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Prevention of the Generalized Shwartzman Reaction and Endotoxin Lethality by Polymyxin B Localized in Tissues

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Pretreatment with multiple doses of polymyxin B and colistimethate was evaluated as to its ability to sequester sufficient antibiotic in tissues to neutralize the effects of endotoxin in three animal models. Animals were challenged with endotoxin 24, 48, or 72 h after the last dose of antibiotic when there was minimal or not detectable drug in serum. Pretreatment with polymyxin B was successful in preventing the generalized Shwartzman reaction in rabbits and reducing endotoxin lethality in mice; however, large doses (20 mg per kg per day for 2 or 4 days) were required. Prolongation by more than 24 h of the interval between the last dose of polymyxin B and endotoxin challenge resulted in reduction or loss of protection. Dogs were unable to tolerate the high polymyxin B dosage which was protective in the mouse and rabbit. Lower, nontoxic doses of polymyxin B in dogs did not prevent endotoxin shock and lethality, even when challenged as soon as 1 h after the last dose. Pretreatment with colistimethate was ineffective in all three animal models.

Gram-negative bacteremia is frequently complicated by shock and intravascular coagulation. Prevention of these complications would significantly reduce the high mortality associated with these infections. Since bacterial endotoxin has been chiefly implicated in producing these complications, numerous attempts have been made to prevent or modify the toxic effect of endotoxin in various animal models. Polymyxin B sulfate administered simultaneously with endotoxin has been shown to reduce endotoxin lethality in chicken embryos (7, 15) and adrenalectomized mice (13) and to prevent the generalized Shwartzman reaction in rabbits (5, 14). Sodium colistimethate has similar activity but is less effective than polymyxin B (4, 15).

Previous studies in this laboratory have demonstrated that the polymyxin group of antibiotics bind to mammalian tissues (8). Accumulation of drug in tissues, but not in serum, occurs when repeated injections are given at dosage levels not associated with toxicity (6, 9). The studies reported here were performed to determine whether polymyxin B and colistimethate, restricted almost entirely to tissues, would also prevent endotoxin toxicity in several animal models.

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MATERIALS AND METHODS

Endotoxin. *Escherichia coli* 0127:B8 lipopolysaccharide, Boivin type (Difco), was used in all studies. It was prepared fresh before each study in pyrogen-free physiological saline solution.

Antibiotics. Polymyxin B sulfate (Aerosporin, Burroughs-Wellcome and Co.) and sodium colistimethate (Colymycin M, Warner-Chilcott) were prepared at various concentrations in pyrogen-free physiological saline solution.

Generalized Shwartzman reaction. New Zealand white rabbits weighing 1 to 1.5 kg were pretreated intramuscularly with polymyxin B at 5 or 10 mg per kg of body weight twice daily for 4 days. Colistimethate was likewise administered twice daily at 10 or 20 mg per kg of body weight. Twenty-four or 48 h after the last dose of antibiotic, pretreated rabbits and a comparable number of controls received two intravenous injections of endotoxin (0.05 mg/kg) spaced 24 h apart. Survivors were killed by exsanguination 24 h after the second injection of endotoxin. The renal Shwartzman reaction (acute cortical necrosis) was usually evident on gross inspection, but all kidneys were studied microscopically, under code, by a pathologist.

Endotoxin mouse lethality. ARS HA/ICR albino mice weighing 25 to 30 g were pretreated subcutaneously once daily for 2 or 4 days with polymyxin B at 250 or 500 μg per mouse (approximately 10 and 20 mg

per kg of body weight). Colistimethate was likewise administered once daily for 4 days at 1,000 μg per mouse (approximately 40 mg per kg of body weight). Twenty-four, 48, or 72 h after the last dose of antibiotic, groups of pretreated mice and a comparable number of controls were challenged intravenously with various concentrations of endotoxin ranging from 200 to 1,200 μg per mouse. Animals were observed for 72 h, and the percent mortality was determined for each concentration of endotoxin.

Canine endotoxin shock and lethality. Mongrel dogs weighing 9.0 to 15.1 kg were used for these studies. Initially dogs were studied in pairs; by random choice one was pretreated intramuscularly with polymyxin B at 2.5 or 5 mg/kg twice daily for 4 days. Twenty-four hours after the last dose of antibiotic, the pretreated and untreated dogs were anesthetized with pentobarbital (25 to 30 mg/kg). The femoral artery and vein were cannulated for pressure monitoring, fluid replacement, and blood sampling. After a short control period, both dogs were given a single bolus of endotoxin at 2 mg/kg (approximately 1.5 mean lethal doses [LD_{50}]) into the inferior vena cava.

In later studies the protocol was simplified by using awake dogs without pressure monitoring. These dogs were pretreated intramuscularly with polymyxin B at 2.5 mg/kg twice daily for 4 days. Colistimethate was likewise administered at 10 mg/kg twice daily for 4 days. At 1 or 24 h after the last dose of antibiotic, pretreated dogs and a comparable number of controls received a single bolus of endotoxin at 2 mg/kg into a peripheral vein. Animals were observed for 72 h and mortality was recorded.

Antibiotic concentrations in tissues and serum. Pairs of rabbits and five to eight mice pretreated at similar dosage schedules of polymyxin B and colistimethate were killed by exsanguination at 24, 48, and 72 h after the last dose of drug for measurement of antibiotic concentrations in serum and several tissues (liver, kidney, and muscle). In addition, the majority of rabbits and all dogs had blood samples obtained from an ear vein and peripheral vein, respectively, just before endotoxin challenge.

Serum was separated and stored at −20 C until assayed. Tissues were freshly prepared as 25% homogenates in water (wet weight) and stored at −20 C until assayed. Antibiotic levels in serum and in tissues were assayed by the method previously reported by this laboratory (6, 10). All values were expressed in terms of micrograms per milliliter of serum or micrograms per gram (wet weight) of tissue. Blood samples were also analyzed routinely for electrolytes, glucose, cholesterol, blood urea nitrogen, creatinine, uric acid, lactic dehydrogenase, and glutamic oxaloacetic transaminase with an SMA 1260 Auto-Analyzer (Technicon Corporation, Adsley, N.Y.).

RESULTS

Generalized Shwartzman reaction. The effect of pretreatment with polymyxin B and colistimethate on the development of the generalized Shwartzman reaction is shown in Table 1. Some animals died after the first injection of endotoxin, but in each case histological examination of the kidney revealed no evidence of acute cortical necrosis. Only those animals receiving two injections of endotoxin were included in statistical comparisons.

Pretreatment with polymyxin B at 10 mg/kg twice daily for 4 days significantly reduced the occurrence of the renal Shwartzman lesion from 42 to 11% when the initial endotoxin dose was administered 24 h after the last dose of antibiotic. Protection was lost if initial endotoxin challenge was delayed until 48 h after the last dose of antibiotic. Pretreatment for 4 days with colistimethate at both dosage levels and polymyxin B at a lower dosage level were ineffective in reducing the occurrence of the renal Shwartzman lesion.

Serum antibiotic levels were determined in a large number of rabbits just before the first dose of endotoxin to determine whether protection was due to circulating antibiotic. No antibiotic was detected in serum 24 or 48 h after colistimethate pretreatment. However, 64% of animals tested that received polymyxin B at 10 mg/kg twice daily for 4 days had measurable serum levels 24 h after the last dose (Fig. 1). Three of 16 rabbits with detectable blood levels of polymyxin B developed acute cortical necrosis, whereas only one of nine animals with undetectable serum concentrations developed the lesion. Although the number of animals with acute cortical necrosis was small, the presence of circulating polymyxin B did not correlate with protection. Serum levels of polymyxin B were consistently undetectable at 48 h and at the lower dosage level.

Endotoxin mouse lethality. Endotoxin dose-percent mortality curves were constructed for pretreated and control mice, with each point representing a group of at least eight mice. From these data, the LD_{50} and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (11) and are shown in Fig. 2. By the same method all endotoxin dose-percent mortality curves were shown to be relatively parallel. Pretreatment with polymyxin B at 500 μg per mouse per day for 4 days significantly raised the endotoxin LD_{50} from 327 to 698 μg per mouse when challenged 24 h after the last dose of antibiotic. Protection was less but still present 48 h after the last dose, but not at 72 h. Polymyxin B at 500 μg per mouse per day for only 2 days raised the endotoxin LD_{50} from 314 to 578 μg per mouse when challenged 24 h after the last dose. Protection was not present at 48 h. Four days of polymyxin B at 250 μg and colistimethate at 1,000 μg per mouse per day were both ineffective in preventing endotoxin

TABLE 1. *Effect of pretreatment for 4 days with polymyxin B and colistimethate on the generalized Shwartzman reaction (acute cortical necrosis) in rabbits*

Drug (dosage)[a]	Time[b] of endotoxin challenge	Pretreated			Control			
		No.[c]	No. with ACN[d]	% with ACN	No.	No. with ACN	% with ACN	*P* value[e]
Polymyxin B (10 mg/kg)	24	36	4	11.1	31	13	41.9	<0.01
	48	34	14	41.1	33	19	57.5	NS[f]
Polymyxin B (5 mg/kg)	24	35	13	37.1	36	16	44.4	NS
Colistimethate (20 mg/kg)	24	33	10	30.3	32	13	40.6	NS
Colistimethate (10 mg/kg)	24	17	8	47.1	12	6	50.0	NS

[a] All doses were given twice daily.
[b] Hours between the last dose of antibiotic and the first dose of endotoxin.
[c] Number of animals receiving both injections of endotoxin.
[d] ACN, Acute cortical necrosis.
[e] Determined by chi-square test with Yates correction.
[f] NS, not significant.

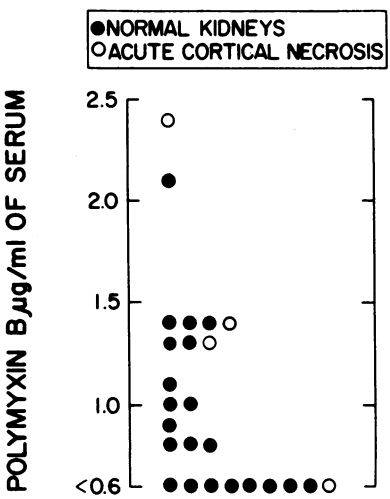

FIG. 1. *Relationship between the serum concentration of polymyxin B at the time of endotoxin challenge and the development of acute cortical necrosis.*

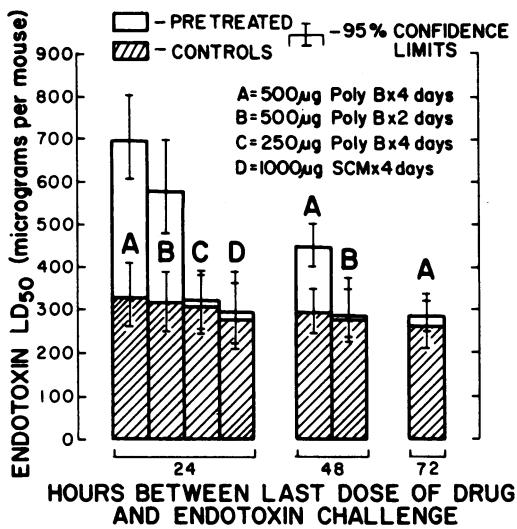

FIG. 2. *Effect of pretreatment of albino mice with different dosages of polymyxin B (poly B) or colistimethate (SCM) on the LD_{50} of endotoxin administered 24, 48, or 72 h after the last dose of antibiotic.*

lethality. Although it was not possible to measure serum levels in all mice, antibiotic was undetectable in the serum of the 38 mice sacrificed for tissue studies at 24, 48, and 72 h after the last dose of antibiotic.

Canine endotoxin shock and lethality. Endotoxin challenge at 2 mg/kg was lethal to all 23 control dogs. Only three animals were studied after pretreatment with polymyxin B at 5 mg/kg twice daily for 4 days. All these animals had elevated levels of blood urea nitrogen and creatinine at the time of endotoxin challenge. A fourth animal died 6 h after the last dose of antibiotic. Pretreatment was ineffective in preventing endotoxin lethality in these animals.

Pretreatment of 16 dogs with polymyxin B at 2.5 mg/kg twice daily for 4 days did not produce clinical or laboratory evidence of toxicity. Endotoxin challege 24 h after the last dose of antibiotic was lethal to all four anesthetized dogs and four of five awake dogs. Furthermore, there was no difference in the arterial blood pressure response of the anesthetized pretreated and control animals after endotoxin administration. Both groups of four animals demonstrated an immediate severe drop in pressure followed by recovery in a few minutes. This was followed in 1 to 2 h by a gradual but progressive drop in pressure until the animal died. Polymyxin B pretreatment did not prevent mortality in six of seven awake dogs that were challenged with endotoxin as soon 1 h after the last dose of

antibiotic. Serum concentrations of polymyxin B at the time of endotoxin administration ranged from 10.8 to 19.4 μg/ml.

Colistimethate at 10 mg/kg twice daily for 4 days was likewise nontoxic to the four animals studied. Pretreatment with this drug was also ineffective in preventing lethality when endotoxin was administered 24 h after the last dose of antibiotic.

Antibiotic tissue levels in rabbits and mice. The concentrations of polymyxin B and colistimethate recovered as free and bound drug from three representative rabbit and mouse tissues at 24, 48, and 72 h after various dosage schedules are shown in Tables 2 and 3. Pretreatment with colistimethate, which was not protective in both animal models, produced tissue levels of bound drug that were higher than or equivalent to concentrations of polymyxin B associated with significant protection. A large percentage of polymyxin B in tissue existed as free, unbound drug. In contrast, only a small percentage of colistimethate was present as free drug.

DISCUSSION

The ability of polymyxin B to neutralize the effects of endotoxin in various animal models has been well established (4, 5, 7, 13-15). Recently, Sieber and associates have presented data that suggest that polymyxin B will also reduce endotoxin-induced neutropenia in humans (17). The major problem with all of these studies is that polymyxin B had to be administered simultaneously with endotoxin in order to prevent toxicity. Delay in the administration of polymyxin B has resulted in loss of protection (5, 7), probably because endotoxin is rapidly cleared from the circulation into tissues (2, 3). This suggests that the polymyxins will have minimal therapeutic application in patients already undergoing the effects of endotoxin.

The prophylactic use of polymyxin B, however, has not been adequately evaluated. In chicken embryos, polymyxin B administered within 6 h before endotoxin challenge prevented lethality; challenge with endotoxin 24 h after polymyxin B administration was not protective (7). In the present study an attempt was made to take advantage of the observation that the polymyxins accumulate in tissues when given in repeated doses. Neutralization of endotoxin by tissue-associated drug would provide a rational basis for prophylaxis.

Although this approach proved successful in

TABLE 2. *Concentrations of bound and free drug recovered from rabbit tissues at 24, 48 or 72 h after pretreatment with polymyxin B or colistimethate*

Drug[b]	Hours after last dose	Bound drug (μg/g)			Free drug (μg/g)		
		Liver	Kidney	Muscle	Liver	Kidney	Muscle
Polymyxin B	24	45.2	64.9	45.6	33.6	39.9	118.0
	48	39.6	33.1	38.6	33.2	40.0	97.1
	72	29.2	27.4	19.2	19.6	28.0	24.4
Colistimethate	24	107.0	53.5	106.5	8.3	6.9	8.4
	48	53.0	26.4	53.0	6.8	5.8	6.9

[a] Values represent average of two animals.
[b] Doses of 10 mg/kg were given twice daily for 4 days.

TABLE 3. *Concentrations of bound and free drug recovered from mouse tissue[a] at 24, 48 or 72 h after pretreatment with polymyxin B and colistimethate at different dosages*

Drug (dosage)	Hours after last dose	Bound drug (μg/g)			Free drug (μg/g)		
		Liver	Kidney	Muscle	Liver	Kidney	Muscle
Polymyxin B (500 μg daily for 4 days)	24	38.5	34.3	48.7	29.1	20.0	56.9
	48	32.0	24.1	29.0	15.0	18.4	37.0
	72	16.6	10.6	13.3	8.8	7.2	14.0
Polymyxin B (500 μg daily for 2 days)	24	32.8	23.7	29.7	28.0	19.5	34.9
	48	16.0	14.1	24.3	9.4	12.0	18.4
Polymyxin B (250 μg daily for 4 days)	24	14.7	11.9	16.3	14.0	8.4	24.4
Colistimethate (1,000 μg daily for 4 days	24	46.2	39.6	57.2	8.4	7.0	10.2

[a] Values represent average of duplicate determinations on tissues pooled from five to eight animals.

the rabbit and mouse models, extremely large doses of polymyxin B were required to provide protective tissue concentrations lasting 24 to 48 h. For example, protection against the generalized Shwartzman reaction in rabbits and prevention of endotoxin lethality in mice both necessitated the daily administration of polymyxin B at 20 mg per kg of body weight. This is four times the currently recommended dose for humans. The inability of polymyxin B pretreatment to prevent canine mortality probably was due to the inability of the dog to tolerate high doses of polymyxin B without the relatively rapid development of toxicity.

Since the polymyxin antibiotics exist in tissues in both a bound and free form (9), protection against the effects of endotoxin demonstrated in these studies may be explained in two ways: (i) bound drug may prevent the attachment of endotoxin to specific cell membrane receptors; and (ii) free drug may directly interact with endotoxin, neutralizing its toxicity.

Lipopolysaccharide particles have been shown to attach to mammalian membranes (16), and Springer and associates have actually isolated an endotoxin receptor from the human red-cell membrane (18). Basic proteins inhibit the binding of endotoxin to this receptor, and it is possible that polymyxins may also bind to the receptor.

Bader and Teuber have recently shown that polymyxin B forms complexes with endotoxin by electrostatic and possibly hydrophobic interactions (1). The negatively charged ketodeoxyoctonate-lipid A region of the molecule appears to be the binding site for polymyxin B. Lipid A has also been shown to be the biologically active component of endotoxin (12).

Colistimethate was consistently ineffective in preventing endotoxin toxicity in the three models studied, even though tissue concentrations of bound colistimethate and polymyxin B were very similar. The ability of these drugs to bind to tissue cell membranes cannot be extrapolated to binding at the sites of action of endotoxin which are still unknown. In contrast to polymyxin B, only a small percentage of colistimethate was recovered from tissues as free, unbound drug. This suggests that protection may be dependent on the amount of free drug in tissue. The ineffectiveness of colistimethate, however, could also be explained by a lower binding affinity for endotoxin or by binding to tissue receptors separate from those for polymyxin B.

Because of the possible importance of free drug, dogs were also challenged with endotoxin 1 h after the last pretreatment dose of polymyxin B. At that time free drug was present in both tissues and plasma. However, this regimen, at the dosage used, was also ineffective.

It seems reasonable to conclude from the results of this and other studies that the potential value of the polymyxins in treatment or prophylaxis of endotoxemia in man is limited by problems of dosage, toxicity, and timing. It would seem more appropriate to explore the use of less toxic substances such as human antiserum to endotoxin (19).

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