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Direct Evidence for Antipseudomonal Activity of Macrolides: Exposure-Dependent Bactericidal Activity and Inhibition of Protein Synthesis by Erythromycin, Clarithromycin, and Azithromycin

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Several previous investigators have reported that long-term administration of certain macrolides is efficacious in patients with persistent pulmonary *Pseudomonas aeruginosa* infections, even though the clinically achievable concentrations of these medications are far below their MICs. In the present study, we examined how sub-MICs of macrolide antibiotics affect the viability of and protein synthesis in several strains of *P. aeruginosa*. We report that 48 h, but not 12 or 24 h, of growth on agar containing a clinically achievable concentration of azithromycin (0.5 μg/ml, 1/128 the MIC) significantly reduces the viability of strain PAO-1. Similar effects were seen with erythromycin and clarithromycin at 2 μg/ml (1/128 and 1/64 the respective MICs), whereas josamycin, oleandomycin, ceftazidime, tobramycin, minocycline, and ofloxacin had no effect on viability, even following 48 h of incubation with concentrations representing relatively high fractions of their MICs. The bactericidal activity of azithromycin seen following 48 h of incubation was not limited to strain PAO-1 but was also seen against 13 of 14 clinical isolates, including both mucoid and nonmucoid strains. Although viability was not decreased prior to 48 h, we found that 4 μg of azithromycin per ml inhibits protein synthesis after as little as 12 h and that protein synthesis continues to decrease in a time-dependent manner. We likewise found that *P. aeruginosa* accumulates azithromycin intracellularly over the period from 12 to 36 h. These results suggested that sub-MICs of certain macrolides are bactericidal to *P. aeruginosa* when the bacteria are exposed to these antibiotics for longer periods. Exposure-dependent intracellular accumulation of the antibiotic and inhibition of protein synthesis may partially account for the antipseudomonal activity of macrolides over relatively prolonged incubation periods.

Pseudomonas aeruginosa is one of the most important bacterial pathogens in patients with chronic pulmonary diseases such as cystic fibrosis (24, 34) and diffuse panbronchiolitis (12). Despite treatment with potent antibiotics, infection of these patients' lungs by *P. aeruginosa* typically leads to death by respiratory failure or other complications. As a result, it is extremely important to inhibit the organism or eradicate the organism from such patients.

Bacterial susceptibility to antimicrobial agents is determined in vitro by measuring the antibiotic concentration required to inhibit growth under defined conditions, typically, incubation of an inoculum of 10^5 to 10^6 CFU for 18 to 24 h at 35°C. Although the results of these in vitro tests are usually consistent with clinical responses, this is not always the case. One explanation for the occasional discrepancy may rest on the increasing number of reports (2, 5, 33) indicating that subinhibitory levels of antibiotics can affect bacteria in ways distinct from their recognized antimicrobial actions. Since the amount of antibiotic at a bacterial infection site may fall below inhibitory levels during the course of therapy, it is tempting to speculate that some of these subinhibitory effects may contribute to the clinical efficacies of certain antibiotics, particularly when they are administered for prolonged periods.

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Administration of certain macrolide antibiotics, erythromycin (EM), clarithromycin (CAM), and azithromycin (AZM), for periods of several months or years has been reported to improve the clinical symptoms and prognosis of patients with chronic *P. aeruginosa* infections (15–17, 28). This may be considered paradoxical, since laboratory susceptibility testing of these antibiotics at their usual therapeutic doses has demonstrated neither bactericidal nor bacteriostatic activity against *P. aeruginosa*. It has been speculated that the clinical efficacies of these macrolides may be an indirect result of the effects on the organism's virulence factors (15, 19–21), host defense systems (6, 7, 14, 22, 29), or both (11).

We have recently reported that subinhibitory levels of EM, CAM, and AZM enhance the susceptibility of *P. aeruginosa* to serum bactericidal activity by altering cell surface structures (30, 31). In the course of those experiments, we noticed that exposure time is a critical factor: Serum susceptibility is significantly enhanced by 36 h of exposure to the macrolides, but not by exposures of 12 or 24 h. We considered this to be potentially significant since, even though concentrations may never reach laboratory-derived MICs, prolonged administration of macrolides leads to the exposure of the infecting organisms to these antibiotics for far more than 24 h. Thus, it seems plausible that only studies reflecting the long-term interactions between macrolides and *P. aeruginosa* that are typical in vivo can truly elucidate the mechanisms underlying the clinical efficacies of these antibiotics.

In the present study, we examined the effect of sub-MICs of

macrolide antibiotics on the viability of *P. aeruginosa* during incubation periods exceeding 24 h. In addition, to better understand the clinical efficacy of macrolides against persistent pulmonary infections caused by *P. aeruginosa*, we examined this organism's time-dependent accumulation of AZM and the influence of macrolide antibiotics on its rate of protein synthesis.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO-1 and PA-103 were kindly provided by B. H. Iglewski (University of Rochester School of Medicine and Dentistry, Rochester, N.Y.). Additionally, 14 clinical isolates, 7 of the mucoid phenotype isolated from patients with cystic fibrosis and kindly provided by P. H. Edelstein (Department of Pathology and Laboratory Medicine and Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia) and 7 of the nonmucoid phenotype isolated from patients with diffuse panbronchiolitis at Toho University Hospital, Tokyo, Japan, were used in the present study.

Antibiotics and susceptibility testing. The following antibiotics were kindly provided by the indicated manufacturers: the 14-membered macrolide antibiotics EM (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), CAM (Taisho Pharmaceutical Co., Ltd., Tokyo, Japan), and oleandomycin (OM; Pfizer Laboratories, Groton, Conn.); the 16-membered macrolide antibiotic josamycin (JM; Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan); and the 15-membered macrolide antibiotic AZM (Pfizer Laboratories, Groton, Conn.). Other classes of antibiotics, ceftazidime (CAZ; Tanabe Pharmaceutical Co., Ltd., Osaka, Japan), ofloxacin (OFLX; Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), minocycline (MINO; Lederle Japan, Ltd., Tokyo, Japan), tobramycin (TOB; Shionogi Pharmaceutical Co., Ltd., Osaka, Japan), and clindamycin (CLDM; Japan Upjohn Co., Ltd., Tokyo, Japan), were also used. The MICs for *P. aeruginosa* were determined by using serial twofold dilutions of each antibiotic in Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). Approximately 10^5 log-phase *P. aeruginosa* organisms were plated onto the antibiotic-containing agar, and the MICs were the lowest antibiotic concentration that inhibited visible bacterial growth after 24 h of incubation at 35°C (23).

Viability determinations. After growth on Mueller-Hinton agar with or without antibiotics, the bacteria were harvested at various intervals and were suspended in physiological saline. Each bacterial suspension was adjusted to an optical density at 660 nm of 0.100. The viable bacterial counts in these suspensions were determined with serially diluted samples spread onto Mueller-Hinton agar and then incubated overnight at 35°C. Bacterial viability was expressed as a percentage of that for bacteria grown on agar without antibiotics.

The viability of *P. aeruginosa* grown in the presence or absence of AZM was also examined by using the Live/dead BacLightTM Viability Kit (Molecular Probes, Inc., Eugene, Oreg.), which distinguishes live and dead cells on the basis of differences in the spectral characteristics of nucleic acid stains and in the ability of the stains to penetrate bacterial cell membranes. Briefly, the bacteria were grown for 48 h on agar to which, in some cases, 4 μg of AZM per ml had been added. They were then suspended in physiological saline, washed by centrifugation, and stained with the stain provided with the kit according to the manufacturer's directions. Viability was assessed by fluorescence microscopy, with green cells counted as viable and red cells counted as nonviable.

AZM concentrations in bacteria. The concentrations of AZM in *P. aeruginosa* PAO-1 were determined by high-performance liquid chromatography (HPLC) with an electrochemical detector (L-EDD-6A; Shimazu Corporation, Kyoto, Japan). The bacteria were grown for 12, 24, or 36 h on agar to which 10 μg of AZM per ml had been added. They were then suspended in phosphate-buffered saline (50 mmol/liter; pH 6.4), washed two times by centrifugation, and sonicated for 2 min. Finally, the supernatants were processed for AZM concentration determination as described by Sawada et al. (27). The protein concentrations in these samples were determined by a protein assay (Bio-Rad Laboratories, Richmond, Calif.). The AZM concentrations in bacteria were expressed as micrograms per milligram of protein.

Protein synthesis rates. Rates of protein synthesis in *P. aeruginosa* PAO-1 were determined by a pulse-labeling technique. The bacteria were grown on agar, to which antibiotics were added in some cases, and were then suspended in M9 minimal medium. Pulse labeling was initiated by the addition of 10 μCi of [^{35}S] methionine to 5 ml of medium, as described by Yamashino et al. (35). Samples of 0.3 ml were taken at intervals of 5, 10, 15, 30, and 60 min, and protein was precipitated by the addition of trichloroacetic acid to a final concentration of 5%. This was followed by incubation on ice for 15 min. The precipitates were collected by centrifuging at 10,000 × *g* for 3 min, washed with acetone, and then dissolved in a solution containing 1% sodium dodecyl sulfate (SDS), 50 mmol of Tris-HCl (pH 8.0) per liter, and 1 mmol of EDTA per liter. These redissolved samples were analyzed electrophoretically on SDS–10% polyacrylamide gels, with migration followed by fluorography.

Statistics. Student's *t* test was used to compare means, with significance assessed at the 95% confidence level.

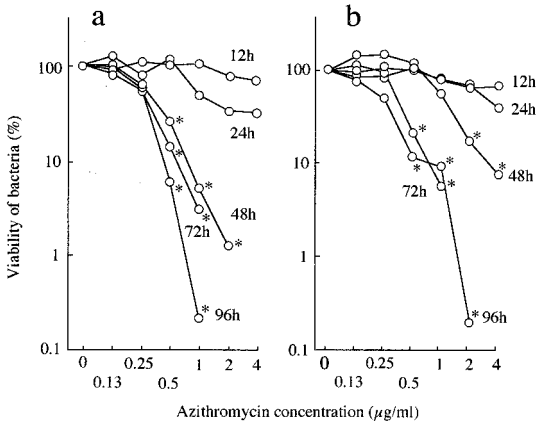

FIG. 1. Change in viability of *P. aeruginosa* following exposure to AZM for various incubation times. Strains PAO-1 (a) and PA-103 (b) were grown on agar with increasing doses of azithromycin for 12 to 96 h, and the viability of each bacterial suspension was examined ($n = 3$). *, $P < 0.05$, compared with the control.

RESULTS

MICs for *P. aeruginosa*. The measured MICs of the macrolide antibiotics AZM, EM, CAM, JM, and OM for *P. aeruginosa* PAO-1 were 128, 256, 128, >256, and >256 μg/ml, respectively. The MICs of other classes of antibiotics, CAZ, TOB, MINO, OFLX, and CLDM, for strain PAO-1 were 16, 0.5, 32, 0.5, and >256 μg/ml, respectively. The MIC of AZM for strain PA-103 was likewise 128 μg/ml, while the MIC of this antibiotic for 14 clinically isolated strains varied between 64 and 256 μg/ml.

Effects of incubation time on viability. AZM at 4 μg/ml had no apparent effect on the viability of strain PAO-1 or PA-103 during incubation times of 12 or 24 h (Fig. 1). However, longer incubation times produced a significant decrease in the viability of both strains, the extent of which depended on both the incubation time and the AZM concentration. For example, the viability of strain PAO-1 grown for 48 h in the presence of 0.5 μg of AZM per ml (1/256 the MIC) was approximately 20% of that of the control bacteria, but was only about 1% of that of the control bacteria when the agar contained 2 μg of AZM per ml (1/64 the MIC). Extending the incubation time to 96 h, however, allowed 1 μg of AZM per ml to reduce the viability to less than 1% of that of the control.

Microscopic examination of viability of bacteria grown on agar with AZM. On microscopic examination, bacteria grown on agar containing 4 μg of AZM per ml were slightly longer than those grown on antibiotic-free agar. Vital staining revealed that 77.8% ± 6.4% of the cells grown without AZM, but only 2.5% ± 0.4% of those grown on AZM-containing agar, were viable. Although there are slight quantitative differences, these observations largely confirm those presented in Fig. 1: AZM at levels significantly below the MIC is bactericidal to *P. aeruginosa* when the exposure time is at least 48 h.

Change of viability of strain PAO-1 grown on agar with various antibiotics. To investigate whether this exposure-dependent bactericidal effect is specific to AZM, we examined the effects of 48 h of exposure to several other antibiotics belonging to different classes on the viability of strain PAO-1 (Fig. 2). The macrolide antibiotics EM and CAM exhibited dose-dependent bactericidal activity, although they were less potent than AZM. Whereas 0.5 μg of AZM per ml was sufficient to reduce viability below 10%, EM and CAM concentrations of 8 and 4 μg/ml, respectively, were required to achieve

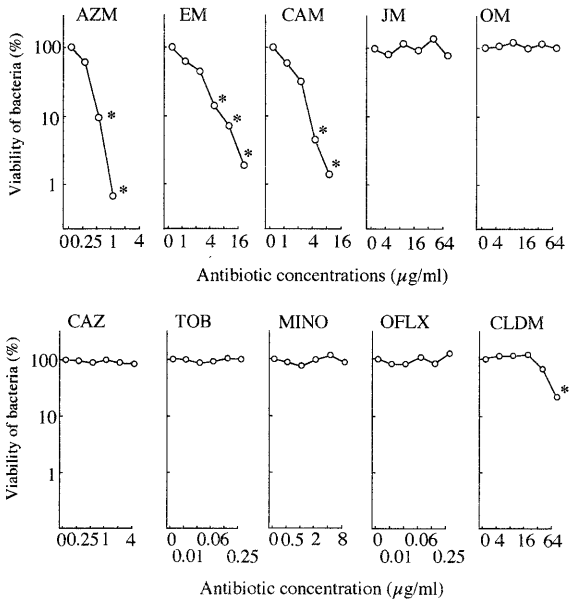

FIG. 2. Change in viability of *P. aeruginosa* PAO-1 following exposure to various antibiotics. Bacteria were grown for 48 h on agar with the indicated concentrations of antibiotics, and the viability of each bacterial suspension was examined. *, $P < 0.05$, compared with the control.

this same result. In contrast, no bactericidal activity was observed for the macrolide antibiotics JM and OM, even at concentrations as high as 64 μg/ml. Likewise, the nonmacrolide antibiotics CAZ, MINO, OFLX, and TOB showed no activity at high fractions of their respective MICs. However, CLDM did produce a slight decrease in bacterial viability at a concentration of 64 μg/ml.

Effects of AZM on viability of 14 strains of clinical isolates of *P. aeruginosa*. AZM's long-term bactericidal activity against 14 clinically isolated strains of *P. aeruginosa*, mucoid type (n = 7) and nonmucoid type (n = 7), was also examined (Fig. 3). Two mucoid strains were extremely susceptible: AZM at 0.5 μg/ml decreased their viability to less than 10% of that for control bacteria. In the other mucoid strains, a concentration-dependent decrease in viability was first observed with AZM at 2 μg/ml. Nonmucoid strains were generally more susceptible than mucoid strains: A concentration-dependent decrease in viability was observable beginning at 1 μg/ml for six strains. The last nonmucoid strain was completely resistant, however, with no evidence of decreased viability even at AZM concentrations as high as 16 μg/ml. Thus, although the strains tested showed various degrees of susceptibility, 48 h of incubation with AZM at levels significantly below the MIC was bactericidal to 13 of 14 clinically isolated *P. aeruginosa* strains tested.

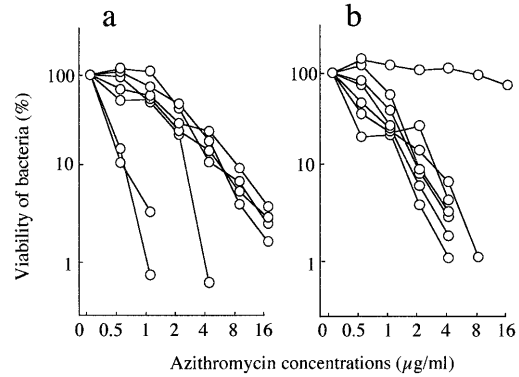

FIG. 3. Change in viability of mucoid (n = 7) (a) and nonmucoid (n = 7) (b) strains of *P. aeruginosa* following exposure to AZM. Bacteria were grown on agar with the indicated concentrations of AZM for 48 h, and the viability of each bacterial suspension was examined.

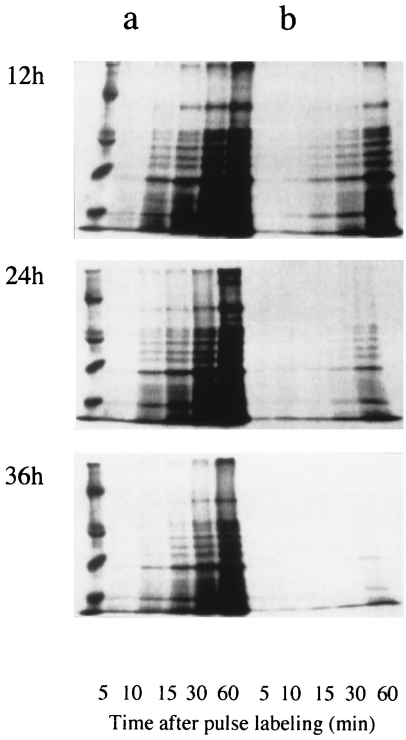

FIG. 4. Protein synthesis of *P. aeruginosa* PAO-1 grown on agar with or without AZM. Bacteria were grown on agar with (b) or without (a) 4 μg of AZM per ml for 12, 24, or 36 h. Protein synthesis of each bacterial suspension was examined at 5, 10, 15, 30, and 60 min after pulse-labeling with [^{35}S]methionine.

Intracellular accumulation of AZM. The concentrations of AZM in strain PAO-1 grown on agar containing 10 μg of this antibiotic per ml for periods of 12, 24, or 36 h were 0.190, 0.445, and 1.709 μg/ml of protein, respectively. These results indicate that strain PAO-1 accumulates AZM in a time-dependent manner.

Protein synthesis in bacteria grown on agar with AZM. Incubation of *P. aeruginosa* PAO-1 with 4 μg of AZM per ml for periods as short as 12 h clearly inhibited protein synthesis (Fig. 4). This inhibition increased in a time-dependent fashion when the incubation period was extended to 24 or 36 h. Combining these results with those presented in Fig. 1, it is clear that AZM inhibits protein synthesis before any decrease in viability can be observed. To confirm the importance of the incubation time for AZM-mediated inhibition of protein synthesis, 8 μg of AZM per ml was added to the bacterial suspension 1 min before pulse-labeling. As expected, the nearly simultaneous addition of AZM did not inhibit protein synthesis (data not shown). Thus, inhibition of *P. aeruginosa* protein synthesis by AZM takes place through a time-dependent process.

DISCUSSION

In contrast to previous reports showing the indirect effects of macrolides on *P. aeruginosa*, the present study is the first to

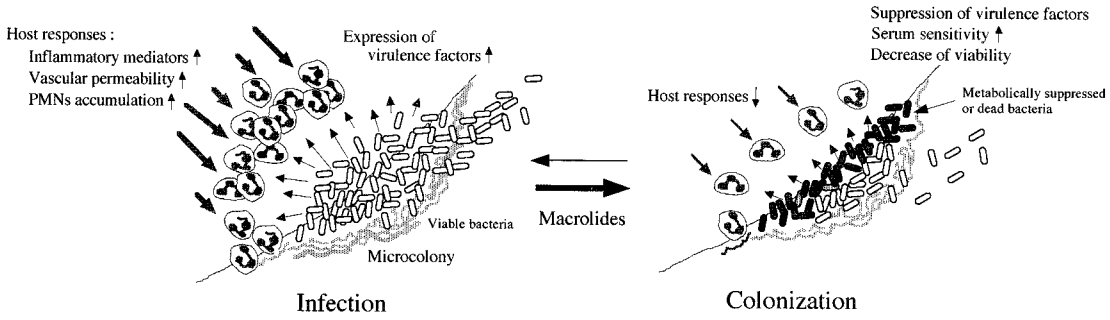

FIG. 5. Speculative scheme of "infection to colonization-promoting activity" as a possible mechanism for the efficacy of macrolides against persistent pulmonary infection with *P. aeruginosa*. PMNs, polymorphonuclear leukocytes.

show a direct antipseudomonal activity of macrolides at clinically achievable concentrations. These experiments confirm that exposure time is a critical factor for macrolide-mediated bactericidal activity against this organism.

Specifically, our results indicate that an exposure period of 48 h or longer allows the macrolide antibiotics AZM, EM, and CAM to demonstrate bactericidal activity against *P. aeruginosa* at concentrations far below the MICs. On the other hand, JM and OM show no similar activity. The differences in activity do not correlate with obvious structural differences between the antibiotics. Although EM and CAM are very similar, differing only in one side-chain residue of the macrolide aglycone ring, AZM is quite different: It has a 15-member macrolide ring, in contrast to the 14-member rings of EM and CAM with a nitrogen atom at position 9a. Conversely, OM lacks activity, despite sharing the 14-member ring structure of EM and CAM. Thus, the antipseudomonal activities of EM, CAM, and AZM may be related to structural aspects such as substitutions on the lactone ring and/or their sugar compositions rather than to the number of atoms in the macrocyclic nucleus.

Of the other nonmacrolide antibiotics tested, TOB, MINO, and CLDM inhibit protein synthesis, although they act through mechanisms different from those of the macrolides. Nevertheless, CLDM was the only other antibiotic to reduce the viability of *P. aeruginosa* when the drug was tested at very high concentrations. The present study indicated that AZM inhibits protein synthesis before any decrease in viability can be observed. These results suggest that the ability of certain macrolides to reduce viability following prolonged incubation may be associated with the inhibition of protein synthesis in *P. aeruginosa*. The inhibition of protein synthesis may be a reporter for the loss of viability. In this regard, time course experiments in which DNA, RNA, and protein synthesis are followed may be necessary for determining whether the inhibition of protein synthesis is the primary event.

Several investigators have reported that long-term macrolide therapy is clinically efficacious against *P. aeruginosa* infections (15–17, 28). The majority of these cases, however, were in diffuse panbronchiolitis patients infected with nonmucoid strains of the organism. In cystic fibrosis patients, on the other hand, up to 80% of those with chronic *P. aeruginosa* infections harbor mucoid strains (3, 4); unfortunately, adequate clinical trials of long-term macrolide therapy have not been conducted with patients with this type of infection. It is thus significant that the in vitro results of the present study suggest that macrolides exhibit bactericidal activity against clinical isolates of the mucoid as well as the nonmucoid phenotype. Accordingly, long-term macrolide therapy appears to be a potentially attractive choice for the treatment of lung infections caused by *P. aeruginosa* without regard to the organism's phenotype.

Macrolides have been reported to suppress the production of several bacterial exoproducts, including exoenzymes (11, 15, 19, 20), exopolysaccharide (13, 16), and pigment (20). Since protein synthesis may be required for several steps in the expression of the factors, our results demonstrating exposure-dependent inhibition of protein synthesis support these reports. Indeed, we found that macrolides strongly inhibit synthesis of the stress protein GroE (data not shown), which is known to play a critical role in the assembly, transport, and secretion of several exoproducts (8, 10). We accordingly suggest that short macrolide exposures may suppress the synthesis of nonessential bacterial products without affecting viability, while longer exposures may be lethal.

The outer membrane of gram-negative bacteria acts as a permeability barrier against hydrophobic antibiotics, including macrolides (25, 26). On the basis of the results of experiments with *Escherichia coli* and *Salmonella typhimurium* mutants affecting lipid A and lipopolysaccharide inner core synthesis, Vaara (32) has suggested that EM, CAM, and AZM use the hydrophobic pathway to cross the outer membrane. Similarly, Capobianco and Goldman (1) have reported that in *Haemophilus influenzae* macrolide entry into the cell is by passive diffusion, not by an active transport process. We have previously reported that 48 h of exposure to EM alters the hydrophobicity of *P. aeruginosa* (30); this is associated with changes in lipopolysaccharide and outer membrane proteins (31). In the present study, AZM was observed to accumulate intracellularly in a time-dependent manner. These results suggest that macrolide exposure induces changes in the cell surface structures of bacteria, which may in turn facilitate macrolide entry and allow the antibiotic to accumulate within the cell. Further studies on the mechanisms underlying the exposure-dependent accumulation of macrolides, for example, on the transport process and on the role, if any, of the internal ribosomal binding site in *P. aeruginosa*, are required.

To explain the effects of long-term macrolide therapy on *P. aeruginosa*, we propose the speculative scheme from the standpoint of a macrolide's effect on bacteria (Fig. 5). In the respiratory tracts or alveolar spaces of patients with persistent *P. aeruginosa* infections, the bacteria live on the surface of respiratory cells. There they are associated with secreted mucus and host-cell debris in the form of microcolonies or biofilm (9, 18). As the bacteria multiply, they express virulence factors that may injure host cells and induce local host responses, for example, the production of inflammatory mediators, increases in vascular permeability, and leukocyte accumulation. When macrolides are administered, the bacteria are exposed to these antibiotics; populations directly adhering to epithelial cells may experience particularly high concentrations because of the antibiotic concentration gradient. Under these conditions, sub-

MICs of the drug may, as reported previously, suppress the virulence of *P. aeruginosa*. Moreover, in patients undergoing macrolide therapy for prolonged periods, bacteria continuously exposed to the antibiotic may be sensitized to the serum bactericidal effect. Still further, bacteria closely associated with host cells may gradually lose their viability as a consequence of the direct antipseudomonal bactericidal activities of these medications. This may be expected to produce clinical improvement, even though bacteria may continue to be expectorated for a time as a sign of colonization. Thus, we speculate that long-term macrolide therapy may shift the host-pathogen interaction from infection to a relatively benign colonization state and possibly even to eradication in some patients. This hypothesis explains quite well the frequent clinical observation that long-term macrolide therapy leads to improvements in clinical symptoms and laboratory data prior to any observable bacteriological response. It may accordingly be the promotion of a shift from infection to colonization that underlies the efficacy of long-term macrolide therapy ("infection to colonization-promoting activity").

In conclusion, we report evidence for a direct antipseudomonal bactericidal activity of certain macrolides. These data may explain, at least in part, the clinical efficacy of macrolide antibiotics against persistent *P. aeruginosa* infection in the lung, a site where bacteria are continuously exposed to these antibiotics, even though the concentrations remain far below the MICs determined by using 24-h incubation times.

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