leakage, needles were passed subcutaneously for a short distance prior to entering the peritoneal cavity.

In order to estimate a delay of antimicrobial treatment so that an otherwise adequate dose of ampicillin would not prevent death, 16 animals were infected ip with 9.6 × 10⁶ cfu of *HibE*. Treatment was delayed for 3, 6, 9, or 12 h after infection. When the delay was 12 h after infection, ampicillin did not decrease mortality. Survival and *in vivo* endotoxin release studies were initiated 12 h after *HibE* infection unless indicated otherwise.

Statistical analysis. Statistical analysis was performed with the two-tailed Fisher's exact test for survival at predetermined times and for the presence of bacteremia. Endotoxin activities and colony counts in the *in vivo* experiments were not Gaussian in distribution, therefore, results were analyzed with the Wilcoxon Mann Whitney rank sum test.

RESULTS

Effect of antibiotics on in vitro release of endotoxin by Hib in broth. Endotoxin activities in brain heart infusion broth, NAD/hemin supplement, and antibiotic solutions were 20, 10, and ≤0.02 ng/ml, respectively. The results of two separate experiments examining the *in vitro* release of endotoxin by *HibE* after exposure to antibiotics at peak serum levels attainable in humans are shown in Table 1. Prior to the addition of antibiotics initial colony counts were 4.3 and 4.7 × 10⁸ cfu/ml and filtered supernatants demonstrated endotoxin activity of 1000 ng/ml. Refrigeration at 4° C halted bacterial multiplication at 2 and 6 h and a 3 to 4 log loss of viability was seen at 22 h; the activity of cell free endotoxin did not increase during the first 6 h and tripled at 22 h. In comparison, colony counts in the incubated control sample increased 1 log by 2 h and there was a 20-fold increase in endotoxin activity observed at 22 h.

Addition of ampicillin or chloramphenicol reduced colony counts by 2 logs and increased the cell-free endotoxin activity at least 25-fold at 6 h compared to the values found for the refrigerated controls and 5-fold against incubated control samples. Polymyxin B was the most effective bactericidal antibiotic and decreased the level of cell free endotoxin activity to one-sixth of the initial level. Addition of colistinmethanate was associated with a reduction in bacterial viability at 22 h similar to untreated controls but with a reduction in cell-free endotoxin activity.

The results of identical experiments after addition of low doses of polymyxin B at 0.1, 0.01, or 0.001 µg/ml, or a combination of 100 µg/ml ampicillin and 0.1 µg/ml polymyxin B *versus* ampicillin alone are shown in Table 2. These low concentrations of polymyxin B alone or in combination with ampicillin exerted only minimal effects on colony forming units or endotoxin activities.

Determination of in vivo antimicrobial effects of ampicillin and low dose polymyxin B. Thirty-two infant rats were infected intraperitoneally with between 1.5 to 2.4 × 10⁶ cfu *HibE*. Twelve animals were treated with ampicillin alone at 0.5 mg/kg/dose ip and 20 with polymyxin B alone at 0.0125 mg/kg/dose ip. (This low dose of polymyxin B was selected on the basis of preliminary experiments which demonstrated that this dose was not effective in eradicating *Hib* in this model which was critical to studying the non antibiotic related activity of polymyxin B in overwhelming *Hib* infection.) A total of three doses of antibiotics were given 3 h apart and antibiotic treatment was initiated 3 h after intraperitoneal injection of *HibE*. Consecutive quantitative blood

Table 1. *Effect of antibiotics on colony counts and release of endotoxin in Hib broth cultures*

	Time of incubation after addition of antibiotics*					
	2 h		6 h		22 h	
	cfu/ml	Endotoxin activity (ng/ml)	cfu/ml	Endotoxin activity (ng/ml)	cfu/ml	Endotoxin activity (ng/ml)
Antibiotics						
Ampicillin (100 µg/ml)	5.8×10^6	9,500	1.3×10^6	25,000	5.0×10^1	45,000
Chloramphenicol (25 µg/ml)	6.1×10^6	15,000	3.0×10^5	35,000	7.6×10^3	50,000
Colistinmethanate (5 µg/ml)	3.2×10^9	3,000	4.4×10^9	5,000	5.0×10^4	2,000
Polymyxin B (7 µg/ml)	NG†	150	Ng	400	Ng	150
No antibiotics added						
Control incubated at 35° C	2.8×10^9	3,000	6.3×10^9	5,000	5.0×10^4	20,000
Control refrigerated	4.8×10^8	1,000	6.2×10^8	1,000	1.3×10^4	3,000

* Results are the means of two separate experiments. Broths were incubated for two hours prior to the addition of antibiotics at which time colony counts in all samples were approximately 4×10^8 cfu/ml and endotoxin activity in filtered, sterile supernatants was 1000 ng/ml.

† No growth.

Table 2. *Effect of ampicillin or polymyxin B alone or in combination on colony count and release of endotoxin from Hib broth cultures**

	Time of incubation after addition of antibiotics			
	2 h		6 h	
	Colony count (cfu/ml)	Endotoxin activity (ng/ml)	Colony count (cfu/ml)	Endotoxin activity (ng/ml)
Control incubated at 35° C	4.0×10^9	4,000	5.0×10^9	7,500
Ampicillin 100 µg/ml	4.4×10^7	20,000	5.9×10^6	30,000
Polymyxin B 0.1 µg/ml	3.1×10^9	5,000	4.9×10^9	3,000
0.01 µg/ml	3.8×10^9	5,500	5.6×10^9	5,500
0.001 µg/ml	3.2×10^9	6,000	7.2×10^9	4,500
Ampicillin 100 µg/ml + Polymyxin B 0.1 µg/ml	8.7×10^6	15,000	3.5×10^6	20,000

* Results are the means of two separate experiments. Broths were incubated for 2 h prior to the addition of antibiotics at which time the colony counts were 7.7×10^8 cfu/ml and endotoxin activity in filtered, sterile supernatants was 3000 ng/ml.

cultures were obtained from tail veins at 8 and between 18 and 20 h after infection in a random sample of 6 ampicillin and 14 polymyxin B-treated animals. Mortality was monitored every 3 h for 27 h after infection was initiated. At 5 h after initiation of treatment no growth was noted from cultures plated at a 10^{-5} dilution in ampicillin-treated animals, whereas in the polymyxin B group all blood cultures were positive containing from 2×10^6 to 7×10^8 cfu/ml ($p = 0.0001$). Eighteen to 20 h after infection, all 12 animals in the ampicillin group remained alive, whereas seven of 20 animals in the polymyxin B group had died ($p = 0.05$). Survivors that had sequential blood cultures performed in the polymyxin B group ($n = 9$) continued to have positive blood cultures with bacterial concentrations ranging from 1×10^7 to 6.4×10^8 cfu/ml. In contrast, only two of six sequentially sampled animals in the ampicillin group had positive blood cultures (2 and 7×10^6 cfu/ml) ($p = 0.048$).

These results confirmed that a 0.5 mg/kg dose of ampicillin administered three times every three hours was protective if initiated within 3 h of infection. In contrast, a low dose (0.0125 mg/kg) of polymyxin B was not effective in preventing bacteremia and one-third of these animals died.

Efficacy of Ampicillin or subinhibitory polymyxin B on survival when treatment was delayed for 12 h after infection. Infant rats were infected ip with 4.7×10^6 HibE (range 1.6 to 7×10^6 cfu). All inoculations were performed at 2100 h. Twelve h after infection was initiated, surviving animals were weighed and randomly assigned to antimicrobial treatment groups. Care was taken to assign approximately equal numbers of infant rats in

each litter to two or three treatment modalities to control for possible differences related to maternal protective factors, *ie.* milk or nursing behavior. Antimicrobial treatment consisted of one of the following five regimens: PBS (16 animals), ampicillin at 0.5 mg/kg/dose (low dose, 30 animals) or at 500 mg/kg/dose (high dose, 45 animals), polymyxin B at 0.0125 mg/kg/dose (17 animals), or a combination of high dose ampicillin plus polymyxin B at 0.0125 mg/kg/dose (27 animals). Antibiotics were first administered 12 h after the ip injection of HibE and were given three times at three hour intervals. Antibiotics were diluted in PBS such that a volume of 0.1 ml/10 g body weight resulted in the appropriate dose in mg/kg. Intraperitoneal antibiotic injections were alternated between the right and left sides. Deaths were recorded hourly for 23 h and again at 36 h after infection.

The mortality of untreated animals 12 h after ip infection was 18.3%, confirming the advanced state of infection prior to institution of therapy. The observed death rates at 17, 20, and 36 h after infection in all five treatment groups are shown in Table 3. Polymyxin B alone at 0.0125 mg/kg/dose every 3 h resulted in a significantly greater survival at 17 h ($p = 0.009$) and 20 h ($p = 0.003$) after infection compared to ampicillin at a 0.5 mg/kg/dose given at the same time and interval. The addition of ampicillin at 500 mg/kg/dose to the same polymyxin B regimen did not result in further improvements of survival but the combination was superior to ampicillin (500 mg/kg) alone at 36 h after infection ($p = 0.026$).

Colony counts and free endotoxin activity after antibiotic administration. The effects of the antibiotics on bacterial counts

Table 3. A. Effect of antimicrobial therapy on mortality of overwhelming *Hib* infection in infant rats at 17 h after treatment

Antibiotic (dose mg/kg)	(n)	Alive/dead	% Alive	Time after infection (h)
Control (PBS)	(16)	8/8	50	17
Ampicillin (0.5)	(30)	8/22	27	17
Ampicillin (500)	(45)	21/24	47	17
Polymyxin B (0.0125)*	(17)	12/5	71	17
Polymyxin B (0.0125)† and ampicillin (500)	(27)	17/10	63	17

* $p = 0.009$, polymyxin B alone compared to ampicillin 0.5 mg/kg alone.

† $p = 0.01$, polymyxin B and ampicillin (500 mg/kg) versus ampicillin (0.5 mg/kg).

Table 3. B. Effect of antimicrobial therapy on mortality of overwhelming *Hib* infection in infant rats at 20 h after treatment

Antibiotic (dose mg/kg)	(n)	Alive/dead	% Alive	Time after infection (h)
Control (PBS)	(16)	4/12	25	20
Ampicillin (0.5)	(30)	5/25	17	20
Ampicillin (500)	(45)	16/29	36	20
Polymyxin B (0.0125)*	(17)	11/6	65	20
Polymyxin B (0.0125)† and ampicillin (500)	(27)	14/13	52	20

* $p = 0.003$, polymyxin B alone compared to ampicillin 0.5 mg/kg alone.

† $p = 0.01$ polymyxin B and ampicillin (500 mg/kg) versus ampicillin (0.5 mg/kg).

Table 3. C. Effect of antimicrobial therapy on mortality of overwhelming *Hib* infection in infant rats at 36 h after treatment

Antibiotic (dose mg/kg)	(n)	Alive/dead	% Alive	Time after infection (h)
Control (PBS)	(16)	3/13	19	36
Ampicillin (0.5)	(30)	4/26	14	36
Ampicillin (500)	(45)	9/36	20	36
Polymyxin B (0.0125)	(17)	6/11	36	36
Polymyxin B (0.0125)* and ampicillin (500)	(27)	13/14	49	36

* $p = 0.026$, polymyxin/ampicillin 500 mg/kg combination against ampicillin 500 mg/kg alone.

and the release of endotoxin were examined in animals receiving ampicillin 0.5 mg/kg and polymyxin B 0.0125 mg/kg. Quantitative bacterial and endotoxin concentrations were determined 2 h after the second dose of antibiotics in 11 surviving animals in each group. The results are shown in Table 4. Ampicillin-treated animals had a significantly decreased blood concentration of *HibE* compared to polymyxin B-treated animals (4×10^6 versus 288×10^6) ($p = 0.0007$). The endotoxin activity did not differ significantly.

In order to avoid the bias present due to preexisting treatment dependent mortality rates in this above mentioned group, bacterial quantitation and endotoxin activities were determined within 30 to 45 min after the administration of a single dose of antibiotic in a separate experiment. Seventeen animals were given 0.1 ml/10 g body weight of PBS, six received 500 mg/kg

ampicillin, and seven animals received 0.0125 mg/kg polymyxin B. Animals receiving high dose ampicillin had a significantly lower ($p = 0.023$) number of *HibE* in their blood than the PBS-treated animals, however, the endotoxin activity was significantly greater ($p = 0.006$) in the ampicillin-treated animals than in the PBS treated group (Table 5). The polymyxin B-treated group did not differ from the control or ampicillin groups in any of the parameters. Thus, *in vivo* endotoxin activity was found to be significantly elevated at 30 min after a dose of ampicillin compared with saline-treated animals.

DISCUSSION

Our study demonstrates that ampicillin causes the release of endotoxin from *HibE* *in vitro* and *in vivo*, in the infant rat. A very low dose of polymyxin B, a potent neutralizing agent of endotoxin, promoted the survival of infected animals, when compared to treatment with ampicillin alone. Improved survival was also apparent when polymyxin B was given in conjunction with ampicillin. The addition of ampicillin in this later combination did not enhance survival over polymyxin B alone during the first 11 h of treatment, but by eradicating the infecting organism, no further mortality was observed after its discontinuation. The protective action of polymyxin B became apparent in a clinically relevant animal model, *i.e.* initiation of treatment in an advanced state of septicemia.

The observed *in vitro* and *in vivo* release of endotoxin by ampicillin and *in vitro* release of endotoxin by chloramphenicol, both commonly used antimicrobial agents in the treatment of invasive *Hib* disease, could explain the sudden clinical deterioration seen in some patients following institution of antimicrobial therapy. Other investigators have demonstrated the release

Table 4. Colony counts and endotoxin activity in infant rat model of overwhelming *Hib* infection following two doses of low dose ampicillin or polymyxin B

Antibiotic (dose)	Colony Count (10^6 cfu/ml)		Endotoxin activity (ng/ml)	
	Mean	SD	Mean	SD
Ampicillin (0.5 mg/kg)* (n = 11)	4 ± 9.3	0–30	105 ± 132	0–384
Polymyxin B (0.0125 mg/kg)* (n = 11)	288 ± 413	1.9–1400	87 ± 80	0–230
	$p = 0.0007†$		$p = 0.34†$	

* Antibiotic therapy initiated 12 hours after infection. Two doses were administered 3 h apart. Values were determined 2 h after the second dose.

† p values by Wilcoxon Mann Whitney Rank Sum Test.

Table 5. Colony counts and endotoxin activity in infant rat model of overwhelming *Hib* infection following sham treatment, a single dose of polymyxin B or high dose ampicillin

Treatment (dose)	Colony Count (10^6 cfu/ml)		Endotoxin activity (ng/ml)	
	Mean	SD	Mean	SD
Control-PBS (0.1 mg/kg)* (n = 17)	210 ± 200	12–740	19 ± 20	0–70
Ampicillin (500 mg/kg)* (n = 6)	35 ± 49	0.4–131	57 ± 23	40–90
	$p = 0.023†$		$p = 0.006†$	
Polymyxin B (0.0125 mg/kg)* (n = 6)	218 ± 278	1–800	26 ± 37	0–100

* Dose given 12 h after intraperitoneal infection. Blood was obtained 30–45 min after administration.

† p values by Wilcoxon Mann Whitney Rank Sum Test, ampicillin versus control.

of endotoxin by antibiotics from *Neisseria meningitidis* and *E. coli* *in vitro* (19, 20) and from *E. coli* in infected rabbits (3). It is presently unknown if release of endotoxin either spontaneously, by the bactericidal action of serum, or by antibiotics is important in the pathogenesis or complications of human infections.

Antibodies directed against the core region of LPS in mice infected with *Haemophilus influenzae* type b (6) and in humans with gram negative sepsis (2) have been successful in improving survival. Polymyxin B binds and neutralizes endotoxin and appears to be an effective alternative to antibody to core LPS in neutralizing endotoxin (10, 15). Higher doses of polymyxin B than the ones used in our experiments were beneficial in experiments examining the mitigation of a range of biological actions due to purified endotoxin (8, 9, 11, 14, 16, 17, 24).

In two other studies polymyxin B was given after an *in vivo* infection had been established (21, 22). These investigators used polymyxin B resistant organisms in order to distinguish between antimicrobial and endotoxin neutralizing activity. In one study, adult mice were infected with a *N. meningitidis* strain resistant to polymyxin B. Treatment (5 mg/kg) was initiated 3 h after infection and in 800 animals polymyxin B resulted in increased survival compared to untreated animals ($p < 0.05$) (21). In the other study adult rabbits were infected with a polymyxin B-resistant *Pasteurella multocida* strain (22). Polymyxin B treatment (5 mg/kg) was initiated 6 h after infection and 10 untreated animals were compared. The polymyxin B-treated animals exhibited lower serum endotoxin concentrations, higher platelet counts, and equal degrees of bacteremia, but overall mortality compared to penicillin-treated animals (10,000 U/kg) was not lower. A penicillin-polymyxin B combination seemed to be superior but the difference was not significant ($p > 0.05$).

We were able to separate the antimicrobial properties from the endotoxin neutralizing action of polymyxin B by comparing an effective ampicillin regimen to a dose of polymyxin B with negligible *in vivo* antimicrobial activity. In addition, we chose a state of disease at which time otherwise effective antimicrobial treatment was no longer efficacious in terms of survival. This experimental design simulates more closely the clinical conditions found in septic shock due to gram-negative organisms. The low dose of polymyxin B that was used in this experiment might have been advantageous by not triggering the release of histamine (a side effect of polymyxin B), to which animals that have received endotoxin are even more susceptible (23).

As described by Corrigan and Kiernat (22) polymyxin B may have neutralized endotoxin in circulation as the mechanism for prolonging survival. However, we could not convincingly demonstrate this with our low dose of polymyxin B at either 30 min after administration (the time for distribution might have been too short) or at 120 min after two doses, when a substantial number of ampicillin-treated animals had died and were unavailable for valid comparison. Alternatively, polymyxin B might have neutralized some of the toxic effects of endotoxin which are not associated with decreased endotoxin activity as measured by the limulus lysate assay, *i.e.* polymyxin B inhibiting C-kinase activity (24). Increased survival seen with low doses of polymyxin B in a model of overwhelming infection with *Hib* may indicate the potential for such adjunct therapy for gram-negative infections in humans.

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REFERENCES

1. Luderitz O, Freudenberg MA, Galanos C, Lehman V, Rietschel ET, Shaw DW 1982. Lipopolysaccharides of gram-negative bacteria. In: Bronner F, Kleinzeller A (eds) Current Topics in Membranes and Transport, Vol 17. Academic Press, New York, pp 79-151
2. Ziegler EJ, McCutchan JA, Fierer J, Glauser MP, Sadoff JC, Douglas H, Braude AI 1982. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. *N Engl J Med* 307:1225-1230
3. Shenep JL, Mogan KA 1984. Kinetics of endotoxin release during antibiotic therapy for experimental gram-negative bacterial sepsis. *J Infect Dis* 150:380-387
4. Shenep JL, Barton RP, Mogan KA 1985. Role of antibiotic class in the rate of liberation of endotoxin during therapy for experimental gram-negative bacterial sepsis. *J Infect Dis* 151:1012-1018
5. Flesher AR, Insel RA 1978. Characterization of lipopolysaccharide of *Haemophilus influenzae*. *J Infect Dis* 138:719-730
6. Marks MI, Ziegler EJ, Douglas H, Corbell LB, Braude AI 1982. Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* lipopolysaccharide. *J Clin Invest* 69:742-749
7. Rifkind D, Palmer JD 1966. Neutralization of endotoxin toxicity in chick embryos by antibiotics. *J Bacteriol* 92:815-819
8. Rifkind D 1967. Prevention by polymyxin B of endotoxin lethality in mice. *J Bacteriol* 93:1463-1464
9. Corrigan JJ, Sieber OF, Ratajczak H, Bennet BB 1974. Modification of human neutrophil response to endotoxin with polymyxin B sulfate. *J Infect Dis* 130:384-387
10. Cooperstock MS 1974. Inactivation of endotoxin by polymyxin B. *Antimicrob Agents Chemother* 6:422-425
11. Craig WA, Turner JH, Kunin CM 1974. Prevention of the generalized Schwartzman reaction and endotoxin lethality by polymyxin B localized in tissues. *Infect Immun* 10:287-292
12. Palmer JD, Rifkind D 1974. Neutralization of the hemodynamic effects of endotoxin by polymyxin B. *Surg Gynecol Obstet* 138:755-759
13. Bannatyne BM, Harnett NM, K-Y, Biggar WD 1977. Inhibition of the biologic effects of endotoxin on neutrophils by polymyxin B sulfate. *J Infect Dis* 136:469-474
14. From AHL, Fong JSC, Good RA 1979. Polymyxin B sulfate modification of bacterial endotoxin: effects on the development of endotoxin shock in dogs. *Infect Immun* 23:660-664
15. Copperstock M, Riegle L 1981. Polymyxin B inactivation of lipopolysaccharide in vaccines of gram-negative bacteria. *Infect Immun* 33:315-318
16. Hughes B, Madan BR, Parratt JR 1981. Polymyxin B sulfate protects cats against the haemodynamic and metabolic effects of *E. coli* endotoxin. *Br J Pharmacol* 74:701-707
17. Friberger P, Knos M, Mellstam L 1982. A quantitative endotoxin assay utilizing LAL and a chromogenic substrate. In: Watson SW, Levin J, Novitsky TJ (eds) Endotoxins and Their Detection with the Limulus Amebocyte Lysate Test. Alan R. Liss, Inc., New York, pp 195-206
18. Johnson KG, Perry MB 1975. Improved technique for the preparation of bacterial lipopolysaccharides. *Can J Microbiol* 22:29-34
19. Anderson BM, Solberg O 1980. The endotoxin-liberating effect of antibiotics on meningococci *in vitro*. *Acta Pathol Microbiol Scand [B]* 88:231-236
20. Goto H, Nakamura S 1980. Liberation of endotoxin from *Escherichia coli* by addition of antibiotics. *J Exp Med [Jpn]* 50:35-43
21. Bannatyne RM, Harnett NM, Cheung R 1977. Protective effect of polymyxin B sulfate in experimental meningococcal infection in mice. *Can J Microbiol* 23:1526-1528
22. Corrigan JJ, Kiernat JF 1979. Effect of polymyxin B sulfate on endotoxin activity in a gram-negative septicemia model. *Pediatr Res* 13:48-51
23. Urbaschek B 1966. The histamine sensitizing effect of endotoxin. *Ann NY Acad Sci* 133:709-711
24. Nel AE, Wooten MW, Goldschmidt-Clermont PJ, Miller PJ, Stevenson HC, Galbraith RM 1985. Polymyxin B causes coordinate inhibitions of phorbol ester-induced C-kinase activity and proliferation of B lymphocytes. *Biochem Biophys Res Commun* 128:1364-1372