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Sulfatide mediates attachment of *Pseudomonas aeruginosa* to human pharyngeal epithelial cells

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SUMMARY

Pseudomonas aeruginosa infections are particularly common in people with cystic fibrosis and despite regular treatment with antibiotics, lung damage due to chronic infection with *P. aeruginosa* remains the major cause of death in those patients. In order to initiate an infection, *P. aeruginosa* needs contact with the respiratory epithelial surface and by means of its adhesins i.e., fimbria, hemagglutinins,etc., it recognizes and adheres to the corresponding epithelial receptors. We treated *P. aeruginosa* strains isolated from sputum of cystic fibrosis patients with several glycolipids such as sulfatide, sulfated ganglioside mixture (GM1a, GD1b, GT1b), asialo-GM_1 and galactocerebrosides to determine their effect on attachment with pharyngeal epithelial cells. Sulfated ganglioside mixture and sulfatide inhibited the attachment of *P. aeruginosa* significantly, whereas asialo-GM_1, Gal-Cer and sodium sulfite had no effect on attachment inhibition. This finding suggests that sulfated glycoconjugates found in the extracellular matrix, in mucus and on the surface of epithelial cells of human trachea and lung mediates attachment of *P. aeruginosa.*

KEY WORDS: Pseudomonas aeruginosa, attachment, epithelial cells, sulfatide, asialo-GM_1

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Pseudomonas aeruginosa is an opportunistic pathogen that is unable to cause disease in healthy immunocompetent people, but can infect people whose specific or nonspecific defenses have been impaired. *P. aeruginosa* infections are particularly common in people with cystic fibrosis (CF), a genetic disease associated with a defect in chloride secretion, which is characterized by production of mucin that has an altered ionic composition and is unusually thick. CF is due to mutations of CF gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFRT), a chloride channel localized to the apical surface of epithelial cell membranes. CF subjects show reduced mucociliary clearance, perhaps related to the primary defect in CFTR, this in turn predisposes to infection. Despite regular treatment with antibiotics, lung damage due to chronic infection with *P. aeruginosa* remains the major cause of death in CF patients (Davis *et al.*, 1996).

The pathogenicity of respiratory infection commences with the colonization of pharyngeal epithelial cells after successful attachment of bacteria. For *P. aeruginosa,* numerous studies have implicated flagella and pili as bacterial adhesins binding specifically to terminal or internal *N*-acetylgalactosamine residues that are linked to β1-4 galactose (Gal) residues unsubstituted with sialyl residues (Krivan *et al.*,1988). Such structures are found in the asialo-GM_1 receptor on host cells and the number of asialo-GM_1 recep-

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tors is reported to be increased on respiratory epithelial cells from patients with CF (Saiman *et al.*, 1993). Reports suggesting an involvement of asialo-GM_1 as a receptor for *P. aeruginosa* revealed contradictory results (Saiman *et al.*, 1993, Schroeder *et al.*, 2001). Since sulfatides are found in the extracellular matrix, in mucus and on the surface of epithelial cells of human trachea and lung, and previously shown as receptors for respiratory pathogens like *Bordetella pertussis*, and *Haemophilus influenzae*, therefore it is reasonable to assume that as a respiratory pathogen *P.aeruginosa* might utilize sulfatide as a molecule to attach with host cells (Hannah *et al.*, 1994, Hartman *et al.*, 2001). Therefore, this study was done to establish the effects of sulfatide and sulfated ganglioside mixture on the attachment of *P. aeruginosa* to human nasopharyngeal epithelial cells.

Strains of *P. aeruginosa* 511, 544, 361, 551, 532, 83, isolated from sputum of cystic fibrosis patients, were used in the study. Strain 361 was mainly used for attachment inhibition assay and antibody production. Isolates were preserved on beads in a cryopreservative (Protect STC, UK) at -45°C, and for attachment inhibition assay, bacteria were grown on Brain Heart Infusion agar (Merck KgaA, Darmstadt, Germany) for 18 h at 37°C. The glycoconjugates used in this study were natural sulfated gangliosides mixture, sulfatides (3-SO_3-GalCer) and galactosylceramide (GalCer). The sulfated gangliosides mixture was prepared from gangliosides purified from bovine brains and sulfated derivatives were prepared by successive treatment with sulfurtrioxide trimethylamine complex in dimethylformamide at 50°C for 20 h and with trifluoroacetic acid in dichloromethane followed by gel filtration through Sephadex LH-20 (Hanna *et al.*, 1991). The sulfated gangliosides mixture used in this study contains sulfated form of GM1a, GD1a, GD1b and GT1b. The structures of the per-*O*-sulfated gangliosides were supported by the positive ion fast bombardment (FAB) mass spectrometry (JMS-SX102, JEOL, Tokyo, Japan). Sulfatide was isolated from fresh bovine brain as described elsewhere (Suziki *et al.*, 1993). Galactosylceramide is a desulfated form of sulfatide. Sodium sulfite (Na_2SO_3) were purchased from Sigma Chemical (St, Louis, MO). Pharyngeal epithelial cells were collected from a consented, healthy adult female subject by scraping the oropharynx with a cotton swab. Cells from the swab were collected in 1/15 mM phosphate buffer solution (PBS), pH 7.2, and washed three times by centrifugation at 80Xg, each time for 10 min at room temperature. Finally, oropharyngeal cells were adjusted to a concentration of $2.5x10^4$ cells/ml. The attachment inhibition assay was done as described before with modification (Ahmed *et al.*,1996). *P. aeruginosa* strain 361 organisms, at a concentration of $1x10^8$ cfu/ml, were treated with different concentrations of sulfatide, sulfated ganglioside mixture, asialoGM_1, Gal-Cer or sodium sulfite for 30 min at 37°C. Bacteria were then mixed with the cells at equal ratios and incubated at 37°C for 30 min. Unattached bacteria were separated by a total of five washings in phosphate buffer solution (PBS) using centrifugation at 80X g for 10 min at room temperature. Finally, the cells were collected on a glass by cytospin (Shandon, UK). Smears were gram stained and viewed under an oil- immersion lens of a light microscope to count the number of attached bacteria on 50 successive cells. Polyclonal antibody production against strain 361 was accomplished as described previously by Luo *et al.* (Luo *et al.*, 1997). Briefly, 8-10 weeks old BALB/c mice were primed intraperitoneally by incomplete Freund's adjuvant. 7-10 days later, 10^8 heat-killed bacteria were injected intraperitoneally. Mice were administered bacteria two more times at three-week intervals. After the last injection, blood was collected from the tail vein, and serum antibody titer was determined by slide agglutination. Then, 1-3 million SP2/O mouse myeloma cells were injected intraperitoneally to induce ascites production. Ascitic fluid was collected 7-10 days later, and mouse polyclonal antibody titer was reevaluated by slide agglutination. TLC was performed according to previously described methods (Stromberg *et al.*, 1990). Briefly, sulfatide, Asialo GM_1, and GalCer were separated on a thin layer plate (Polygram, Sil G, Macherey- Nagel) with a solvent system of chloroform: methanol: water (65: 35: 7, by volume). The plate was dried and then blocked with 1% bovine serum albumin (BSA; Sigma) in phosphate buffer solution (PBS) by shaking at room temperature for 2 h. After 5 washes with PBS, the plate was incubated overnight at 4°C with bacterial suspension

($1x10^8$ cfu/ml). All the strains given above were tested separately. After 5 washes with PBS, the plate was incubated for 2 h at.
4°C with antibody against *P. aeruginosa* diluted in 0.1% BSA-PBS. After 5 washes with PBS, the plate was treated with horseradish peroxidase - conjugated secondary antibody (Anti- mouse IgG peroxidase conjugate, Sigma) diluted 0.1% BSA-PBS (1:100) for 2 h at 4°C. The plate was then washed five times with PBS and incubated with peroxidase substrate solution. The development of color reaction was observed by examination with the naked eye. All data were expressed as mean ± SD. Kruskal-Walls one - way ANOVA was used for comparisons. Mann-Whitney U test was used as post-hoc. Differences were considered as significant with a *P* value <0.05
Effects of glycoconjugates on the attachment of *P. aeruginosa,* strain 361 is shown in the table 1. At a very low concentration, sulfated ganglioside mixture inhibited the attachment of *P. aeruginosa* significantly, in a dose dependent manner. Sulfatide inhibited the attachment of *P. aeruginosa* in 10 μg/ml concentration only. Asialo- GM_1, GalCer and sodium sulfite had no effect on attachment inhibition. Reactivity was observed in any of the six clinical strains with sulfatide, asialo GM_1, and GalCer on TLC plates.
The initial step of bacterial infection, preceding chronic colonization is the adherence of bacteria to epithelial cells. In normal airways, a thin layer of mucus covers surface epithelium and bacteria entrapped in the gel phase of mucus are then rapidly cleared out from the airways. In order to initiate an infection, *P. aeruginosa* should be in contact with respiratory epithelial surface and by way of its adhesins i.e., fimbriae, hemagglutinins, etc., should recognize and adhere to the corresponding epithelial receptors (Doig *et al.*,1998, Glick *et al.*, 1987). Understanding the cellular events that allow the adherence of *P. aeruginosa* to respiratory cells is fundamental to eradicate this pathogen, which may become very resistant to several antibiotics. Among the population at high risk of developing *P. aeruginosa* respiratory infections, patients admitted to intensive care units with respiratory assistance and cystic fibrosis patients represent the most exposed population. Bacterial surface lectins, which are typically located on fimbriae, have been shown to be critical for the initiation of

TABLE 1 - *Effects of different glycoconjugates on the attachment of* P. aeruginosa.

Substances	*Concentration (μg/ml)*	*Attachment*	*Control*	*p value*
Sulfated ganglioside mixture	10	2.0 + 0.9		<0.05
	1.0	5.0 + 0.6		<0.05
	0.1	7.6 + 1.6		<0.05
	0		10.3 ± 0.1	
Sulfatide	10	7.8 ± 2.3		<0.05
	1.0	9.6 ± 3.1		NS
	0.1	10.3 ± 2.8		NS
	0		13.0 ± 2.6	
Asialo GM1	10	13.8 ± 2.3		NS
	1.0	13.0 ± 0.8		NS
	0.1	13.4 ± 1.1.		NS
	0		13.0 ± 1.1	
GalCer	10	9.8 ± 2.8		NS
	0		10.4 ± 2.8	
Sodium sulfite	10	10.4 ± 1.8		NS
	0		10.4 ± 2.8	

Attachment is expressed as the number of bacteria (mean±SD) attached per pharyngeal epithelial cell. Each experiment was performed five times. NS, not significant.

infection by mediating bacterial adherence to epithelial cells through lectin- carbohydrate interactions (Sharon *et al.*, 1987).

In order to understand attachment and pathogenesis and to characterize receptors, different methods including thin layer chromatography have been used successfully (Ahmed *et al.*, 2000, Gilbert *et al.*, 1993, Krivan *et al.*, 1988,Stromberg *et al.*, 1990) . Asialo - GM_1 was shown as a major receptor for attachment of *P. aeruginosa* to the cells and the number of asialo-GM_1 receptors was also reported to be increased on respiratory epithelial cells from patients with cystic fibrosis (Comolli *et al.*, 1999,Gupta *et al.*, 1994,Saiman *et al.*, 1993.) However, asialo-GM_1 had no effect on attachment inhibition and no reactivity was observed on TLC plates in our study. Previous studies implicating asialo-GM_1 as cellular adhesin for *P. aeruginosa* have been conducted with laboratory strains PAO1, PA103, ATCC 19660 and cell lines like MDCK, nasal polyp cells, etc. Shroeder *et al.* (Shroder *et al.*, 2001) used PAO1 and eight clinical strains isolated from different materials and demonstrated that asialo-GM_1 was a receptor for laboratory strain but not for fresh clinical isolates.

On the other hand, in an *in vitro* model of wound repair of respiratory epithelium, asialo-GM_1 is reported to be expressed only during repair process and CF respiratory epithelium cells apically express more asialo- GM1 residues than non-CF cell with relation to an increased affinity for *P. aeruginosa* (Benztman *et al.*, 1996). Such asialylated receptors are not particularly numerous on the normal epithelial surface, they are more abundant in cells expressing mutant CF transmembrane conductance regulator (Saiman *et al.*, 1993). Our findings, which were concluded by using strains isolated from clinical materials and pharyngeal cells obtained from a healthy volunteer, were correlated with these results. Sulfated glycolipids have been shown to occur in large amounts in human trachea and lung, and *Bordetella pertussis* and *Haemophilus influenzae* were shown to bind avidly to sulfatides (Hannah *et al.*, 1994, Hartman *et al.*, 2001). Attachment to sulfated glycoconjugates, which are present throughout the respiratory tract, could effectively secure *P. aeruginosa* to the site where productive infection can be initiated. We demonstrated that sulfated ganglioside mixture and sulfatide inhibited the attachment of *P. aeruginosa* significantly in pharyngeal epithelial cells. Unfortunately, we could not confirm this finding with thin layer chromatography (TLC), which was used successfully to identify carbohydrate receptors for different bacteria (Ahmed *et al.*, 1996, Bentzmann *et al.*, 1996, Hannah *et al.*, 1994). It is possible that weak binding between *P. aeruginosa* and sulfatide occurred on the TLC plate and that the interaction was disturbed by the many washes that are required for the TLC assay. In the TLC plate, glycoconjugates are presented as polyvalent, while in attachment inhibition assay as used in the present study, the glycoconjugates are in monovalent form. Therefore, we hypothesize that *P. aeruginosa* recognize sulfatide in the monovalent form. Sulfatide was demonstrated previously to bind to gp120 molecules of HIV-1 and allow HIV-1 infection of $CD4^-$ neural and intestinal cells, and high level of anti-sulfatide antibodies were detected in patients with neuropathy (Fantini *et al.*, 1998, Lopate *et al.*, 2005,16). If sulfatide is the receptor for *P. aeruginosa*, our preliminary data should be confirmed by using anti-sulfatide antibodies to determine presence of sulfatide on the surface of pharyngeal cells. Validating the level of such antibodies in the sera of CF patients chronically colonized by *P. aeruginosa* would also be interesting.

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