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Antibacterial Synergism of Polymyxin B Nonapeptide and Hydrophobic Antibiotics in Experimental Gram-Negative Infections in Mice

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Polymyxin B nonapeptide, derived by cleavage of the fatty acyl diaminobutyric acid from polymyxin B, is considerably less toxic, lacks bactericidal activity, and retains its ability to render gram-negative bacteria susceptible to several antibiotics by permeabilizing their outer membranes. The peptide rendered all 53 polymyxin-susceptible strains tested more susceptible to novobiocin, lowering the MIC of novobiocin eightfold or more. The combination of polymyxin B nonapeptide with novobiocin or with erythromycin administered intraperitoneally in multiple doses synergistically protected mice infected with gram-negative bacteria. This combination may be clinically useful because of the apparent rarity of the acquisition of resistance.

Resistance to hydrophobic antibiotics in gram-negative bacteria may be either due to a decrease in the penetration of the antibiotic through the outer membrane or due to specific mechanisms, such as gene mutation or acquisition of resistance genes. The distinction between the two types of resistance is important, because it allows the search for antibiotics to which the development of specific mechanisms of resistance are rare but to which the bacterial membrane is impermeable. Such active but nonpenetrating antibiotics may gain clinical use when combined with membrane-active agents that increase their penetration. Polycations at sublethal concentrations increase the permeability of the outer membranes of gram-negative bacteria and render these microorganisms susceptible to many hydrophobic antibiotics (15–18). Among the various polycations tested, polymyxin B nonapeptide (PMBN), derived by cleaving the fatty acid moiety from polymyxin B, has been studied the most. PMBN lacks antibacterial activity but renders most polymyxin-susceptible gram-negative bacteria susceptible to hydrophobic antibiotics by disorganizing the bacterial outer membranes to enhance antibiotic penetration (17–19). In the present study, we sought to further determine the incidence of strains that exhibit permeability-associated resistance in assays with PMBN and to examine the therapeutic efficacy of hydrophobic antibiotics in combination with PMBN in experimental gram-negative infections in mice.

All clinical isolates were obtained from various body sites at regional outpatient clinics (see Table 2). The bacteria were grown on agar slants (nutrient agar; Difco Laboratories, Detroit, Mich.) and kept at 4°C for up to 2 weeks. The MICs of each antibiotic were determined as described elsewhere (7). In brief, 10 μl of a bacterial suspension (approximately 10^5 CFU) from an overnight culture in isotonic Sensitest broth (Oxoid) was inoculated into microtiter plate wells each containing 100 μl of a serial twofold dilution of the test antibiotic(s) in broth. Each test was done in triplicate, and the MIC was defined as the concentration at which there was no visible growth. ICR female mice were infected intraperitoneally with 0.5 ml of phosphate-buffered saline (0.1 M NaCl, 0.02 M PO_4; pH 7.2) containing either 5×10^5 CFU of *Klebsiella pneumoniae* serotype K2 (1) harvested from the logarithmic phase of growth in nutrient broth (Difco) at 37°C or 1×10^8 CFU of *Pseudomonas aeruginosa* 33347 (kindly provided by Nehama Garber, Bar-Ilan University, Ramat-Gan, Israel) harvested from agar slants after overnight growth at 37°C (8).

The efficacy of PMBN either alone or in combination with other antibiotics was evaluated following intraperitoneal administration of 0.5 ml of water containing various amounts of the antibiotics to groups of 6 to 20 mice at the desired time intervals after bacterial challenge. The number of dead mice was counted 1 week later. The 50% lethal doses of the bacteria in mice were determined as described previously (10) to be 2×10^5 and 5×10^7 CFU for *K. pneumoniae* and *P. aeruginosa*, respectively.

PMBN rendered all polymyxin-susceptible strains susceptible to six different antibiotics tested, except for one *P. aeruginosa* strain resistant to nafcillin and one *Escherichia coli* and two *K. pneumoniae* strains resistant to vancomycin (Table 1). These resistant strains probably possess mechanisms of resistance unrelated to permeability. This presumption is strengthened by the inability of the deacylated peptide to lower the MIC of ampicillin against three penicillinase-producing strains.

Further studies focused on the ability of PMBN to reduce the MIC of novobiocin for 55 clinical isolates belonging to nine bacterial species and exhibiting multiple resistance patterns. PMBN caused a significant reduction in the MIC of novobiocin for all 53 polymyxin-susceptible strains tested, including 7 quinolone-resistant strains (Table 2). The MIC of norfloxacin for six quinolone-resistant strains was 600 μg/ml, and PMBN did not cause a significant reduction in this MIC (data not shown), suggesting that resistance to quinolones is probably due to mechanisms other than permeability. We conclude that resistance to novobiocin in gram-negative bacteria is probably solely due to the inability of the antibiotic to penetrate the outer membrane. In a separate study, we found that 30 μg of PMBN per ml reduced the MIC of novobiocin for *P. aeruginosa* (blood isolate) from 250 to 0.05 and 0.1 μg/ml in the presence of 10 and 5% normal human serum, respectively.

Previous studies showed that a single intravenous adminis-

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TABLE 1. Effect of PMBN on the MICs of antibiotics for 11 strains of gram-negative bacteria

Strain	Presence of PMBN[a] at 30 μg/ml	MIC (μg/ml) of[b]:					
		Ampicillin	Erythromycin	Lincomycin	Nafcillin	Novobiocin	Vancomycin
Escherichia coli							
EC1	−	>1,000	32	256	>1,000	256	128
	+	>1,000	1	2	128	0.1	8
EC2	−	NT[c]	64	256	256	128	128
	+	NT	1	8	64	2	32
EC3	−	NT	32	NT	>1,000	128	128
	+	NT	2	NT	256	8	64
EC4	−	NT	64	NT	>1,000	128	128
	+	NT	2	NT	16	2	32
Klebsiella pneumoniae							
KL1	−	>1,000	256	64	>1,000	256	512
	+	>1,000	32	2	256	0.1	256
KL2	−	NT	256	>1,000	>1,000	256	256
	+	NT	16	2	256	16	256
Pseudomonas aeruginosa							
PS1	−	>1,000	512	>1,000	>1,000	>1,000	>1,000
	+	512	1	2	16	0.1	2
PS2	−	NT	16	>1,000	512	512	512
	+	NT	2	16	256	2	32
PS3	−	NT	512	>1,000	>1,000	>1,000	>1,000
	+	NT	0.5	64	64	8	0.1
PS4	−	NT	NT	NT	>1,000	>1,000	512
	+	NT	NT	NT	1	2	16
Salmonella typhimurium ST1	−	NT	256	256	>1,000	512	256
	+	NT	16	4	128	4	64

[a] PMBN was prepared by enzymatic deacylation of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) as described previously (5, 18) with either ficin in buffer or papain in distilled water. Identical results were obtained with PMBN prepared by hydrolysis with papain in distilled water. Both procedures yielded identical batches of PMBN exhibiting similar in vitro biological activities. The purity of the PMBN obtained by the two methods was ascertained by high-pressure liquid-gas chromatography as described previously (9).

[b] Antimicrobial powders for the determination of MICs were provided by Bristol Myers Co., Wallingford, Conn. (ampicillin); Abbott Laboratories, North Chicago, Ill. (erythromycin); Upjohn s.a., Puurs, Belgium (lincomycin); and Sigma (nafcillin, novobiocin, and vancomycin). All strains tested for ampicillin susceptibility were penicillinase producers, as assayed by methods described previously (13).

[c] NT, not tested.

TABLE 2. Effect of PMBN on the MIC of novobiocin for clinical isolates of gram-negative bacteria[a]

Organism (no. of isolates)[b]	Test agent(s)	MIC (μg/ml)		% of isolates susceptible at breakpoint concn (≤16 μg/ml)
		Range	50%[c]	
Acinetobacter spp. (7)	Novobiocin	32–250		0
	Novobiocin + PMBN	1.6–4		100
Escherichia coli (14)	Novobiocin	64–125	125	0
	Novobiocin + PMBN	0.2–2	0.5	100
Enterobacter spp. (6)	Novobiocin	125–500		0
	Novobiocin + PMBN	0.01–4		100
Klebsiella pneumoniae (10)	Novobiocin	16–250	125	10
	Novobiocin + PMBN	0.05–25	1.6	100
Proteus mirabilis (2)	Novobiocin	125		0
	Novobiocin + PMBN	125		0
Pseudomonas aeruginosa (15)	Novobiocin	16–1,000	520	6
	Novobiocin + PMBN	0.05–8	2	100
Salmonella typhimurium (1)	Novobiocin	250		0
	Novobiocin + PMBN	3		100

[a] All isolates were resistant to >350 μg of PMBN per ml. PMBN was prepared by hydrolysis with ficin as described previously (5, 18). PMBN was added at 30 μg/ml, except for *Pseudomonas* strains, for which it was added at 7.5 μg/ml.

[b] The sources of the isolates were as follows: *Acinetobacter* spp., six from urine and one from a wound; *E. coli*, four from departmental stock, eight from urine, and two from wounds; *Enterobacter* spp., four from blood, one from a wound, and one from sputum; *K. pneumoniae*, five from urine, three from sputum, and two from wounds; *P. mirabilis*, two from urine; *P. aeruginosa*, nine from urine, three from wounds, two from sputum, and one from blood; and *S. typhimurium*, one from departmental stock. With the exception of the two *P. mirabilis* isolates, all isolates were susceptible to polymyxin B. The antibiotic susceptibilities of the isolates were tested with Sensi-Discs (Becton Dickinson and Co., Rutherford, N.J.) according to the manufacturer's instructions as described elsewhere (2) and according to the guidelines of the National Committee for Clinical Laboratory Standards for classifying isolates as susceptible or resistant (12). All isolates were resistant to clindamycin, erythromycin, nafcillin, rifampin, and vancomycin. A total of 5 isolates of *Acinetobacter* spp. and 2 isolates of *P. aeruginosa* were resistant to at least three of the antibiotics listed below, 13 were resistant to only two, and 6 were resistant to none: ampicillin, chloramphenicol, cefotaxime, carbenicillin, gentamicin, kanamycin, nalidixic acid, norfloxacin, streptomycin, tetracycline, and tobramycin.

[c] 50%, MIC for 50% of isolates tested.

TABLE 3. Protection by PMBN, novobiocin, and erythromycin of mice against a challenge with *P. aeruginosa* or *K. pneumoniae*

Antibiotic	Amount (mg/kg) of intraperitoneal drug administration at indicated time after challenge:			No. of surviving mice/total no. challenged with:	
	4 h	1 day	2 days	*K. pneumoniae*	*P. aeruginosa*
None				0/6	0/10
PMBN[a]	50	50		NT[b]	0/6
Novobiocin	25	25		NT	0/8
Novobiocin + PMBN	25 + 50			NT	0/6
Novobiocin + PMBN	25 + 50	25 + 50		NT	10/10
PMBN	4	2[c]	2	0/6	NT
Erythromycin	10	10[c]	10	0/18	NT
Erythromycin + PMBN	10 + 4			0/6	NT
Erythromycin + PMBN	10 + 4	10 + 2[c]	10 + 2	10/18	NT

[a] PMBN was prepared by hydrolysis of polymyxin B with papain (see Table 1, footnote *a*).
[b] NT, not tested.
[c] The amounts indicated were administered twice on day 1 after challenge.

tration of PMBN (200 mg/kg of body weight) in combination with erythromycin to mice shortly after a challenge with a lethal dose of *P. aeruginosa* or *E. coli* failed to protect the animals (11). We used *P. aeruginosa* and *K. pneumoniae* strains which were resistant to >1,000 μg of novobiocin per ml and >200 μg of erythromycin per ml, respectively. The addition of PMBN to the medium rendered the strains susceptible to <32 μg of these hydrophobic antibiotics per ml. Untreated mice challenged with *P. aeruginosa* developed septicemia and died within 24 to 48 hours; untreated mice died 3 to 6 days after challenge with *K. pneumoniae*. A single intraperitoneal PMBN dose in combination with either erythromycin or novobiocin did not protect mice challenged with *K. pneumoniae* or *P. aeruginosa* (Table 3). In contrast, multiple doses administered over two or more consecutive days protected all mice challenged with *P. aeruginosa* and afforded partial, albeit significant, protection to mice challenged with *K. pneumoniae*.

Our studies suggest that the combination of PMBN and novobiocin should be considered for clinical use against gram-negative infections because (i) although PMBN activity is restricted only to polymyxin-susceptible strains (11, 16), natural resistance to polymyxins is limited to only a few gram-negative bacterial species and acquired resistance is rare (14); (ii) acquired specific resistance to novobiocin in gram-negative bacteria has not been reported (3), and once the outer membrane becomes permeable, novobiocin resistance is not anticipated; (iii) PMBN is highly active in vitro and renders gram-negative bacteria susceptible to hydrophobic antibiotics at concentrations as low as 1 μg/ml (19), even against strains exhibiting multiple resistance patterns; (iv) the combination of PMBN with novobiocin (or other hydrophobic antibiotics), when administered in multiple doses, protects mice challenged with a lethal dose of gram-negative bacteria; and (v) while the 50% lethal dose of polymyxin B or colistin in mice is in the range of 6 to 10 mg/kg, colistin nonapeptide is 15 times less toxic than the parent molecule (4), and doses as high as 200 mg of PMBN per kg are not lethal (11). Furthermore, 3 mg of PMBN per kg administered intravenously to dogs and rats over a long period of time does not cause a neuromuscular blockade or neurotoxic or nephrotoxic effects, all of which have been observed in animals administered the same dose of polymyxin B (6). Both novobiocin and polymyxins have been restricted for clinical use because of their high toxicity and because virtually all gram-negative strains are resistant to novobiocin (3). In view of our results, these restrictions should be reconsidered, as the combination of polymyxin B nonapeptide and novobiocin at relatively low concentrations is active in vitro, affords clinical protection to lethally infected mice, and is devoid of severe toxicity; most importantly, the development of resistance to this combination is rare.

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