Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface

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ABSTRACT Chronic colonization and infection of the lung with *Pseudomonas aeruginosa* is the major cause of morbidity and mortality in cystic fibrosis (CF) patients. We found that polarized CF bronchial and pancreatic epithelia bound *P. aeruginosa* in a reversible and dose-dependent manner. There was significantly greater binding to CF bronchial and pancreatic cells than to their matched pairs rescued with the wild-type CF transmembrane conductance regulator. Bound *P. aeruginosa* were easily displaced by unlabeled *P. aeruginosa* but not by *Escherichia coli*, an organism that does not cause significant pulmonary disease in CF. In contrast, *Staphylococcus aureus*, a frequent pathogen in CF, could effectively displace bound *P. aeruginosa* from its receptor. We found undersialylation of apical proteins and a higher concentration of asialoganglioside 1 (aGM₁) in apical membranes of CF compared with rescued epithelia. Incubation of *P. aeruginosa* with aGM₁ reduced its binding, as did treatment of the epithelia with the tetrasaccharide moiety of this ganglioside (Gal_β1-3GalNAc_β1-4Gal_β1-4Glc). Finally, an antibody to aGM₁ effectively displaced *P. aeruginosa* from its binding site and blocked binding of *S. aureus* to CF cells but not to rescued cells. These results show that the tetrasaccharide of aGM₁ is a receptor for *P. aeruginosa* and *S. aureus* and that its increased abundance in the apical membrane of CF epithelia makes it a likely contributor to the pathogenesis of bacterial infections in the CF lung.

Colonization of the respiratory tract by *Pseudomonas* is present in most patients with cystic fibrosis (CF) (1). This infection occurs in infancy before significant inflammatory damage to the lung generates bronchiectasis (2). Younger patients often have antecedent pulmonary infections with *Staphylococcus aureus*, and they are frequently colonized by both *S. aureus* and *Pseudomonas aeruginosa*. Loss of function of the CF gene product [CF transmembrane conductance regulator (CFTR)] is responsible for chronic infection with *Pseudomonas* (3, 4) because the severity of the defect in CFTR function correlates both with the age at colonization (5) and with the extent of binding of *Pseudomonas* to respiratory cells taken from CF patients (6). However, the identification of CFTR as an apical membrane Cl⁻ channel has not suggested why CF children are so readily infected with *Pseudomonas*.

Adherence of bacteria to specific cellular receptors is the initial event in many infectious diseases (7–9). In an *in vitro* assay, *P. aeruginosa* bound to asialoganglioside 1 (aGM₁) and aGM₂ but not to the sialylated homologues GM₁ and GM₂ (10). Respiratory cells from CF patients had more aGM₁ than normal cells (11). In fact, undersialylation is also found in mucus glycoproteins of clinical specimens and in secretions from immortalized CF cell lines (12). We recently proposed that loss of the CFTR Cl⁻ channel from intracellular glyco-

sylation compartments impairs sialyltransferase activity (13, 14). Thus, we hypothesized that defective sialylation of gangliosides generates *Pseudomonas* receptors (13, 14), and we show here that there are a larger number of these receptors on the apical surfaces of CF epithelia.

MATERIALS AND METHODS

Cells and Bacteria. CF bronchial epithelial cells, IB3 (genotype ΔF508/W1282X), and a matched rescued control cell line, C38, were from P. Zeitlin, Johns Hopkins University (15). These cells were maintained in LCH-8 medium (Biofluids, Rockville, MD) at 37°C in 5% CO₂/95% air. The second CF epithelial cell line, CFPAC (genotype ΔF508/ΔF508), and its matched rescued control cell line, CFPAC/CFTR, were from M. Drumm, University of Michigan (16). These cells were maintained in minimal essential medium (GIBCO)/10% fetal calf serum at 37°C with 5% CO₂/95% air. *P. aeruginosa* PAO1 is nonmucoid laboratory strain, grown in M9 medium at 37°C with aeration to late-logarithmic phase (OD_{490–180}). A non-typeable clinical strain of *S. aureus* was obtained from a patient with pneumonia (from S. Daugherty, Columbia University). *Escherichia coli* HB101 is a nonpilated laboratory strain. Both strains were grown in Luria broth (LB) at 37°C with aeration, pelleted, and resuspended in phosphate-buffered saline (PBS) to the same concentration as PAO1 strain.

Adhesion Assay. The apical adhesion assay was modified from Saiman and Prince (11). All binding of *P. aeruginosa* is reported as specific binding, defined as total binding minus that which was not displaced by 50-fold excess unlabeled bacteria. All assays were done in duplicate or triplicate, and results were averaged. Bronchial cells were seeded at a density of 2.5×10^5 cells per well onto collagen-coated transwell filters (Costar 3401). CFPAC cells were seeded at a density of 1×10^5 cells per well onto transwell filters (Costar 3404); the cells were grown to confluence and labeled with [³H]leucine (0.2 μCi per well; 1 Ci = 37 GBq). Before bacteria were added, the monolayers were washed three times with assay medium (Eagle's minimal essential medium/10 mM Hepes without fetal calf serum or antibiotics), and a final 0.3-ml vol was placed apically and 1 ml was placed basolaterally. Epithelial cells were treated with trypsin/EDTA and counted in a hemocytometer; their ³H counts were used to obtain a cell per ³H count. A 10-ml aliquot of PAO1 strain was labeled for 20 min with 150 μCi of [³⁵S]methionine, pelleted, and washed three times in M9 medium, before resuspension in assay medium. Unlabeled *P. aeruginosa* were also washed in M9 medium and resuspended in assay medium at 50-fold concentration of the labeled bacteria. Bacteria were serially diluted and plated for colony counts. The ³⁵S counts of labeled *P. aeruginosa* were used to establish the number of bacteria per ³⁵S count. Labeled bacteria were added to all wells at 37°C, and

FIG. 1. Specific binding of *P. aeruginosa* to CF epithelial cells and matched rescued cells [CFPAC and rescued control pair ($n = 5$); CF bronchial and rescued control pair ($n = 4$)]. About 1×10^7 *Pseudomonas* were added to each well in all sets of experiments.

unlabeled bacteria were added to parallel wells to determine displaceable binding. The final concentration of labeled *P. aeruginosa* was $1-5 \times 10^7$ bacteria per well. After incubation for 1 hr, the medium was removed from the transwell by using gentle suction, and the filters were washed with assay medium three times apically and basolaterally with gentle shaking to remove unbound bacteria. The transwell filters were then cut out, and radioactivity was counted. A determination of pseudomonas (^{35}S) per epithelial cell (^3H) was calculated.

Competition Assays. Galactose, GalNAc (Sigma), and the tetrasaccharide of aGM₁, Gal_{β1-3}GalNAc_{β1-4}Gal_{β1-4}Glc (Bio-Carb, Lund, Sweden), were added immediately before labeled bacteria at the noted concentrations. The complete ganglioside, a glycolipid with a hydrophobic ceramide tail, can partition into the epithelial lipid bilayer, thereby creating new receptors on the cell surface. To study the effect of aGM₁ on *P. aeruginosa* binding to the epithelia, labeled bacteria were pretreated with aGM₁ (Matreya, Pleasant Gap, PA). aGM₁ was first reconstituted in PBS at 0.5 mg/ml and sonicated for 30 sec with a Branson sonicator; the bacteria were allowed to bind to it for 60 min after which they were pelleted and washed five times with M9 medium. Untreated *P. aeruginosa* were also prepared in parallel, and equal counts of either aGM₁-treated or untreated *Pseudomonas* were then added to each well; the assay was continued as described above.

To demonstrate the role of aGM₁ in bacterial adherence, some epithelia were pretreated with a polyclonal rabbit anti-aGM₁ antibody (Wako Biochemicals, Richmond, VA; 1 mg/ml) for 60 min at room temperature. Labeled *P. aeruginosa* or

S. aureus were then added, and the adherence assay was done. A polyclonal rabbit anti-mouse antibody (Zymed; 75 mg/ml) was used as a control antibody.

To determine reversibility of binding, labeled *P. aeruginosa* were bound to epithelial cells for 60 min without unlabeled bacteria and washed three times. Assay medium alone (0.3 ml) was then added to the apical transwell compartment with or without anti-aGM₁ (6 $\mu\text{g}/\text{ml}$) and incubated at 37°C and washed three times.

Adhesion Assay with *S. aureus*. Ten milliliters of *S. aureus* was labeled for direct adhesion assay with 650 μCi of [^{35}S]methionine in PBS for 60 min and washed three times in PBS. The final concentration used was 1×10^5 cells per well. Fab₂ was prepared by digesting anti-aGM₁ with pepsin, which was applied to a protein A column. SDS/7% PAGE of the effluent showed a single band of the appropriate molecular weight (17).

Immunofluorescence. Confluent cells were fixed in 4% (wt/vol) paraformaldehyde and washed with PBS/50 mM ammonium chloride. Anti-aGM₁ was added at 1:100 for 60 min, and cells were washed; fluorescein isothiocyanate goat anti-rabbit antibody (Zymed) was then added at 1:500 for 60 min. Cells were photographed at $\times 60$ oil immersion. To identify the apical surface, cells were double-stained first as above with anti-aGM₁ followed by secondary tetramethylrhodamine β isothiocyanate goat anti-rabbit antibody (Jackson ImmunoResearch) and then with lectin (*Triticum vulgaris*) labeled with fluorescein isothiocyanate (Sigma) at 1:100 for 1 hr.

RESULTS AND DISCUSSION

We characterized epithelial receptors responsible for *P. aeruginosa* adherence using confluent, polarized epithelial monolayers of CFPAC cells, a pancreatic cell line, and IB3 cells, a CF bronchial cell line. The number of bacteria bound increased linearly when 1×10^6 to 5×10^8 bacteria were added per well, but at higher concentration no saturation occurred. Rather, the number of bound bacteria increased exponentially. Microscopy revealed that while individual bacteria adhered to the cell surface at low concentrations, at higher concentrations *P. aeruginosa* were seen adhering to each other, forming colonies. The exponential increase in binding without saturation thus results from self-adherence at high concentrations, a phenomenon known to occur *in vivo*. It was therefore necessary to use concentrations of bacteria that would result in binding of only a few bacteria per cell to avoid self-agglutination.

Specific binding was defined as that displaced by a 50-fold excess of unlabeled bacteria. Unlike polarized monolayers, nonconfluent cells bound many-fold more *P. aeruginosa* per cell, but these nonconfluent-cell-bound bacteria were not

FIG. 2. Displacement of [^{35}S]methionine-labeled *P. aeruginosa* (1×10^7 cells per well) with increased amounts of unlabeled *P. aeruginosa* (●), *E. coli* (Δ), or *S. aureus* (□). A single experiment, representative of two others, is shown.

FIG. 3. Inhibition of binding of *Pseudomonas* by various reagents. Percentage inhibition of total binding is shown. (A) CFPAC cells: anti-aGM₁ antibody (Ab), 6 μ g ($n = 5$); aGM₁, 25 mM ($n = 3$); galactose, 25 mM ($n = 3$); GalNAc, 25 mM ($n = 3$); galactose + GalNAc, 25 mM each ($n = 3$). (B) Bronchial cells: anti-aGM₁ Ab, 6 μ g ($n = 7$); aGM₁, 25 mM ($n = 3$); galactose, 25 mM ($n = 3$); GalNAc, 25 mM ($n = 3$); galactose + GalNAc, 25 mM each ($n = 3$); Tetra (aGM₁ tetrasaccharide), 0.05 mM ($n = 5$), 0.125 mM ($n = 2$), and 250 mM ($n = 2$).

readily displaced by unlabeled bacteria, suggesting that the basolateral surface exposed in nonconfluent monolayers has many "nonspecific" binding sites. Prebound ³⁵S-labeled *P. aeruginosa* dissociated slowly from the apical surface of polarized cells with a $t_{1/2}$ of 45 min, and the addition of 50-fold excess of unlabeled *P. aeruginosa* caused rapid dissociation of

all but 10–20% of the initially bound bacteria. This nonspecific binding of *P. aeruginosa* was constant in each assay and did not increase with increased amounts of *P. aeruginosa* added. These data suggest that the apical surface of epithelial cells, exposed in the airway, have specific and reversible binding sites for *Pseudomonas*.

Polarized CF epithelial cells, grown on transwell filters, bound significantly more *P. aeruginosa* per cell (over the linear range of specific *P. aeruginosa* binding) compared with their matched cell lines that have been "rescued" by transfection with the wild-type CFTR. CFPAC cells bound $51 \pm 11\%$ more *P. aeruginosa* per cell than rescued CFPAC/CFTR cells ($n = 16$, $P < 0.001$). Similarly, bronchial CF cells bound $82 \pm 26\%$ more bacteria per cell than their rescued CFTR pairs ($n = 9$, $P < 0.01$) (Fig. 1). CF bronchial cells had a higher number of binding sites than the pancreatic cell line. To test whether the binding is specific to *P. aeruginosa*, we measured the propensity of *E. coli* to compete with *Pseudomonas* for apical binding sites. *E. coli* was chosen because it does not contribute significantly to CF pulmonary disease. The number of unlabeled bacteria required to displace 50% of bound ³⁵S-labeled *P. aeruginosa* (IC₅₀) was 20×10^7 for *Pseudomonas* but was 140×10^7 for *E. coli* ($n = 3$) (Fig. 2). In contrast, *S. aureus*, a pulmonary pathogen in CF, displaced bound *Pseudomonas* at a similar IC₅₀ (*Pseudomonas*, 7.5×10^7 ; *S. aureus*, 10×10^7 ; $n = 4$). These data demonstrate the expression of a specific bacterial receptor and that the two common CF pathogens bind to the same receptor.

Pretreatment of bacteria with the entire aGM₁ glycolipid blocked *P. aeruginosa* binding to the apical cell surface of CFPAC cells and almost abolished binding to CF bronchial cells (Fig. 3). In contrast, treatment of bacteria with aGM₁ was ineffective in blocking *Pseudomonas* binding to the rescued CFPAC cells ($106 \pm 24\%$, $n = 3$) or rescued bronchial cells, (155% , $n = 1$). Similarly, a polyclonal antibody to aGM₁, which does not react to GM₁, inhibited *P. aeruginosa* binding to CF cells in a dose-dependent manner, reaching statistical significance at 3 μ g/ml (Fig. 4), whereas a control polyclonal antibody was ineffective. Fab₂ fragments were as effective as whole anti-aGM₁ molecules. Anti-aGM₁ was also able to displace *P. aeruginosa* already bound to the cell surface; 20 min after addition of the antibody, 71% of bound *P. aeruginosa* dissociated, compared with only 36% without treatment. In contrast, this antibody was ineffective at any concentration (*P*

FIG. 4. The effect of anti-aGM₁ on bacterial binding to CF cells. *P. aeruginosa* bound is expressed as a percentage of the total binding. (A) CFPAC epithelial cells: Effect of control antibody (Ab) ($\square$) ($n = 1$) and anti-aGM₁ ($\blacksquare$) ($n = 5$) on *P. aeruginosa* binding. Effect of Fab₂ of anti-aGM₁ ($\circ$) on *S. aureus* binding. Anti-aGM₁ did not significantly effect binding of *P. aeruginosa* to CFPAC/CFTR cells ($n = 6$). Similarly the Fab₂ of anti-aGM₁ did not block *S. aureus* binding to CFPAC/CFTR ($n = 1$). (B) Bronchial cells: $\blacksquare$, *P. aeruginosa* binding to bronchial CF cells with increasing titers of anti-aGM₁ ($n = 7$); $\circ$, binding of *S. aureus* to bronchial CF cells with increased titers of Fab₂ of anti-aGM₁ ($n = 1$). *Pseudomonas* binding to rescued bronchial cells (C38) in the presence of anti-aGM₁ was not significantly affected ($n = 3$). *S. aureus* binding to C38 was also not significantly affected by addition of Fab₂ ($n = 1$).

FIG. 5. Binding of anti-aGM₁ antibodies to the surface of CFPAC cells (Left) and its matched rescued pair (CFPAC/CFTR) (Right).

> 0.05) in blocking binding to rescued cells. These data together with our studies using the ganglioside, indicate a paucity of these receptors in rescued cells. In fact, anti-aGM₁ antibodies decorated the apical membrane of both CF cell lines, but there was markedly less staining to the rescued pair (Fig. 5).

To determine whether the complex sugar of aGM₁ (a tetrasaccharide, Gal_β1-3GalNAc_β1-4Gal_β1-4Glc) is the receptor, we tested its effect on *P. aeruginosa* binding and found that 0.25 mM blocked 57% of the binding, whereas the monosaccharides galactose and GalNAc were ineffective at these concentrations ($2 \pm 12\%$ and $1 \pm 8\%$, respectively; $n = 2$). Higher concentration of the monosaccharides (25 mM) blocked binding to CF cells slightly, but their effect was not additive (Fig. 3). The addition of the monosaccharides to the rescued cell line CFPAC/CFTR did not significantly block binding (galactose, 25 mM, $98 \pm 0\%$; GalNAc, 25 mM, $127 \pm 28\%$; $n = 2$). We conclude that it is the sugar moiety of aGM₁ that is the specific receptor for *P. aeruginosa* on the apical surface of CF cells.

That *S. aureus* was effective in displacing *P. aeruginosa* from the apical membrane and could also bind to aGM₁ *in vitro* (10) suggested that the two organisms share the same receptor. Using Fab₂ fragments of anti-aGM₁ antibodies (to eliminate spurious binding produced by interaction of protein A of *S. aureus* to Fc), we found that they blocked binding of ³⁵S-labeled *S. aureus* to both types of CF cells, with greater blockade in the CF bronchial cell line. In contrast, these Fab₂ had no significant effect on *S. aureus* binding to either type of rescued cell. Further, similar to their effect on *Pseudomonas*, high concentrations of monosaccharides decreased *S. aureus* binding to CFPAC cells [galactose (25 mM), 10%; GalNAc (25 mM), 27%; and galactose + GalNAc (25 mM), 22% ($n = 1$)]. These studies suggest that tetrasaccharide of aGM₁ is a specific apical receptor for both bacterial species in CF cells. Whether this complex carbohydrate is present only on aGM₁ or is also present on apical glycoproteins will need to be determined.

We recently found that CF epithelia undersialylate only apically secreted proteins, whereas basolaterally secreted proteins are normally sialylated. In fact, wild-type CFTR traverses an apical sialylation compartment *en route* to the apical plasma membrane, whereas it is absent from apical organelles in CF cells. The results presented above suggest that the generation of specific receptors for *Pseudomonas* and other important CF pathogens results from a generalized defect in sialylation.

Identification of a *P. aeruginosa* receptor on the apical cell surface has important therapeutic implications for patients with CF. An antibody to aGM₁ could prevent *P. aeruginosa* binding, as well as dislodge bacteria that were already bound to the airway surface. Recent animal studies demonstrate that anti-aGM₁ can prevent *P. aeruginosa* from binding to damaged cornea (18), and antibodies to *Pseudomonas* surface antigens

can prevent lung and pleural damage when instilled into the lungs (19). Interception of bacterial binding by antireceptor antibodies might reduce the bacterial load and ultimately modulate the cascade of events that leads to inflammation and lung damage (20). The recent use of inhaled Fab to viral surface antigens demonstrated efficient and prolonged protection against Rous sarcoma virus infection (21), suggesting a therapeutic model for antibody therapy.

The identification of the sugar moiety of aGM₁ as the receptor for *Pseudomonas* raises the possibility that ligands to this sugar or other compounds resembling the receptor in three-dimensional structure might prevent the initial colonization with *P. aeruginosa* and *S. aureus* and serve as another type of antibacterial therapy.

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