*Pseudomonas aeruginosa*-mediated damage requires distinct receptors at the apical and basolateral surfaces of polarized epithelium

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Running title: Distinct receptors mediate *P. aeruginosa* binding

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Abstract

_Pseudomonas aeruginosa_, an important opportunistic pathogen of man, exploits epithelial damage to establish an infection. We have rigorously explored the role of N-glycoproteins and heparan sulfate proteoglycans (HSPGs) in _P. aeruginosa_-mediated attachment and subsequent downstream events at the apical (AP) and basolateral (BL) surface of polarized epithelium. We demonstrate that the N-glycan chains at the AP surface are necessary and sufficient for binding, invasion, and cytotoxicity to kidney (MDCK) and airway (Calu-3) cells grown at various states of polarization on Transwell filters. Upregulation of N-glycosylation enhanced binding, whereas pharmacologic inhibition of N-glycosylation or infection of MDCK cells defective in N-glycosylation resulted in decreased binding. In contrast, at the BL surface, the HS moiety of HSPGs mediated _P. aeruginosa_ binding, cytotoxicity, and invasion. In incompletely-polarized epithelium, HSPG abundance was increased at the AP surface, explaining its increased susceptibility to _P. aeruginosa_ colonization and damage. Using MDCK cells grown as 3-dimensional cysts as a model for epithelial organs, we show that _P. aeruginosa_ specifically co-localized with HS-rich areas at the BL membrane but with complex N-glycans at the AP surface. Finally, _P. aeruginosa_ bound to HS chains and N-glycans coated onto plastic surfaces, showing the highest binding affinity towards isolated HS chains. Together, these findings demonstrate that _P. aeruginosa_ recognizes distinct receptors on the AP and BL surface of polarized epithelium. Changes in the composition of N-glycan chains and/or in the distribution of HSPGs may explain the susceptibility of damaged epithelium to _P. aeruginosa_.

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Introduction

Ninety five percent of all infectious agents enter through mucosal surfaces of gastrointestinal, genito-urinary, and respiratory tracts (reviewed in (35)). These mucosal surfaces are usually lined by a single layer of epithelial cells, which serves as the primary barrier against the entry of most infectious agents and can be considered a primary component of the innate immune system.

Epithelial cells form highly polarized cell layers, with apical (AP) and basolateral (BL) surfaces that exhibit distinct protein, lipid, and glycoconjugate compositions. *Pseudomonas aeruginosa* is a ubiquitous opportunistic pathogen of man that exploits injured mucosa to cause acute and chronic infections with high morbidity and mortality (reviewed in (26, 31). In the setting of epithelial injury and immunocompromise, this gram-negative pathogen causes serious infections in patients with extensive burns, corneal trauma, catheter-related bladder injury, or on ventilators. In addition, *P. aeruginosa* chronically colonizes the lungs of patients with cystic fibrosis (4), leading to severe pulmonary damage and death. Despite aggressive antibiotic therapy, the fatality rate in many *P. aeruginosa* infections is 40%, and new approaches to treatment are even more critical now that antibiotic resistance is widespread among *P. aeruginosa* isolates.

The first step in establishing *P. aeruginosa* infection is receptor-mediated binding to the injured epithelium on the AP and/or BL surface, leading to bacterial internalization and/or direct host injury, as well as dissemination to distant tissues and organs. Glycoconjugates, including glycolipids, glycosylated proteins, and proteoglycans, are candidate receptors for *P. aeruginosa*.
Their long carbohydrate chains are prominently displayed on the surface, exhibit distinct AP and BL localization, and serve as receptors for many microorganisms (3). For *P. aeruginosa*, however, conclusive *in vitro* or *in vivo* data is missing. For example, the predilection of *P. aeruginosa* for injured epithelium has been attributed to increased levels of asialo-GM1 on the AP surface of regenerating cells (11, 23, 43, 44), though it remains controversial whether asialo-GM1 and other glycosphingolipids bind *P. aeruginosa* (13, 49). Furthermore, secreted O-glycoproteins, mucins, have been associated with the binding of *P. aeruginosa* to the AP surface (23, 37). N-glycosylated proteins, in which mannose (Man), glucose (Glc), N-acetylglucosamine (GlcN), and fucose are attached to core proteins to form high mannose, complex, and hybrid N-glycans, are also candidate receptors. For example, the N-glycoproteins CFTR and CD95, have been shown to function as receptors for bacterial binding and internalization (20). However, the role of CFTR as binding receptor for *P. aeruginosa* remains controversial (42).

In contrast to N-glycoproteins, which are present at the AP and BL surface, heparan sulfate proteoglycans (HSPGs) are preferentially expressed on the BL surface of the polarized epithelium (3) and could serve as BL receptors for *P. aeruginosa*. HSPGs are heterogeneous structures that are composed of a core protein and one or more covalently attached heparan sulfate (HS) chains. In addition to variability in the number of HS repeating units and the identity of the core proteins, HS chains are further modified by sulfation at the N, 2, 3, and/or 6 positions, giving rise to enormous combinatorial diversity. The primary HSPG families include syndecans, transmembrane proteins located at the BL surface; perlecan and agrin, secreted HSPGs associated with the extracellular matrix; and glycosylphosphatidylinositol-anchored glypicans, found at the AP surface. HSPGs are known to mediate binding of various bacterial and viral
pathogens (2, 14, 21, 25). They have previously been postulated to modulate adhesion of *P. aeruginosa* to incompletely polarized epithelial respiratory cells (41) and to the exposed basement membrane of mouse cornea (9), but direct evidence for this function is lacking.

In this work, we have rigorously explored the role of N-glycan chains of glycoproteins and HS chains of HSPGs in *P. aeruginosa* infection at the AP and BL surface of polarized kidney and airway epithelial cells, and how changes in their structure and/or expression affect bacterial binding and downstream events including internalization and cell damage. Using 2D and 3D cell cultures, we show that N-glycans are necessary and sufficient for binding, entry, and cytotoxicity at the AP surface of polarized epithelium. Enhanced expression and/or expression of more complex N-glycans, which can occur in damaged epithelium, increases *P. aeruginosa* infection at the AP surface. We further establish that HS chains of HSPGs are necessary and sufficient to mediate binding, invasion, and cytotoxicity on the BL surface in polarized cells and that sulfation is a critical determinant. Finally, we show that in incompletely-polarized cells, a model of tissue injury, HSPGs are upregulated at the AP surface, which leads to enhanced susceptibility to binding and subsequent tissue damage by *P. aeruginosa*. Together our results provide a basis for the increased susceptibility of acute or chronically injured tissue to *P. aeruginosa* infections, and they raise the possibility that well studied molecules, such as N-glycans and HS, might be useful therapeutic targets for the treatment of *P. aeruginosa* infections.

Materials and Methods
**Bacterial strains and electroporation**

*P. aeruginosa* strain K (PAK; obtained from J. Mattick, University of Queensland, Brisbane, Australia) was routinely grown shaking overnight in Luria-Bertani broth (LB broth) at 37°C. To create a plasmid which constitutively produces GFP, the pnpT2-GFP fragment from p519ngfg (33) was excised with HindIII and ExoRI and cloned into the corresponding sites of pUCP20 to yield pnpT2-GFP-pUCP20. This plasmid was introduced into PAK by electroporation with a Bio-Rad Gene Pulser II with settings of 1.6V/25µF/200Ω. The resulting strain is PAK-GFP.

**2D and 3D Cell Culture**

MDCK clone II and ConA' MDCK cells obtained from Dr. Keith Mostov (University of California, San Francisco, CA) were maintained in minimal essential medium (51) supplemented with 5% fetal bovine serum (FBS; Invitrogen) at 37°C with 5% CO₂. Calu-3 cells were obtained from the ATCC (Rockville, MD) and maintained in MEM supplemented with 10% FBS and L-glutamate at 37°C with 5% CO₂. Cells were grown as 2D monolayers on 12-mm Transwell filters (3-µm pore size; Corning Incorporated). For all experiments, cells were plated under conditions such that they formed confluent monolayers that exhibited basic features of early polarized cells, including polarized distribution of AP and BL membrane proteins and functional tight junctions that were impermeable to small molecules such as FITC-inulin (data not shown and see figure 4). Cells were grown for different lengths of time to model different states of polarization. For incompletely-polarized confluent monolayers, MDCK cells were seeded at 0.7 x 10⁶ cells/well and cultured for 24 h. For well-polarized confluent monolayers, MDCK cells were seeded at 0.3 x 10⁶ cells/well and cultured for 5 days. For incompletely-polarized confluent Calu-3 cell monolayers, Calu-3 cells were seeded at 1.5 x 10⁶ cells/well and cultured for 2 days. For well-
polarized confluent Calu-3 cell monolayers, Calu-3 cells were seeded at 1 x 10^6 cells/well and cultured for 7 days.

For some experiments, MDCK or Calu-3 cells grown as 2D cultures on Transwell filters were treated with the following reagents. None of these treatments inhibited bacterial growth (data not shown). To inhibit N-glycosylation, cells were pre-treated with 0.1-1 µg/ml of tunicamycin (Sigma-Aldrich) for 16 h in MEM supplemented with 5% FBS (MDCK and ConA\(^\text{r}\) cells) or 10% FBS (Calu-3 cells). For competition blocking with proteoglycans, cells were pretreated with 0.1-10 µg/ml of heparin or chondroitin sulfate (Sigma-Aldrich) at 37°C for 1 h in serum-free MEM. To remove proteoglycans, cells were treated with 1-200 mU of heparinase III (Sigma-Aldrich) or chondroitinase ABC (Sigma-Aldrich) in Hank’s Buffered Salt Solution (HBSS) containing 0.1% BSA at 37°C for 2 h. Proteoglycan desulfation was performed by overnight incubation of cells with 10 mM sodium chlorate in MEM containing 5% FBS (MDCK and ConA\(^\text{r}\) cells) or 10% FBS (Calu-3 cells). Resulfation was accomplished by adding 1 mM sodium sulfate to sodium chlorate-containing media. Upregulation of N-glycosylation was performed according to (46), with the following modifications. Cells were grown in the presence of 1mM Man, Glc, or Gal (Sigma-Aldrich) in MEM with 5% (MDCK and ConA\(^\text{r}\) cells) or 10% (Calu-3) FBS for 1 week (long upregulation of N-glycosylation) or for 1 day (brief upregulation of N-glycosylation). Cells were stained with FITC-Concanavalin A (FITC-ConA; Sigma Aldrich) to assess cell surface N-glycosylation. Cell surface proteoglycans were visualized by immunofluorescence staining with HS antibody (10E4; Seikagaku) or with FITC-WFA (a chondroitin sulfate-specific lectin from Wisteria floribunda; Sigma Aldrich).
For 3D cell culture, MDCK or ConA\textsuperscript{+} MDCK cells were grown as cysts in Matrigel/collagen as previously described (39, 52) with the following modifications. A single cell suspension of $4 \times 10^4$ cells/ml was added to a solution of buffered, liquefied collagen (3 mg/ml). 250 μl of cells in collagen I were plated in 8-well coverglass chambers (Nalge Nunc International) that had been previously covered with 100% Matrigel. The Matrigel served as a solid base to which the cysts attached prior to collagen solidification. The collagen was allowed to solidify into a gel by incubation at 37 °C prior to the addition of MEM containing 10% FBS. Over the 5 day growth period, individual cells in the collagen matrix proliferated to form cysts, with the AP membrane facing the lumen and the BL membrane facing the surrounding collagen (BL-side out cysts). For some experiments, anti-beta-1 antibody AIIB2 (1:100 dilution in the collagen I solution and or in MEM; a kind gift of Dr. Caroline Damsky at the University of California, San Francisco) was included for the entire culture period. Under these conditions, polarized AP-side out cysts were formed, although the structures are less well organized than in the BL-side out cysts (39). For bacterial infections MDCK cysts grown in Matrigel/collagen were treated with collagenase type VII (Sigma-Aldrich) at 100 U/ml in PBS for 15 min at 37 °C to digest the collagen gel. For some experiments, cysts were treated with heparinase III in HBSS containing 0.1% BSA at 37°C for 3 h, washed, and resuspended in serum-free MEM for bacterial infections.

**Bacterial Adhesion and Invasion Assays on 2D monolayers**

PAK grown overnight in LB broth to stationary phase were diluted in 50 μl of serum-free MEM and added to cells at an MOI of 20. To infect the AP side of 2D monolayers grown on Transwells, the bacteria were added to the AP chamber. For BL infections, the Transwell insert was placed directly onto 50 μl of serum-free MEM containing PAK. After 1 h of infection at
37°C, adhesion and invasion assays were performed as described previously (27). Briefly, for the adhesion assay, cells were washed in PBS to remove non-adherent bacteria and lysed in 1 ml Ca$^{2+}$ Mg$^{2+}$-free PBS with 0.25% Triton X-100 (Sigma-Aldrich) for 30 min. For invasion assays, before lysis, cells were first incubated in serum-free MEM containing 0.4 mg/ml amikacin (Fisher Scientific) for 2 h. After lysis, cells were removed from the Transwell filters by gentle scraping. Bacteria were enumerated by plating serial dilutions of cell lysates to LB plates and counting colony-forming units (cfu). All assays were carried out on triplicate wells, and results are reported as the average of three to four experiments. Because of well established variability in adhesion and invasion assays, data were normalized to 100% of bacterial adhesion or internalization at the AP surface of cultured epithelial cells to allow comparison between experiments performed on different days. Consistent with previous reports, approximately 10% of the PAK inoculum binds to cells whereas 1-2% of the inoculum is internalized (29).

**Bacterial Infection of 3D Cysts**

Prior to bacterial infections, cysts were briefly treated with type VII collagenase (100 U/ml in PBS for 15 min at 37 °C), which removed the thin layer of collagen the coated the cysts and allowed the bacteria to access the surface of the cysts. GFP-expressing PAK (10$^7$) suspended in serum-free MEM were incubated with the cysts for 2 h. Cells were washed with PBS to remove non-bound bacteria and fixed in PBS containing 1% paraformaldehyde at 37°C for 0.5 h. Bacterial adherence and co-localization with specific surface markers are described below.

**Immunofluorescence Microscopy and Image Analysis**
HS chains were stained with anti-heparan sulfate antibody (10E4), tight junctions with anti-ZO-1 (R40.76) antibody (a gift from B. Stevenson, University of Edmonton, Alberta, Canada), actin filaments were stained with AlexaFluor594-phalloidin (Invitrogen) and mannose residues were stained by FITC-ConA. AlexaFluor488- or 647-conjugated secondary antibodies were obtained from Invitrogen. Transwell grown monolayers were fixed in PBS containing 1% paraformaldehyde at 37°C for 0.5 h. After washing, cells were incubated with primary antibodies overnight at 4°C and, afterwards, with fluorescent secondary antibodies for 2 h at room temperature. Filters were excised and mounted on microscope slides (Fisher Scientific) in mounting medium (Vector Laboratories, Inc). Cysts, grown in 8-well coverglass chambers, were fixed and stained with antibodies directly in chambers. Cysts were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 2 h at room temperature.

Samples were examined with a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.). Images and 3D reconstructions were acquired by and processed in Meta 510 software. Image J analysis was performed on TIFF files.

Bacterial binding to 3D cysts and co-localization with surface markers was quantified using the Image J plugin Voxel counter on 3D reconstructions of TIFF images acquired with Meta 510 software. Voxel Counter (ImageJ plugin) was used to quantify the volume of bound 3D bacterial aggregates and a minimum volume was set as a threshold to enable automated cell counting using the 3D Object Counter (ImageJ plugin). Any bacterial aggregate above the threshold was counted as one. The surface area of membrane regions either enriched or depleted of HS or N-glycans (as determined by staining with an anti-HS antibody or with FITC-ConA, respectively)
was measured in pixels by ImageJ, and the number of bacterial aggregates bound was normalized per pixel of each specific surface area. The percentage (compared to total) of bacterial aggregates bound to each specific region was determined. For each treatment, 40-50 cysts were quantified.

In vitro Binding Assay

Sugars, N-glycans, and glycosaminoglycans (Sigma Aldrich; 0.1-10 µg in 0.2 ml ddH₂O) were added to 96-well plastic plate (Falcon, Becton Dickinson Labware) and incubated overnight at 37°C until evaporated. Wells were washed with ddH₂O and blocked in 0.1% BSA for 0.5 h at room temperature. Bound glycans were stained with 1% Toluidine blue (Sigma Aldrich) and absorbance was measured at 630 nm. The absorbance of known concentration of glycans was used as the standard curve and the concentration of bound glycans (µg/well) was calculated.

Stationary phase grown PAK-GFP (10⁷ in 0.1 ml ddH₂O) were added to wells and incubated for 2 h at room temperature. Non-adherent bacteria were removed by washing with ddH₂O. Bound PAK-GFP was quantified using a SpectraMax 340PC plate reader using SOFTmaxPro software (Molecular Devices) at λex = 480 nm and λem = 530 nm. PAK-GFP bound to non-coated wells was used as a control and subtracted out as background. All assays were carried out on triplicate wells, and results are reported as the average of four experiments.

Cytotoxicity Assay

Epithelial cell cytotoxicity was quantified by colorimetric quantification of lactate dehydrogenase (LDH) release using a commercially available kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) according to manufacturer’s instructions and as previously
described (17) with the following modifications. For both AP and BL infections, Transwell filters were placed on 50 μl drop of media only (for AP infections) or media plus bacteria (for BL infections). Transwell-grown cells were infected with PAK (MOI of 20) for 5 h at the AP or BL surface and 50 μl of the supernatant was collected from the AP chamber every 1 h. The absorbance of the sample was measured at 490 nm on SpectraMax 340PC plate reader using SOFTmaxPro software. Results were normalized to 100% of LDH release in infected cells. Assays were carried out on triplicate wells, and the results are reported as the average of three to four experiments.

**Transepithelial Permeability**

Transwell grown cells were infected with PAK for 5 h at the AP surface. Non-adherent bacteria were removed by washing with PBS. FITC-inulin (Sigma-Aldrich; 100 μg/ml) in PBS was added to the AP chamber, and PBS alone was added to the BL chamber of Transwell plates. Cells were incubated at 37°C and 100-μl samples were collected from the BL chamber every 0.5 h. Fluorescence was quantified using a fluorescence plate reader (λ:ex = 480 nm and λ:em = 530 nm; SpectraMax 340PC plate reader using SOFTmaxPro software). Known dilutions of FITC-inulin were used as a standard curve.

**Statistical Analysis**

Data are expressed as means ± SD (standard deviation). Statistical significance was estimated by paired Student’s t-test. Differences were considered to be significant at P < 0.05.
Results

Modulations of specific enzymes in N-glycosylation pathway affect the expression of N-glycan chains on the cell surface

Previous studies have shown that *P. aeruginosa* cytotoxicity was diminished in filter-grown confluent monolayers of concanavalin A resistant Madin-Darby Canine Kidney (ConA<sup>r</sup> MDCK) cells compared to wild type (wt) MDCK cells (1). While the exact defect in the glycosylation pathway in ConA<sup>r</sup> MDCK cells is not known, there are alterations in the Man core of its N-linked carbohydrates, which impair the formation of more complex N-linked carbohydrate structures (34). We hypothesized that surface-exposed complex N-glycans are important determinants of *P. aeruginosa* adhesion to an injured epithelium, thus explaining the reduced susceptibility of ConA<sup>r</sup> MDCK cells to *P. aeruginosa*-mediated damage. Others have suggested that *P. aeruginosa* binding and entry into ConA<sup>r</sup> MDCK cells is diminished because these cells form hyperpolarized monolayers (15).

To test our hypothesis, we utilized MDCK or human airway epithelial (Calu-3) cells grown as confluent monolayers on Transwell filters for varying lengths of time. We have shown that under these conditions, MDCK cells form functional tight and adherens junctions within 24 h and show polarized distribution of many AP and BL markers, such as gp135 and gp 58((28) and Fig. S1). With increasing time in culture, they exhibit enhanced polarized distribution of markers such as predominantly BL-expressed epidermal growth factor (EGFR; Fig S1). We confirmed that cells
grown for 1 day (MDCK cells) or 2 days (Calu-3 cells) formed confluent monolayers with functional tight and adherens junctions, as evidenced by impermeability to apically applied FITC-inulin (Fig. S1), a low molecular weight molecule that is not able to diffuse through functional tight junctions (1). Epithelial polarity continued to increase at later times in culture (5 days for MDCK cells and 7 days for Calu-3 cells) as evidenced by increased polarized distribution of AP and BL markers, such as HSPGs and EGFR (see Fig. 2 and Fig. S1). For ease of nomenclature, we refer to cells cultured for 1 day (MDCK) or 2 days (Calu-3) as “incompletely-polarized” monolayers and 5 days (MDCK) or 7 days (Calu-3) as “well-polarized” monolayers. We emphasize that under the conditions of our experiments, incompletely-polarized monolayers describes confluent monolayers with functional tight and adherens junctions but with incompletely polarized distribution of some AP and BL markers.

We qualitatively and quantitatively assessed the amount of surface-exposed Man in N-glycan chains in wt MDCK, ConA r MDCK, and Calu-3 cells at various states of polarization by confocal microscopy and fluorescent plate assays using FITC-conjugated ConA, a lectin that specifically recognizes nonreducing terminal α-D-glucosyl and α-D-mannosyl groups. The results obtained in incompletely-polarized cells (Fig. S2A) were similar to those seen in well-polarized cells (Fig. 1). FITC-ConA bound more efficiently to well-polarized wt MDCK than to ConA r MDCK cells at both the AP and BL surface (Figs 1A and B). FITC-ConA binding to the AP and BL surface of Calu-3 cells was of the same intensity as binding to wt MDCK cells. We used Man or Glc (a Man precursor) supplementation to complement the defect in ConA r MDCK or to augment N-glycosylation in wt MDCK and Calu-3 cells. Addition of these sugars is known to upregulate the activity of phosphomannomutase (PMM) and phosphomannose isomerase.
(PMI), enzymes that play a critical role in maintaining the supply of D-Man derivatives required for N-glycosylation (46). Man or Glc supplementation restored the binding of FITC-ConA to ConA$^r$ MDCK cells (Figs. 1A and B). Increased binding of FITC-ConA to Man residues on N-glycan chains was observed only after 5-6 days of treatment with exogenous Man, eliminating the possibility that the exogenous Man simply adhered to the cell surface and facilitated the binding of the lectin. Addition of galactose (Gal), which is not involved in Man synthesis, did not restore binding of FITC-ConA. Moreover, binding of FITC-ConA was enhanced in wt MDCK and Calu-3 cells cultured with Man or Glc (but not Gal) compared to untreated cells. We verified that MDCK and Calu-3 cells with normal or enhanced levels of Man were sensitive to killing by ConA added to the growth medium (Fig. S2). ConA$^r$ MDCK cells grown in the presence of Man or Glc (but not Gal) lost their normal resistance to the lectin and showed enhanced sensitivity to ConA (Fig. S3).

Several conclusions that are important for our subsequent work can be drawn from these experiments. First, well- and incompletely-polarized ConA$^r$ MDCK cells have decreased levels of surface exposed Man and, thus, N-linked glycans on both the AP and BL surface. Second, Man or Glc supplementation is able to restore the cell surface expression of N-glycans in these cells. It is also sufficient to increase the expression of more complex N-glycans on the AP and BL surface of wt MDCK and Calu-3 cells. Third, and finally, the surface presentation of N-glycans does not change as cells become more polarized (compare Fig. 1B to Fig. S2A).

The expression level and structure of N-glycan chains modulate *P. aeruginosa* binding at the AP surface of well- and incompletely-polarized epithelium.
To address the hypothesis that modifications in the expression and structure of N-glycan chains alter interactions of *P. aeruginosa* with the host epithelium, we assayed the effect of various modifications in N-glycosylation on bacterial adhesion to host epithelial cells. MDCK, ConA' MDCK and Calu-3 cells were grown on Transwell filters as well- or incompletely-polarized monolayers with or without sugar supplementation. Augmentation of N-glycosylation by addition of Man or Glc (but not Gal) to ConA' MDCK cells increased bacterial binding nearly 3-fold, to at least wt levels, at the AP surface of well-polarized cells (Fig. 1C). Likewise, addition of Man or Glc (but not Gal) to wt MDCK cells (Fig. 1C) or Calu-3 cells (Fig. 1D) was sufficient to increase bacterial binding at the AP surface. Similar results were observed in incompletely-polarized cells (Figs. S2B and C). None of these treatments affected bacterial binding at the BL surface of ConA' or wt MDCK cells (Fig. 1C), or Calu-3 cells (Fig. 1D), eliminating the possibility that these treatments non-specifically affected bacterial binding.

We next pre-treated cells with tunicamycin, a well characterized inhibitor of GlcN phosphotransferase, the enzyme that catalyzes the first step of N-glycoprotein synthesis. In control experiments, we determined that tunicamycin treatment reduced the amount of N-glycans on the AP and BL cell surface of well- and incompletely-polarized wt MDCK and Calu-3 cells, roughly to similar levels as untreated ConA' MDCK cells (Fig. S4). As expected, tunicamycin treatment had minimal effect on FITC-ConA binding to ConA' MDCK cells, which already express less N-glycans on their surface. Tunicamycin treatment of well-polarized wt MDCK or Calu-3 cells decreased *P. aeruginosa* adhesion to the AP surface in a dose-dependent manner, up to 2-fold (Figs. 1E and F), indicating that N-glycans may function as AP binding receptors. Drug treatment did not further decrease bacterial binding to the AP surface of ConA' MDCK cells.
(Fig. 1E), which was already decreased approximately 2-fold compared to wt cells, strengthening our conclusion that N-glycosylation is defective in these cells. \textit{P. aeruginosa} adhesion to the BL surface was not affected in tunicamycin-treated wt or ConA' MDCK cells (Fig. 1E), or in tunicamycin-treated Calu-3 cells (Fig. 1F). Similar results were observed in incompletely-polarized cells (Figs. S1D and E).

Consistent with previously published results (15, 28), we found that \textit{P. aeruginosa} bound more efficiently to the AP surface of incompletely-polarized cells than to the AP surface of well-polarized cells (Fig. S5). We determined if alteration in the expression of N-glycans in incompletely-polarized cells is responsible for the increased binding of \textit{P. aeruginosa} to the AP surface. Bacterial binding to the AP surface of incompletely-polarized cells was 2-fold higher than binding to well-polarized kidney and lung cells (Fig. S6A). Treatment with tunicamycin did not change the binding ratio. These results establish that N-glycans are important determinants of \textit{P. aeruginosa} binding to the AP surface of polarized epithelium. However, enhanced bacterial binding to the AP surface of incompletely-polarized cells is N-glycan independent; otherwise, tunicamycin treatment would have abrogated the increased binding. Together, these results firmly establish that an increase in the abundance and/or in the length and complexity of N-glycan chains enhances \textit{P. aeruginosa} binding at the AP surface independently of the state of epithelial polarization.

**HSPGs are expressed preferentially at the BL surface of well-polarized but on both surfaces of incompletely-polarized epithelium**
In contrast to N-glycoproteins, which are present at the AP and BL surface, HSPGs are preferentially expressed on the BL surface of the polarized epithelium (3). We considered the possibility that (i) HS chains of HSPGs modulate *P. aeruginosa* attachment to the BL surface of mucosal surfaces and/or (ii) enhanced AP expression of HS explain the increased binding of *P. aeruginosa* to the AP surface of incompletely-polarized epithelium, since the increase is independent of N-glycans.

We first assessed surface expression of HS qualitatively and quantitatively at the AP and BL surface of MDCK cells and Calu-3 cells at various stages of polarization, using a commercially available antibody that recognizes the HS moiety of HSPGs independently of the identity of the core protein. Confocal microscopy revealed that the HS antibody preferentially bound to the BL surface of well-polarized Calu-3 cells (Fig. 2A) as well as to the BL surface of well-polarized wt MDCK and ConA\textsuperscript{r} MDCK cells (data not shown). In contrast, the HS antibody bound approximately equally to the AP and BL surfaces of incompletely-polarized cells (Fig. 2). Both polarization models had functional tight junctions, as evidenced by Z0-1 staining (Fig. 2A) and by the lack of permeability to apically applied FITC-inulin (data not shown). Quantification of bound FITC-conjugated antibody confirmed that HSPGs were largely restricted to the BL surface of well-polarized wt MDCK, ConA\textsuperscript{r} MDCK cells, and Calu-3 cells (Fig. 2B), whereas in incompletely-polarized cells, there were approximately equivalent levels at both the AP and BL surfaces (Fig. 2C). Together, the experiments demonstrate that HS chains of HSPGs fulfil the characteristics predicted for a BL receptor in multiple cell lines. There is preferential presentation of HSPGs at the BL surface of fully polarized cells while there is increased presentation of HSPGs at the AP surface of incompletely-polarized cells.
HS chains of HSPGs contribute to *P. aeruginosa* binding at the BL surface of well-polarized epithelium and at both surfaces of incompletely-polarized epithelium

We utilized comprehensive approaches to determine the role of HSPGs in bacterial adherence to the AP and BL surface of well- and incompletely-polarized epithelial monolayers. First, we added excess heparin to block the interaction between *P. aeruginosa* and HS chains on the cell surface. In well-polarized epithelium, addition of heparin inhibited bacterial binding in a dose-dependent manner at the BL surface of wt MDCK, ConA_r MDCK, and Calu3 cells (Figs. 3A and C). At 10 µg/ml heparin, binding was decreased approximately by ∼50% compared to cells without the addition of heparin. As expected, exogenous addition of heparin had minimal or no effect on *P. aeruginosa* binding at the AP surface of well-polarized cells (Figs. 3A and C). The results were distinctly different in incompletely-polarized cells. Addition of heparin reduced bacterial adhesion by 50% at both the AP and BL surface of incompletely-polarized wt MDCK (Fig. 3B) and Calu-3 cells (Fig. 3D). Notably, heparin also decreased bacterial attachment to the AP surface of incompletely-polarized ConA_r MDCK cells (Fig. 3B). To rule out non-specific charge effects, we demonstrated that addition of another highly negatively charged glycosaminoglycan chain, chondroitin sulfate (CS), had no effect on bacterial binding to either surface in any of these cell types (Figs. S7A-D).

We further examined the role of HS chains in mediating the interaction between *P. aeruginosa* and HSPGs in well- and incompletely-polarized cells. Pre-treatment of cells with heparinase III, an enzyme that cleaves HS chains, reduced bacterial adhesion to the BL surface of well-polarized wt MDCK, ConA_r MDCK, and Calu3 cells in a dose-dependent manner, up to 50% at
200 mU (Figs. 3E and G). Minimal effect was observed on bacterial binding at the AP surface. In incompletely-polarized cells, heparinase III treatment decreased bacterial attachment to both the AP and BL surface of all three cell lines (Figs. 3F and H). Enzymatic removal of CS by chondroitinase ABC did not affect bacterial attachment, confirming the specific role of HS in mediating interactions with *P. aeruginosa* (Figs. S7E-H). We qualitatively and quantitatively confirmed the specificity of enzymatic treatment with heparinase III by showing decreased HS antibody staining at the BL surface of well-polarized wt MDCK, ConA MDCK and Calu-3 cells, and at the AP and BL surface of incompletely-polarized cells (Fig. S8). Likewise, the efficacy of chondroitinase ABC treatment was confirmed by staining with FITC-conjugated WFA, a lectin that binds to the N-acetylgalactosamine moiety in CS (data not shown). Treatment with chondroitinase ABC did not attenuate the staining of the HS antibody (data not shown).

The sulfate moieties on HS chains are major contributors to their net negative charge and may provide the basis for ionic forces that underlie non-covalent interactions between HSPGs and *P. aeruginosa*. To study the role of sulfation in bacterial binding, we pre-treated wt MDCK, ConA MDCK, and Calu-3 cells with sodium chlorate, an inhibitor of sulfate adenyltransferase and HS sulfation. As shown in Figure 4, chemical desulfation of HS reduced bacterial binding over 2-fold at the BL surface of well-polarized cells and at both surfaces in incompletely-polarized cells. Resulfation by addition of sodium sulfate to chlorate-treated cells (53) restored bacterial binding to well- and incompletely-polarized epithelium. The efficiency of desulfation and resulfation was monitored by staining with the anti-HS antibody (data not shown).
In summary, our results suggest that HS chains with intact sulfate groups play an important role in binding of *P. aeruginosa* to the BL surface of the polarized epithelium. Importantly, all three treatments that affected HSPGs (addition of excess exogenous heparin, removal of HS chains by digestion with heparinase III, or removal of sulfate residues by chemical desulfation) reduced the bacterial binding to the AP surface of incompletely-polarized cells approximately 2-fold for all three cell lines when compared to well-polarized cells (Fig. S6B). We conclude that the increased presence of HSPGs on the AP surface of incompletely-polarized cells accounts at least in part for their increased susceptibility to *P. aeruginosa* infection.

*P. aeruginosa* binds directly to isolated HS and N-glycan chains *in vitro*

If N-glycans and HS chains serve as receptors for *P. aeruginosa* binding, then it should be possible to demonstrate direct binding in vitro. We established an in vitro binding affinity assay where informative glycosylated molecules were coated onto 96-well plastic plates at different concentrations, GFP-conjugated bacteria was then added to coated plates, and fluorescence was measured after 1h. As shown in Figure 5, *P. aeruginosa* bound in a dose-dependent manner to HS or to a complex hybrid N-glycan chain ((Gal-GlcN)_4Man_3(GlcN)_2), with the strongest binding to HS. Minimal or no binding was observed to other glycosaminoglycans: alternatively sulfated CS chains or to non-sulfated hyaluronic acid (HA). These results suggest that (i) *P. aeruginosa* binds to specific sulfated sequences in HS chain and that (ii) the anionic charge provided by sulfate groups is necessary for the interaction. Moreover, no binding was observed to wells coated with GlcN, which is one of the sugar residues present in N-glycan chains. Our findings suggest that single sugars are not sufficient and that recognition of specific ordered combinations of sugar sequences along N-glycan chain are required for bacterial adhesion.
Together, these results strongly indicate that HS and N-glycans can function as binding receptors for *P. aeruginosa*.

*P. aeruginosa*-induced host cell injury or internalization into host cells is mediated by binding to N-glycans at the AP surface or HS at the BL surface

Following binding to epithelial cells, *P. aeruginosa* is able to enter into cells and/or, at later time points, cause type III secretion-dependent cytotoxicity. In order to study the extent of host cell damage after bacterial attachment to N-glycans or HS, variously treated cells were incubated with bacteria for 5 h and standard LDH release assays (which measures cell damage) and the FITC-inulin monolayer permeability (which measures integrity of tight junctions) assays were performed. We first studied the role of N-glycans by pre-treating cells with tunicamycin to inhibit N-glycosylation or by exogenous addition of Man to increase N-glycosylation. At up to 5 hours post infection, after AP addition of bacteria to tunicamycin-treated cells, LDH release was reduced by 50-70% in both well- and incompletely-polarized wt MDCK and Calu-3 cells (Fig. 6 and Figs S8A-H). In contrast, Man supplementation augmented bacterial-induced cytotoxicity at the AP surface of well- and incompletely-polarized monolayers, as evidenced by increased LDH release. Bacterial-induced cell death correlated well with the loss of epithelial barrier as measured by FITC-inulin diffusion through epithelial monolayer (Fig. S10 and data not shown).

As expected, ConA⁺ MDCK cells were resistant to bacterial killing at the AP surface, and tunicamycin treatment did not further decrease LDH release (Fig. 6 and Figs. S9I-L) or affect FITC-inulin diffusion (data not shown). However, incubation with Man increased the sensitivity of ConA⁺ MDCK cells to bacterial-induced injury at the AP surface (Fig. 6). Consistent with our
binding data (see Fig. 1), cytotoxicity induced by bacteria added to the BL surface was unaffected by tunicamycin treatment or Man supplementation (Fig. 6 and Fig. S9).

Next, we investigated the role of HS chains of HSPGs in promoting host injury upon *P. aeruginosa* binding. Heparinase III digestion or HSPG desulfation decreased LDH release upon BL addition of bacteria to well-polarized wt MDCK, ConA* MDCK, and Calu-3 cells (Figs. 6A, C and Fig. S9). Importantly, these treatments also decreased cytotoxicity at the AP surface of incompletely-polarized cells (Figs. 6B, D and Fig. S9). Resulfation of chemically desulfated HS chains restored cytotoxicity when bacteria were added to the BL surface of well-polarized cells or to both surfaces of incompletely-polarized cells (Figs. 6A-D). As expected, decreased cytotoxicity correlated with decreased FITC-inulin diffusion (Fig. S10 and data not shown).

In order to examine the role of N-glycans and HS chains in *P. aeruginosa* entry into host cells, variously pre-treated cells were infected for 1 h and standard bacterial internalization assays were performed. Bacterial internalization was reduced at the AP membrane of well-polarized ConA* MDCK cells compared to wt MDCK cells, but internalization at the BL surface was equivalent in the two cell lines (Figs. 7A and C). It was restored to wt levels in ConA* MDCK cells supplemented with Man or Glc (Fig. 7A). An increase in bacterial internalization at the AP surface was observed in well-polarized wt MDCK or in Calu-3 cells expressing more complex N-glycans after supplementation with Man or Glc (Figs. 7A, B). Addition of Man or Glc did not enhance internalization at the BL surface of MDCK, ConA* MDCK, or Calu-3 cells (Figs. 7A and B). Inhibition of N-glycosylation with tunicamycin reduced bacterial entry at the AP surface of well-polarized MDCK and Calu-3 cells, but did not further decrease bacterial internalization.
into ConA\(^{\dagger}\) MDCK (Figs. 7C and D). Tunicamycin had no effect on \textit{P. aeruginosa}
internalization from the BL surface of any of these cell types. Similar results were observed in incompletely-polarized monolayers (data not shown). Finally, enzymatic cleavage of HS by heparinase III reduced bacterial invasion in a dose-dependent manner on both the AP and BL surface of incompletely-polarized but only at the BL surface of well-polarized cells (Figs. 7E-H). Chondroitinase ABC treatment had no effect on invasion under any of these conditions (data not shown).

Importantly, bacterial internalization at the AP surface of incompletely-polarized cells was 2-fold higher than in well-polarized cells (Fig. S6C). This 2-fold difference was still observed after tunicamycin treatment, again indicating that the enhanced internalization observed at the AP surface of incompletely-polarized cells in not due to upregulation of N-glycans at the AP surface. However, pre-treatment of incompletely-polarized cells with heparinase III reduced bacterial internalization at the AP surface to levels observed in well-polarized cells. Together, these data confirm that \textit{P. aeruginosa}-induced binding and subsequent host injury and entry to well-polarized epithelium are mediated by bacterial binding to HS chains at the BL surface and to N-glycans at the AP surface. In contrast, these events are mediated by HS at the BL surface and by both HS and N-glycans at the AP surface in incompletely-polarized epithelium. An increase in the abundance and complexity of N-glycan chains enhances \textit{P. aeruginosa} binding at the AP surface of both well- and incompletely-polarized epithelium. However, the enhancement of bacterial internalization on the AP surface of incompletely-polarized cells compared to well-polarized cells is HS-dependent.
**P. aeruginosa** co-localizes with N-glycans at the AP or HS chains at the BL membrane of 3D cysts.

MDCK cells grown in collagen or Matrigel form highly polarized 3D clonal cysts comprised of a layer of well-polarized cells surrounding a simple lumen, with the AP side facing the lumen and the BL side facing the surrounding collagen (BL-side out cysts). This system recapitulates the organization of simple epithelial tissues (5). It also affords the possibility of studying microbe interactions with the BL surface of the mucosal barrier in the absence of a Transwell filter. If an antibody to the extracellular domain of integrin is present during growth, cysts with opposite polarity are formed, in which the AP surface faces outward (AP-side out cysts), allowing direct comparison with BL-side out cysts (52). These cysts, while less well organized, are as well-polarized as the BL-side out cysts. We used this model to test whether **P. aeruginosa** binding to the outside surface of highly polarized BL-side out and AP-side out cysts occurs at HSPG-rich or N-glycan-rich regions. Without additional manipulations, the 3D cysts are not representative of incompletely-polarized epithelium.

We first examined the distribution of HS chains and N-glycans in cysts. An antibody to HS exhibited uniform staining of the outer membrane of BL-side out cysts, consistent with the known polarized distribution of HSPGs in the BL membrane (Fig. 8A, left panel). In AP-side out cysts, it showed a chicken-wire-like pattern of staining the inner BL membrane (Fig. 8A, right panel). Heparinase III treatment of BL-side out cysts resulted in patchy staining of HS when compared to untreated cysts, affording us the possibility of correlating bacterial binding to HS-rich patches (Figs. 8C and D). N-glycans, revealed by staining with FITC-ConA, were found on the BL surface of BL-side out cysts and both surfaces of AP-side out cysts (Fig. 8B). Staining of
the luminal surface of BL-side out cysts with FITC-ConA was not detectable since the lectin could not penetrate through well-polarized cells. As expected, ConA'-MDCK BL-side out (Figs. 8E, F) and AP-side out cysts (Fig. 8I) exhibited decreased staining with FITC-ConA when compared to wt MDCK cells, with patchy distribution of N-glycans. Brief Man supplementation of ConA'-MDCK cysts resulted in increased surface FITC-ConA staining (Fig. 8J). However, the uniform staining seen in wt MDCK cysts was not achieved since the sugar was added for shorter times (1 day) when compared to complete supplementation (1 week). Together, these studies suggest that the distribution of N-glycans and HPSGs was similar in MDCK cells grown as 3D cysts or as well-polarized 2D monolayers.

We used heparinase III treated BL side-out MDCK cells cysts and Mannose-supplemented AP-side out ConA'-MDCK cysts, to ask whether *P. aeruginosa* binding to cysts correlated with HS- or N-glycan-rich regions at the BL or AP surface. As BL-side out cysts show a uniform distribution of HSPGs on the BL surface, we briefly exposed these cysts to heparinase III and then correlated the binding of GFP-expressing *P. aeruginosa* with HS-rich or HS-poor patches. For comparison, we determined the association of binding to Man-rich regions using cysts formed from ConA'-MDCK cells or ConA'-MDCK cells briefly supplemented with Man (1 day), where N-glycosylation is upregulated but not restored to wt levels. Under these conditions a non-uniform distribution of N-glycans is observed, as determined by binding of FITC-ConA. As shown in Figure 8G, significantly more GFP-expressing bacteria co-localized to HS-rich patches (70%) than to HS-poor patches (30%) in BL-side out wt MDCK cysts. Only 25% *P. aeruginosa* bound to Man-rich patches expressed on the BL membrane of BL-side out ConA'-MDCK wt cysts (data not shown) or ConA'-MDCK cysts briefly grown in the presence of Man (Fig. 8H).
We also tested whether bacteria bound preferentially to Man-rich regions on the AP surface of AP-side out cysts, by comparing binding to AP-side out ConA\textsuperscript{+} MDCK cysts grown in the absence or presence of supplemental Man. As shown in Figure 8K, only ~30\% of PAK-GFP co-localized with Man-rich regions in AP-side out of ConA\textsuperscript{+} MDCK cysts. When the cysts were supplemented briefly with Man, the fraction of \textit{P. aeruginosa} co-localizing with more complex N-glycans increased to 70\% (compare panels K and L). In summary, in 3D cysts, \textit{P. aeruginosa} bound preferentially to more complex N-glycans on the AP surface or with HS chains of HSPGs on the BL surface, supporting our hypothesis that these molecules are specific binding partners for \textit{P. aeruginosa}.

**Discussion**

Successful opportunistic pathogens, of which \textit{P. aeruginosa} is a prime example, exploit specific niches in the host in order to facilitate attachment, colonization, damage, and dissemination. Our work was inspired by the fact that long carbohydrate chains of various glycoconjugates, including HSPGs and N-glycoproteins, could potentially serve as bacterial receptors since they are prominent cell surface exposed structures at the mucosal epithelium. We extensively characterized the distribution of HSPGs and the structure of N-glycan chains in cultured epithelial cells grown at various states of polarization. As \textit{P. aeruginosa} requires pre-existing epithelial damage and loss of at least some degree of polarity in order to cause disease, these in vitro epithelial cell culture systems recapitulate important aspects of human infections and serve...
as a useful model to further dissect mechanisms of disease. Using comprehensive and multi-
faceted approaches, we demonstrated that complex N-glycan chains are necessary and sufficient
to mediate *P. aeruginosa* binding at the AP surface, whereas the HS moieties of HSPGs mediate
binding at the BL surface of the polarized epithelium. During epithelial injury and
dedifferentiation, HSPG presentation at the AP surface is increased, explaining at least in part the
predilection of this important pathogen for such injured tissues. Changes in the composition of
N-glycan chains and/or in the polarized segregation of HSPGs could contribute to the
pathogenesis of acute and chronic diseases.

We first focused on N-glycosylation, as increased or altered expression of N-glycans could result
in an enhanced susceptibility to *P. aeruginosa* infections in the setting of acute or chronic injury.
Using chemical and enzymatic inhibitors, we showed that N-glycans are important contributors
to *P. aeruginosa* binding, entry, and damage at the AP surface of airway and kidney cells grown
at varying states of polarity as confluent 2D monolayers. ConA \(^\text{r}\) MDCK cells, which are
defective in N-glycosylation, were particularly informative in identifying the role of N-glycans
in *P. aeruginosa* binding and subsequent internalization and host injury. Although the specific
defect in these cells is not known, the fact that N-glycosylation could be restored by growing
these cells in the presence of excess Man or Glc suggested that they are defective in the activity
of PMM and PMI enzymes (46). When *P. aeruginosa* was added to the AP surface of ConA \(^\text{r}\)
MDCK cells, decreased binding, entry, and cytotoxicity was observed compared to wt cells,
whereas no difference was observed at the BL surface. While it has previously been suggested
that ConA \(^\text{r}\) MDCK cells form more highly polarized monolayers, which leads to resistance to *P.
aeruginosa* infection (15), we found no evidence for altered polarity in these cells under our
experimental conditions. Importantly, when ConA \(^\text{r}\) MDCK cells were grown in the presence of
Man or Glc P. aeruginosa binding, entry, and cytotoxicity was restored to wild type levels at the AP surface. We could also enhance the binding and subsequent damage specifically at the AP surface of wt MDCK and airway epithelial cells grown in the presence of excess Man or Glc. We demonstrated increased co-localization of the bacteria with more complex N-glycan patches on the AP membrane of highly organized 3D cysts grown in the presence of Man to upregulate N-glycosylation. Finally, P. aeruginosa preferentially bound in vitro to a mixture of complex N-glycans over an individual sugar. Together, these results suggest that N-glycan chains of one or more N-glycosylated proteins at the AP surface serve as important receptors for AP binding of P. aeruginosa. The N-glycosylated molecule is unlikely to be the previously identified receptors CD95 (Fas receptor), integrin or fibronectin, as these molecules are preferentially expressed at the BL surface (50). Likewise, it is unlikely to be CFTR, since similar results were obtained with Calu-3 cells, which express high levels of CFTR at the AP surface, and wt MDCK, which express very little CFTR (18). Future studies will be aimed at identifying this important molecule. In summary, N-glycans and particular enzymes in N-glycosylation pathway could be potential targets for novel therapeutic approaches in treatment of P. aeruginosa infections. Most importantly, simple sugars could be used to either modify the course of the disease or competitively inhibit bacterial binding and subsequent infection, a line of treatment currently being investigated.

While these results identified N-glycan chains as AP receptors, they did not reveal the identity of the BL receptor or the receptor that is upregulated in incompletely-polarized cells. We therefore examined whether HSPGs, which are abundantly expressed on the BL surface (3), can serve as specific BL receptors for P. aeruginosa. We demonstrated that HSPGs are upregulated in...
incompletely-polarized cells and lose their polarized distribution. Competitive inhibition, enzymatic removal or desulfation of HS chains decreased *P. aeruginosa* binding, entry, and cytotoxicity at the BL surface of well-polarized cells and at both surfaces in incompletely-polarized cells. We corroborated these results by showing that *P. aeruginosa* binds to HS *in vitro* with the highest affinity among all tested compounds and that it preferentially binds to HS-rich patches on the BL surface of 3D cysts.

If N-glycans can function as binding receptors and are found on both the AP and the BL surface, why do they not contribute to *P. aeruginosa* binding to the BL surface? First, the identity of the protein core that is N-glycosylated may also contribute to binding specificity and the BL N-glycoproteins may not function as binding receptors or they may not be readily accessible. In addition, or alternatively, the binding affinity to HSPGs may be higher than to N-glycan chains (as suggested by our *in vitro* data). While our results demonstrate that AP N-glycans can function as receptors, in normal hosts with well-polarized intact mucosal barriers, *P. aeruginosa* does not cause disease. This observation likely reflects the importance of local defense mechanisms, such as a functional innate immune system. However, upregulation of AP HSPGs, particularly in the context of altered local innate immune responses, may offer an explanation for the increased binding and subsequent damage seen when *P. aeruginosa* infects incompletely-polarized but intact monolayers, such as might be seen during regenerating epithelium. In more severe injury, when the monolayer is disrupted, increased access to HSPGs on the BL surface may also contribute the enhanced susceptibility to infection. Interestingly, it has been shown that inhibition of shedding of the HSPG syndecan-1 or degradation of the shed HS chains attenuates mouse lung infection (40). These results suggest an additional possible role for secreted HSPGs.
in mediating microbial virulence. They also indirectly suggest that *P. aeruginosa* binds to HS chains on syndecan-1, thus, supporting work presented here.

Flagella and type IV pili are the predominant *P. aeruginosa* adhesins. Studies are underway to determine their binding specificity towards N-glycans and HSPGs and whether they play different roles in adhesion at the AP versus BL surface. Previous studies have suggested that glycosphingolipids may serve as AP receptors for type IV pili, (10, 47), although these findings remain controversial (13). Flagellar components have been shown to bind to Lewis x derivatives that are found on secreted mucins (48). Moreover, Toll-like receptor 5, predominantly found on the AP surface, has been found to bind flagellin (24). Finally, there can be flagella- and pili-independent bacterial binding to the cell surface since two different lectins have been described in *P. aeruginosa*, which bind specifically to either Fuc or Gal, sugars present in both N- and O-glycan chains (8, 22).

There is a great deal of potential to use heparin or heparin-like structures as drugs to treat a wide range of disorders, including respiratory diseases (12, 30). Although heparin has been in use as an anti-coagulant for over 70 years, heparan sulfate-like structures are attracting considerable interest as a source of new therapeutics since it has been proposed that the main purpose of heparin is in a defensive mechanism at sites of tissue injury against invading bacteria and other foreign materials (36). HSPGs are attractive as therapeutic targets since a wide range of viral and bacterial pathogens are known to bind to HS chains (2, 14, 21, 25), and our work further shows that the sulfate moieties of HSPGs function as *P. aeruginosa* binding receptors. Drugs that
disrupt HSPG-pathogen interactions may be an effective strategy for preventing or treating acute
P. aeruginosa infections, particularly as inhaled therapy for pneumonia.

An important recent advance in understanding epithelial cell biology is the ability to grow
epithelial cells as 3D cysts in a collagen/matrigel matrix, which recapitulates the development
and structural organization of tubular structures such as the lung alveolus. (5). While there are a
few reports of studying the interactions of pathogens with epithelial cells grown as aggregates,
(7, 38), to the best of our knowledge, our study is the first that examines the interaction of
bacteria with epithelial cells grown as organized 3D cysts. This approach required that we
overcome several technical challenges. First, the cysts had to be briefly treated with collagenase
in order to expose their surface. Second, bacterial binding had to be quantified by direct confocal
microscopy, as the bacteria also bind avidly to the collagen/matrigel matrix. Third, during the 7
days it takes for mature cysts to form, the cells become highly polarized, and bacterial binding is
limited. In addition, N-glycans and HSPGs are expressed at high levels in a highly polarized
manner, with uniform distribution on the AP and BL sides, respectively. Therefore, we either
exposed the BL surface of BL-side out cysts to heparinase III (to correlate binding to HS-rich
domains) or utilized ConA MDCK cells (to correlate binding to Man-rich domains). Altogether,
cysts provide a promising avenue for further study of host-pathogen interactions in vitro, in a
biologically relevant context, particularly as they relate to microbes that infect tubular or lumenal
structures. Future directions will include observing these events by time-lapse confocal
microscopy to delineate the temporal and spatial events that occur during bacterial attachment,
internalization and host damage.
While our work examined epithelial cells are various states of polarization as a model for acute
or chronic epithelial injury, upregulation of N-glycosylation and/or altered expression or
localization of HSPGs may be specifically relevant for the chronic *P. aeruginosa* infections that
result in end stage lung disease in patients with CF. CFTR itself is highly glycosylated protein,
and there is published data that suggests that the sugar composition of membrane glycoproteins
and secreted mucins is altered in CF, though the exact mechanism remains unknown (45). We
are currently testing whether N-glycosylation or HSPGs are upregulated in lung tissues from
patients with CF.

Another possible way in which upregulation of HSPG expression could affect both chronic and
acute *P. aeruginosa* infections is through enhancing the binding and dimerization of growth
factors and their receptors as well as by stabilizing these receptors. Of particular relevance are
the epidermal growth factor (EGF) and its receptor, EGFR. EGFR activation is important in
wound repair. In addition, EGFR has been shown to be hyperphosphorylated in CF (6). HSPGs
also bind and stabilize the activity of IL-8, which may play a role in IL-8-driven
hyperinflammatory responses in CF (16, 19). Thus, upregulation of HSPGs in acute and chronic
epithelial injury may amplify *P. aeruginosa*-induced changes in growth factor signalling. In
preliminary studies, we have found that *P. aeruginosa* activates EGFR in a HS-dependent
manner.

The studies presented here on the pathogenesis of *P. aeruginosa* infections provide key insights
into how changes in the presentation of AP and BL molecules contribute to acute and chronic *P.
aeruginosa* infections. The use of cultured epithelial cells grown as 3D cysts provides new and
powerful in vitro tools that have the potential to offer better insights into understanding complex mechanism(s) involved in the establishment of the infection and subsequent organ damage. Our data shows an important role for HS chains and N-glycans in mediating microbial adherence, entry and cytotoxicity, and provides a basis for designing therapeutic strategies based on these interactions. The use of combined therapeutic strategies that target N-glycosylation, HSPG synthesis, or polarized segregation of these molecules may provide powerful new therapeutic approaches to correct the abnormal epithelial architecture that occurs in the setting of chronic lung disease, including CF, in order to improve clinical outcome. In summary, our work lays fertile ground for better understanding the connection between specific physiological alterations in various acute and chronic disorders and the predilection to infections by P. aeruginosa.

Acknowledgments

This work was supported by the Elizabeth Nash Memorial Fellowship of the Cystic Fibrosis Research Inc (IB) and the National Institutes of Health (P01 AI053194 (JE and KM); R01 AI065902 (JE); and R01 DK067153 (KM)). We thank Armando Lemus for his kind gift of pnpT2-GFP-pUCP20. We thank members of our laboratories for experimental suggestions and for advice and comments on the paper.

References


**Figure Legends**

**Figure 1.** *P. aeruginosa* adhesion to the AP surface is N-glycan-dependent. A-B; Addition of Man or Glc, but not Gal, to the growth medium increases levels of Man on the cell surface. MDCK, ConA⁺ and Calu-3 cells were cultured in the presence or absence of the indicated sugar. Shown are well-polarized monolayers grown on Transwells that were fixed without permeabilization and stained from either the AP or BL surface with FITC-ConA, which binds to Man in N-glycan chains. ConA⁺ cells have less Man on the AP and BL surface when compared to wt MDCK cells, but addition of Man or Glc, restores FITC-ConA binding to wt levels. Addition of Man or Glc enhances FITC-ConA binding to MDCK and Calu-3 cells at both the AP and BL surface. A; 3D reconstruction of Z stack images acquired by confocal microscopy. Shown are an AP and a BL projection of well-polarized monolayers. Man is stained with FITC-
ConA (green) and actin is stained with phalloidin (red). B; FITC-ConA fluorescence (in arbitrary units) of well-polarized cells treated with indicated sugars measured in a fluorescent plate reader. Shown are the mean +/- SD for 3 separate experiments. * P < 0.05 compared to AP untreated MDCK cells. * P < 0.05 compared to AP untreated ConA cells. * P < 0.05 compared to AP untreated Calu-3 cells. ** P < 0.05 compared to BL untreated MDCK cells. *** P < 0.05 compared to BL untreated ConA cells. ++ P < 0.05 compared to BL untreated Calu-3 cells. C-D; Addition of Man or Glc is sufficient to enhance *P. aeruginosa* binding. Cells were grown in the presence or absence of the indicated sugar, bacteria were added for 1 h to the AP or BL surface of well-polarized cells grown on Transwells, and standard adhesion assays were performed. Shown are the mean +/- SD for 3 separate experiments. C; *P. aeruginosa* binding to well-polarized MDCK and ConA cells, normalized to AP infected untreated MDCK cells. D; *P. aeruginosa* binding to well-polarized Calu-3 cells, normalized to AP infected untreated Calu-3 cells. E-F; Tunicamycin inhibits bacterial binding to the AP surface of wt MDCK and Calu-3 cells in a dose-dependent manner. Cells were pre-treated with the indicated amounts of tunicamycin to inhibit N-glycosylation. Bacterial binding was measured as described above. E; *P. aeruginosa* binding to well-polarized MDCK and ConA cells, normalized to AP infected untreated MDCK cells (point “0”). F; *P. aeruginosa* binding to well-polarized Calu-3 cells, normalized to AP infected untreated Calu-3 cells (point “0”). * P < 0.05 compared to AP infected untreated MDCK cells. * P < 0.05 compared to AP infected untreated ConA cells. * P < 0.05 compared to AP infected untreated Calu-3 cells.

**Figure 2. HSPGs are expressed on the AP surface in incompletely-polarized cells.** In well-polarized monolayers grown on Transwells, there is a greater amount of HSPGs on the BL surface.
surface compared to the AP surface. In incompletely-polarized monolayers, there is increased
expression of HSPGs on the AP surface. A; 3D reconstruction of Z stack images acquired by
confocal microscopy. Shown are an AP and a BL projection of well- and incompletely-polarized
Calu-3 cells stained with an antibody against HS chain of HSPGs (FITC-HS; purple), phalloidin
(red) to visualize actin, and ZO-1 (green) to visualize tight junctions. B-C; FITC-HS
fluorescence (in arbitrary units) measured in a fluorescent plate reader. B; Well-polarized or C;
incompletely-polarized MDCK, ConA', and Calu-3 cells stained with FITC-HS added to the AP
or BL surface. Shown are the mean +/- SD for 3 separate experiments. * P < 0.05 compared to
AP stained MDCK cells. * P < 0.05 compared to AP stained ConA' cells. + P < 0.05 compared
to AP stained Calu-3 cells.

**Figure 3. HS chains mediate *P. aeruginosa* adhesion to the BL surface and they contribute
to binding to the AP surface in incompletely-polarized cells.** Competitive inhibition with
heparin (A-D) or enzymatic removal of HS chains with heparinase III (E-H) inhibits *P.
aeruginosa* binding to the BL surface of well- and incompletely-polarized cells and to the AP
surface of incompletely-polarized cells. Cells were pre-treated with the indicated amount of
heparin or heparinase III, bacteria were added for 1 h and standard adhesion assays were
performed. Shown are the mean +/- SD for 3 separate experiments. A; *P. aeruginosa* binding to
well-polarized and B; incompletely-polarized MDCK and ConA' cells pre-treated with
increasing amounts of heparin, normalized to AP infected untreated MDCK cells. C; *P.
aeruginosa* binding to well-polarized and D; incompletely-polarized Calu-3 pre-treated with
increasing amounts of heparin, normalized to AP infected untreated Calu-3 cells. E; *P.
aeruginosa* binding to well-polarized and F; incompletely-polarized MDCK and ConA' cells pre-
treated with increasing doses of heparinase III, normalized to AP infected untreated MDCK cells.  

**Figure 4.** *P. aeruginosa* binding to HS is dependent on the sulfation of HS chains. Chemical inhibition of sulfation inhibits *P. aeruginosa* binding to the BL surface of well- and incompletely-polarized cells and to the AP surface of incompletely-polarized cells. Cells were pre-treated with sodium chlorate for desulfation (desulf) of HS chains, and sulfation was restored (resulf) by treatment with sodium sulfate. Bacteria were added for 1 h and standard adhesion assays were performed. Shown are the mean +/- SD for 3 separate experiments.  

A; *P. aeruginosa* binding to well-polarized and B; incompletely-polarized MDCK and ConA\(^{+}\) cells, normalized to AP infected untreated MDCK cells.  

C; *P. aeruginosa* binding to well-polarized and D; incompletely-polarized Calu-3 cells, normalized to AP infected untreated Calu-3 cells. 

+ P < 0.05 compared to AP infected untreated MDCK cells.  

\(^{+}\) P < 0.05 compared to AP infected untreated ConA\(^{+}\) cells.  

++ P < 0.05 compared to BL infected untreated MDCK cells.  

\(\times\) P < 0.05 compared to BL infected untreated ConA\(^{+}\) cells.  

++ P < 0.05 compared to BL infected untreated Calu-3 cells.
Figure 5. *P. aeruginosa* directly binds *in vitro* to isolated HS and N-glycans chains in a dose-dependent manner. 96-well plastic plates were coated overnight with increasing concentrations of the indicated molecules. GFP-conjugated bacteria were added for 1 h and fluorescence was quantified in a fluorescent plate reader. Control bacterial binding to non-coated wells was set as a background and the percent of binding above control is indicated. Shown are the mean +/- SD for 4 separate experiments HS: heparan sulfate; CS-4: 4-O-sulfated chondroitin sulfate; CS-6: 6-O-sulfated chondroitin sulfate; HA: hyaluronic acid; GlcN: N-acetylglucosamine.

Figure 6. Bacterial cytotoxicity is mediated upon *P. aeruginosa* binding to N-glycans on the AP surface, to HSPGs on the BL surface of polarized cells, and to both molecules on the AP surface of incompletely-polarized cells. Cells were pre-treated with mannose (man) to upregulate N-glycosylation, with tunicamycin (32) to inhibit N-glycosylation, with heparinase III (hepIII) to remove HS chains, desulfated with sodium chlorate (desulf), or resulfated with sodium sulfate (resulf). Host cell treatments are color-coded: modifications of HSPGs are shades of red and modifications of N-glycans are shades of blue. Bacteria were added for 5 h and standard LDH release assays were performed. Shown are the mean +/- SD for 4 separate experiments. A; Percentage of LDH release in well-polarized and B; incompletely-polarized MDCK and ConA<sup>r</sup> cells, normalized to AP infected untreated MDCK cells. C; Percentage of LDH release in well-polarized and D; incompletely-polarized Calu-3 cells, normalized to AP infected untreated Calu-3 cells. * P < 0.05 compared to AP infected untreated MDCK cells. † P < 0.05 compared to AP infected untreated ConA<sup>r</sup> cells. ‡ P < 0.05 compared to AP infected untreated Calu-3 cells. ** P < 0.05 compared to BL infected untreated MDCK cells. *** P < 0.05
compared to BL infected untreated ConA' cells. ↑↑ P < 0.05 compared to BL infected untreated Calu-3 cells.

Figure 7. *P. aeruginosa* internalization is dependent on bacterial binding to N-glycans on the AP surface, HSPGs on the BL surface of well-polarized cells, and to both molecules at the AP surface of incompletely-polarized cells. Cells were pre-treated with the indicated sugars to upregulate N-glycosylation (A, B), with the indicated concentrations of tunicamycin to inhibit N-glycosylation (C, D), or with the indicated amount of heparinase III to remove HS chains (E, G). Bacteria were added for 1 h and standard invasion assays were performed. Shown are the mean +/- SD for 3 separate experiments. A-B; Man and Glc are sufficient to enhance bacterial invasion from the AP surface of MDCK, ConA', and Calu-3 cells. A; *P. aeruginosa* internalization to well-polarized MDCK and ConA' cells, normalized to AP infected untreated MDCK cells. B; *P. aeruginosa* internalization to well-polarized Calu-3 cells, normalized to AP infected untreated cells. C-D; Tunicamycin inhibits invasion in a dose-dependent manner from the AP surface of well-polarized MDCK and Calu-3 cells. C; *P. aeruginosa* internalization to well-polarized and incompletely-polarized MDCK and ConA' cells, normalized to AP infected untreated MDCK cells. D; *P. aeruginosa* internalization to well-polarized Calu-3 cells, normalized to AP infected untreated cells. E-H; Heparinase III treatment inhibits invasion from the BL surface of well- and incompletely-polarized MDCK, ConA', and Calu-3 cells and from the AP surface of incompletely-polarized cells. E; *P. aeruginosa* binding to well-polarized and F; incompletely-polarized MDCK and ConA' cells, normalized to AP infected untreated MDCK cells. G; *P. aeruginosa* binding to well-polarized and H; incompletely-polarized Calu-3 cells, normalized to AP infected untreated cells. * P < 0.05 compared to AP infected untreated MDCK cells. x P <
0.05 compared to AP infected untreated ConA\textsuperscript{r} cells. \textsuperscript{+} P < 0.05 compared to AP infected untreated Calu-3 cells. \textsuperscript{**} P < 0.05 compared to BL infected untreated MDCK cells. \textsuperscript{***} P < 0.05 compared to BL infected untreated ConA\textsuperscript{r} cells. \textsuperscript{++} P < 0.05 compared to BL infected untreated Calu-3 cells.

** Figure 8. *P. aeruginosa* co-localizes with HS-rich patches on the exposed BL membrane and with N-glycan-rich patches on the exposed AP membrane of highly polarized 3D cysts. **

A; B; Representative X-Y confocal sections of BL-side out or AP-side out MDCK cysts cultured for 5 days stained with an anti-HS antibody (HS; purple) or with ConA (Man; purple), and with phalloidin (Actin; red). A; BL-side out (left panel) or AP-side out (right panel) MDCK cysts show preferential localization of HSPGs to the BL surface of the cyst. B; BL-side out (left panel) or AP-side out (right panel) MDCK cysts show localization of Man to both the AP and BL surface of the cyst. C-F; 3D reconstructions of Z stack images acquired by confocal microscopy. HS is stained with an anti-HS antibody (HS; purple), Man is stained with ConA (Man; purple), actin is stained with phalloidin (Actin; red). C; BL-side out MDCK cysts show uniform staining of HS at the exposed BL membrane. D; MDCK cysts treated with heparinase III and infected with GFP-PAK for 2 h (green) show non-uniform patchy HS staining, and the bacteria bind preferentially to HS-rich areas (purple). E; BL-side out wt MDCK cysts show uniform staining of Man at the exposed BL membrane. F; BL-side out ConA\textsuperscript{r} MDCK cysts briefly treated with Man (to induce expression of more complex N-glycans) and infected with GFP-PAK for 2 h (green) show non-uniform patchy Man staining, and the bacteria do not bind preferentially to Man-rich areas (purple). G; Quantification of GFP-PAK bound to HS-rich versus HS-poor patches in BL-side out MDCK cysts pre-treated with heparinase III. H; Quantification of
bacteria bound to Man-rich versus Man-poor patches in BL-side out ConA^r cysts briefly treated with Man. I-J; 3D reconstructions of Z stack images of ConA^r MDCK cysts acquired by confocal microscopy. Man is stained with ConA (purple) and actin is stained with phalloidin (red). I; AP-side out ConA^r cysts infected with PAK-GFP show non-uniform patchy staining of Man at the exposed AP membrane, and the bacteria do not bind preferentially to Man-rich areas. J; ConA^r cysts briefly treated with Man and infected with GFP-PAK (green) show non-uniform patchy Man staining, and the bacteria bind preferentially to Man-rich areas (purple). K; Quantification of GFP-PAK bound to Man-rich versus Man-poor patches in AP-side out ConA^r cysts. L; Quantification of GFP-PAK bound to Man-rich versus Man-poor patches in AP-side out ConA^r cysts briefly treated with Man. * P < 0.01.
Figure 1

A

wt MDCK  AP  BL
ConA
ConA+man
ConA+glc
ConA+gal
Calu-3
Calu-3+man
Calu-3+glc
Calu-3+gal

Man/Actin

B

FITC-ConA (fluorescence)

un  man  glc  gal

MDCK  ConA  Calu3
AP  BL

C

adhesion (% of control)

un  man  glc  gal

MDCK  ConA  MDCK  ConA
AP  BL

D

adhesion (% of control)

un  man  glc  gal

Calu-3  Calu-3
AP  BL

E

adhesion (% of control)

MDCK  ConA

0  0.1  0.5  1

0  0.1  0.5  1

0  0.1  0.5  1

0  0.1  0.5  1

F

adhesion (% of control)

Calu-3

0  0.1  0.5  1

0  0.1  0.5  1

0  0.1  0.5  1

0  0.1  0.5  1
Figure 2

A

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</tr>
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</table>

B

![Bar chart](image7.png)

C

![Bar chart](image8.png)
Figure 3

A

well-polarized

• MDCK  □ ConA

adhesion (% of control)

0  0.1  1  5  10  0  0.1  1  5  10

heparin (µg/ml)

AP  BL

B

incompletely-polarized

• MDCK  □ ConA

adhesion (% of control)

0  0.1  1  5  10  0  0.1  1  5  10

heparin (µg/ml)

AP  BL

C

well-polarized

• Calu-3

adhesion (% of control)

0  0.1  1  5  10  0  0.1  1  5  10

heparinase III (mU)

AP  BL

D

incompletely-polarized

• Calu-3

adhesion (% of control)

0  0.1  1  5  10  0  0.1  1  5  10

heparinase III (mU)

AP  BL

E

well-polarized

• MDCK  □ ConA

adhesion (% of control)

0  1  10  100  200  0  1  10  100  200

heparinase III (mU)

AP  BL

F

incompletely-polarized

• MDCK  □ ConA

adhesion (% of control)

0  1  10  100  200  0  1  10  100  200

heparinase III (mU)

AP  BL

G

well-polarized

• Calu-3

adhesion (% of control)

0  1  10  100  200  0  1  10  100  200

heparinase III (mU)

AP  BL

H

incompletely-polarized

• Calu-3

adhesion (% of control)

0  1  10  100  200  0  1  10  100  200

heparinase III (mU)

AP  BL
Figure 4

A

well-polarized

un □ desulf □ resulf

adhesion (% of control)

MDCK ConA

AP BL

B

incompletely-polarized

un □ desulf □ resulf

adhesion (% of control)

MDCK ConA

AP BL

C

well-polarized

un □ desulf □ resulf

adhesion (% of control)

Calu-3

AP BL

D

incompletely-polarized

un □ desulf □ resulf

adhesion (% of control)

Calu-3

AP BL
Figure 5

[Graph showing the percentage of bound glycans (above control) as a function of glycan concentration (μg/well) for different glycans: HS, CS-4, CS-6, N-glycan, and GlcN. The graph includes error bars for each data point.]
Figure 6

A) well-polarized

B) incompletely-polarized

C) well-polarized

D) incompletely-polarized

Legend:

- uninfected
- infected
- man
- tun
- heplI
- desulf
- resulf
Figure 8

A. BL-out, AP-out
   HS/Actin

B. BL-out, AP-out
   Man/Actin

C. BL-out
   HS/Actin
   PAK/HS/Actin

D. BL-out
   Man/Actin
   PAK/Man/Actin

G. HepIII-treated BL-out MDCK cysts
   bacterial aggregates / surface area (% of total)
   HS-rich, HS-poor

H. HepIII-treated BL-out MDCK cysts
   bacterial aggregates / surface area (% of total)
   Man-rich, Man-poor

I. AP-out
   PAK/Man/Actin
   PAK/Man/Actin

K. AP-out ConA cysts
   bacterial aggregates / surface area (% of total)
   Man-rich, Man-poor

L. Man-treated AP-out ConA cysts
   bacterial aggregates / surface area (% of total)
   Man-rich, Man-poor