Pseudomonas aeruginosa Pili Bind to AsialoGM1 Which Is Increased on the Surface of Cystic Fibrosis Epithelial Cells

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Abstract

The basis for the unique association of Pseudomonas aeruginosa and the cystic fibrosis (CF) lung has remained obscure despite major advances in the understanding of the molecular genetic cause of this disease. There is evidence to suggest that abnormalities in CF transmembrane conductance regulator function result in alterations in the glycosylation of epithelial components. The number of asialoGM1 residues, as representative of a class of glycolipids which contain a GalNAcβ1-4Gal sequence for P. aeruginosa attachment, was quantified by flow cytometric studies of respiratory epithelial cells in primary culture from both CF patients and normal subjects. Supernatals asialoGM1 was detected on 12% of the CF cells as compared with 2.9% of the cells from normal control subjects (P = 0.03, \( x^2 = 4.73 \)), and more asialoGM1 residues were exposed on CF cells after modification by P. aeruginosa exoproducts. AsialoGM1, but not the sialylated glycolipid GM1, was demonstrated to be a receptor for \(^{125}\text{I}\)-labeled P. aeruginosa pilin, a major adhesin for this organism, and exogenous asialoGM1 was found to competitively inhibit P. aeruginosa adherence to epithelial cells, thus, confirming the biological role of the asialoGM1 receptor. Quantitative and qualitative differences in the sialylation of superficial glycolipids in CF epithelial cells may directly contribute to the colonization of the CF lung by P. aeruginosa. (J. Clin. Invest. 1993. 92:1875–1880.)

Key words: bacterial adherence • pathogenesis • sialylation

Introduction

A major consequence of the expression of an abnormal cystic fibrosis transmembrane conductance regulator (CFTR) in CF is the chronic pulmonary infection due to Pseudomonas aeruginosa. It is well established that children with CF are frequently colonized with P. aeruginosa, while normal children are not. This colonization leads to chronic infection and a subsequent immune response which eventually results in destruction of the lung, and ultimately pulmonary failure (1). The factors which contribute to the pathogenesis of this infection have not been well defined, but are likely to include properties of the epithelium which are attributable to CFTR dysfunction (2) as well as unique properties of P. aeruginosa.

CF epithelial cells in primary culture have been shown to bind approximately twice as many P. aeruginosa as respiratory epithelial cells from normal subjects (3). Although the biochemical nature of the relevant Pseudomonas receptors on the CF cells have not been identified, in vitro binding studies using thin layer chromatography and bacterial overlay technique, have demonstrated that P. aeruginosa recognizes a GalNAcβ1-4Gal sequence present in asialoGM1, asialoGM2, asialoGM1, and related glycolipids (4). Conflicting reports suggest that lactosylceramide and GM1 also can function to bind P. aeruginosa in vitro (5). Several studies have suggested that the glycosylation and/or sulfation of superficial glycoconjugates may be altered in CF as a consequence of abnormal CFTR function (6, 7). Sialylation of proteins and lipids in CF epithelial cells has been shown to be reduced and may be a consequence of defective acidification of the trans-Golgi in airway cells due to limitations of Cl\(^-\) transport (6). CFTR has been demonstrated to be functional in endosomes although its role in acidification has not been consistent in all cell types examined (8). Thus, the CF epithelial cell may contain less fully sialylated glycolipids than the normal cell, which in turn may provide increased numbers of receptors for P. aeruginosa attachment. In this report asialoGM1 is shown to be recognized as a receptor for a major P. aeruginosa adhesin, pilin, and the number of asialoGM1 receptors is shown to be increased on the surface of CF respiratory epithelial cells.

Methods

Bacterial strains and culture conditions. The P. aeruginosa strain PA1244 and its isogenic pil\(^-\) mutant PA1244/NP were obtained from S. Lory (University of Washington, Seattle, WA). The construction of this mutant strain has been previously described (9). In brief, gene replacement techniques were used to replace the wild-type pilin gene by homologous recombination with a cloned copy of the pilin gene interrupted by a tetracycline resistance cartridge. The phenotype of the pil\(^-\) mutant PA1244/NP was confirmed by resistance to phase P04, as well as by electron microscopy. The adherence properties of the parental strain PA1244 have been shown to be typical of nonmucoid strains of P. aeruginosa (10). Strains were grown in M9 minimal media at 37°C with aeration (11). Unless specified all chemicals were obtained from the Sigma Chemical Co. (St Louis, MO).

Iodination of pilin. PA1244 pilin was generously provided by J. Sadoff (Walter Reade Army Hospital, Washington, DC). Purity was confirmed by electron microscopy and by SDS-PAGE. Radioiodination was performed using the method of Markwell (12). Five Iodo-beads (Pierce Chemical Corp., Rockville, IL) were placed in 2 mL of Na\(^{131}\)I (NEN Research Products, Boston, MA) to which 200 \( \mu \)g of pilin suspended in 200 \( \mu \)L of PBS were added for 30 min at 25°C. An additional 200 \( \mu \)L of PBS, pH 7, was then added to the reaction mixture. The radiolabeled pilin was collected using a Sephadex PD-10 GM25 col-
umn (Pharmacia, Inc., Piscataway, NJ) equilibrated with NaPO₄, pH 7, with BSA 1 mg/ml. Incorporation was 20%. To verify that the pilin subunits were intact after radiolabeling, the labeled pilin was electrophoresed on an SDS-polyacrylamide gel and autoradiographed. The presence of a single band at 18,000 daltons with minimal degradation was evident.

Thin-layer chromatography (TLC). Commercially prepared ganglioside standards for TLC were obtained (Bio Carb Chemicals, Lund, Sweden). A 5-mg aliquot of a mixture of 21% GM1 (Galβ1-3GalNAcβ1→4[NeuAcα2-3]Galβ1-4Glcβ1-Cer), 40% GD1a (Neu2-3 Galβ1-3GalNAcβ1→4[NeuAcα2-3]Galβ1-4Glcβ1-1Cer), 16% GD1b (Galβ1-3GalNAcβ1→4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glcβ1-1Cer), 16% GT1b (Neu2-3Galβ1-3GalNAcβ1→4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glcβ1-1Cer), and 5 μg of asialoGM were separated by chromatography using a glass-backed silica high-performance TLC plate (E. Merck, Darmstadt, FRG). The chromatogram was developed in chloroform/methanol/aqueous 0.2% CaCl₂ (50:45:10). After chromatography the plates were air-dried and dipped for 20 s in 0.25% polyisobutyl-methacrylate dissolved in chloroform/hexane (1:10) and dried overnight. The attachment of 125I-labeled pilin to the separated glycolipids was determined using a modification of the method described by Krivan et al. (13). The fixed chromatogram was sprayed with BSA-PBS (1% BSA in PBS, pH 7.4) then blocked in this solution for 1 h. 125I-labeled pilin suspended in BSA-PBS were applied to the chromatogram, covered with parafilm, and incubated at 4°C overnight. The plates were rinsed repeatedly with PBS washes, dried under a heat lamp, and exposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY). The glycolipids were visualized with orcinol ferric chloride.

Epithelial cell culture. Primary cultures of nasal poly epithelial cells from CF patients (CFNP) with the genotypes, ΔF508/W1282, unknown/unknown (not any of the common mutations), or normal subjects (NHNP) were isolated and cultivated using the protease method previously described (3). Genotyping was performed in the laboratory of Dr. Gregory Highsmith, University of North Carolina. Confluent monolayers of immortalized CF epithelial cells (imCF) derived from nasal polytissue and immortalized human tracheal cells (imHTR) were cultivated using the techniques previously published (3). These cell lines are SV40-transformed clones (CFNP-E90- and 3HTEO-) whose epithelial properties have been established (14).

Flow cytomtery. A fluorescence-activated cell sorter (FACScan, Becton Dickinson & Co., Mountain View, CA) was used to determine the distribution of asialoGM1 and GM1 on the epithelial cell surface. Confluent monolayers in tissue culture media, M9 media, or after 60 min of incubation with 0.5 μl of P. aeruginosa PAO1 0.5 ml of M9 media showed complete adherence at 4°C for 20 min. The washed cells were resuspended in PBS, pH 7.4, 1% BSA, 0.02% azide and separated into 1 × 10⁷ cell aliquots. The presence of asialoGM1 was quantified with rabbit anti-asialoGM1 antibody used in a 1:10 dilution (Wako Chemicals USA, Richmond, VA) detected using biotin labeled goat anti-rabbit F(ab)², incubated with the cells at a 1:1,000 dilution. The addition of each antibody was followed by four PBS/BSA/azide washes. Bound antibody was tagged with streptavidin-conjugated phycocerythrin (Molecular Probes, Inc., Eugene, OR) (4 μl of 1:50 dilution per sample) which fluoresces at 585 nM. After four washes the cells were fixed in 1% paraformaldehyde. Approximately 100,000 cells were used and 10,000 events recorded.

To confirm the specificity of the anti-asialoGM1 antibody, a thin-layer chromatogram of asialoGM1, GM1, GD1a, GD1b, and GTb was overlaid with the antibody which was detected with peroxidase labeled anti-rabbit IgG, as described below. The presence of GM1 on the surface of the epithelial cells was detected by the binding of cholera toxin. The cells were incubated with 10 or 100 ng/ml of the β subunit of cholera toxin conjugated to fluorescein isothiocyanate (FITC-CTB) (List Biochemicals, Campbell, CA) for 30 min at 5°C. The cells were washed with PBS/BSA/azide and fixed with 1% paraformaldehyde. 10,000 cells/sample were analyzed by flow cytomtery monitoring fluorescence at 540 nM. Separate aliquots of epithelial cells were used to quantify each of fluorescent markers.

Adherence assay. The number of P. aeruginosa binding to the epithelial monolayers was quantified using the assay system as has been described (10). The Pseudomonas strains were metabolically labeled with [35S]methionine (New England Nuclear) to a specific activity of ~100 CFU/cpm, washed with 10 mM NaCl, and incubated with the monolayer for 2 h at 37°C. Nonadherent organisms were rinsed off the monolayers with three successive PBS rinses. The epithelial cells and adherent Pseudomonas were solubilized in 0.5 ml of 2% SDS and scintillations counted (Tricarb liquid scintillation spectrometer, Packard Instruments, Inc., Downers Grove, IL). The scintillations associated with a 100-μl aliquot of the initial 5 × 10⁴ CFU/ml suspension of organisms were counted to determine the CFU/cpm. The added inocula were standardized to 5 × 10⁴ CFU/ml/well for the purpose of data comparison. All experiments were done in triplicate and a mean, standard deviation (SD), and standard error (SE) were determined. Statistical analysis was performed using StatView software (Abacus Concepts, Inc., Berkeley, CA).

Binding competition experiments. The radioactively labeled organisms were preincubated with 0.01–0.5 mg/ml of GM1, asialoGM1 (aGM1) (gangliotetraosylceramide), sialic acid (N-acetylmuramic acid) (Bio Carb Chemicals) or PBS as a control for 60 min at 25°C before the addition of the bacteria to the monolayer. To test the effect of cholera toxin (CT) or the B subunit of cholera toxin (CTB) (Calbiochem Corp., La Jolla, CA) 1–100 nM of CT suspended in PBS was incubated with the monolayer for 30 min at 37°C. The monolayers were rinsed with PBS 0.5 ml per well to remove unbound CT, radiolabeled organisms were added and adherence quantified as described above.

Results

PA1244 pilin binds to asialoGM1. The pilii of P. aeruginosa have been demonstrated to be a major adhesin, implicated in the pathogenesis of infection of the respiratory tract (15–18). We sought to determine if the GalNAcβ1-4Gal residue as present in asialoGM1 acts as the receptor for P. aeruginosa pilin. Purified 125I-labeled pilin from P. aeruginosa strain PA1244 was incubated with commercially obtained glycolipid standards which had been separated by thin layer chromatography as described by Krivan et al. (4). The resulting autoradiograph demonstrated that asialoGM1, but not the sialylated forms of the glycolipid core was able to bind pilin (Fig. 1). Thus pili appear to recognize the GalNAcβ1-4Gal sequence which is exposed in asialoGM1, but is sialylated, and therefore unavailable for binding in GM1 and the other glycolipids tested.

AsialoGM1 functions as a receptor for pilated P. aeruginosa on respiratory epithelial cells. The distribution of the GalNAcβ1-4Gal receptor as presented in asialoGM1 residues on epithelial cells, and its accessibility to microorganisms was examined. The binding of the 35S-labeled PA1244 was compared with that of the isogenic pil− mutant PA1244/NP (Fig. 2). Under control conditions the adherence of the pilated strain was twice that of the pil− mutant in that 9% of the added PA1244 inoculum versus 4.6% of the added PA1244/NP bound to monolayers of immortalized CF respiratory epithelial cells. Competitive inhibition experiments were performed by preincubating the bacteria in the presence of exogenous asialoGM1, washing, and performing an adherence assay. A dose-response relationship was shown for strain PAO1 in which 0.01 mg/ml of asialoGM1 blocked 9%, 0.1, and 0.5 mg/ml of asialoGM1 blocked 33% of binding as compared to a control. For
strain PA1244 adherence was decreased by 45% by exogenous 0.1 mg/ml asialoGM1 (unpaired two-tailed t test P < 0.008). The amount of PA1244 adherence which was blocked by asialoGM1 was equivalent to the fraction of binding attributed to the pilin adhesin. If the monolayers, as opposed to the organisms, were preincubated with asialoGM1 at 37°C, adherence of PA1244 was proportionally increased. The incorporation of the glycolipid into the epithelial cell membrane thereby producing neoreceptors was confirmed by fluorescence microscopy (data not shown). Neither the sialylated glycolipid GM1 nor sialic acid alone blocked the adherence of PA1244.

Adherence of the nonpiliated strain PA1244/NP decreased in the presence of GM1 (P < 0.002) or sialic acid (P < 0.0015).

These results are consistent with the expression of non–pilus adhesins which have been suggested to have a role in the recognition of siaIylated receptors (9, 10). The overall adherence properties of PA1244, reflect the dominant binding specificity of pili. However, for strains which do not express pili, it is possible that GM1 or sialic acid alone could function as a receptor for non–pilus adhesins.

PA1244 and PA1244/NP binding in the presence of cholera toxin (CT). The role of GM1 as a possible P. aeruginosa receptor was examined by determining if CT, a well-established ligand for GM1, competes for Pseudomonas receptors on the epithelial cell surface. The binding of CT is highly specific for GM1 (19) with minimal binding to GD1b (20) and no binding to asialoGM1 (19). In the presence of either CT or CTB there was no evidence of competitive inhibition of PA1244 or PA1244/NP binding to the monolayers (Fig. 3), even when greater than saturating amounts of CT or CTB (up to 100 nM) were used (data not shown). CTB consists of the subunit of cholera toxin which recognizes GM1 but does not have enzymatic activity (20). As neither CT, nor CTB interfered with Pseudomonas binding these results were not a consequence of any toxic effects of CT on the epithelial cells. Thus, it seems unlikely that GM1 functions as a major receptor for either the pilus or a non–pilus adhesin of PA1244.

More asialoGM1 residues are available on the surface of CF epithelial cells than on normal epithelial cells. Having identified asialoGM1 as a receptor for the major P. aeruginosa adhesin, pilin, we sought to quantify superficial asialoGM1 on respiratory epithelial cells in primary culture, from both CF patients and normal control subjects. Flow cytometric studies were performed on primary respiratory epithelial cells obtained from two CF patients and three normal controls. In a representative study, superficial asialoGM1 residues were present on 12% of the primary CF airway cells (Fig. 4 A); as compared with 2.9% of the cells from normal control subjects (P = 0.03, χ² = 4.73) (Fig. 4 B). The distribution of GM1 on

![Figure 2](image1)

*Figure 2.* Binding competition studies. The adherence of a standard inoculum of 5 × 10⁷ CFU/ml of (A) PA1244 and (B) PA1244/NP; to imCF monolayers in the presence of: PBS (control), asialoGM1 (aGM1), GM1, or sialic acid (NANA). Data are mean±SE (n = 3).

![Figure 3](image2)

*Figure 3.* PA1244 and PA1244/NP adherence to imCF monolayers pretreated with CT or CTB. A standard inoculum of 5 × 10⁷ CFU/ml of (A) PA1244 or (B) PA1244/NP was incubated with immortalized CF epithelial cell monolayers pretreated with PBS (control), 1 nM CT, 10 nM CT or CTB. Data are mean±SE (n = 3). The SE of some of the points are contained within the symbol of the mean.
Figure 4. FACS analysis of asialoGM1 and GM1 on the surface of primary CF (genotype ΔF508/W1282) and NHNP epithelial cells. The number of cells with asialoGM1 labeled is shown corresponding with the events recorded in the UR field. (A) CFNP cells. (B) Normal control epithelial cells. The effects of bacterial exoproducts on the availability of asialoGM1 was tested by incubating the monolayers with stationary...
these cells was also different; 76% of the CF cells had superficial GM1 labeled with FITC-CTB (Fig. 4 E) as compared with 83% of the cells from normal subjects (Fig. 4 F). This observation, similarly, suggests inherent differences in sialylation. The number of airway epithelial cells with accessible asialoGM1 residues is significantly greater in the cells obtained from CF patients, than in the respiratory epithelial cells from normal subjects.

P. aeruginosa produce a number of extracellular products including a neuraminidase which can modify epithelial surfaces (3, 21). The number of asialoGM1 and GM1 residues were quantified after the epithelial cells were exposed to P. aeruginosa stationary phase culture supernatants. The effect of the P. aeruginosa exoproteins was to increase the number of asialoGM1 residues on the CF cells from 12% to 17% (Fig. 4, A and C), an effect paralleled by a decrease in the number of available GM1 residues from 76% to 65% (Fig. 4, E and G). Pseudomonas exoproteins did not increase the number of available asialoGM1 residues on the normal cells (2.87–3.60%) (Fig. 4, B and D). When expressed as a ratio of (asialoGM1)/ (asialoGM1 + GM1), the relative number of cells potentially expressing an asialoGM1 receptor was fourfold greater for the CF cells (0.13) than for the cells from normal subjects (0.03).

A FACS analysis of asialoGM1 and GM1 on the surface of the immortalized cells lines was also performed. As P. aeruginosa adherence to the imCF and imHTr cells was previously shown to be equivalent (3), we predicted that these SV40 transformed cells would express similar amounts of superficial asialoGM1. Flow cytometry studies demonstrated that 1.7% of the imCF cells had superficial asialoGM1 residues which increased to 2.4% after exposure to bacterial supernatant; not significantly different than the 1.25–1.87% of imHTr cells which were identified by anti-asialoGM1 antibody before and after exposure to bacterial supernatant. The number of immortalized cells with GM1 residues was also equivalent with 55.5% of the imCF and 55.4% of the imHTr cells identified with FITC-CTB. These SV40-transformed cell lines were useful for defining likely receptors, but may not express the patterns of superficial glycosylation present on primary epithelial cells as SV40 infection is well known to modify cell surface properties (3).

Discussion

The pathogenesis of infection in CF, historically, has been ascribed to interactions between P. aeruginosa and viscous mucin. The heavily glycosylated mucin glycoproteins within the respiratory tract were initially implicated as potential receptors for P. aeruginosa infection (22), although the bacterial ligand responsible for this interaction was not identified. However, since these initial observations were made, extensive studies have failed to identify specific receptors for P. aeruginosa in mucin and instead, suggest that the affinity of P. aeruginosa for any mucin, including that from CF patients, is nonspecific (23, 24).

An extensive literature documents the importance of pili as a virulence factor of many gram-negative bacteria (25), including P. aeruginosa. Pili are thought to be the major adhesin associated with the initial colonization of the respiratory tract by environmental strains of P. aeruginosa (16, 18). P. aeruginosa and other human pathogens such as Vibrio cholera express polar pili of the NMephe type which bind directly to epithelial cells (15). Numerous in vitro assays demonstrate that pili are responsible for ~ 50% of the binding attributed to whole Pseudomonas organisms (10, 15, 18). As pilated and nonpiliated strains of P. aeruginosa have equivalent affinity for purified human mucin (22), pilin-mediated attachment in the respiratory tract is more likely to involve a component of the epithelial cell membrane. The TLC experiments reported here confirm the previous studies of both Baker et al. (5) and Krivan et al. (13) and further suggest that pilin is the dominant adhesin responsible for the specific glycolipid binding displayed by the intact organisms used in their studies. Although the interpretation of TLC binding studies that utilize polysialobutylmethacrylate must be made with caution (26), the observed recognition of asialoGM1 by purified pilin is entirely consistent with the P. aeruginosa adherence studies in which the binding of pilated strains, but not isogenic pil− mutants could be inhibited by exogenous asialoGM1. As demonstrated in this report the GalNAcβ1-4Gal sequence found in asialo-glycolipids may function as a specific receptor for P. aeruginosa pili on the surface of the CF epithelial cell.

Other properties unique to P. aeruginosa, other than binding specificity, must be implicated in the pathogenesis of this infection. There are bacterial pathogens which are not associated with disease in CF but which recognize the same GalNAcβ1-4Gal receptor (4). The availability of asialoGM1 receptors on 17% of CF cells but only 3% of normal cells after exposure to accumulated Pseudomonas exoproteins suggests that the ability of the organism to modify the epithelium may be important in establishing infection. The activity of P. aeruginosa exoproteins, which are specifically expressed in the hyperosmolar milieu of the CF lung may further expose receptors on other gangliosides (21). The effects of the bacterial neuraminidase in vivo may be significantly enhanced in the presence of surfactant within the lung (A. Prince, unpublished observations), as has been observed for other bacterial neuraminidases in the presence of detergents (27). Thus, we postulate that the availability of P. aeruginosa receptors on the epithelium facilitates the establishment of an initial nidus of infection by transiently inspired organisms. A few adherent organisms can then proliferate to colonize the lung more efficiently due to the elaboration of specific exoproteins.

The increased amount of superficial asialo-glycolipid in CF airway cells as quantified by flow cytometry in this report is consistent with previous studies which used biochemical techniques to quantify sialylation, and found CF cells to be relatively less sialylated than normal cells (6). Analyses of CF secretions have similarly revealed changes in sulfation (7), altered ratios of fucose to sialic acid in tracheal secretions (28) and less highly sialylated salivary glycoconjugates (29). Although the numerical differences in asialoGM1 content on the surface of CF and normal cells in these studies were small, this glycolipid species is representative of a class of glycolipids, in-

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Phase P. aeruginosa PAO1 culture supernatants, before dissociation of the monolayers from the tissue culture plates. (C) CFNP cells. (D) Normal control epithelial cells. The presence of GM1 was detected using CTB conjugated to fluorescein isothiocyanate. (E) CFNP cells. (F) Normal control epithelial cells. After exposure to PAO1 culture supernatants: (G) CFNP cells; (H) Normal control epithelial cells.
cluding asialoGM2, asialoCad, and fucosylasialoGM1 which also have accessible GalNAcβ1-4Gal receptor sequences to bind P. aeruginosa. The presence of superficial asialo-glycolipid receptors on CF cells correlates with the clinical phenotype of P. aeruginosa colonization in CF patients; with in vitro data quantifying increased Pseudomonas adherence to primary CF cells as compared with normal epithelial cells in culture (3); with the demonstration that asialoGM1 blocks the attachment of pilated organisms; and with the observation that Pseudomonas neuraminidase enhances P. aeruginosa binding specifically to CF cells (21).

It remains to be established exactly how CFTR dysfunction is directly or indirectly associated with levels of protein and glycolipid sialylation. Depending upon the pH optima for certain sialyltransferases, the level of CFTR function in specific cell populations and organelles may be critical in determining the overall glycosylation of mature proteins and lipids. It seems unlikely that a general defect in endosomal acidification is a consequence of CFTR mutations, but that the relative expression of CFTR in different populations of cell types and in discrete subcellular compartments may be responsible for local changes in acidification, as has been observed in some reports (6). Studies to define the role of CFTR-dependent chloride transport in specific organelles and resultant sialyltransferase activity in respiratory epithelial cells should allow a direct correlation to be made between CFTR mutations and the pathogenesis of Pseudomonas infection in CF.

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