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Comparative In Vitro Exoenzyme-Suppressing Activities of Azithromycin and Other Macrolide Antibiotics against *Pseudomonas aeruginosa*

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The inhibitory effects of azithromycin (AZM), a new 15-membered macrolide antibiotic, on the production of exotoxin A, total protease, elastase, and phospholipase C by *Pseudomonas aeruginosa* were determined, and the virulence-suppressing effects of AZM were compared with those of erythromycin (EM), roxithromycin (RXM), and rokitamycin (RKM). The effect of exposure of *P. aeruginosa* PA103 or B16 in cultures to sub-MICs of these macrolide antibiotics on the production of exoenzymes was determined. AZM suppressed the in vitro production of extracellular and intracellular exotoxin A by *P. aeruginosa* PA103 more than did EM, even at a concentration of only 2 μg/ml. At concentrations of between 4 and 32 μg/ml, AZM also inhibited total protease, elastase, and phospholipase C production by *P. aeruginosa* B16 more than did EM, RXM, and RKM. AZM was effective in suppressing exotoxin A and total protease production through 24 h of incubation in the presence of drug at sub-MICs, but it had no significant effect on either the growth of *P. aeruginosa* or its total protein production. Moreover, at a concentration of 4 μg/ml, AZM suppressed exoenzyme production by other strains of *P. aeruginosa* more than did EM. These findings indicate that AZM, EM, RXM, and RKM each has an inhibitory effect on exoenzyme production separate from the antimicrobial effect and that, of these macrolides, AZM has the strongest virulence-suppressing effect.

Pseudomonas aeruginosa is an opportunistic pathogen which frequently causes severe septicemia in immunocompromised hosts (4, 5). It is also the principal pulmonary pathogen in patients with cystic fibrosis (24, 33) and diffuse panbronchiolitis (16). *P. aeruginosa* exoenzymes, including exotoxin A, alkaline protease, elastase, and phospholipase C, are known to be virulence factors of importance in the various types of *P. aeruginosa* infections (26). The findings of our previous studies have suggested that *P. aeruginosa* isolates from blood cultures produce large quantities of exotoxin A and total protease (10) and that the production of exotoxin A plays an important role in the pathogenesis of endogenous *P. aeruginosa* septicemia in mice (13).

It has recently been reported that treatment with erythromycin (EM) improves the clinical symptoms and prognosis of patients with chronic pulmonary infections (18). Kita et al. (18) have shown that erythromycin stearate (EMS) suppresses the production of protease and leukocidin by *P. aeruginosa* without affecting cell growth and speculated that EMS may affect the virulence factors of this organism (18) and host defense mechanisms (7, 9, 17). More recently, we have shown that erythromycin lactobionate (EML) suppresses the in vitro production of exotoxin A, total protease, elastase, and phospholipase C and that EML inhibits the production of exotoxin A and total protease more than it does the production of elastase and phospholipase C (14). In that study, we suggested that the virulence-suppressing effect of EML was one of the mechanisms that protects mice against developing *P. aeruginosa* septicemia (14).

Azithromycin (AZM), a newly developed 15-membered macrolide, has a spectrum of antimicrobial activity similar to that of EM, which is a 14-membered macrolide (25). AZM has a broad spectrum of antimicrobial activity, which includes most gram-positive bacteria (22) and certain other organisms, including *Mycoplasma* spp. (30) and *Legionella* spp. (6). In contrast, members of the family *Enterobacteriaceae* and *P. aeruginosa* are generally resistant to AZM and other macrolide antibiotics (29). In the present study, we found that sub-MICs of AZM inhibit exotoxin A, total protease, elastase, and phospholipase C production by *P. aeruginosa* without affecting either the growth of *P. aeruginosa* or its total protein production. The effects of AZM on exoenzyme production by *P. aeruginosa* were compared with those of EM, roxithromycin (RXM; a 14-membered macrolide), and rokitamycin (RKM; a 16-membered macrolide). We found that, of the macrolides studied, sub-MICs of AZM were the most effective in suppressing exoenzyme production.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the study were *P. aeruginosa* PA103 (19) and B16 (10). *P. aeruginosa* PA103 was kindly provided by B. H. Iglewski, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. This strain, which was clinically isolated from sputum, is known to be a hyperproducer of exotoxin A (13), but it produces very small quantities of alkaline protease and no elastase (27). *P. aeruginosa* B16 was a clinical isolate from a blood sample obtained at Nagasaki University Hospital, Nagasaki, Japan; it

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produces relatively large amounts of exotoxin A, total protease, elastase, and phospholipase C (10). To investigate whether AZM is more able than EM to suppress exoenzyme production by other strains of *P. aeruginosa*, an additional 15 strains from the blood of patients at Nagasaki University Hospital were used. Six strains produced exotoxin A, and nine strains produced relatively large amounts of total protease, elastase, and phospholipase C.

Antibiotics. The following macrolide antibiotics were kindly provided by the indicated manufacturers: AZM, Pfizer Laboratories, Groton, Conn.; EM base, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan; RXM, Roussel Morishita Co., Ltd., Osaka, Japan; and RKM, Asahi Chemical Industry Co., Ltd., Tokyo, Japan. Also studied were the following antibiotics representing the nonmacrolide classes: ofloxacin (OFLX; Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), gentamicin sulfate (GM; Schering-Plough K. K., Osaka, Japan), and ceftazidime (CAZ; Tanabe Pharmaceutical Co., Ltd., Osaka, Japan).

In vitro susceptibility. The MICs of antibiotics were determined by an agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with calcium and magnesium at concentrations of 50 and 25 mg/liter, respectively. Approximately 10^5 logarithmic-phase organisms were inoculated onto agar containing antibiotics, and the MICs, defined as the lowest concentrations of antibiotics that inhibited the visible growth of bacteria (23), were determined.

Assay of total protein. The total protein concentration was determined by the method of Lowry et al. (21), with bovine serum albumin used as a standard.

Assay of exotoxin A. Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) dialysate (TSBD-C) medium (20) was used as the growth medium for measurement of exotoxin A. *P. aeruginosa* PA103, adjusted to 10^7 CFU/ml by reference to a standard curve relating optimal density to bacterial count, was grown in TSBD-C medium with or without (control) antibiotics at 32°C (20). After culture, the supernatant was removed from the culture fluid and was assayed for extracellular exotoxin A. The cell sediments were washed twice with 0.1 M sodium phosphate buffer saline (pH 7.0) to remove the residual culture medium. After disruption of the cells with a cell disruptor (SONIFIRE 185 type; Branson Ultrasonic Co., Danbury, Conn.), a crude enzyme solution was obtained from the supernatant resulting from centrifugation at 15,000 × *g* for 30 min at 4°C. We assayed the levels of exotoxin A in the disrupted cells. Measurement of exotoxin A was performed by the enzyme-linked immunosorbent assay (ELISA) method (14). Anti-exotoxin A antiserum was obtained from a Japanese White rabbit (Viotek Co., Ltd., Saga, Japan) immunized three times with purified *P. aeruginosa* exotoxin A (List Biological Laboratories Inc., Campbell, Calif.). Goat anti-exotoxin A antibody (List) and rabbit anti-exotoxin A antiserum were used in a sandwich-type ELISA. The values obtained were compared with those obtained on a standard curve derived by use of purified *P. aeruginosa* exotoxin A (List) (14).

Assay of protease activity. *P. aeruginosa*, adjusted to 10^6 CFU/ml, was grown in brain heart infusion broth with or without (control) antibiotics at 37°C. Total protease activity was assayed by using the hide powder azure dye (Sigma Chemical Co., St. Louis, Mo.) release method (14, 35). Total protease activity was determined by comparing A_{595} values for the resultant supernatants with those obtained on a standard curve prepared with purified *P. aeruginosa* alkaline protease (Nagase Biochemicals, Ltd., Osaka, Japan).

Elastase activity was assayed by the elastin Congo red (Sigma) method (31). The A_{495} was read. The A_{495} values obtained were compared with those obtained on a standard curve derived by use of purified *P. aeruginosa* elastase (Nagase).

Assay of phospholipase C activity. *P. aeruginosa*, adjusted to 10^7 CFU/ml, was grown in tryptose minimal medium (2) with or without (control) antibiotics at 32°C for assay of phospholipase C activity. Phospholipase C activity was measured by using *p*-nitrophenylphosphorylcholine as a substrate (2). The A_{405} of the supernatants was read with a microplate autoreader (MR600; Dynatech Laboratories, Chantilly, Va.). The values obtained were compared with those obtained on a standard curve prepared with purified *Clostridium perfringens* phospholipase C (Sigma) (14).

Statistics. Bonferroni's test was used to compare group means; P values of <0.05 were considered to indicate significant differences between groups.

RESULTS

MICs of AZM and other macrolide antibiotics for *P. aeruginosa* PA103, B16, and other strains. The MICs of the macrolide antibiotics for both *P. aeruginosa* PA103 and B16 were 256, 512, 1,024, and 512 μg/ml for AZM, EM, RXM, and RKM, respectively. The MICs of AZM and EM for 15 other strains of *P. aeruginosa* were >64 μg/ml. The MICs of OFLX, GM, and CAZ for *P. aeruginosa* B16 were 1.0, 4.0, and 4.0 μg/ml, respectively.

Effects of various concentrations of AZM on extracellular exotoxin A production by *P. aeruginosa* PA103. As shown in Table 1, AZM suppressed exotoxin A production by *P. aeruginosa* PA103 in supernatants after 24 h of incubation. Extracellular exotoxin A production was suppressed by AZM in a concentration-dependent fashion. AZM was more effective than EM, RXM, ($P < 0.01$) and RKM ($P < 0.01$) at concentrations of between 2 and 16 μg/ml at suppressing extracellular exotoxin A production. No significant reduction in the numbers of viable cells or the amounts of total protein in the supernatants was observed for concentrations of AZM of between 0 and 16 μg/ml.

Effects of time on extracellular exotoxin A production by and growth of *P. aeruginosa* PA103. Figure 1 portrays exotoxin A production by and growth of *P. aeruginosa* PA103 in supernatants during the 24-h incubation period. Extracellular exotoxin A production was detected during the stationary phase, and suppression of exotoxin A production was achieved with 2 μg of AZM per ml. Suppression of exotoxin A production by AZM (2 μg/ml) was greater than that by EM (2 μg/ml) through 24 h (Fig. 1A). Bacterial growth was not inhibited by AZM (2 μg/ml) between 16 and 24 h in the stationary phase (Fig. 1B).

Effects of various concentrations of AZM on intracellular exotoxin A production by *P. aeruginosa* PA103. AZM was also found to have suppressed exotoxin A production by *P. aeruginosa* PA103 in cells after 24 h of incubation. The inhibition of intracellular exotoxin A production by AZM occurred in a concentration-dependent fashion. AZM was more effective in decreasing the ratio of intracellular exotoxin A production to total protein than was EM at concentrations of between 2 and 4 μg/ml (Table 1).

Effects of various concentrations of AZM on total protease production by *P. aeruginosa* B16 in supernatants. The effects of AZM on total protease production by *P. aeruginosa* B16 after 24 h of incubation are given in Table 1. AZM suppressed total protease production by *P. aeruginosa* B16 in a concentration-dependent fashion. AZM was more effective in the suppression of total protease production than were EM, RXM (*P*

TABLE 1. Effects of macrolide antibiotics on exoenzyme production by *P. aeruginosa* over 24 h of culture

Exoenzyme in sample	Antibiotic	Concn (μg/ml)	Exoenzyme production[a]	% of control
Exotoxin A in supernatant[b]	None		12.5 ± 2.8	100
	AZM	2	3.9 ± 0.5[c]	31
		4[c]	1.9 ± 0.3	15
		8[c]	0.92 ± 0.1	7
		16[c]	0.16 ± 0.04	1
	EM	2	5.3 ± 0.9	42
		4	2.6 ± 0.5	21
		8	2.0 ± 0.4	16
		16	0.3 ± 0.05	2
	RXM	2	7.8 ± 0.7	62
		4	6.8 ± 0.5	54
		8	3.8 ± 0.4	30
		16	0.94 ± 0.3	8
	RKM	2	7.0 ± 0.9	56
		4	3.7 ± 0.4	30
		8	2.8 ± 0.3	22
		16	0.64 ± 0.1	5
Exotoxin A in disrupted cells[b]	None		$9.8 \pm 0.8 \times 10^{-4}$	100
	AZM	2	$2.6 \pm 0.4 \times 10^{-4}$	26
		4	$1.3 \pm 0.2 \times 10^{-4}$	13
	EM	2	$3.5 \pm 0.4 \times 10^{-4}$	36
		4	$1.9 \pm 0.4 \times 10^{-4}$	19
Total protease in supernatant[d]	None		33.2 ± 6.7	100
	AZM	4	11.1 ± 2.7[c]	33
		8	3.1 ± 0.4[c]	9
		16	1.6 ± 0.4[c]	5
		32	ND[e]	0
	EM	4	18.8 ± 4.1	57
		8	12.5 ± 1.2	38
		16	10.4 ± 0.59	31
		32	7.5 ± 0.4	23
	RXM	4	30.8 ± 4.5	93
		8	20.1 ± 3.3	63
		16	13.2 ± 1.9	40
		32	9.4 ± 2.7	28
	RKM	4	29.4 ± 5.1	89
		8	20.3 ± 3.8	63
		16	13.5 ± 2.5	39
		32	10.7 ± 1.8	31

[a] Values are means ± standard deviations for five replicate flasks. Values are in micrograms per milliliter for exotoxin A in supernatant and total protease in supernatant and express the ratio of exotoxin A to total protein in disrupted cells.
[b] *P. aeruginosa* PA103 was grown with various concentrations of macrolide antibiotics.
[c] Significantly less than the value for the same concentrations of RXM and RKM ($P < 0.01$).
[d] *P. aeruginosa* B16 was grown with various concentrations of macrolide antibiotics.
[e] ND, not detected.

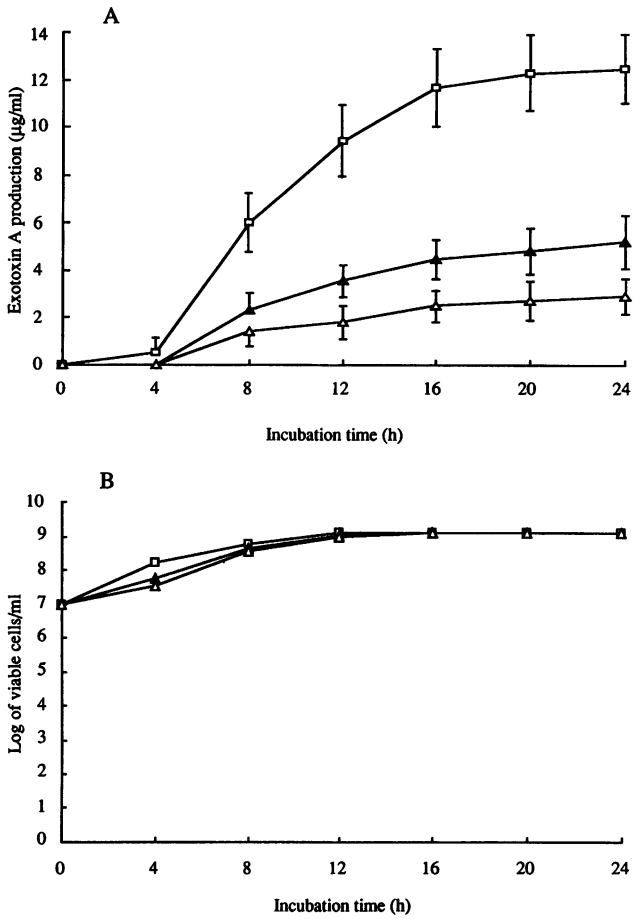

FIG. 1. Effect of growth time on exotoxin A production by *P. aeruginosa* PA103 (A) and growth of *P. aeruginosa* (B) in supernatant with or without antibiotics. Symbols: □, no antibiotics (control); △, AZM at 2 μg/ml; ▲, EM at 2 μg/ml. Values are the means ± standard errors of results of three separate experiments.

< 0.01), and RKM ($P < 0.01$). AZM at concentrations of between 0 and 32 μg/ml had no effect on the growth of *P. aeruginosa* B16 or the amounts of total protein present in the supernatants.

Effects of time on total protease production by and growth of *P. aeruginosa* B16. Total protease production by and growth of *P. aeruginosa* B16 in supernatants over a 24-h period of incubation were determined. Total protease production was detected in the stationary phase, and suppression was achieved with 4 μg of AZM per ml. AZM (4 μg/ml) inhibited total protease production more than EM (4 μg/ml) did through a 24-h period of incubation (Fig. 2A). No significant reduction in the numbers of viable cells by AZM (4 μg/ml) was observed between 16 and 24 h in the stationary phase (Fig. 2B).

Effects of various concentrations of AZM on elastase and phospholipase C production by *P. aeruginosa* B16 in supernatants. AZM, EM, RXM, and RKM were each found to have suppressed elastase (Fig. 3A) and phospholipase C (Fig. 3B) production in supernatants after 24 h of incubation, with suppression occurring in a concentration-dependent fashion. Of the macrolides tested, AZM at concentrations of between 4 and 32 μg/ml was the most effective at suppressing elastase and phospholipase C production. AZM suppressed the production of elastase and phospholipase C less than it did that of exotoxin A.

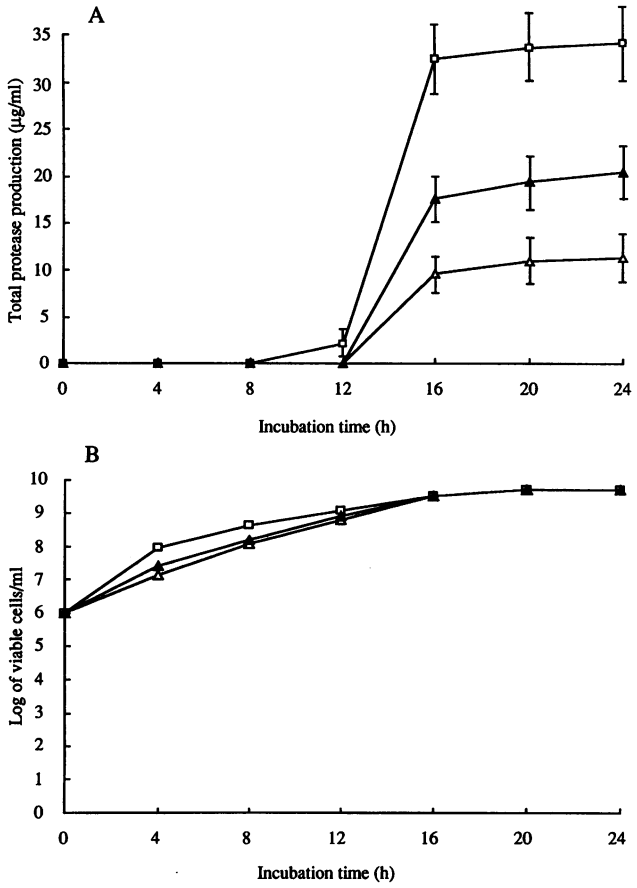

FIG. 2. Effect of growth time on total protease production by *P. aeruginosa* B16 (A) and growth of *P. aeruginosa* (B) in supernatant with or without antibiotics. Symbols: □, no antibiotics (control); △, AZM at 4 μg/ml; ▲, EM at 4 μg/ml. Values are the means ± standard errors of results of three separate experiments.

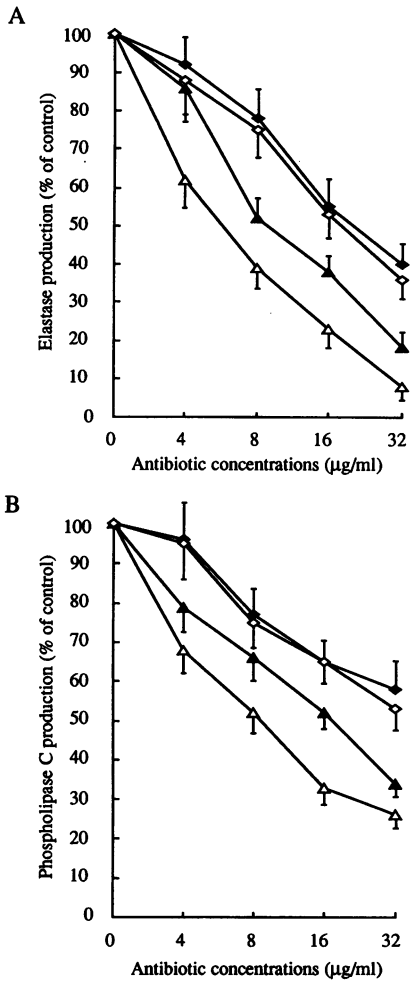

FIG. 3. Inhibition of elastase (A) and phospholipase C (B) production by *P. aeruginosa* B16 with the macrolide at concentrations of between 0 and 32 μg/ml. Values are the mean ± standard error percentages of exoenzyme production in three replicate flasks compared with that in controls. Symbols: △, AZM; ▲, EM; ◆, RXM; ◇, RKM.

Effects of AZM on exoenzyme production by other strains of *P. aeruginosa* in supernatants. At a concentration of 4 μg/ml, AZM suppressed exotoxin A production by six strains of *P. aeruginosa* in supernatants after 24 h of culture more than EM did (Fig. 4A). The mean proportion of inhibition of exotoxin A production by AZM compared with that in controls was 12%. On the other hand, the mean proportion of inhibition by EM was 35%. AZM (4 μg/ml) also inhibited total protease (Fig. 4B), elastase (Fig. 4C), and phospholipase C (Fig. 4D) production by nine strains of *P. aeruginosa* more than EM (4 μg/ml) did. The exoenzyme-suppressing activity of AZM against almost all other strains of *P. aeruginosa* was stronger than that of EM.

Effects of typical anti-*P. aeruginosa* agents on exoenzyme production in supernatants. As shown in Table 2, we evaluated the effects of nonmacrolide antibiotics on exoenzyme production by *P. aeruginosa* B16 after 24 h of incubation. Weak exoenzyme-suppressing activities of 0.1 μg of OFLX per ml (1/10th the MIC), 0.4 μg of GM per ml (1/10th the MIC), and 0.4 μg of CAZ per ml (1/10th the MIC) were observed.

DISCUSSION

The addition of AZM and other macrolide antibiotics had no influence on exoenzyme activities after the bacteria were already grown (data not shown), indicating that macrolides suppress exoenzyme production by *P. aeruginosa* and have no intrinsic antiexoenzyme effects. For the exoenzyme assay, inoculation of 10^6 or 10^7 CFU of bacteria per ml was needed to achieve sufficient quantities of exoenzyme. When the starting inoculum was low, small amounts of exotoxin A and phospholipase C were produced even in control samples; even so, exoenzyme production by *P. aeruginosa* was suppressed. Sub-MICs of AZM inhibited the production of exoenzymes, including exotoxin A, total protease, elastase, and phospholipase C, but they had no effect on the growth of *P. aeruginosa* or its total protein production. In general, the in vitro virulence-suppressing activity of AZM was greater than that of EM, RXM, or RKM. Even at a concentration of only 2 μg/ml, AZM inhibited extracellular exotoxin A production in supernatants and decreased the intracellular exotoxin A levels measured in disrupted cells. This finding suggests that AZM has no effect on the extracellular release of exotoxin A and that AZM probably suppresses the synthesis of exotoxin A. Various antipseudomonal agents have exoenzyme-suppressing activities at sub-MICs (12). However, AZM inhibited exoenzyme

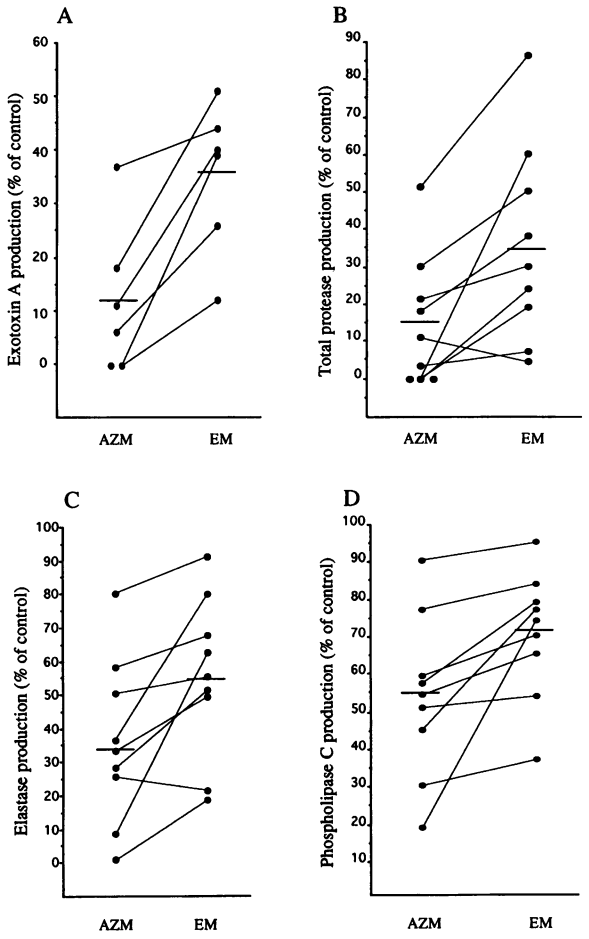

FIG. 4. Effect of AZM on exoenzyme production by other strains of *P. aeruginosa* in the supernatant. With or without 4 μg of macrolide per ml, six strains were incubated for assay of exotoxin A (A) and nine strains were incubated for assay of total protease (B), elastase (C), and phospholipase C (D). The bars indicate mean exoenzyme production levels compared with that in controls in each group. The results are expressed as the mean percentages of exoenzyme production in three replicate flasks.

production at a concentration of 1/10th the MIC more than the other classes of antibiotics did.

Some investigators have reported a role for the exoenzymes of *P. aeruginosa* in the pathogenesis of infections caused by this bacterium. There is evidence that *P. aeruginosa* exotoxin A and elastase are virulent in animal models of acute and chronic lung infections (3, 34, 36). Azghani et al. (1) have shown that the elastase produced by *P. aeruginosa* increases alveolar epithelial permeability in guinea pigs and noted that elastase is a virulence factor of potential importance in the pathogenesis of acute lung infections. Snell et al. (32) showed that alkaline protease and elastase are associated with virulence in *P. aeruginosa* infections in a burned mouse model. Woods et al. (35) reported that levels of *P. aeruginosa* exoproducts vary significantly, depending on the site of isolation. Elevated levels of elastase have been demonstrated in strains isolated from patients with acute lung infections, phospholipase C levels were found to be elevated in isolates from the urinary tract and blood, and exotoxin A levels have been found to be elevated in isolates from blood (35). We also observed that *P. aeruginosa* isolates from blood produce higher levels of exotoxin A, total protease, and elastase than strains from any other site (10). Pollack et al. (28) reported that antitoxin was present at a high titer in the sera of patients who had recovered from serious pseudomonas infections (28).

TABLE 2. Effects of nonmacrolide antibiotics on exoenzyme production by *P. aeruginosa* B16

Antibiotic[a] (1/10th the MIC)	Exoenzyme production in supernatant (% of control)[b]			
	Exotoxin A	Total protease	Elastase	Phospholipase C
AZM	3 ± 1.3	2 ± 0.9	18 ± 4	27 ± 6
OFLX	42 ± 7	28 ± 5	66 ± 8	93 ± 12
GM	77 ± 8	52 ± 7	70 ± 12	96 ± 16
CAZ	86 ± 15	60 ± 10	69 ± 9	76 ± 11

[a] *P. aeruginosa* B16 was grown for 24 h with AZM (25.6 μg/ml), OFLX (0.1 μg/ml), GM (0.4 μg/ml), CAZ (0.4 μg/ml), or no antibiotic (control).

[b] The percentages of exoenzyme production compared with those in controls are expressed as means ± standard deviations (n = 3).

It has been suggested that decreases in *P. aeruginosa* elastase, exoenzyme S, or both induced by ciprofloxacin, tobramycin, and ceftazidime might play an important role in the prevention of histological damage in rat lungs (12). The suppression of elastase and leukocidin production by *P. aeruginosa* is one reason that long-term administration of EM is useful in the treatment of chronic lower respiratory tract infections (18). We reported (14, 15) that EML reduces the rate of mortality of mice with *P. aeruginosa* bacteremia and that suppression of exotoxin A and total protease production by EML probably contribute to the protection of mice from *P. aeruginosa* bacteremia. Consequently, AZM may be useful clinically for the treatment of chronic respiratory tract and other *P. aeruginosa* infections, and the high degree of suppression of exoenzymes by AZM may make this macrolide more useful than EM and other macrolide antibiotics in the treatment of *P. aeruginosa* infections. In the present study, AZM was found to suppress exoenzyme production at lower concentrations than was the case for EM or the other macrolide antibiotics tested. Furthermore, AZM has been found to be more acid stable and to reach higher concentrations than EM in plasma and tissue (8, 11). The in vivo half-life of AZM is longer than those of EM and other macrolide antibiotics (11).

In conclusion, we demonstrated that the suppression of production of exoenzymes by *P. aeruginosa* can be achieved by administration of sub-MICs of AZM, a new macrolide antibiotic. Our findings showed that AZM is an excellent virulence-suppressing agent.

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