ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1984, p. 701–705
0066-4804/84/060701-05$02.00/0

Vol. 25, No. 6

Susceptibility of Gram-Negative Bacteria to Polymyxin B Nonapeptide

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Received 28 October 1983/Accepted 16 March 1984

Subinhibitory concentrations of polymyxin B nonapeptide sensitized all 21 polymyxin-susceptible gram-negative bacterial strains studied to hydrophobic antibiotics such as fusidic acid, novobiocin, and erythromycin. The susceptibility increases were usually 30- to 300-fold. The strains included representatives of *Escherichia coli* with different O- and K-antigens, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Salmonella typhimurium*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, and *Pseudomonas maltophilia*. In contrast, polymyxin-resistant strains (*Proteus mirabilis*, *Proteus vulgaris*, *Morganella morganii*, *Providencia stuartii*, and *Serratia marcescens*) were resistant to the action of polymyxin B nonapeptide.

Polymyxin is an amphipathic cationic decapeptide and a potent bactericidal agent against most gram-negative bacteria. Derivatives of polymyxin which lack the fatty acid residue (deacylpolymyxins) or both the fatty acid residue and the terminal diaminobutyric acid (polymyxin nonapeptides) have drastically decreased antibacterial action (1, 9, 15, 18). Vaara and Vaara, however, have recently shown that although polymyxin B nonapeptide (PMBN) had a very low or absent antibacterial activity against smooth *Salmonella typhimurium* and *Escherichia coli*, it was remarkably active in sensitizing them to hydrophobic agents such as novobiocin, fusidic acid, erythromycin, clindamycin, nafcillin, rifampin, and actinomycin D. In fact, PMBN made the smooth strains as susceptible to the agents as are deep rough (Re) mutants with a very abnormal outer membrane (20–22).

Further studies showed that PMBN interacts with lipopolysaccharide (LPS) on the surface of the outer membrane and expands the surface area of the outer membrane as indicated by characteristic protrusions seen by electron microscopy (19, 21). Finally, PMBN has been shown to sensitize *E. coli* to the lethal membranolytic action of the complement system in normal sera of guinea pigs, rats, rabbits, and humans (22; M. Vaara, P. Viljanen, T. Vaara, and P. H. Mäkelä, J. Immunol., in press).

These findings suggested a new approach for studying the details of the molecular organization of the outer membrane as well as the molecular mechanisms of the serum resistance of gram-negative bacteria. PMBN might also be of value in microbiological research as a means to increase bacterial permeability to a variety of hydrophobic agents, e.g., mutagens, antimetabolites, and inhibitors. Last, the findings might act as a basis for therapeutic applications; polymyxin nonapeptide is reported to be less toxic to eucaryotes than the parent compound (2, 3).

The findings evoked, however, numerous immediate questions to be answered. One of them concerns the spectrum of PMBN. The target of PMBN is the acidic lipid A part of LPS (19), which is also the target for polymyxin (17, 23). Therefore it could be expected that all gram-negative bacteria which are inherently susceptible to the bactericidal action of polymyxin would be susceptible to the action of PMBN as well. The results in the present communication show this expectation to be true.

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MATERIALS AND METHODS

Bacterial strains. Smooth *S. typhimurium* SL696 (29) and *E. coli* IH3080 (O18:K1) are strains used in our earlier studies on the action of PMBN (20–22). The seven other encapsulated smooth *E. coli* strains were isolated from urine of patients with urinary tract infections (25) and selected for the present study as representatives of the most common *E. coli* serotypes found in urinary tract infections. The serotypes of these strains are presented in the context of Table 2.

The other bacterial species included the following isolates from this Institute: *Klebsiella pneumoniae* (two strains), *Klebsiella oxytoca*, *Enterobacter cloacae* (two strains), *Enterobacter agglomerans*, *Proteus mirabilis*, *Proteus vulgaris*, *Morganella morganii*, *Providencia stuartii*, *Serratia marcescens*, *Pseudomonas aeruginosa* (two strains), and *Acinetobacter calcoaceticus* var. *anitratus*. These strains were from clinical specimens (blood, urine, wound pus, external ear exudate) and their identification was verified by the API 20E system (API System S.A., Montalieu Vercieu, France) and, when applicable, by the OxiFerm system (Hoffmann-La Roche, Basel, Switzerland). The *Pseudomonas maltophilia* strain (our strain code, RHP1050) was a laboratory quality control strain from Central Public Health Laboratory, London, England. *K. pneumoniae* strains PCC7823 (capsular serotype 2 [6]) and PCC7824 (Caroli strain [4]), both mouse-virulent strains, were a kind gift from L. Chedid, Institut Pasteur, Paris, France. The *P. mirabilis*, *P. vulgaris*, *M. morganii*, *P. stuartii*, and *S. marcescens* strains were resistant to polymyxin (growth inhibition zone, below 20 mm around a polymyxin-containing disk; tested on IsoSensitest agar [Oxoid, Hampshire, England]; Neo-Sensitabs disks, diameter 9 mm [A/S Rosco, Taatsrup, Denmark]).

Testing the PMBN-induced susceptibility to antibiotics. The MICs of the antibiotics in the presence of PMBN were measured essentially as described previously (20, 22). However, to make the test more suitable for simultaneous testing of numerous bacterial strains, the inoculum was grown to stationary growth phase on L agar (without glucose) (12) instead of growth to early exponential phase in L broth (12). Furthermore, because PMBN, in contrast to several other polycations (20), is not precipitated in L broth, this medium was used as the growth medium in the sensitivity test. Shortly, bacterial colonies grown overnight on L agar were

suspended in 0.9% NaCl, washed in this diluent, and resuspended to a final absorbance of 120 Klett units (Klett-Summerson colorimeter, red filter; corresponding to ca. 10^9 bacteria per ml) in the same diluent. This suspension was used to inoculate (with ca. 10^4 bacteria per ml) L broth containing 0.1, 0.3, 1, 3, 10, 30, 100, or 300 μg of the antibiotic to be tested per ml. Samples (200 μl) of this inoculated medium were pipetted into wells of a microtiter plate (Titertek; Flow Laboratories, Inc., Rockville, Md.). Each well already contained increasing amounts of PMBN. The plates were incubated at 37°C for 18 h. The lowest concentration of an antibiotic that completely inhibited visible growth was recorded and interpreted as the MIC.

Preparation of PMBN. PMBN was prepared by an enzymatic hydrolysis of polymyxin B as described previously (20) (see also reference 2). The method was, however, modified to scale up the preparation. Polymyxin B sulfate (4 g; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 80 ml of deionized water. Papain (590 mg, corresponding to 500 mg of protein [EC 3.4.22.2]; type IV; Sigma) was dissolved in 10 ml of water. The solutions were combined, 5 drops of toluene was added, and the mixture was incubated for 48 h at 37°C in a rotary shaker (220 rpm). The mixture was then stirred in boiling water for 5 min and the precipitate formed (denatured papain) was removed by centrifugation and filtration through a Millex-HA filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The solution was adjusted to pH 2 with HCl and washed twice with 40 ml of *n*-butanol, then adjusted to pH 9 with NaOH and washed twice with *n*-butanol, and neutralized (pH 7). To remove possible anionic impurities, the solution was run through an Amberlite IRA-410 anion exchange column (OH-form; BDH Chemicals, Poole, England) with 0.05 M pyridine (adjusted to pH 7.0 with acetic acid) as eluent. The eluent was then concentrated, desalted in a Sephadex G-10 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and lyophilized. Purity analysis gave the same results as those found previously (polymyxin B content less than 0.25%) (20).

Antibiotics. Ampicillin and carbenicillin (sodium salts) were from Astra, Södertälje, Sweden. Chlortetracycline hydrochloride was from Lederle Laboratories, Wayne, N.J. Tetracycline hydrochloride was from Orion, Helsinki, Finland. Rifampin was from Ciba-Geigy, Basel, Switzerland. The sources of other antibiotics were described previously (20). The stock solution of rifampin was prepared by dissolving 10 mg of rifampin with 1 ml of methanol, after which deionized water was added to a final volume of 10 ml. Erythromycin was dissolved as described previously (20). Other antibiotics were readily dissolved in deionized water.

RESULTS

Sensitization assay. We slightly modified our earlier assay to facilitate the screening of numerous bacterial strains (see above). PMBN was able to sensitize our standard strain *E. coli* IH3080 (O18:K1) to a number of antibiotics by a factor of 30 to 300 (Table 1). Common to all these antibiotics is that they are hydrophobic and believed to traverse the outer membrane through the hydrophobic pathway of diffusion (14). Much less sensitization was found for penicillin G as also noted by us previously (20). Penicillin G is a relatively hydrophilic molecule and is believed to penetrate the outer membrane through porin pores (14). We also tested some other antibiotics (ampicillin, carbenicillin, and tetracyclines) that use the porin pores (7, 14). As expected, the sensitization factor for these antibiotics also was very low (Table 1). Accordingly, we chose three hydrophobic antibiotics (fusidic acid, novobiocin, and erythromycin) for representative probes to screen the effect of PMBN.

***E. coli* strains.** Several smooth encapsulated clinical isolates of different serotypes were used (Table 2). Regarding the direct antibacterial activity of PMBN, PMBN concentrations as low as 1 μg/ml slightly slowed the growth of all strains, as earlier reported by us for *E. coli* IH3080 (20). However, PMBN concentrations up to 30 μg/ml (IH11019) or 100 μg/ml (other strains) allowed full growth when the incubation was for 18 h. Regarding the sensitizing activity, PMBN increased the susceptibility of all the strains to the probe antibiotics in a manner very similar to that seen with standard strain IH3080. Thus, a PMBN concentration of 1 μg/ml increased the susceptibilities by a factor of 10 to 30, and a concentration of 30 μg/ml by a factor of 30 to 300.

Other polymyxin-susceptible enteric bacteria and *Acinetobacter* sp. The smooth *S. typhimurium* strain was very susceptible to the PMBN-induced sensitization (susceptibility increase, ca. 30- to 100-fold) (Table 2), as shown before by a slightly different method (20). Also, the five *Klebsiella* strains were very susceptible (susceptibility increase, ca. 30- to 300-fold), whereas the two *E. cloacae* isolates were somewhat less susceptible (susceptibility increase, 10- to 30-fold). The growth of *E. agglomerans* was inhibited at a PMBN concentration of 30 μg/ml; subinhibitory concentrations of PMBN sensitized the strain to antibiotics by a factor of 10 to 30. The *A. calcoaceticus* strain was effectively sensitized to fusidic acid (susceptibility increase, 100-fold) (Table 2). This strain was also susceptible to novobiocin and erythromycin in the absence of PMBN.

***Pseudomonas* strains.** PMBN alone, without any antibiotics, retarded the growth of the *Pseudomonas* strains. After 18 h of incubation, practically no visual growth was found for *Pseudomonas* strains at PMBN concentrations ≥1 μg/ml. Good growth was achieved in 24 to 32 h at 30 μg of PMBN per ml; hence, the sensitization assays were done with an incubation time of 32 h. In these conditions, PMBN sensitized both of the tested *P. aeruginosa* strains drastically to the probe antibiotics, the susceptibility increase being even at a PMBN concentration of 1 μg/ml as high as 300-fold (Table 2). The *P. maltophilia* strain also was sensitized.

Polymyxin-resistant enteric bacteria. Table 3 shows that

TABLE 1. MICs of various antibiotics against *E. coli* IH3080 (O18:K1) in the presence of PMBN[a]

Antibiotic	MIC (μg/ml) at a PMBN concn (μg/ml) of:						Sensitization index[b]
	0	0.3	1	3	10	30	
Fusidic acid	300	30	10	10	3	1	300
Rifampin	10	1	0.3	0.3	0.1	0.03	300
Novobiocin	30	10	3	1	0.3	0.3	100
Erythromycin	100	30	10	10	3	1	100
Clindamycin	≥100	30	10	10	10	3	≥30
Nafcillin	≥1,000	300	300	100	100	30	≥30
Actinomycin D	≥100	≥100	30	30	10	3	≥30
Carbenicillin	10	10	10	3	1	1	10
Ampicillin	3	3	3	3	3	1	3
Penicillin G	10	10	10	10	10	3	3
Chlortetracycline	1	1	1	1	1	0.3	3
Tetracycline	1	1	1	1	1	1	0

[a] The MIC was defined as the lowest concentration of antibiotic that prevented visible growth of the test bacteria incubated in L broth for 18 h at 37°C, starting from an inoculum of 10^4 cells per ml.

[b] The approximate ratio of the MIC in the absence of PMBN to that obtained with the highest concentration of PMBN used.

TABLE 2. MICs of fusidic acid, novobiocin, and erythromycin against various polymyxin-susceptible bacteria in the presence of PMBN[a]

Organism and serotype	Strain	MIC (μg/ml) in the presence of the indicated concn (μg/ml) of PMBN								
		Fusidic acid			Novobiocin			Erythromycin		
		0	1	30	0	1	30	0	1	30
E. coli										
O18:K1	IH 3080	300	10	1	30	3	0.3	100	10	1
O4:K12	IH11051	100	10	1	30	10	1	ND[b]	ND	ND
O4:K12	IH11167	100	10	1	≥100	10	1	100	10	1
O2:K1	IH11055	300	30	1	30	3	1	ND	ND	ND
O6:K2	IH11431	300	10	1	30	10	0.3	100	10	3
O6:K2	IH11019	300	10	0[c]	30	3	0[c]	ND	ND	ND
O18:K5	IH11038	100	10	1	30	3	0.3	≥300	30	1
O75:K5	IH11030	300	10	1	30	3	0.3	100	10	1
S. typhimurium	SL696	100	10	3	≥100	3	1	ND	ND	ND
K. pneumoniae	IH19050	≥1,000	100	10	≥100	10	1	≥300	100	10
K. pneumoniae	IH19051	300	10	1	≥100	3	0.3	100	30	3
K. pneumoniae	PCC7823	100	10	3	10	3	0.3	ND	ND	ND
K. pneumoniae	PCC7824	300	30	3	3	1	0.3	ND	ND	ND
K. oxytoca	IH19052	300	10	1	30	1	0.3	≥300	30	10
E. cloacae	IH19048	300	30	10	≥100	30	3	≥300	100	30
E. cloacae	IH19049	300	100	30	≥100	30	10	ND	ND	ND
E. agglomerans	IH19053	100	3	0[c]	≥100	10	0[c]	ND	ND	ND
A. calcoaceticus	IH19063	100	1	1	3	1	0.3	ND	ND	ND
P. aeruginosa	IH19059	300	1	1	≥100	1	1	≥300	3	10
P. aeruginosa	IH19060	300	3	1	≥100	1	1	≥300	3	10
P. maltophilia	RHP1050	30	10	1	30	3	1	ND	ND	ND

[a] The MIC was defined as the lowest concentration of antibiotic that prevented visible growth of the bacteria incubated in L broth at 37°C for 18 h (or, in the case of *Pseudomonas* strains, for 32 h), starting from an inoculum of 10^4 cells per ml.
[b] ND, Not done.
[c] 0, PMBN (30 μg/ml) alone prevented growth.

PMBN affected very slightly, if at all, the antibiotic susceptibilities of *P. mirabilis*, *P. vulgaris*, *M. morganii*, *P. stuartii*, and *S. marcescens*, all known to be inherently resistant to polymyxin (1, 8, 18). Two of the strains (*P. mirabilis* IH19055 and *P. vulgaris* IH19061) were, however, susceptible to novobiocin even in the absence of PMBN.

DISCUSSION

The present paper shows that the susceptibility of a particular gram-negative bacterium to polymyxin is a good predictor of the susceptibility of that bacterium to the outer membrane permeability-increasing action of PMBN. Thus, PMBN rendered all of the 21 polymyxin-susceptible strains susceptible to the hydrophobic probe antibiotics (fusidic acid, novobiocin, and erythromycin; Table 2). The strains included representatives of *E. coli*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *E. agglomerans*, *S. typhimurium*, *A. calcoaceticus*, *P. aeruginosa*, and *P. maltophilia*. The susceptibility increase achieved was usually 30- to 300-fold.

All the different serotypes of *E. coli* were equally susceptible to PMBN. Consistent with this finding is our earlier observation that smooth *E. coli* (O18:K1) and *S. typhimur-*

TABLE 3. MICs of fusidic acid and novobiocin against various polymyxin-resistant bacteria in the presence of PMBN[a]

Organism	Strain	MIC (μg/ml) in the presence of the indicated concn (μg/ml) of PMBN					
		Fusidic acid			Novobiocin		
		0	1	30	0	1	30
P. mirabilis	IH19055	300	300	300	1	1	1
P. vulgaris	IH19061	300	300	300	3	3	3
M. morganii	IH19057	300	300	300	≥100	≥100	30
P. stuartii	IH19058	300	100	100	≥100	30	30
S. marcescens	IH19054	300	100	30	≥100	30	30

[a] The MIC was defined as the lowest concentration of antibiotic that prevented visible growth of the test bacteria incubated in L broth for 18 h at 37°C, starting from an inoculum of 10^4 cells per ml.

ium are as susceptible to PMBN as the rough strains *E. coli* K-12 and *S. typhimurium* chemotype Rb_2 (19, 24). PMBN thus seems to have free access to its target in the outer membrane, irrespective of the nature of the capsular or somatic (LPS) antigen. The results also indicate that acidic capsules such as the sialic acid-containing K1 do not inactivate PMBN to an extent that would inhibit its biological activity.

Pseudomonas strains (with some notable exceptions) are known to be very susceptible to polymyxin (1, 8, 18), and they proved to be extremely susceptible to PMBN as well. PMBN not only sensitized the *Pseudomonas* strains to antibiotics but also slowed their growth (Table 2). The mechanism for this latter action was not studied. It is, however, known that heavy outer membrane perturbations such as that caused by EDTA can activate even a lytic process in *P. aeruginosa* (10, 16). Because the polymyxin content of the PMBN preparation was below 0.25%, it is rather unlikely that the growth retardation was due to contaminating polymyxin.

In contrast to the polymyxin-susceptible strains, all the polymyxin-resistant strains (representatives of *P. mirabilis*, *P. vulgaris*, *M. morganii*, *P. stuartii*, and *S. marcescens*) were resistant to the effects of PMBN (Table 3). These data are consistent with our earlier finding showing that polymyxin-resistant *pmrA* mutants of *S. typhimurium* are resistant to the effect of PMBN (19). Moreover, we have recently found that polymyxin-resistant bacteria (*P. mirabilis*, and the *pmrA* strains of *S. typhimurium*) bind significantly less radiolabeled PMBN than do polymyxin-susceptible bacteria (*E. coli*, *S. typhimurium*, *P. aeruginosa*) (M. Vaara and P. Viljanen, manuscript in preparation). Interestingly, *Proteus* and *Serratia* strains are resistant also to the cationic bactericidal protein (BPI protein) isolated from the granules of polymorphonuclear leukocytes and claimed to be an important host defense factor (27, 28). Moreover, this protein, like polymyxin or PMBN, is inactive against gram-positive bacteria, binds to the outer membrane of susceptible bacteria, and complexes with isolated LPS (5, 26). Put together, these data might suggest that the BPI protein has the same outer membrane target (acidic lipid A) as polymyxin and PMBN.

The set of gram-negative bacteria used in the present study did not include strains of *Haemophilus* (which are known as polymyxin susceptible) (1, 8) or *Neisseria* (polymyxin resistant) (1, 8). They were excluded because they are inherently very susceptible to hydrophobic agents and, accordingly, allow hydrophobic diffusion through their outer membrane even in the absence of PMBN (1, 8, 11, 13, 14). We shall, however, show elsewhere that *Haemophilus influenzae* (capsular type b) is susceptible to the synergistic bactericidal action of PMBN plus serum, as are most strains of *E. coli*, *Klebsiella*, and *Enterobacter*, and that strains of *Neisseria meningitidis* and *Neisseria gonorrhoea* are resistant to PMBN plus serum as are the *Proteus* strains (manuscript in preparation).

ACKNOWLEDGMENTS

We thank P. H. Mäkelä, T. Vaara, and P. Koivuranta-Vaara for critically reading the manuscript and for useful advice and discussion.

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