Adjunctive Treatment with Low-Dose Polymyxin B Improves Operant Response Rates and Latency in Infant Rats with *Haemophilus influenzae* Type b Meningitis¹

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ABSTRACT. Polymyxin B given in conjunction with ampicillin protects infant rats against death from overwhelming *Haemophilus influenzae* type b infection. This study was undertaken to examine whether polymyxin B would mitigate the effects of brain damage caused by meningitis. Six- to 7-d-old Sprague-Dawley rats were infected subcutaneously into the nape with 10^7 cfu *Haemophilus influenzae* type b strain Eagan. This dose consistently caused bacteremia (1.2×10^5 cfu/mL) and meningitis (0.5×10^5 cfu/mL) in pilot studies. Twenty-four h after infection, all animals received intraperitoneal treatment consisting in either ampicillin alone (400 mg/kg $\times$ 4 q 3 h) repeated 12 h later, $n = 15$ or combined with polymyxin B (0.1 mg/kg/dose) $n = 16$. At age 2 mo, they were taken off *ad libitum* feeding and maintained at 80% of their wt. They were then conditioned to receive a food pellet by pressing a lever (continuous reinforcement). The next day, the time lapse between placement and pressing the lever for the first time was recorded (conditioned operant response or latency). Three wk later, the animals were put in the test chamber again and the time to press the lever (latency), as well as the time required to obtain 100 pellets (rate) were recorded. Animals who received polymyxin B had a significantly shorter reaction time; mean 34 s, SEM $\pm$ 5.7 versus mean 88 s, SEM $\pm$ 26.3, $P \leq 0.05$ and performed significantly faster in obtaining 100 pellets; mean 925 s, SEM $\pm$ 72.1 versus mean 1283 s, SEM $\pm$ 126.3, $p \leq 0.02$ (analysis of variance, Scheffé test). Adjunctive antiinflammatory therapy with low dose polymyxin B appears to be a promising, inexpensive, and readily available mode of treatment for further study with eventual future application in the treatment of children with *H. influenzae* type b meningitis. (*Pediatr Res* 26: 496-499, 1989)

Approximately 18 000 pediatric cases of invasive infection with *Haemophilus influenzae* type b per year are recorded in the United States, and 900 to 1200 of these children die (1-3). Most children with *H. influenzae* type b meningitis are under 5 y of age and approximately 75% are less than 18 mo of age. These younger children cannot be protected by currently approved vaccines (3-5). Permanent sequelae from meningitis have been observed in one-third to one-half of survivors in the past (6, 7)

Received March 3, 1989; accepted July 7, 1989.

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¹ Presented in part as an oral presentation at the 28th ICAAC Meeting held in Los Angeles, CA (abstr 871).

and more recently in 8% with possibly lowered IQ scores in 28% (8). However, many of the children learn to compensate for their diminished intellectual capacity with additional help at school and at home (9).

Further advances in antimicrobial agents are unlikely to improve outcome. Efforts have therefore focused on modulating the possibly deleterious inflammatory response by the host (10).

The lipid A portion of lipopolysaccharide or endotoxin is postulated to contribute to the stimulation of such inflammatory reactions in *H. influenzae* type b infection and the exposure of gram-negative organisms to antimicrobials causes an additional release of cell wall components (11, 12). This might explain the development of endotoxic shock that develops after the administration of antibiotics in some cases of severe *H. influenzae* type b infection (13).

Approaches have been explored to neutralize endotoxin with specific antibodies (14), to block endotoxin receptors with the lipid A precursor, lipid X (15, 16), and to use dexamethasone as nonspecific antiinflammatory agent (10, 17). Another way is to aim for the chemical neutralization of endotoxin. An agent that is approved for use in humans, polymyxin B, was successful in this respect by decreasing mortality in infant rats when given concomitantly at low doses with antimicrobial treatment in overwhelming infection due to *H. influenzae* type b (18). The next study was designed to address the question of whether such an approach could improve the neurofunctional outcome.

MATERIALS AND METHODS

Experimental infection and treatment. The pups were born to time pregnant, specific pathogen-free outbred albino Sprague-Dawley rats obtained from Charles River Breeding Laboratories, Wilmington, MA 01887. The infection was set at age 6 to 7 d by injecting $\sim 10^7$ cfu of *H. influenzae* type b strain Eagan suspended in 0.1 mL PBS, 0.1% gelatin into the nape. The strain, kindly provided by Dr. T. J. Inzana (Baylor College of Medicine, Houston, TX), was grown to log phase and washed as previously described (18).

Its minimal inhibitory concentrations for ampicillin and polymyxin B were 0.1 and 0.003 μ g/mL, respectively. Ampicillin and polymyxin B did not have a synergistic effect *in vitro* (18). The theoretical possibility, that subinhibitory concentrations of polymyxin B might lead to a more rapid cidal action by ampicillin without influencing the mic of ampicillin was considered to be unlikely.

Twenty-four h after injection, all animals received 400 mg/kg/dose ampicillin (polycillin-N, Bristol Meyers, Syracuse, NY) injections intraperitoneally. This dose was diluted in 0.15 mL of NS and given at a q 3 h $\times$ 4 schedule which was repeated 12 h

later. The adjunctive treatment group (about half of all animals in each litter, randomly selected before infection) received 0.1 mg/kg/dose of polymyxin B sulfate (Aerosporin, Burroughs-Wellcome, Research Triangle Park, NC) mixed with ampicillin at the same dose, route, volume, and schedule described above. This dose of ampicillin is 120 times higher than the one of polymyxin B in relation to the MIC's of the organism.

Control studies done before the experiment showed that the 10^7 cfu inoculum had produced bacteremia in all of six pups when cultured 24 h after inoculation (mean 1.2×10^5 cfu/mL, median 1.6×10^5 , range $0.3-2 \times 10^5$) and meningitis (mean 0.5×10^5 cfu/mL, median 1.3×10^5 , range $0.09-2 \times 10^5$). The treatment schedule of q 3 h $\times$ 4 with and without polymyxin B added, sterilized blood, and CSF in another nine control animals cultured 48 h after treatment, confirmed previous experience with a similar schedule (18). A tenth animal was found to have one colony on a plate inoculated with undiluted CSF. Because of this one animal, we chose to repeat the treatment schedule after a 12-h interval in all study animals. The study animals that received the tested 10^7 cfu inoculum and treatment schedules were not cultured because the cardiac puncture and dissection to obtain CSF from the cisterna magna (under pentobarbital) were considered too invasive and likely to introduce additional variables.

A noninfected control group was not included in the study, because it seemed difficult to rule out spread of subclinical infection within a litter containing infected pups. Furthermore, the competition of these healthy animals for maternal care could have influenced our results. A single litter was infected each month to allow for sufficient time to condition the individual animals as described below.

Training and later performance tests. At the time of weaning, the animals were put in individual cages marked with a random number. The individual color markers for the two treatment groups became undetectable after a period of 2 wk due to shedding and growth of new hairs. This allowed the blinding with respect to previous treatment of the one person who performed and recorded the training and memory performance tests (R.D.R.). When the animals reached 2 mo of age $\pm$ 1 wk, their previously free food supply (Rodent Blox, Wayne Feeds, Chicago, IL 60606) was controlled to reduce their wt to a steady level of 80%. Animals were then placed in an operant conditioning cage (G 2150 Unit, Gerbrands Corp., Arlington, MA) with a provision to deliver food pellets (P.J. Noyes Corp., Lancaster, NH) by pressing a lever. On the first day, the animals were placed in the cage for 40 min for habituation. This was in a room with a low noise level and dim light. On the second day, the animals were fed a food pellet via a remote control switch each time they came close to or touched the lever (continuous

reinforcement). The magazine that delivered a food pellet produced an auditory click. A flash of light was also produced on the equipment control panel with each lever press. This was continued until each animal had learned to press the lever 100 times (acquisition). The time to obtain the first pellet (latency) was recorded for each subject 24 h later (conditioned operant response). Then 3 wk later, the animals were individually placed in the same environment with automatic delivery of a food pellet whenever the lever was pressed. The time until the first press (latency) and the time to obtain 100 pellets (rate) was recorded. The animals' wt at the time of latency testing ranged from 149 to 352 g with a mean of 242.6 in the ampicillin/polymyxin B treated group and from 152 to 349 g with a mean of 212.2 in the group treated with ampicillin alone ($p = 0.24$ by two-tailed Student's t test). Males and females were equally distributed in both treatment groups. A total of 31 animals underwent the operant conditioning with 16 of them having received ampicillin with polymyxin B and 15 ampicillin alone. Seven animals receiving ampicillin and polymyxin B and five animals receiving ampicillin alone belonged to a pilot study. Data analyses were performed both including and excluding this latter group.

Statistical analysis. Analysis of variance was used with the factor treatment at two levels (ampicillin alone, ampicillin with adjunctive polymyxin B) for the three response measures; latency on acquisition and latency and rate on retesting. Pairwise comparisons between favor levels were conducted by the Scheffé test (19).

RESULTS

Animals treated with adjunctive, low dose polymyxin B had significantly shorter latencies and significantly faster rates of operant responding on retesting at three weeks compared to animals in the control group.

No significant difference was found for latency 24 h before training. The statistics describing the responses are given in Table 1. All observations from the retesting sessions that were made 3 wk after training are depicted in Figures 1 and 2. The p values for the significant differences were $p \leq 0.012$, $p \leq 0.048$ for latency and $p \leq 0.02$, $p \leq 0.02$ for rate. The calculation of the last number includes observations from the pilot study.

DISCUSSION

Results of this study suggest that the effects of adjunctive low-dose treatment with the endotoxin neutralizing agent polymyxin B go beyond an increase in survival (18). The choice to investigate low-dose polymyxin B for meningitis, despite its poor penetration into the CSF, was based on speculations on findings in the

Table 1. Results and statistical analysis for three time variables using ANOVA and Scheffé test*

	Training session		Retesting session			
	Latency 24 h after training (s)		Latency at 3 wk (s)		Rate at 3 wk (s)	
	AMP	AMP + PMB	AMP	AMP + PMB	AMP	AMP + PMB
$n = 10/9$						
Mean $\pm$ SEM	986.6 $\pm$ 596.3	107.0 $\pm$ 32.6	55.2 $\pm$ 9.5	25.0 $\pm$ 4.3	1101.7 $\pm$ 87.3	816.6 $\pm$ 66.6
Range	15-6037	24-326	18-107	11-43	810-1593	596-1078
	$p \geq 0.05$		$p \leq 0.012$		$p \leq 0.02$	
All animals (incl. pilot)						
$n = 15/16$						
Mean $\pm$ SEM	739.7 $\pm$ 402.2	786.6 $\pm$ 655.5†	88.3 $\pm$ 26.3	34.6 $\pm$ 5.7	1283.2 $\pm$ 126.3‡	925.8 $\pm$ 72.1‡
Range	15-6037	24-9300	18-435	11-91	810-2785	596-2550
	$p \geq 0.05$		$p \leq 0.048$		$p \leq 0.02$	

* AMP, ampicillin; PMB, polymyxin B.

† Two observations in the pilot group (polymyxin B) were not available.

‡ One high outlier from each treatment group was excluded.

Fig. 1. Times (s) it took animals in the two groups to press the lever the first time during retest session (latency). I, mean and SEM (all observations).

Fig. 2. Times (s) it took animals in the two groups to obtain 100 pellets by pressing the lever during retest session (rate). I, mean and SEM (all observations).

literature suggesting that part of the pathophysiologic process in bacterial meningitis takes place at the vascular level (20–23). Furthermore, we believed that a high and CSF-penetrating dose of polymyxin B (1.5 mg/kg), which has been shown to decrease brain edema and endotoxin activity in rabbits infected with *Escherichia coli* (24), might expose humans to unwanted toxicity (25).

Endotoxin is known to exert a direct detrimental effect on endothelial cells (26–29), which also is mediated through neutrophils (30) and oxidized low-density lipoprotein-endotoxin complexes in humans (31). It causes platelet reactions that in turn damage the endothelium directly (32–36), or the platelets might fail their normal function to maintain endothelial integrity (37).

Numerous studies have documented the mitigating effects of polymyxin B on a wide variety of *in vitro* and *in vivo* reactions to endotoxin. These are thought to be due to neutralization by

binding of polymyxin B to endotoxin molecules. Not all endotoxins and endotoxin preparations although are equally susceptible. Membrane material with endotoxin activity released from *H. influenzae* type B strain Eagan was shown to be neutralized when tested with a chromogenic Limulus lysate assay (18). Less well-characterized effects of polymyxin B on cell membranes and perturbation of signals leading to protein kinase-C activation (38) are potential alternative explanations for the biologic effects of polymyxin B.

Most of the relevant studies describing neutralization are cited in the discussions by Flynn (39) and Walterspiel (18). The literature seems more notable for the paucity of reports describing failure with polymyxin B under various experimental conditions than the many descriptions of its mitigating effects. We did not aim at exploring a specific mechanism of action, but to detect an overall beneficial effect on an observable behavioral function in rats.

The infant rat meningitis model was chosen, because it most closely mimics the histopathology seen in humans (40–42) with documented deficiencies in conditioned behavior of infected animals (40, 43). Lower rates of operant behavior under schedules of continuous reinforcement were found among rats infected at 5 d of age when this model was first described (40). Reinforcement by water was used in that study as compared to the food reinforcement in our study. In another study, active and passive avoidance tasks showed significant impairment in rats inoculated at 6 d of age with a capsulated strain of *H. influenzae*, compared to those pups who were given a nonvirulent, uncapsulated strain (43). The *H. influenzae* infected group also showed increased locomotor activity during habituation to the conditioning chamber (43).

The present observation suggests that adjunctive treatment with polymyxin B in Sprague-Dawley pups resulted in decreased latency and increased rate of operant responding, as adults. These differences were seen during retesting 3 wk after training and can be interpreted as improved learning during the first test for latency and/or better recall. Our model studied the effect of polymyxin B on one rat behavior and maintenance only. Future studies comparing a wider set of observable behaviors might point toward areas of maximum, no, or even detrimental effects of combined therapy.

Adjunctive antiinflammatory therapy with low dose polymyxin B appears to be a promising, inexpensive, and readily available mode of treatment. It might be suitable for more detailed study not only with the goal to decrease mortality from gram-negative sepsis, but also to diminish neurobehavioral sequelae in children with *H. influenzae* type b meningitis.

Acknowledgment. The authors thank Robert D. Ronnlund for expert technical assistance and patience observing the animals and Mae Jean Reeves for typing the manuscript.

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