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Functional Role of Mucoid Exopolysaccharide (Alginate) in Antibiotic-Induced and Polymorphonuclear Leukocyte-Mediated Killing of *Pseudomonas aeruginosa*

ARNOLD S. BAYER,[1,2]* DAVID P. SPEERT,[3] SUSAN PARK,[1] JEREMY TU,[1] MALLORY WITT,[1] CYNTHIA C. NAST,[2,4] AND DEAN C. NORMAN[4,5]

Division of Adult Infectious Diseases, Department of Medicine,[1] and Department of Pathology,[4] Harbor-UCLA Medical Center, Torrance, California 90509; Department of Pediatrics, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada[3]; Geriatric Division, West Los Angeles Veterans Administration Medical Center, Los Angeles, California 90073[5]; and UCLA School of Medicine, Los Angeles, California 90024[2]

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We evaluated in vitro the functional role of mucoid exopolysaccharide (MEP) of *Pseudomonas aeruginosa* in blocking antibiotic-induced and polymorphonuclear leukocyte (PMN)-mediated pseudomonal killing. The serum-resistant *P. aeruginosa* isolates used were mucoid strain 144MR and its nonmucoid revertant, strain 144NM. By timed kill curves, early bactericidal effects of amikacin against mucoid strain 144MR were substantially less than those observed with nonmucoid strain 144NM; this effect was reversible with enzymatic hydrolysis of MEP of strain 144MR by alginase. Also, early tobramycin uptake (15 to 30 min) by mucoid 144MR cells was less than that seen with nonmucoid strain 144NM; pretreatment of 144MR cells with alginase substantially enhanced early tobramycin uptake compared with untreated 144MR cells ($P = 0.08$). In strain 144NM (but not in strain 114MR) there was a notable postantibiotic leukocidal enhancement effect manifested by increased nonopsonic killing following brief exposure of these cells to supra-MIC amikacin; pretreatment of strain 144MR with alginase rendered these cells more susceptible to amikacin-induced postantibiotic leukocidal enhancement. Similarly, direct PMN-mediated nonopsonic killing of mucoid strain 144MR was significantly less than that observed with strain 144NM ($P < 0.05$); pretreatment of 144MR cells with alginase rendered this strain equal to strain 144NM in susceptibility to nonopsonic killing. In addition, exogenous sodium alginate or extracted MEP of strain 144MR interfered with effective nonopsonic killing of strain 144NM by PMNs. Studies also indicated that mucoid strain 144MR was phagocytosed significantly less well than its nonmucoid mate ($P < 0.00001$), an effect reversed by pretreatment of the mucoid cells with alginase. These data confirm that *P. aeruginosa* MEPs functionally decrease the uptake and early bactericidal effect of aminoglycosides in vitro and interfere with effective PMN-mediated nonopsonic phagocytosis and killing of mucoid strains.

The extracellular glycocalyx produced in copious amounts by mucoid strains of *Pseudomonas aeruginosa* has been implicated in morbidity associated with cystic fibrosis (10, 15, 24). The overall biological role of this mucoid exopolysaccharide (MEP) of *P. aeruginosa* remains incompletely defined. It appears that this alginic acid MEP mediates attachment of pseudomonal strains to tracheal epithelium (25), interferes with effective complement-driven PMN chemotaxis (31), renders mucoid variants less susceptible to nonopsonic phagocytosis by macrophages (9, 11, 29), and assists the organism in resisting oxidative intracellular killing via the myeloperoxidase system through 'hypochlorite scavenging' (16). The other putative major function of MEP is to interfere with intracellular antibiotic accumulation, presumably via barrier mechanisms, with resultant diminution of antibiotic-induced growth inhibition and killing. However, the latter protective function of MEP remains controversial (13, 21–23, 27, 28).

The current study was designed to examine the role of MEP in blocking antibiotic-induced and polymorphonuclear leukocyte (PMN)-mediated killing of *P. aeruginosa*. In these studies, we examined pseudomonal strains which were either phenotypically mucoid or had oxygen-upregulated MEP production (3, 4).

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MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* 144NM is a stable, spontaneous, nonmucoid revertant of mucoid strain 144M, a clinical isolate initially cultured from a patient with cystic fibrosis; strain 144MR is a serum-resistant derivative of parent strain 144M obtained by serial passage of strain 144M on increasing concentrations of human serum (26). These strains were kindly provided by Neal Schiller, University of California at Riverside. Strain PA-96, a clinical nonmucoid isolate used in our prior studies of experimental endocarditis (2, 4, 5), exhibits enhanced MEP production at oxygen tensions reflective of the left-sided cardiac circuit (~80 mm Hg) but not at oxygen tensions observed in the right-sided cardiac circuit (~40 mm Hg; [3, 4]).

Antibiotics. Amikacin and tobramycin were supplied by

* Corresponding author.

Bristol Pharmaceuticals (Syracuse, N.Y.) and Eli Lilly Laboratories (Indianapolis, Ind.), respectively. Antibiotic stock solutions of 1,000 μg/ml were kept at −70°C until used on the day of in vitro assays.

Antibiotic susceptibility testing. The MICs of amikacin for the various pseudomonal strains in this study were determined in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) by the macrodilution technique (32) using washed logarithmic-phase organisms at final concentrations of $\sim 10^6$ CFU/ml. The range of final amikacin concentrations was 0.125 to 256 μg/ml. The MIC was the lowest antibiotic concentration resulting in no visible turbidity after 24 h of incubation at 37°C. Final MICs were the means of three separate determinations.

Alginase preparation. The alginase used in the current study was obtained from *Bacillus circulans* (ATCC 15518) as previously described (11). The organism was grown in alginate-yeast extract medium, and the culture supernatant was concentrated by microfiltration. After precipitation by ammonium sulfate and dialysis, the crude enzyme preparation was frozen at −20°C until thawed for use on the day of the study. The specific activity of the enzyme preparation was determined by the yield of unsaturated material after enzyme action on alginate substrates by the periodate-thiobarbituric acid procedure (33). The calculated enzyme activity was 125 U/ml (7.7 U/mg of protein). The specificity of this enzyme is for 1,4-linked polyuronic residues of alginates (17). The enzyme has been shown to lack functional alterations of pseudomonal lipopolysaccharide by its inability to influence nonopsonic phagocytosis of nonmucoid strains (11).

Phagocytic assays. We used a visual inspection assay to assess the relative efficiency of PMN phagocytosis of mucoid strain 144MR versus that of nonmucoid strain 144NM (11). We added either strain at a final concentration of $\sim 10^7$ CFU/ml to PMN-containing polypropylene tubes to achieve a final organism-PMN ratio of ~20:1. In parallel, PMNs were incubated with 144MR cells that had been pretreated with alginase. Tubes were rotated end over end for 60 min at 37°C; after centrifugation and washing of the PMNs to remove adherent but unphagocytosed organisms, cells were deposited on glass slides with a Cytospin 2 centrifuge (Shandon Southern Products Ltd., Astmoor, England) at 600 rpm for 5 min. The slides were air dried, heat fixed, and stained with crystal violet. The number of intracellular bacteria per cell was determined by counting 50 PMNs per experimental run. The mean numbers of mucoid or nonmucoid bacteria per PMN undergoing nonopsonic phagocytosis were calculated from the results of three separate experimental runs. Although the absolute numbers of bacteria undergoing nonopsonic phagocytosis varied somewhat from day to day, the relative quantitative phagocytosis profile for each strain and condition was highly reproducible (e.g., more effective phagocytosis of mucoid strain 144MR by PMNs with than without alginase).

Influence of alginase on the early bactericidal effect of aminoglycosides. We previously showed that aminoglycoside-induced killing of a nonmucoid pseudomonal strain with oxygen-upregulated exopolysaccharide production was substantially slower than for the same strain not preexposed to such conditions (4). To further define the role of MEP in this regard, we examined the early killing kinetics of amikacin (with or without alginase pretreatment) against pseudomonal strains 144MR and 144NM by the timed kill curve technique. Each pseudomonal strain was grown to logarithmic phase (~6 h; optical density at 600 nm, 0.8), washed, and then added in parallel to either amikacin-containing or amikacin-free BHI broth to achieve a final concentration of $\sim 10^6$ CFU/ml and a final amikacin concentration of 1.5 times the MIC for the respective organism. Pilot kill curve studies had shown that at amikacin concentrations greater than or equal to twice the MIC, killing of both strains was rapid and complete by 6 h. In parallel with the above-described studies, logarithmic-phase cells of mucoid strain 144MR were treated for 90 min with alginase (125 U/ml) before exposure to amikacin in the kill curve assay. At 0, 4, 6, and 24 h of incubation at 37°C, 100-μl samples obtained from all growth tubes were quantitatively cultured onto BHI agar. Kill curves compared the $\log_{10}$ CFU of surviving bacteria per milliliter on the vertical axis with incubation time on the horizontal axis. Summary data were the means of quantitative results from three separate experimental runs; in each experimental run, the relative kill curve results were highly reproducible (e.g., enhanced aminoglycoside killing of mucoid strain 144MR with versus without alginase). A significant bactericidal effect was defined as a ≥ 2 $\log_{10}$ difference in bacterial counts when various experimental conditions (e.g., killing of mucoid strain 144MR with or without alginase) were compared.

In vitro postantibiotic effect. The presence of a persistent, sublethal postantibiotic effect (PAE) was determined for amikacin by a modification of the technique of McDonald et al. (19). Pseudomonal test strains 144MR and 144NM, in the late logarithmic growth phase in BHI broth, were diluted 1:10 in fresh BHI broth containing either no antibiotics or amikacin to achieve a final concentration of $\sim 10^7$ CFU/ml and a final drug concentration of 10 or 20 times the respective amikacin MIC. After incubation of these tubes for 2 h at 37°C, the antibiotics were removed by centrifugation of the organism at 15,000 × *g* for 10 min and subsequent washing of the cell pellets twice with phosphate-buffered saline (19); in separate parallel studies, the time of exposure of the organisms to amikacin was shortened to 1 h. After the washing steps, the cell pellets were suspended in fresh, antibiotic-free BHI broth to the original volume (20 ml). Quantitative cultures were obtained from each tube at zero time (preincubation), after 2 and 3 h (before and after antibiotic removal, respectively), hourly for 5 h after antibiotic removal, and at 15 h after antibiotic removal. Curves were then constructed relating time to viable pseudomonal counts. The PAE duration (in hours) was defined as the difference in the time required for counts of antibiotic-exposed and unexposed pseudomonal cells, respectively, to increase by 1 $\log_{10}$ CFU/ml above the number present just after the centrifugation-washing-suspension step (19). For each pseudomonal isolate tested, four separate PAE experimental runs were performed.

Postantibiotic leukocidal enhancement (PALE). We examined the susceptibility of 144MR or 144NM pseudomonal cells to PMN-mediated killing in vitro following brief exposure of growing cells to amikacin by the method of McDonald et al. (20). Blood from healthy volunteers who were not taking any medications was drawn into heparinized tubes. After thorough mixing, the heparinized blood was added to tubes containing Ficoll-Hypaque (Mono-Poly Resolving Medium; Flow Laboratories, McLean, Va.). After centrifugation, the PMN-rich layer was drawn off into 50-ml polypropylene tubes. Contaminating erythrocytes were removed with a lysing buffer. PMNs were washed twice and then suspended in Eagle minimal essential medium (MEM) with 0.1% gelatin. The PMN suspension was adjusted to a concentration of $\sim 10^7$ cells per ml, and viability was determined

by trypan blue exclusion; preparations exhibiting >95% viability were used in these studies. For each experiment, freshly collected PMNs from a single donor were used.

Pooled normal human serum (PNHS), used for opsonization of pseudomonal cells, was prepared from five healthy donors and stored at −70°C until thawed on the day of use. PNHS was used at a final dilution of 10% in opsonophagocytic assays.

For assessment of the PALE, strain 144MR or 144NM was grown to the logarithmic phase, washed twice in phosphate-buffered saline, suspended, and diluted in MEM-gelatin to a final concentration of ~10^7 CFU/ml spectrophotometrically. To the bacterium-containing suspension (0.5 ml) was added 0.5 ml of either amikacin-containing or amikacin-free MEM-gelatin for 10 min at 37°C; final amikacin concentrations were 10 times the MICs. At the end of the 10-min incubation period, tubes were microcentrifuged and the cell pellet was washed twice in phosphate-buffered saline to remove residual amikacin (dilution factor of amikacin, >1,000). Samples (0.1 ml) of amikacin-sensitized or unsensitized pseudomonal cells (~10^7 CFU/ml) were added to 0.9-ml mixtures in MEM of PMN alone, PNHS alone, MEM alone (positive growth control), or PMN-PNHS. The final ratio of pseudomonal cells to PMNs in the reaction mixtures was ~5:1. At 0, 60, 120, and 180 min of incubation at 37°C on a rotator (10 rpm), a 50-μl sample from each reaction tube was added to sterile distilled water to lyse PMNs and briefly sonicated to guarantee singlet bacterial cells. Samples were then quantitatively cultured onto sheep blood agar plates. After incubation at 37°C for 48 h, surviving pseudomonal colonies were counted; survival curves were then constructed comparing mean $\log_{10}$ CFU of surviving pseudomonal cells per milliliter of the various reaction mixtures over time. As done in the PAE study, parallel experiments were run with mucoid pseudomonal strain 144MR pretreated with alginase before performing PALE. Summary data represent the means of at least five separate experimental runs. The comparative results of PMN-mediated pseudomonal killing in PALE (as well as in the other PMN-mediated bactericidal systems below) under various experimental conditions were highly reproducible.

PMN killing of pseudomonal cells in the presence of alginase. We examined the effect of alginase pretreatment on the opsonic and nonopsonic killing of various pseudomonal cells by PMNs. The following strains were studied: PA-96 preexposed for 6 h to a pO_2 of either ~40 or 80 mm Hg, 144NM, and 144MR. Confirmation of MEP upregulation by pO_2s of ~80 mm Hg in strain PA-96 was by the periodate-barbituric acid assay as described above (3, 33). The PMN-mediated killing assay strategies outlined above for the PALE study were used to construct survival curves. In parallel studies, various pseudomonal cells were treated with alginase (125 U/ml) before addition of the organism to the various reaction tubes containing PMNs with or without PNHS. Data were expressed as the means of nine separate experimental runs.

Influence of exogenous alginate on PMN killing of nonmucoid pseudomonal strains. To confirm that alginate itself has an inhibitory effect on PMN-mediated killing of mucoid pseudomonal strains, the above-described PMN bactericidal assays were repeated with nonmucoid strain 144NM with or without exogenous alginate. In parallel studies, either purified seaweed sodium alginate (Sigma Chemicals, St. Louis, Mo.) or extracted MEP from strain 144MR was used at a final concentration of 2.5 mg/ml. The MEP extraction process has been detailed elsewhere (18). Parallel studies in which alginase was coincubated with exogenous sodium alginate in the final PMN bactericidal reaction mixtures were also performed. All results represented the means of at least five separate experimental runs.

Aminoglycoside uptake by mucoid and nonmucoid pseudomonal cells. Aminoglycoside uptake was determined for pseudomonal strains 144NM and 144MR by a modification of the technique of Burns et al. (8). Tobramycin was used instead of amikacin; pilot studies had shown that the indicator organism for detecting aminoglycoside levels by bioassay (*Escherichia coli* ATCC 15236) was severalfold more susceptible to tobramycin than amikacin, generating more linear dose-response standard curves for determination of aminoglycoside levels in the bioassay system.

Pseudomonal strains 144NM and 144MR were grown to logarithmic phase as before in BHI broth and then washed twice in phosphate-buffered saline before being added to fresh tobramycin-containing BHI broth. Final bacterial concentrations were ~10^8 CFU/ml as adjusted spectrophotometrically, and final tobramycin concentrations were 2.5 and 5 μg/ml in parallel studies. After vortexing, samples were removed at 0, 15, 30, and 60 min postincubation at 37°C and quantitatively subcultured onto BHI agar (to ensure no reduction in bacterial cell mass). All tubes were then centrifuged at 10,000 × *g* to pellet the bacterial inoculum, the supernatant was sampled, and the samples were stored at −70°C for determination of residual tobramycin concentrations by bioassay and percent uptake of tobramycin. In parallel studies, tobramycin uptake was determined after pretreatment of 144MR cells with alginase. Summary data represent means of three separate uptake experiments.

Statistical analysis. Analysis of variance was used to compare the differences between sets of continuous data (e.g., the $\log_{10}$ CFU of surviving bacteria per milliliter). The Bonferroni test was used when appropriate for pairwise comparisons, while the nonparametric Kruskal-Wallis test was used to evaluate the results of the analysis of variance. For the phagocytosis data, the mean numbers of bacteria per PMN were transformed to the corresponding square root to yield more normal distribution of the data; which could then be subjected to parametric statistical analyses (7, 11).

RESULTS

Antibiotic susceptibility testing. The amikacin MICs (in micrograms per milliliter) for mucoid strain 144MR at a concentration of ~10^6 CFU/ml was 4 μg/ml; its nonmucoid mate (144NM) was twofold more susceptible to amikacin (MIC, 2 μg/ml). The MIC differences between the strains were identical in the three separate experiments performed.

Phagocytosis of mucoid versus nonmucoid pseudomonal cells. Nonmucoid strain 144NM was phagocytosed significantly better than mucoid strain 144MR (mean square root of the number of bacteria per PMN ± the standard error of the mean [SEM], 2.9 ± 0.24 versus 0.69 ± 0.07, respectively; $P < 0.00001$); alginase pretreatment of strain 144MR significantly enhanced the phagocytosis of this organism by PMNs compared with that of non-alginase-treated 144MR cells (2.03 ± 0.16 versus 0.69 ± 0.07 cells per PMN, respectively; $P < 0.00001$).

Kill curve studies. The amikacin-induced early bactericidal effect against mucoid strain 144MR was substantially less than that observed with nonmucoid strain 144NM (Fig. 1). After 4 h of exposure to amikacin, there were ~2 $\log_{10}$ fewer surviving CFU of nonmucoid strain 144NM per ml than of mucoid strain 144MR. By the end of 6 h of exposure to supra-MIC amikacin concentrations, counts of strain 144NM

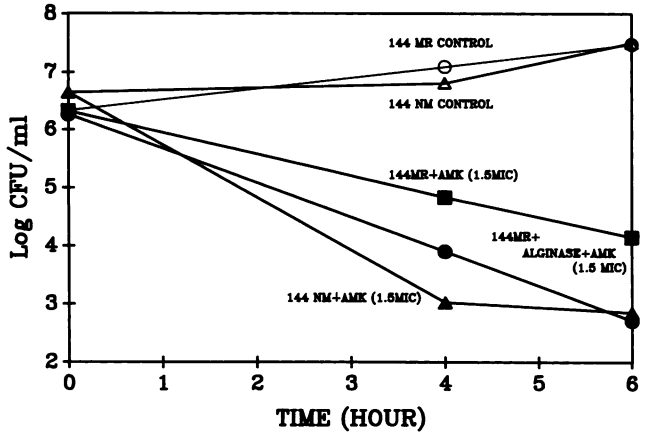

FIG. 1. Effect of alginase pretreatment of mucoid strain 144MR (versus nonmucoid strain 144NM) upon early bactericidal activity of amikacin (AMK) at 1.5 times the respective MIC for each organism. The means of three separate experiments are shown.

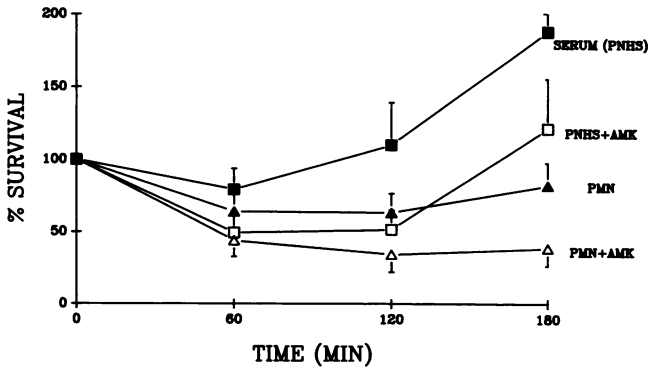

FIG. 2. PALE versus strain 144NM following 10 min of exposure of the organism to amikacin at 10 times the MIC. For example, PNHS+AMK means the growth kinetics of strain 144NM in PNHS following 10 min of exposure to amikacin. PMN+AMK represents the nonopsonic bactericidal efficacy of PMNs for strain 144NM following 10 min of exposure of the strain to amikacin. The means (± SEM) of five separate experiments are shown.

had fallen by ~4 $\log_{10}$ from zero-time values, compared with an ~2 $\log_{10}$ decline for mucoid strain 144MR. Pretreatment of mucoid strain 144M with alginase eliminated this difference in kill curve kinetics between the two strains (Fig. 1). By 24 h of incubation, counts of surviving strain 144NM and 144MR organisms had fallen below 2 $\log_{10}$ (data not shown).

In vitro PAE. There were no significant differences in the durations of the PAE determined for the mucoid and nonmucoid pair of pseudomonal strains. For example, following 2 h of exposure to amikacin at 20 times the respective MIC for each organism, the mean PAE for strain 144NM was 2.26 h versus 2.15 h for strain MR. Similar data were generated for exposure of these two strains to amikacin levels of 10 times the MIC for 2 h. Also, there was no discernible difference between PAE durations for this pair of strains when the antibiotic exposure time was shortened to 1 h from 2 h (data not shown).

PALE. 144NM cells not pretreated with amikacin exhibited modest nonopsonic killing by PMNs; following brief exposure to supra-MIC amikacin concentrations, this nonmucoid strain exhibited enhanced susceptibility to nonopsonic killing by PMNs (Fig. 2). In contrast, mucoid strain 144MR resisted nonopsonic killing by PMNs, with a mean survival of >100% at 180 min (the brief exposure of this strain to amikacin did not substantially enhance PMN nonopsonic killing) (Fig. 3). Pretreatment of strain 144MR with alginase rendered these cells substantially more susceptible to nonopsonic killing by PMNs following brief exposure to amikacin. Opsonic killing of nonmucoid strain 144NM was rapid and nearly complete after 120 min (mean survival at 180 min, ~10%; data not shown), with enhancement of neither the extent nor the rapidity of killing by amikacin pretreatment. Opsonic killing of mucoid strain 144MR was moderately slower and less efficient than that of its nonmucoid counterpart, with mean survival at 180 min of 40% (data not shown); as with strain 144NM, brief pretreatment of strain 144MR with amikacin did not substantially effect its opsonic killing.

Effect of alginase on PMN-mediated killing of pseudomonads. Pretreatment of mucoid strain 144MR with alginase resulted in enhancement of nonopsonic killing to levels seen with PMN-mediated killing of nonmucoid strain 144NM ($P < 0.05$ versus prealginase values; Fig. 4). As seen in the PALE studies described above, pretreatment of mucoid 144MR cells with alginase did not appreciably effect either the rate or extent of opsonic killing.

Addition of either exogenous sodium alginate or MEP extracted from mucoid strain 144MR to the reaction mixtures containing PMNs with or without PNHS and strain 144NM substantially interfered with efficient nonopsonic killing of the latter organism (Fig. 5). Coincubation of either exogenous alginate or MEP with alginase within the reaction mixtures abrogated the inhibitory effects of alginate and MEP upon nonopsonic killing of strain 144NM by PMNs (data not shown). Addition of neither exogenous alginate nor exogenous MEP influenced PMN-mediated killing of strain 144NM via opsonic pathways (data not shown).

Following 6 h of exposure of strain PA-96 to a pO_2 of ~80 mm Hg to upregulate MEP production (1, 2), this organism exhibited PMN-mediated killing curves similar to those of mucoid strain 144MR, manifesting interference predominantly in nonopsonic killing (Fig. 5). In contrast, PA-96 cells exposed in vitro to pO_2s of ~40 mm Hg (reflective of the right cardiac circuit in vivo [4]) exhibited more rapid and complete PMN-induced killing by both opsonic and nonopsonic pathways (e.g., 15% mean survival at 120 min; data not shown). In parallel, following in vitro exposure of PA-96 to

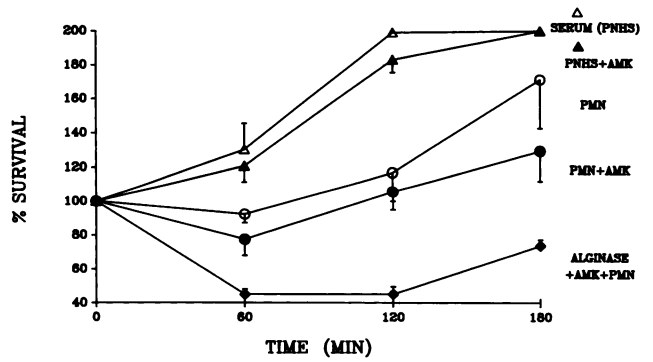

FIG. 3. PALE versus strain 144MR following 10 min of exposure to amikacin at 10 times the MIC. The means (± SEM) of five separate experiments are shown.

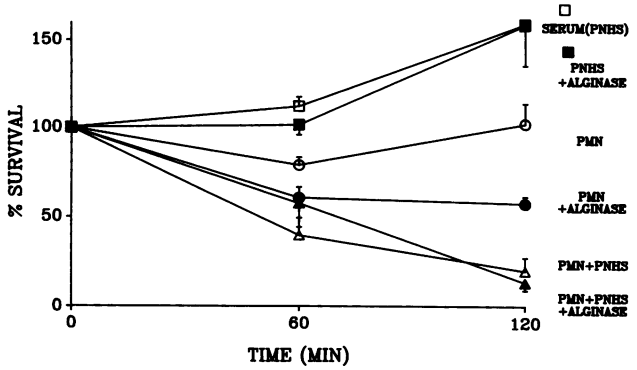

FIG. 4. Effect of alginase on PMN-mediated killing of mucoid strain 144MR. The means of nine separate experiments are shown.

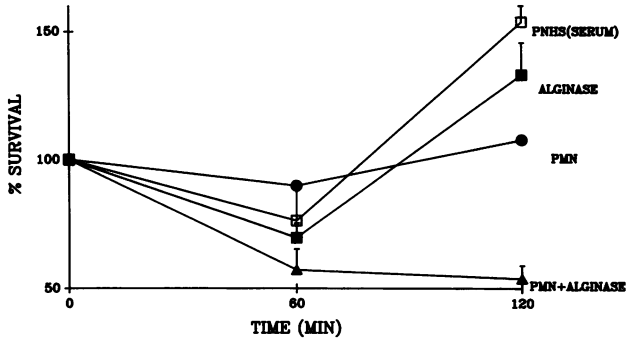

FIG. 6. Effect of alginase on PMN-mediated killing of *P. aeruginosa* PA-96 following enhancement of the MEP production of the strain by exposure to oxygen tensions of ~80 mm Hg for 6 h. The means (± SEM) of nine separate experiments are shown.

a pO_2 of ~80 mm Hg to upregulate MEP production, these cells were exposed to alginase. Alginase pretreatment enhanced the efficiency of nonopsonic killing of this strain by PMNs compared with non-alginase-treated cells (Fig. 6). Alginase pretreatment did not substantially influence the rate or extent of PMN-induced opsonic killing of this strain. These data are very similar to those generated as described above with phenotypically mucoid strain 144MR.

Tobramycin uptake by mucoid and nonmucoid pseudomonal cells. Early tobramycin uptake by nonmucoid strain 144NM was higher than that of its mucoid mate 144MR. By 15 to 30 min of incubation at a final drug concentration of 5 μg/ml, ~80% of the tobramycin was accumulated by strain 144NM; in contrast, strain 144MR accumulated only ~65% of the initial tobramycin in the medium over the same period. Similar differences in tobramycin uptake between these strains were observed at an initial tobramycin concentration of 2.5 μg/ml (data not shown). Pretreatment of strain 144MR with alginase yielded tobramycin uptake curves similar to those observed with strain 144NM; moreover, early tobramycin uptake (15 to 30 min) by alginase-treated 144MR cells was substantially greater than that seen with untreated 144MR cells ($P = 0.08$; Fig. 7).

DISCUSSION

We designed the current study to examine the interaction of MEP with both antimicrobial effects against *P. aeruginosa* and PMN-mediated antipseudomonal killing. Several interesting findings emanated from this investigation. A functional inhibitory effect of MEP on early aminoglycoside-induced killing of mucoid pseudomonal strain 144MR was demonstrated. This mucoid strain, which had an MIC for amikacin which was reproducibly twice that of its nonmucoid partner, 144NM, was more slowly killed by amikacin at supra-MIC drug levels over the first 6 h of incubation (such modest increases in aminoglycoside MICs among mucoid versus nonmucoid *P. aeruginosa* strains have been previously observed [14]). The slow early bactericidal effect rendered by amikacin against mucoid strain 144MR was reversible by pretreating this organism with an MEP-specific hydrolyzing enzyme (alginase). These observations on the relatively slow aminoglycoside bactericidal effect against the mucoid pseudomonal variant parallel those we previously noted in aminoglycoside-induced killing of nonmucoid pseudomonal strains with oxygen-upregulated MEP production (4). The modest increase in the aminoglycoside MIC of the mucoid variant and the eventual effective killing of this strain by amikacin by 24 h of incubation suggest that the functional barrier inhibiting aminoglycoside uptake is not an all-or-none phenomenon. This has been confirmed by Nichols et al. (21) in a theoretical model of tobramycin diffusion into glycocalyx biofilms, in which they found that tobramycin penetration times were about threefold longer in the presence than in the absence of glycocalyx. Our data on

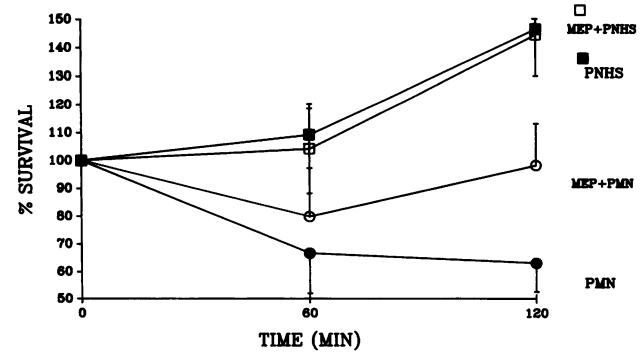

FIG. 5. Effect of exogenous pseudomonal MEP extracted from strain 144MR upon PMN-mediated killing of nonmucoid strain 144NM. The means (± SEM) of five separate experiments are shown.

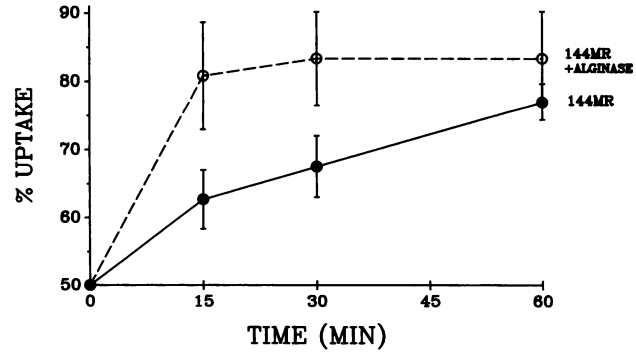

FIG. 7. Effect of alginase pretreatment upon tobramycin uptake by mucoid strain 144MR. The initial tobramycin concentration in the medium was 5 μg/ml. The means (± SEM) of three separate experiments are shown.

tobramycin uptake by mucoid versus nonmucoid pseudomonal strains agree with this concept; although early tobramycin uptake by mucoid cells was clearly less than that by their nonmucoid counterparts, mucoid cells still were able to accumulate ~65% of the tobramycin in the original reaction mixture (versus 80 to 85% by the nonmucoid variant). This modest slowing of tobramycin uptake by the mucoid pseudomonal strain was reversible by pretreatment of such cells with alginase. The in vivo significance of such modest increases in aminoglycoside MICs and modest diminution of aminoglycoside uptakes manifested by mucoid pseudomonal strains remains to be determined. It is possible that such differences between mucoid and nonmucoid pseudomonal strains become more magnified and significant in the lungs of patients with cystic fibrosis. Local pulmonary factors, such as cation-rich endobronchial secretions, large bacterial inocula (6, 31), and growth of mucoid organisms in a biofilm (1, 15), may act synergistically to increase the in vivo MICs for mucoid organisms in this setting, particularly those of aminoglycosides.

The mucoid phenotype of strain 144MR also interfered with the ability of amikacin to induce a measurable PALE effect following brief exposure (10 min) to this agent. The PALE phenomenon depends on antibiotic alteration of the cell surface of the bacteria, presumably to expose ligands involved in either opsonic or nonopsonic phagocytic recognition; the lack of a PALE in mucoid strain 144MR further supports the notion of a functional barrier against aminoglycoside cell wall penetration rendered by MEP. We were not able to demonstrate any functional blockage of the in vitro PAE by the presence of MEP in the mucoid pseudomonal strain in the current investigation. However, the longer aminoglycoside exposure times in the PAE study (1 to 2 h) compared with the PALE study (10 min) may have allowed sufficient drug to penetrate MEP to induce similar PAEs in the mucoid and nonmucoid variants. These data are at variance with those of our previous study (4), in which we showed that the presence of oxygen-upregulated MEP in nonmucoid pseudomonal strain PA-96 was associated with substantial lengthening of the PAE duration compared with that of the nonupregulated PA-96 variant (~3 versus 1.4 h, respectively [4]). This finding suggests that MEPs from different pseudomonal strains differ either in their overall quantitative or qualitative features vis a vis antibiotic barrier function.

The ability of MEP to interfere with direct host cell (PMN)-mediated killing of mucoid pseudomonal strains was also demonstrated in the current study. Nonopsonic PMN killing of the mucoid strain was substantially less than that of its nonmucoid variant. Moreover, addition of either exogenous purified sodium alginate or MEP extracted from the mucoid variant rendered nonmucoid cells similarly resistant to nonopsonic PMN killing. In addition, pretreatment of the mucoid variant with alginase allowed more efficient nonopsonic PMN killing of this strain. Further support of the ability of MEP to interfere with PMN-mediated pseudomonal killing was provided by oxygen-dependent upregulation of MEP production. Exposure to oxygen tensions of ~80 mm Hg rendered phenotypically nonmucoid strain PA-96 more resistant to nonopsonic PMN killing; i.e., its resistance was similar to that of phenotypically mucoid strain 144MR. Treatment of PA-96 cells with alginase following oxygen upregulation of MEP also rendered this strain more susceptible to nonopsonic killing by PMNs.

The above data suggest that phenotypically mucoid pseudomonal strains or nonmucoid strains which are able to upregulate alginate production may achieve a selective survival advantage in vivo by escaping both antibiotic-induced and phagocyte-mediated killing. This may be pertinent both to areas of limited antibiotic diffusion (e.g., abscesses, the central nervous system, osteomyelitic lesions) and to areas of limited PMN and/or opsonizing immunoglobulin G antibody access or function. This may be particularly relevant to cystic fibrosis pulmonary disease associated with persistent airway colonization with mucoid pseudomonal strains (10, 30). In the latter situation, the pseudomonal metalloprotease elastase appears to cleave native immunoglobulin G into Fc and Fab fragments at its carbohydrate-rich domain, potentially rendering immunoglobulin G-mediated opsonophagocytic bactericidal pathways ineffectual (12). Thus, airway PMNs must depend on nonopsonic bactericidal mechanisms to eradicate colonizing strains of *P. aeruginosa*; our data confirm the ability of MEP to interfere with this bacterial clearance pathway. The current investigation suggests that MEP plays a role in protecting mucoid organisms from PMN phagocytosis and eventual PMN-induced intracellular killing; the relative contributions of MEP to interference with antibiotic diffusion, phagocytosis, and intracellular oxidative bacterial killing remain to be elucidated.

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