Sugar administration is an effective adjunctive therapy in the treatment of *Pseudomonas aeruginosa* pneumonia

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1Department of Medicine, University of California, San Francisco, California; 2Microbial Pathogenesis and Host Defense Program, University of California, San Francisco, California; 3Department of Microbiology and Immunology, University of California, San Francisco, California; 4Department of Anesthesiology, University of California, San Francisco, California; and 5The Cardiovascular Research Institute at the University of California, San Francisco, California

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*Bucior I, Abbott J, Song Y, Matthay MA, Engel JN.* Sugar administration is an effective adjunctive therapy in the treatment of *Pseudomonas aeruginosa* pneumonia. Am J Physiol Lung Cell Mol Physiol 305: L352–L363, 2013. First published June 21, 2013; doi:10.1152/ajplung.00387.2012.—Treatment of acute and chronic pulmonary infections caused by opportunistic pathogen *Pseudomonas aeruginosa* is limited by the increasing frequency of multidrug bacterial resistance. Here, we describe a novel adjunctive therapy in which administration of a mix of simple sugars—mannose, fucose, and galactose—inhibits bacterial attachment, limits lung damage, and potentiates conventional antibiotic therapy. The sugar mixture inhibits adhesion of nonmucoid and mucoid *P. aeruginosa* strains to bronchial epithelial cells in vitro. In a murine model of acute pneumonia, treatment with the sugar mixture alone diminishes lung damage, bacterial dissemination to the subpleural alveoli, and neutrophil- and IL-8-driven inflammatory responses. Remarkably, the sugars act synergistically with anti-*Pseudomonas* antibiotics, including β-lactams and quinolones, to further reduce bacterial lung colonization and damage. To probe the mechanism, we examined the effects of sugars in the presence or absence of antibiotics during growth in liquid culture and in an ex vivo infection model utilizing freshly dissected mouse tracheas and lungs. We demonstrate that the sugar mixture induces rapid but reversible formation of bacterial clusters that exhibited enhanced susceptibility to antibiotics compared with individual bacteria. Our findings reveal that sugar inhalation, an inexpensive and safe therapeutic, could be used in combination with conventional antibiotic therapy to more effectively treat *P. aeruginosa* lung infections.

*Pseudomonas aeruginosa* is a leading cause of lung infections, particularly in hospitalized patients and in patients with compromised respiratory systems (33). Indeed, it is the second most common pathogen in hospital-acquired pneumonia, the most frequent cause of ventilator-associated pneumonia, and a frequent cause of acute and chronic infections in individuals with structural lung abnormalities, including patients with chronic obstructive pulmonary disease bronchiectasis, and cystic fibrosis (CF). Infections caused by *P. aeruginosa* are associated with high crude (40–90%) and attributable (30–40%) mortality (17), and selecting effective treatment is made even more challenging by the emergence of widespread multidrug antibiotic resistance (35, 38). Therefore, developing new therapeutic strategies or compounds that would improve existing antibiotic treatments to overcome infections by multidrug-resistant *P. aeruginosa* are of utmost importance.

A particularly attractive therapeutic strategy would be to block the initial binding between *P. aeruginosa* and carbohydrate chains of host cell surface glycoproteins and mucins. These glycan chains are prominently displayed on the cell surface and can potentially serve as receptors for bacterial attachment (7, 12), an early and crucial step in the establishment of *P. aeruginosa* infection. Bacterial binding to the carbohydrate chains can be competitively blocked by treatment with exogenous sugars (10). Indeed, there are several examples in which this approach shows promise for the treatment of bacterial infections. For example, mannosides are effective inhibitors of the uropathogenic *Escherichia coli* lectin FimH (21, 22) and have shown therapeutic promise in murine models for the treatment of *E. coli* urinary tract infections (13, 27). Inhalation of the dry powder form of mannitol has shown promise in the treatment of bronchiectasis and CF (2, 9, 18).

*P. aeruginosa* encodes at least four adhesins that recognize N-glycans, which are predominant on the cell surface (19), and O-glycans, which are predominant on secreted mucins (39). We have recently established that the type IV pilus of *P. aeruginosa* binds to N-glycan chains on the apical (AP) surface of the host epithelium, whereas flagella mediate binding to heparan sulfate chains of heparan sulfate proteoglycans (HSPGs) on the basolateral (BL) surface (7, 8). Upregulation of N-glycosylation, which leads to the expression of more complex N-glycans that contain higher amounts of a major sugar mannose (Man), increases bacterial attachment to epithelial cells (7). In addition, *P. aeruginosa* lectins PA-I (LecA) and PA-II (LecB) can mediate bacterial attachment to both N- and O-glycan chains by binding to galactose (Gal) and fucose (Fuc), respectively (11), and PA-I and PA-II mutants exhibit reduced adhesion and cytotoxicity in vitro (11). Administration of various lectin inhibitors markedly reduces *P. aeruginosa*-induced lung injury and mortality in vivo (11, 20), inhibits biofilm formation, and enhances dispersion of established biofilms (28, 30). Together, these findings suggest that exogenous administration of sugars present in both N- and O-glycans could competitively inhibit carbohydrate-mediated binding of *P. aeruginosa* to the airway epithelium and to mucus.

In this study, we establish that administration of a sugar mixture that consists of the three sugars present in N- and O-glycans, Man, Fuc, and Gal, is an effective adjunctive therapy when used in combination with conventional antibiotics for the treatment of pulmonary infections caused by both nonmucoid and mucoid strains of *P. aeruginosa*. The sugar mixture inhibits bacterial adhesion in vitro and functions syn-
ergistically when administered with conventional antibiotics in a murine model of acute pneumonia. In vitro, the sugar mixture induces formation of bacterial clusters that are more susceptible to antibiotic killing, providing an additional explanation for their efficacy in vivo. These studies introduce a simple, inexpensive, and effective novel form of adjunctive therapy that could aid existing antibiotic treatment of acute and chronic P. aeruginosa infections.

MATERIALS AND METHODS

Bacterial strains. P. aeruginosa strain O1 (PAO1) was obtained from the ATCC (ATCC 15692) and PDO300 was a kind gift of Dr. Daniel Wozniak (the Ohio State University). All strains were routinely grown with shaking overnight in Luria-Bertani broth (LB broth) at 37°C, diluted 1:40, and additionally grown in LB for 2 h to reach exponential growth phase. Green fluorescent protein (GFP)-expressing strains were created by electroporation of the pnpT2-GFP-pUCP20 plasmid as described previously (7).

Cell culture. Normal 16HBE14o− and CF CBE14o− bronchial epithelial cells were a kind gift from Dr. Alan Verkman (University of California San Francisco) and maintained in MEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and L-glutamate at 37°C in 10% fetal bovine serum (FBS; Invitrogen) and L-glutamate at 37°C with 5% CO2. Cells were grown as polarized monolayers on 12-mm Transwell filters (3-μm pore size; Corning) as previously described (7). For experiments, cells were seeded at 0.7 × 104 cells/well on Transwells and cultured for 5 days, including a 2-day growth in an air-liquid phase by removing media from the upper chamber of the Transwell insert.

Bacterial adhesion assays. Exponentially grown bacteria were added to polarized cells at a multiplicity of infection (MOI) of 10. For AP infections, the bacteria in serum-free MEM were added to the AP chamber of cells grown on Transwells. For BL infections, the Transwell insert was placed directly onto 50 μl of serum-free MEM containing bacteria. After 2 h of infection at 37°C, adhesion assays were performed as described previously (7). For competition blocking experiments, with sugars, 30 μM Man, 30 μM Fuc, or 30 μM Gal in H2O, or 15 μM Man + 15 μM Fuc, or 10 μM Man + 10 μM Fuc + 10 μM Gal were added to cells at the time of bacterial infection. Bacterial colony-forming units (CFUs) were enumerated by plating serial dilutions of cell lysates on LB plates. All assays were carried out on triplicate wells, and results are reported as the average of three to five experiments.

Bacterial infection and lung injury measurement in mice. All animal studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee (permit number AN083023). BALB/c mice (male; 6–8 wk old; Taconic Farms) were anesthetized with Avertin (500 mg/kg IP). Blood samples were collected in EDTA tubes for blood cell counts and plasma cytokine measurements. Lung homogenates were serially diluted and counted by using a Hemavet (Drew Scientific, Oxford, CT). BAL was diluted five times, and plasma was diluted two times for concentration measurements. ELISAs were carried out with macrophage inflammatory protein-2 (MIP-2) and Kupffer cell (KC) kits (R&D) according to the manufacturer’s protocol. In vitro bacterial aggregation and antibiotic killing. For aggregation experiments, exponentially grown bacteria (2 × 107–100 μl PAO1-GFP or PDO300-GFP) in 200 μl MEM supplemented with 10% FBS and L-glutamate in the presence or absence of 5 mM Man + 5 mM Fuc + 5 mM Gal, or 15 mM Fuc were placed in eight-well coverglass chambers (Nalge Nunc International) at 37°C, and single-plane XY LCSM images were obtained over a period of 0–16 h. Results are reported as the average of three independent experiments.

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BAL and plasma cytokine measurement. Bronchoalveolar lavage (BAL) fluid was collected at 4 h postinfection (hpi) by infusing 1 ml of sterile PBS (containing 5 mM EDTA) into the lungs of the mice after tracheal cannulation. Gentle suction was applied, and ~80% of the fluid was withdrawn from the lungs. The collected fluid was centrifuged at 1,000 rpm for 10 min. The supernatant was stored immediately at −80°C for protein concentration and for cytokine measurements. The pellet was resuspended in 100 μl of PBS for cytometric preparation before hemolysis of the red blood cells; hemolysis was achieved by adding hypotonic PBS (200 mosmol for 20 s). The total BAL white blood cell number was obtained by using a Beckman Coulter (Coulter, Miami, FL), and the cells were analyzed after H&E staining of the cytosplasm material. Blood neutrophils were counted by using a Hemavet (Drew Scientific, Oxford, CT). BAL was diluted five times, and plasma was diluted two times for concentration measurements. ELISAs were carried out with macrophage inflammatory protein-2 (MIP-2) and Kupffer cell (KC) kits (R&D) according to the manufacturer’s protocol.

In vivo bacterial aggregation and antibiotic killing. For aggregation experiments, exponentially grown bacteria (2 × 107–100 μl PAO1-GFP or PDO300-GFP) in 200 μl MEM supplemented with 10% FBS and L-glutamate in the presence or absence of 5 mM Man + 5 mM Fuc + 5 mM Gal, or 15 mM Fuc were placed in eight-well coverglass chambers (Nalge Nunc International) at 37°C, and single-plane XY LCSM images were obtained. Results are reported as the average of three independent experiments.

Isolation and ex vivo infection of tracheas and lung tissues. Fresh tracheas and lungs were dissected from BALB/c mice after euthanization and immediately sectioned into multiple fragments of ~3 × 4 mm. After sectioning, the tissues were glued to the microscope slides (Fisher Scientific) with a superglue, a circle was drawn around the specimen with Super PAP PEN (Beckman Coulter), and ex vivo infections were performed by putting a drop containing exponentially grown PAO1 or PDO300 (2 × 107–100 per 100 μl) in MEM supplemented with 10% FBS and L-glutamate in the presence or absence of 5 mM Man + 5 mM Fuc + 5 mM Gal on top of each tissue. Infections were carried for 2 h at 37°C with 5% CO2, and 10 μl of cefazidime of ciprofloxacin (5 × MIC) were added to half of the samples for additional 2 h. After 4 h of total infection time, samples were washed three times with PBS and homogenized, and bacteria were enumerated by plating serial dilutions of cell lysates onto LB plates and counting CFUs, or single-plane XY confocal fluorescence images of bacteria above the tissue were obtained. Results are reported as the average of three independent experiments.

Immunofluorescence microscopy and image analysis. GFP-expressing or LIVE/DEAD stained PAO1 and PDO300 were examined by LCSM (LSM 510; Carl Zeiss MicroImaging) at a magnification of ×600. Images were acquired by and processed in Meta 510 software. Image J analysis of bacterial clusters was performed on TIFF files. For bacteria stained with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) in MEM supplemented with 10% FBS and L-glutamate in the presence or absence of 5 mM Man + 5 mM Fuc + 5 mM Gal, or 15 mM Fuc were placed in eight-well coverglass chambers for 2 h. Cefazidime or ciprofloxacin (5 × minimal inhibitory concentration [MIC]) was added for additional 2 h. After a total of 4 h, CFUs were enumerated by plating serial dilutions of cell lysates onto LB plates, or single-plane XY LCSM images were obtained. Results are reported as the average of three independent experiments.

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individual and clustered bacteria. Images were acquired from three separate experiments and four events per each sample were quantified ($n = 12$). For whole lung imaging, lungs infected with GFP-expressing PAO1 or PDO300 were placed on microscope slides immediately after surgical removal with an outer surface facing the objective, and examined with Nikon Eclipse TE2000-E fluorescence microscope, by use of a charge-coupled device camera, at a magnification of $\times 400$.

**Statistical analysis.** Data are expressed as means ± SD (standard deviation). Statistical significance was estimated by Mann-Whitney test using InStat version 3.0b. Differences were considered to be significant at $P < 0.05$.

**RESULTS**

*Addition of a sugar mixture inhibits P. aeruginosa binding to the AP surface of polarized bronchial epithelial cells.* We have previously established that nonmucoid *P. aeruginosa* strains bind preferentially to N-glycan chains at the AP surface of polarized lung epithelium (7). Upregulation of N-glycosylation enhanced bacterial binding whereas enzymatic inhibition or infection of cells defective in N-glycosylation resulted in decreased binding. On the basis of these results, we postulated that addition of simple sugars should competitively inhibit bacterial adhesion to the lung epithelium and that their efficacy could be enhanced in cells with elevated levels of N-glycosylation.

To test this hypothesis, we investigated whether simple sugars, singly or in combination, could competitively inhibit *P. aeruginosa* binding to normal bronchial epithelial cells (16HBE14o−) and to CF bronchial epithelial cells (CFBE14o−). We chose to test Man, Fuc, and Gal, since these are the most common sugars present in major glycan chains: Man is predominant in N-glycans on the cell surface glycoproteins whereas Fuc and Gal are present in both N- and O-glycans found on cell surface glycoproteins and secreted mucins. These sugars are inexpensive, readily available, and well tolerated when used as therapeutic agents in humans (14, 23).

We first determined that CF bronchial cells express more Man and Fuc than normal cells on both the AP and BL surface, as determined by staining with specific fluorescent lectins (data not shown), consistent with published data (36, 41). We infected normal and CF bronchial cells grown as polarized monolayers on Transwell filters with PAO1 or its isogenic mucoid mutant PDO300 (which carries the mucA22 allele) (34), strains representative of acute or chronic infections, respectively. Bacteria were added to the AP or BL surface for 2 h in the presence or absence of sugars, and standard adhesion assays were performed. PAO1 bound more robustly to the BL than to the AP surface of polarized normal bronchial cells (Fig. 1A), consistent with our previously published results (7, 8). PDO300 showed less...
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efficiency binding and no preference for the AP or BL surface (Fig. 1B). This latter finding may result from decreased cell surface type IV pili or flagella on the mucoid strain, the overproduction of alginate, and/or contribution of the minor bacterial adhesins, PA-I and PA-II (1, 24). Importantly, addition of the mixture of Man, Fuc, and Gal (Man+Fuc+Gal) or Man and Fuc (Man+Fuc) decreased binding of PAO1 and PDO300 to the AP surface of normal bronchial cells by 50 and 25%, respectively (Figs. 1, A and B). Man+Fuc+Gal did not decrease PAO1 binding to the BL surface, consistent with our previous observation that binding of nonmucoid strains to the BL surface is primarily mediated by HSPGs (6, 7). However, addition of Man+Fuc+Gal resulted in a small but statistically significant decrease in binding of PDO300 to the BL surface (Fig. 1B). Addition of single sugars did not have any effect on PAO1 or PDO300 binding to polarized normal bronchial epithelium.

The binding of PAO1 and PDO300 to the AP surface of polarized CF bronchial cells was increased by 1.6- and 3.9-fold, respectively, compared with bacterial binding to normal bronchial cells (compare Fig. 1, A vs. C, and 1, B vs. 1D), consistent with increased expression of Man and Fuc in CF cells. Binding to the BL surface of CF cells remained unchanged compared with normal bronchial cells and was unaffected by the addition of sugars (Fig. 1, C and D). Addition of the sugar mixtures, either Man+Fuc+Gal or Man+Fuc, inhibited binding of either strain to the AP surface of CF bronchial cells to a greater degree, 75%, compared with 60% as was seen with normal cells (Fig. 1, A and C). Addition of single sugars (Fuc or Gal) had a small inhibitory effect on bacterial binding to CF cells (Figs. 1, C and D). Together, the results suggest that a mixture of sugars that are key components of N- and O-glycosylated proteins effectively inhibits binding of nonmucoid and mucoid P. aeruginosa strains to polarized epithelium. The mixture of Man+Fuc+Gal was the most potent.

Administration of the sugar mixture attenuates bacterial-induced lung damage and acts synergistically with antibiotics in a murine model of acute pneumonia. On the basis of our results in the cell culture system, we postulated that the mixture of simple sugars (Man+Fuc+Gal) could be an effective adjunctive antiadhesive therapy in P. aeruginosa lung infections. To investigate this hypothesis, we utilized a well-established murine acute pneumonia model: 2 × 10⁸ PAO1 or PDO300 were injected intratracheally into BALB/c mice. At 2 hpi, half of the mice were injected IP with ceftazidime, a β-lactam antibiotic that inhibits bacterial cell wall synthesis, or ciprofloxacin, a fluoroquinolone drug class that inhibits DNA topoisomerases. Another 2 h (a total of 4 hpi), the mice were euthanized, and lung bacterial burden, damage, and inflammation were assessed. We analyzed this early time point to focus on initial steps of infection, such as bacterial binding, and to avoid potential confounding indirect effects of the sugar mixture that may occur during later stages of infection.

The bacterial burden in lung homogenates from mice infected with PAO1 in the presence of the sugar mixture was similar to that observed in lung homogenates from mice infected in the absence of sugars (Figs. 2A and 3A). However, we noted decreases in 1) lung injury as quantified by measurements of excess lung water (Fig. 2B), 2) lung inflammation as qualitatively assessed by H&E staining of lung tissues (Fig. 2E), and 3) bacterial dissemination to the subpleural alveoli as assessed by imaging fluorescent bacteria (PAO1-GFP) on the outer surface of the intact lung (Fig. 2F). Together, these results suggested that the sugar mixture alone could be exerting a protective effect. As expected, IP injection of ceftazidime or ciprofloxacin decreased the lung bacterial burden by three to four orders of magnitude over that of untreated mice (Figs. 2A and 3A). Excess lung water levels (Figs. 2B and 3B), lung inflammation (Fig. 2E), and bacterial dissemination (Fig. 2F) were also decreased in antibiotic-treated mice. A remarkable synergistic effect was observed when mice were infected in the presence of the sugar mixture followed by antibiotic treatment. The lung bacterial burden was decreased by six to nine orders of magnitude (Figs. 2A and 3A), and there was a further statistically significant decrease in lung CFU (Figs. 2A and 3A), excess lung water (Figs. 2B and 3B), and qualitative lung inflammation (Fig. 2E), beyond what was seen with antibiotics alone or with the sugar mixture alone. Importantly, administration of a single sugar, Fuc, at the same concentration as the mixture of sugars (15 mM), did not protect from lung damage and did not exhibit synergistic effects with antibiotics (data not shown). These results indicate that administration of a single sugar was not sufficient to inhibit the known multivalent interactions between P. aeruginosa and host glycans.

We observed similar trends with the mucoid strain PDO300, although some minor differences were also noted. The bacterial lung burden in PDO300-infected mice was approximately fivefold lower than for PAO1 (Figs. 2C and 3C). Excess lung water measurements (Figs. 2D and 3D) and histological assessment of inflammation (Fig. 2E) showed much less lung damage compared with PAO1, consistent with the known reduced in vivo virulence of mucoid strains (29). However, administration of the sugar mixture during PDO300 infection still resulted in a twofold decrease in excess lung water compared with untreated mice (Figs. 2D and 3D). Although the MICs of planktonically grown PAO1 vs. PDO300 were indistinguishable for ceftazidime (1 μg/ml) and ciprofloxacin (0.5 μg/ml for PAO1 vs. 1 μg/ml for PDO300), treatment with antibiotics decreased lung bacterial burden by only 2-2.5 orders of magnitude (Figs. 2C and 3C), consistent with the known increased antibiotic resistance of mucoid P. aeruginosa (4). Importantly, we again observed that sugars potentiated the efficacy of antibiotics in infected mice treated with both the sugar mixture and antibiotics; the lung bacterial burden was further diminished by approximately four orders of magnitude over that of untreated mice (Figs. 2C and 3C). In addition, excess lung water (Figs. 2D and 3D) and lung inflammation (Fig. 2E) were decreased. Similarly to PAO1-GFP, dissemination of PDO300-GFP to the subpleural alveoli was inhibited by the sugar mixture, by antibiotics, or by the combination (Fig. 2F). Together, these results indicate that treatment with the sugar mixture protected mice from lung damage and, in combination with either of two different classes of antibiotics, functioned synergistically to further protect mice from infection with and damage caused by either nonmucoid or mucoid P. aeruginosa.

Administration of the sugar mixture attenuates neutrophil- and IL-8 inflammatory-dependent responses in infected mice. Because the presence of the sugar mixture alone had protective effects in the murine model of acute pneumonia and striking synergistic effects in the presence of antibiotics, we measured
inflammatory markers in the BAL fluid for each of the mouse treatment groups. Specifically, we examined BAL bacterial load, protein levels (a marker of epithelial and endothelial permeability), total white blood cells, macrophages, neutrophils, and informative cytokines for each of the treatment groups to determine whether the protective effects were correlated with or could be attributed to a decrease in inflammatory responses.

For PAO1- and PDO300-infected mice treated with ceftazidime, the BAL bacterial load was reduced from $10^4$ or $10^5$ CFUs, respectively, to barely detectable levels (Fig. 4, A and B). However, we found $10^3$-$10^4$ more PAO1 or PDO300 in the BAL from mice infected in the presence of the sugar mixture than in mice infected in the absence of sugars. This finding suggests that the sugar mixture might have inhibited binding of P. aeruginosa to the airway epithelium, leading to more bacteria in BAL fluids in the sugar-exposed mice.

BAL protein levels were significantly lower in mice infected in the presence of the sugar mixture compared with mice infected in the absence of sugars for both PAO1 and PDO300 (Fig. 4C). Likewise, total white blood cells counts and neutrophil counts were significantly lower in BAL from mice infected in the presence of sugars (Fig. 4, D and E), whereas macrophage counts were not affected (Fig. 4E). These results indicate that the decrease in recruitment of white blood cells was largely due to decreased recruitment of neutrophils, consistent
with published data indicating that neutrophils are the primary immune defense and first responders against *P. aeruginosa* infections (15, 26, 31). Furthermore, BAL levels of the MIP-2 and KC chemokines, which are the murine functional homologues of IL-8, were significantly lower in mice infected in the presence of sugars (Figs. 4, G and H), suggesting that IL-8-dependent inflammatory responses in mice infected with PAO1 or PDO300 in the presence of sugars were attenuated. Finally, we observed that treatment with the sugar mixture alone, with antibiotics alone, or with combination of sugars and antibiotics resulted in similar reduction in IL-8-mediated inflammation, including recruitment of neutrophils and release of MIP-2 and KC (Figs. 4, C–H). These results suggest that although sugars and antibiotics acted synergistically to further reduce lung bacterial burden and lung damage (see Fig. 2), this synergistic effect was not a result of the differential recruitment of immune cells.

The sugar mixture acts synergistically with antibiotics during ex vivo infections of tracheas and lung tissues with *P. aeruginosa*. To further investigate the mechanism underlying the synergistic effect of sugars and antibiotics, we compared growth properties and killing of bacteria in liquid culture in the absence of host cells and in the presence of freshly dissected sections of mouse lungs and tracheas. We first determined that the sugars did not affect the rate of PAO1 and PDO300 growth under planktonic conditions (data not shown). Next, we examined the behavior of PAO1 and PDO300 grown statically for 16 h in glass wells in a host-free environment (Fig. 5). In the absence of sugars, bacterial clusters were observed to form at 8–16 h. In contrast, in the presence of sugars (Man+Fuc+Gal), large bacterial clusters formed rapidly, over 2–4 h, and then started to disperse.

We used two methods to determine the effect of sugars on antibiotic-mediated killing. First, we enumerated CFUs of bacteria grown statically in a host-free environment at a 4-h time point to determine whether sugar-exposed bacteria exhibited altered antibiotic sensitivity. Treatment with ceftazidime (Fig. 6A) or ciprofloxacin (data not shown) resulted in a 4-log reduction in CFUs for PAO1 and a 3-log reduction for PDO300. In contrast to our results in the murine model of acute pneumonia, addition of sugars did not potentiate the efficacy of antibiotic killing. Second, we performed live/dead staining to quantitatively assess aggregate formation and to compare the proportions of live to dead bacteria in individual bacteria and in bacterial clusters under the four treatment conditions by use of laser scanning confocal microscopy. Live/dead staining utilizes two different nucleic acid stains to distinguish live bacteria with intact cell membranes (which take up SYTO 9 and fluoresce green) from dead bacteria with compromised membranes (which take up propidium iodide that fluoresces red and displaces SYTO 9 nucleic acid binding). Quantitation of green and red color distribution in single XY plane images showed that, in the absence of sugars, almost no clusters were observed (Fig. 6B; PAO1 or PDO300): 85–95% of the entire population of either PAO1 or PDO300 consisted of individual live bacteria. In contrast, in the presence of sugars, bacterial clusters were formed, comprising 15–20% of the events (Fig. 6B; PAO1+sugars and PDO300+sugars). The clusters were...
variable in size, ranging from a few bacteria to many bacteria (see Fig. 5A that shows the average area size of the bacterial population in pixels). Although we did not observe a synergistic effect of sugars with antibiotics when we quantified total CFUs (Fig. 6A), analysis by live/dead staining revealed additional information. In bacteria treated with sugars and antibiotics, ~70–80% of the entire population of the bacterial clusters stained red whereas only 25–30% of the single bacteria stained red (Fig. 6B; PAO1+sugars+CTAZ and PDO300+sugars+CTAZ). These results indicate that addition of the sugar mixture to in vitro liquid bacterial cultures renders PAO1 and PDO300 clusters more susceptible to killing by antibiotics compared with individual bacteria, although this does not affect overall killing efficiency of antibiotics. Finally, single bacteria are more sensitive to antibiotic-mediated killing in the absence of sugars than in the presence of sugars.
To further study the synergy between the sugar mixture and antibiotics, we developed an ex vivo model of *P. aeruginosa* infection to more closely recapitulate in vivo lung bacterial infections. Freshly dissected mouse tracheas and lungs were immediately sectioned, glued to a glass slide, divided into the same four groups as in the in vivo murine model, and infected for 4 h. Overall, ex vivo infections of tracheas and lung tissues recapitulated the in vivo results. Addition of ceftazidime (Figs. 7, A and C) or ciprofloxacin (data not shown) to tracheas or lung sections infected with PAO1 or PDO300 in the absence of the sugar mixture decreased bacterial CFUs by 3–4 logs. Importantly, the sugar mixture added during bacterial infection acted synergistically with antibiotics to decrease CFUs by an additional /2 logs, similar to trends observed in the murine model.

In control experiments, in which the dissected lung and tracheal sections were fixed prior to addition of bacteria, incubation with sugars did not enhance ceftazidime-mediated bacterial killing (data not shown), suggesting that a host factor may contribute to the ability of sugars to potentiate the effects of the antibiotics.

We also compared the susceptibility of individual bacteria vs. bacterial clusters to antibiotic killing by live/dead staining by quantifying the color distribution in single XY plane images taken above the tissue. In the presence of the sugar mixture, bacterial clusters were more frequent than in the in vitro experiments, comprising 25–30% of the events. Similarly to the in vitro experiments, the bacterial clusters were more sensitive to antibiotic killing than were the individual bacteria: 70–80% of the cluster population stained red whereas ~35% of the single bacteria population stained red (Figs. 7, B and D; PAO1+sugars and PDO300+sugars). Altogether, these results indicate that bacterial clusters exhibit enhanced susceptibility to antibiotic killing in vitro and ex vivo. Finally, the sugar mixture potentiates the efficacy of antibiotics in the environment of a live host tissue, but not a fixed tissue or in the absence of host cells, suggesting that the ability of sugars to enhance killing by antibiotics is at least in part dependent on host cell factors.

**DISCUSSION**

*P. aeruginosa* infections are associated with a high morbidity and mortality rate and are increasingly difficult to treat owing to widespread multidrug resistance (35, 38). Therefore, developing novel therapeutics is of utmost importance, and prevention or reversal of bacterial adherence to the host, which is a critical first step in establishing *P. aeruginosa* infection, is a logical target. In this study, we report that the mixture of Man, Fuc, and Gal inhibits *P. aeruginosa* adhesion to normal and CF bronchial epithelial cells in vitro, whereas the equivalent concentration of a single sugar is without effect. In a murine model of pneumonia caused by either nonmucoid or mucoid *P. aeruginosa*, administration of the sugar mixture alone protects from acute lung injury, limits dissemination to the subpleural alveoli, and decreases IL-8-driven neutrophilic inflammatory responses. When administered in conjunction with systemic antibiotics, the sugar mixture greatly enhances bacterial killing decreasing bacterial lung burden by an additional three to four orders of magnitude compared with the treatment with antibiotics alone. Thus inhalation of simple sugars, a safe and inexpensive treatment that has been used successfully to treat other human diseases (14) could be useful as a novel therapeutic adjunct to enhance the efficiency of conventional antibiotic therapy for acute or chronic pneumonia.

Our work suggests that the sugar mixture attenuated lung damage at least in part by blocking initial binding steps. The
sugars included in the mixture, Man, Fuc, and Gal competitively inhibited *P. aeruginosa* binding in cell culture-based assays. The sugar mixture also likely blocked adhesion in the murine model, since it attenuated bacterial dissemination to the subpleural alveoli and increased recovery of bacteria from BAL. In turn, reduced bacterial binding resulted in decreased lung inflammation and injury, as evidenced by diminished production of KC and MIP-2, reduced recruitment of neutrophils, and less severe histological changes. Our finding that a single sugar at a concentration equivalent to the mixture neither inhibited binding in vitro nor reduced bacterial lung burden or lung damage in vivo suggests that *P. aeruginosa* interacts with multiple sugar residues on glycan chains and that these targets need to be blocked simultaneously to attenuate bacterial binding.

Our in vitro and ex vivo studies revealed an additional mechanism by which the sugars potentiated the effects of antibiotics: the sugar mixture accelerated bacterial aggregation without affecting bacterial growth and enhanced the sensitivity of the bacterial clusters to killing by antibiotics. *P. aeruginosa* multicellular clusters rapidly formed in the presence of the sugar mixture, either in tissue culture medium alone or in the presence of sections of mouse tracheas or lungs. In contrast to biofilms, the bacterial aggregates exhibited increased sensitivity to killing by β-lactam or quinolone antibiotics. Recently, it has been reported that specific metabolic stimuli (mannitol, fructose, and glucose) enhanced uptake of aminoglycosides, but not β-lactams or quinolones, under conditions of both aerobic and anaerobic bacterial growth by generation of proton-motive forces (3). However, this mecha-
Fig. 7. The sugar mixture acts synergistically with antibiotics during *P. aeruginosa* ex vivo infections of tracheas and lung tissues. Tracheas and lungs were isolated from BALB/c mice and infected with $2 \times 10^6$ PAO1 or PDO300 in culture media (inf) or in the presence of sugars solution (inf+sugars) for 4 h. At 2 hpi, $5 \times$ MIC ceftazidime was added to half of the cell culture media group (PAO1+CTAZ or PDO300+CTAZ) or half of the sugar solution group (PAO1+sugars+CTAZ or PDO300+sugars+CTAZ). A: quantitative comparison of PAO1 (solid bars) or PDO300 (shaded bars) CFUs. Shown are means ± SD for 3 independent experiments. B: representative XY confocal images taken from a single plane above a trachea infected with PAO1 (top) and PDO300 (bottom) and stained with LIVE/DEAD bacterial kit (green, live bacteria; red, dead bacteria). Quantitation of green and red color distribution is shown below each image. C: quantitative comparison of PAO1 (solid bars) or PDO300 (shaded bars) CFUs in lung tissues. Shown are means ± SD for 3 independent experiments. D: representative XY confocal images taken from a single plane above a lung infected with PAO1 (top) and PDO300 (bottom) and stained with LIVE/DEAD bacterial kit (green, live bacteria; red, dead bacteria). Quantitation of green and red color distribution is shown below each image.

The mechanisms underlying the ability of sugars to potentiate bacterial killing may comprise a number of intriguing
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possibilities that will necessitate future investigation. At early
time points, the sugars may facilitate multivalent interactions
between bacterial adhesins, such as type IV pili or bacterial
lectins PA-I and PA-II, to form bacterial clusters. The sugars
may facilitate antibiotic binding or uptake. Finally, the sugars
may alter the metabolic state of bacteria in clusters without
enhancing bacterial proliferation, rendering them more suscepti-
tible to antibiotics.

In contrast to the formation of biofilms, bacterial clusters
induced by the sugar mixture are not stable and disperse
rapidly. Although the mechanism for this behavior is not
known, it may be related to the reported observation that
several sugars, including Man, D- and L-Fuc, or D-fructose,
alone inhibit autoaggregation of PAO1 that precedes formation
of biofilms (5). Further studies will be required to determine
whether the sugar mixture, when used in models of biofilm
formation and bacterial infections longer than 4 h, inhibits
formation of biofilms and/or induces biofilm dispersion, as has
been reported for other polysaccharides and glycoconjugates
(28, 30, 37).

Adjunctive sugar therapy may be especially useful in treat-
ment of pulmonary infections with mucoid P. aeruginosa
strains, which exhibit increased intrinsic resistance to antibiot-
ics, and with multidrug-resistant isolates. In the present studies,
the addition of sugars potentiated the antibiotic killing of the
mucoid strain PDO300 by both ceftazidime and ciprofloxacin
in the murine lung infection model or upon cocultivation with
tracheas or lung tissue. CF patients represent a patient popu-
lation in which this approach might be especially attractive,
given that these patients are chronically colonized with multi-
drug-resistant isolates, and even decreasing bacterial lung bur-
den has been shown to be beneficial (16). Indeed, a case study
involving a CF patient has shown that inhalation of Gal and
Fuc in conjunction with standard antibiotic therapy led to more
efficient clearance of P. aeruginosa (40). Furthermore, a small
randomized study in 11 CF patients, without a control group,
has shown that inhalation of Fuc and Gal alone or in combina-
tion with antibiotics for 21 days significantly decreased P.
aeruginosa counts in sputum and TNF-α levels in sputum and
peripheral blood cells, although there was no change in lung
function measurements (23).

In summary, the studies presented here provide strong evi-
dence that sugar inhalation may be a simple, safe, and effective
adjunctive therapy to increase the efficacy of current antibiotic
regimens in the treatment of P. aeruginosa lung infections.
Although other sugars could also be considered for therapeutic
uses, Man, Fuc, and Gal are inexpensive and have previously
been shown to be well tolerated when used as therapeutic
agents in humans (14, 23). Combined antibiotic and sugar
therapy may be especially useful in the treatment of lung
infections with multidrug-resistant P. aeruginosa and of chronic
pulmonary infections with mucoid strains seen in CF patients.
Further studies are needed to evaluate whether this combined
therapy can reduce mortality or prevent ongoing colonization
and reduce P. aeruginosa airway burden in these vulnerable
patient populations. This strategy may also be useful in the
Treatment of other bacterial and viral lung infections.

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AUTHOR CONTRIBUTIONS

I.B., Y.S., M.A.M., and J.N.E. conception and design of research; I.B., J.A.,
and Y.S. performed experiments; I.B. and J.N.E. analyzed data; I.B. and J.N.E.
interpreted results of experiments; I.B. prepared figures; I.B. drafted manu-
script; I.B., M.A.M., and J.N.E. edited and revised manuscript; I.B., J.A., Y.S.,
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