Laboratory and clinical *Pseudomonas aeruginosa* strains do not bind glycosphingolipids *in vitro* or during type IV pili-mediated initial host cell attachment

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The glycosphingolipids (GSLs) gangliotriaosylceramide (Gg₃) and gangliotetraosylceramide (Gg₄) have been implicated as receptors for type IV pili (T4P)-mediated *Pseudomonas aeruginosa* epithelial cell attachment. Since *P. aeruginosa* T4P are divided into five groups, the authors determined whether GSLs in general, and Gg₃ and Gg₄ in particular, are specifically bound and required for host epithelial cell attachment of clinical and laboratory strains within these groups. An enterohaemorrhagic *Escherichia coli* strain, CL56, known to bind to both Gg₃ and Gg₄, provided a positive control. TLC overlay showed no binding of more than 12 *P. aeruginosa* strains to either Gg₃ or Gg₄ (or other GSLs), while CL56 Gg₃/Gg₄ binding was readily detectable. GSL ELISA similarly demonstrated no significant *P. aeruginosa* binding to Gg₃ or Gg₄, compared with CL56. Using a selective chemical inhibitor, epithelial cell GSL synthesis was abrogated, and Gg₃ and Gg₄ expression deleted, but *P. aeruginosa* attachment was not impaired. Target cell attachment was mediated by T4P, since non-piliated, but flagellated, mutants were unable to bind to the target cells. CFTR (cystic fibrosis transmembrane conductance regulator) has also been implicated as a receptor; however, in this work, overexpression of CFTR had no effect on *P. aeruginosa* binding. It is concluded that neither Gg₃ nor Gg₄ are specifically recognized by *P. aeruginosa*, and that endogenous GSLs do not have a role in the attachment of live intact *P. aeruginosa* to cultured lung epithelial cells. In contrast to whole piliated *P. aeruginosa*, T4P sheared from such bacteria showed significant Gg₃ and Gg₄ binding, which may explain the results of other studies.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a Gram-negative bacterium responsible for a variety of opportunistic infections in several clinical settings. Primary among these is lung infection in cystic fibrosis (CF) patients, which is associated with considerable morbidity and mortality (Davies, 2002).

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DAPI, 4′,6-diamidino-2-phenylindole dilactate; EHEC, enterohaemorrhagic *E. coli*; ER, endoplasmic reticulum; FBS, fetal bovine serum; Gb₃, globotriaosylceramide; Gb₄, globotetraosylceramide; Gg₃, gangliotriaosylceramide; Gg₄, gangliotetraosylceramide; GSL, glycosphingolipid; HA, haemagglutinin; PIBM, polyisobutylmethacrylate; P₄, 1-phenyl-2-palmitylaminom-3-pyrrolidino-1-propanol; RELISA, receptor ELISA; SGC, sulfaglycolactosylceramide; T4P, type IV pili; wt, wild-type.

*P. aeruginosa*, together with *Burkholderia cepacia*, is a principal cause of death of such individuals (Gibson et al., 2003). *P. aeruginosa* expresses long polar type IV pili (T4P), which are involved in adherence to cells (Irvin et al., 1989) and a variety of artificial materials (Wall & Kaiser, 1999). The T4P of *P. aeruginosa* have been recently classified into five phylogenetically distinct groups based on amino acid sequence, and presence of accessory genes potentially involved in post-translational modification of the pilins (Kus et al., 2004). The finding that *P. aeruginosa* expressing type IV pili belonging to group 1 are significantly over-represented among isolates from the CF population (Kus et al., 2004) prompted the current study to determine whether differential receptor binding specificity might explain this epidemiological correlation. The binding of T4P in *in vitro* assays to the glycosphingolipids (GSLs) gangliotriaosylceramide (GalNAcβ₁-4Galβ₁-4Glc ceramide, Gg₃) and gangliotetraosylceramide (Galβ₁-3GalNAcβ₁-4Gal
β-4Glc ceramide, Gg₄) (also commonly termed asialoGM2 and asialoGM1, respectively) (Comolli et al., 1999b; Gupta et al., 1994; Lee et al., 1994; Saiman & Prince, 1993; Schweizer et al., 1998) has implicated these molecules as cellular receptors for adhesion of P. aeruginosa (Baker et al., 1990; de Bentzmann et al., 1996; Hazlett et al., 1993; Krivan et al., 1988a; Rampal et al., 1991a). These GSLs contain the common sequence GalNAcβ1-4Gal, which has been defined as the minimal T4P recognition epitope or adhesintope (Campbell et al., 1997; Lee et al., 1996). Receptor ELISA (RELISA) assays based on the binding to this disaccharide sequence have been used to study the mechanism of T4P receptor binding, which is mediated by a C-terminal disulfide loop domain exposed on the terminal pilin subunit. Several studies have implicated these GSLs, particularly Gg₄, as the host cell receptor for P. aeruginosa, but much of this work has employed indirect techniques, such as inhibition of binding by anti-Gg₄ antibodies (Davies et al., 1999; De Bentzmann et al., 1996; Hazlett et al., 1993; Hobden et al., 1996). Some scepticism has arisen with respect to the role that Gg₄ might play as a receptor for P. aeruginosa (Schroeder et al., 2001), and the possibility that other molecules (Chen & Hazlett, 2000; Kirschnek et al., 2005; Wu et al., 1995, 1996), including the CF transmembrane conductance regulator (CFTR) chloride transducer itself (Pier et al., 1997), serve as receptors has been suggested. Anti-Gg₄ antibodies have been shown to react directly with the bacterium (Schroeder et al., 2001), providing an alternative explanation for earlier findings. Gg₄ has been reported to be increased in cells containing non-functional CFTR (Bryan et al., 1998; De Bentzmann et al., 1996; Saiman & Prince, 1993) due to reduced sialylation (Poschet et al., 2001), providing an attractive hypothesis for the increased colonization of respiratory epithelium of CF patients.

As a prelude to investigating the potential role of T4P heterogeneity in clinical pathogy, we investigated the glycolipid-binding specificity of laboratory and clinical P. aeruginosa strains. In this work, we show that none of the tested P. aeruginosa strains specifically bound GSLs, and that selective depletion of GSLs from target respiratory epithelial cells had no effect on P. aeruginosa binding. These results demonstrate that GSLs are unlikely to be involved as specific receptors in P. aeruginosa host cell attachment. We provide a potential explanation for the results of previous binding studies by demonstration of a distinct difference in the GSL binding of bacteria-attached versus cell-free T4P.

**METHODS**

**Cell lines and cell culture.** IB3-1 (ATCC CRL-2777) is a compound heterozygote cell line containing the ∆F508 mutation, and a nonsense mutation, W1282X, with a premature termination signal. The S9 (ATCC CRL 2779) cell line is derived from the IB3-1 cell line, in which the CF phenotype has been corrected by transfection with wild-type (wt) adeno-associated viral CFTR (Virella-Lowell et al., 2004). IB3-1 and S9 cells were maintained in LHC-8 serum-free medium containing glutamine (Biosource), supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics (10,000 μg streptomycin ml⁻¹ and 10,000 IU penicillin; Wisent), and incubated at 37°C in 5% CO₂.

BHK cell lines were transfected with a vector expressing the haemagglutinin (HA)-tagged AF508 CFTR mutation, a vector expressing the HA-tagged wt gene encoding CFTR, or the vector alone (Haardt et al., 1999). All BHK cell lines were a generous gift of Dr G. Lukacs, Hospital for Sick Children, Toronto, ON, Canada. BHK AF508, BHK wt CFTR, and mock-transfected BHK cells, were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F12 50/50 mix (DMEM/F12 50/50) (Wisent), supplemented with 5% FBS, 1% antibiotics, and 250 μg methotrexate ml⁻¹. Under these conditions >95% of the CFTR expressed in BHK wt CFTR cells is the correctly folded mature glycosylated form expressed at the cell surface (Sharma et al., 2004). **Antibodies.** Anti-Gg₃ is a mAb (IgM) produced by 2D4, which is a mouse B cell hybridoma cell line (ATCC TIB-185). Anti-Gg₄ is a mAb (IgM) produced by SH34, which is also a mouse B-cell hybridoma cell line (ATCC CRL-2405). Rabbit anti-PilA is a polyclonal antibody that was raised against purified P. aeruginosa T4P in our laboratory. Mouse anti-CFTR mAb, which recognizes a C-terminal epitope in the region of residues 1370–1380, was purchased from Chemicon. Alexa 488 goat anti-mouse IgG antibodies, Alexa 488 goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgM and DAPI (4′,6-diamidino-2-phenylindole dilactate) were purchased from Molecular Probes. TRITC-conjugated goat anti-rabbit IgG, and alkaline-phosphatase-conjugated goat anti-rabbit antibody, were purchased from Sigma-Aldrich. Horseradish-peroxidase-conjugated goat anti-mouse antibody was from Bio-Rad. **P4-mediated depletion of GSLs.** P₄ (1-phenyl-2-palmitoylaminohexadecanoyl-2-propanol; Lee et al., 1999) was a generous gift from Dr J. Shayman, University of Michigan. IB3-1 and S9 cells were maintained for >10 days in a medium containing 1 μM P₄ reconstituted in DMSO. BHK ∆F508 and BHK wt CFTR were grown in 2 μM P₄. **Bacterial strains.** P. aeruginosa strains (PAO1, PAK, PAKnp, PAKpuiT, PAKpuiA, PA103, PAKFlc, HSCP2, PA14, 1244, PA13, 1457, 1123, 5196 and C27330) were maintained as glycerol stocks at −80°C, and were grown on Luria–Bertani (LB) agar plates, or Pseudomonas isolation agar plates, overnight at 37°C. Cells were removed with a sterile toothpick, and resuspended in RPMI supplemented with 5% FBS and 25 μM HEPES buffer to an OD600 of 0.05 (equivalent to approx. 5 × 10⁷ cells, as determined by plate counts). Enterobacteriaceae Escherichia coli (EHEC) CL56 was maintained in LB.

**Host cell lipid extraction and saponification.** Approximately 10⁷ cells were shaken vigorously overnight in 15 ml 2:1 chloroform:methanol (C:M) mixture in a glass tube. The mixture was filtered, and the collected lipids were dried, and resuspended in 2:1 C:M to give an equivalent of 5 × 10⁸ cells in 20 ml C:M. Total cellular lipid extract was saponified to isolate the glycolipid fraction. The lipid residue was resuspended in 1 M NaOH in methanol for 2 h at 37°C. The mixture was neutralized with 2 M acetic acid. A small volume of water was added, and the mixture applied onto a C-18 column (Sep-Pak Plus C-18; Waters). The column was washed with water, and glycolipids were eluted with methanol, dried under a stream of nitrogen gas, and resuspended in 2:1 C:M to the desired concentration. **Assay of GSL binding by TLC overlay with radiolabelled bacteria.** Binding of P. aeruginosa to Gg₄ and Gg₅, and other GSLs, was assayed by the TLC overlay procedure described by Krivan et al. (1988a), with minor changes. Bacteria were metabolically radiolabelled with [²⁵S]methionine [Amerham; 1 μCi ml⁻¹ (1 kBq ml⁻¹) in Davis minimal medium (DMM)] for 22 h at 37°C, with no shaking. Viability of greater than 95% (as determined by viable plate
counts) was maintained. Gg4 and Gg5 (2 μg of each) were applied to aluminium-backed silica gel plates (Alugram Sil G/UV 254). Total saponified lipid extracts from approximately 7 × 10^8 IB3-1 and S9 cells were similarly applied to TLC plates. Plates were first pre-cleared in chloroform and methanol in the ratio 98:2, followed by application of the GSLs, and separation in chloroform, methanol and water, in a 60:40:9 ratio. One of the plates was stained with orcinol colour reagent (Sigma), and incubated at 110 °C for 10 min. The remaining plates were air-dried, and dipped in a solution of 0·3 % polyisobutylmethacrylate (PIBM; Aldrich) in acetone for 1 min. PIBM treatment is widely used to enhance ligand binding, but we have found this step to be unnecessary (Yu & Lingwood, 1992). Preliminary studies showed that PIBM reduced E. coli CL56 GSL binding, and, therefore, it was omitted for this organism. The GSL binding of Pseudomonas strains was not affected by PIBM pretreatment. The TLC plates were then dried, and blocked with 2 % BSA in 100 mM tricine-buffered saline (TBS) for 2 h at 37 °C. Log phase labelled bacteria (1 × 10^8 c.p.m. ml ^{-1}) were resuspended in 100 mM TBS at a concentration of approximately 10^9 ml ^{-1}. Plates were covered with the suspension of radiolabelled bacteria for 4 h at 37 °C, with slow shaking (20 r.p.m.). Unbound bacteria were washed off, and the plates were dried completely before being exposed to a phosphor screen (Molecular Dynamics) for 6 days.

**Solid-phase GSL-binding assay (RELISA).** Glycolipids were serially diluted in 50 μl methanol in the wells of flat-bottomed polystyrene plates (Evergreen Scientific). Plates were left to dry overnight at 4 °C. The wells were blocked with 50 μl 2 % BSA in TBS for 1 h at 37 °C, after which they were washed five times with TBS. A 50 μl volume of *P. aeruginosa* culture (in stationary phase, and taken from agar plates), containing approximately 10^10 bacteria ml ^{-1} in TBS, was added to each well. The plates were covered with Parafilm, and incubated at 37 °C for 2 h, with slow shaking (20 r.p.m.). Unbound bacteria were washed off with TBS. A polyclonal antibody against the pilin was used to monitor the binding of *P. aeruginosa* to glycolipids. Wells were filled with 50 μl of a 1/1000 dilution of this antibody, overnight at 4 °C. This was followed by incubation with 50 μl of a 1/2000 dilution of goat anti-rabbit alkaline phosphatase secondary antibody (peroxidase-conjugated antibodies cannot be used with catalase-positive *P. aeruginosa*) for 1 h at room temperature. Unbound secondary antibody was washed off, and 50 μl 1 mg phosphatase substrate ml ^{-1} in 1 M diethanolamine buffer (0·5 mM MgCl2, pH 9·8) (Sigma) was added to each well. After 30 min incubation at room temperature, absorbance readings were recorded at 405 nm. CL56 was detected with an anti-whole-cell CL56 mAb, followed by a goat anti-mouse secondary antibody conjugated to horseradish peroxidase.

**Bacterial–host-cell adherence assay.** IB3-1 and S9 cells, or BHK cell lines, were seeded onto 18 mm tissue-culture-treated glass coverslips (Fisher), and grown overnight at 37 °C, 5 % CO2, to 70–80 % confluency. The monolayers were then washed three times with PBS, and fixed with 4 % paraformaldehyde for 30 min on ice, and washed twice with PBS. The cells were then permeabilized with 0·1 % Triton X-100 for 20 min (except for assays staining for surface glycolipid expression of cells), then blocked with 1 % BSA for 1 h at room temperature. The appropriate primary antibody – anti-Gg4 (1:2); anti-Gg5 (1:10) or anti-CFTR (1:500) – was incubated with the monolayers for 1 h on ice, and washed three times with PBS. The coverslips were then mounted on glass slides using DakoCytomation fluorescent mounting medium.

**Glycolipid and CFTR immunofluorescence staining.** IB3-1 and S9 cells, or BHK cell lines, were seeded onto 18 mm tissue-culture-treated glass coverslips (Fisher), and grown overnight at 37 °C, 5 % CO2, to 70–80 % confluency. The monolayers were then washed three times with PBS, followed by addition of the secondary antibody Alexa 488 goat anti-rabbit (or anti mouse) IgG, used at 1:500 dilution in 1 % BSA. The cells were also stained with rhodamine–phalloidin (also a 1:500 dilution in 1 % BSA), and incubated for 1 h on ice, along with the secondary antibody. The cells were then washed with PBS, and the coverslips were mounted on glass slides using glass slides using DakoCytomation fluorescent mounting medium.

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under these conditions no cell attachment was observed for the T4P-deleted mutant organism. *Pseudomonas* cell binding increased with time (5–30 min), but not temperature (room temperature versus 37 °C) (not shown).

The cells were then permeabilized with 0·1 % Triton X-100 for 20 min, and blocked with 1 % BSA for 1 h at room temperature. Rabbit anti-PilA antiserum was used at 1:500 dilution in 1 % BSA to detect bacterial pilin. Any cell binding of the T4P-deficient strain PAKpilT (and PAK control) was detected using a mouse anti-O6 LPS mAb (from Dr J. Lam, University of Guelph, Guelph, ON, Canada). The effect of preincubation of PAK and PAKpilT with 0·5 % BSA or 0·5 % gelatin (30 min at room temperature) on S9 cell binding was also compared.

Coverslips were incubated with primary antibody on ice for 1 h, or at 4 °C overnight. The cells were then washed twice with PBS, followed by addition of the secondary antibody Alexa 488 goat anti-rabbit (or anti mouse) IgG, used at 1:500 dilution in 1 % BSA. The cells were also stained with rhodamine–phalloidin (also a 1:500 dilution in 1 % BSA), and incubated for 1 h on ice, along with the secondary antibody. The cells were then washed with PBS, and the coverslips were mounted on glass slides using DakoCytomation fluorescent mounting medium.

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**Bacterial adherence and glycolipid fluorescent co-staining.** The bacterial adherence assay was performed as described above. The rabbit anti-PilA antibody was used at 1:500 dilution in 1 % BSA to detect bacteria, while mAb anti-Gg4 was diluted 1:2 in 1 % BSA. The fixed bacteria-bound cells were incubated with primary antibodies on ice for 1 h, or at 4 °C overnight. The cells were then washed three times with PBS, followed by addition of the secondary antibody, which was either TRITC-conjugated goat anti-mouse IgM (for anti-Gg4 or anti-Gg5 detection) or Alexa 488 goat anti-mouse IgG (for anti-CFTR) – and rhodamine–phalloidin (again, only for cells that had been permeabilized) at 1:500 dilution in 1 % BSA. The cells were incubated for 1 h on ice, and washed three times with PBS. The coverslips were then mounted on glass slides using DakoCytomation fluorescent mounting medium.

Fluorescent images were taken using a Zeiss LSM 510 confocal microscope under a × 63 oil-immersion objective. The images were recorded in LSM 510 Meta, and composites were prepared using Adobe Photoshop 7.0.

**Preparation of cell-free pili.** *P. aeruginosa* was streaked in grid pattern on DMM agar plates containing glucose, and grown overnight. The cells were scraped off the plates, resuspended in PBS, and

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depilated by vortexing for 1 min. Cells were separated from the supernatant by centrifugation for 30 min at 15,000 g. The supernatant was made 0·1 M with respect to MgCl₂, and incubated at 4°C overnight. The resulting precipitate, which contained the pili, was harvested by centrifugation at 15,000 g for 30 min at 4°C.

RESULTS

GSL content of target eukaryotic cells

The human CF respiratory epithelial cell line IB3-1, and the corresponding S9 cell line reconstituted with a wt gene encoding CFTR, have been used to assess the interaction between P. aeruginosa and host cells (Virella-Lowell et al., 2004). However, the glycolipid content of these cells has not previously been characterized. Fig. 1 shows TLC analysis of the GSL content of these two cell lines. The overall GSL composition (Fig. 1A) of the cells was very similar. Gg₃ and Gg₄ have been implicated as P. aeruginosa host cell receptors, and it has been suggested that these receptors are upregulated in CFTR mutant epithelial cells (De Bentzmann et al., 1996). Using anti-Gg₃ and anti-Gg₄ TLC immunostaining, both IB3-1 and S9 cell lines were found to contain very little Gg₃. Anti-Gg₄ recognized a faster migrating species (comigrating with Gg₅), in addition to Gg₄, in both extracts; both species were elevated approximately twofold in IB3-1 cells. In order to later test for the importance of GSLs in P. aeruginosa host cell binding, the GSL extracts from cells treated with P4, an inhibitor of glucosylceramide synthase (Lee et al., 1999), which therefore prevents the synthesis of most GSLs, were also tested. Both the anti-Gg₄ and the weak anti-Gg₃ staining were missing in the extract of P4-treated cells (Fig. 1C). Mock-transfected BHK cells, and BHK cells transfected with wt or ΔF508 CFTR, were also tested for Gg₃ and Gg₄ expression, but none expressed detectable levels of either GSL (not shown).

P. aeruginosa–GSL binding in vitro

The neutral GSL extracts from S9 and IB3-1 cells were used, together with GSL standards, including Gg₃ and Gg₄, to screen for the potential GSL binding by laboratory and clinical P. aeruginosa isolates. Bacteria were metabolically labelled with [³⁵S]methionine, following overnight growth in DMM. After incubation with exponential-phase bacteria, the plates were washed, and autoradiography was used to detect any bound organisms. The autoradiograms were uniformly negative for all P. aeruginosa strains tested (Fig. 2). Omission of PIBM treatment of the plate (used to optimize GSL presentation for ligand binding; Yiu & Lingwood, 1992) made no difference to the results (not shown). Some non-specific binding at the TLC solvent front was seen, but no binding to the GSL fraction, including the Gg₃ and Gg₄ glycolipid standards, was observed. In contrast, the control EHEC strain CL56, previously demonstrated to show Gg₃ and Gg₄ binding (Barnett-Foster et al., 1999), adhered to these GSLs (both the standards and within the cell extracts, Fig. 2B) under the same conditions used for P. aeruginosa. Of note is the fact that CL56 binding to Gg₄ was more apparent in the IB3-1 than the S9 cell extract. Similarly, CL56 binding to Gg₄ and Gg₃ was demonstrated by RELISA, but no significant binding for a selection of the P. aeruginosa strains was observed in this assay (Fig. 3). It has been reported that BSA inhibits P. aeruginosa–GSL binding by an unknown mechanism (Ramphal et al., 1991a). We found that use of gelatin as an alternative blocker did not increase P. aeruginosa–GSL binding (Fig. 3). Also, pre-incubation of bacteria with BSA had no effect on P. aeruginosa–cell binding (see below).

Host cell GSL depletion

If binding to Gg₃ and Gg₄ plays a role in Pseudomonas host cell binding, and our TLC overlay and RELISA assays were of insufficient sensitivity for detection, depletion of the host cell GSL content should reduce Pseudomonas host cell binding. TLC staining of GSLs with anti-Gg₄ and anti-Gg₃ was eliminated following cell treatment with P4 (Fig. 1). Similarly, cell staining with anti-Gg₃ and anti-Gg₄ was lost after growth in P4 (Fig. 4). In contrast, anti-sulfagalactosylceramide (SGC) staining was retained.

Fig. 1. GSLs of S9 and IB3-1 human lung epithelial cell lines grown with and without P4. (A) Orcinol chemical detection of GSLs, (B) immunodetection of Gg₃, and (C) immunodetection of Gg₄. Lanes: 1, standard Gg₃ (1 μg); 2, standard Gg₄ (1 μg); 3 and 5, saponified GSL extract from S9 cells; 4 and 6, saponified GSL extract from IB3-1 cells. Lanes: 3 and 4, untreated cells; 5 and 6, P4-treated cells. Densitometry showed a 1·9-fold increase in Gg₃ in IB3-1 versus S9 cells.
SGC is a GSL derived from galactosylceramide, and therefore not susceptible to P4 inhibition. Anti-Gg4 staining of IB3-1 and S9 cells was significantly different. Gg4 was only expressed on the surface of IB3-1 cells (Fig. 4B versus F) in punctate domains, consistent with lipid rafts. Only when cells were permeabilized could Gg4 be detected in S9 cells. Both intracellular and cell-surface anti-Gg4 staining were eliminated after P4 treatment. Anti-Gg3 cell staining was not above background levels (not shown).

**Effect of host cell GSL depletion on P. aeruginosa attachment**

P. aeruginosa does not bind GSLs could be seen, but we observed no preferential binding of Pseudomonas to either host cell type (Fig. 5A versus C). In addition, the effect of P4 depletion of GSLs on P. aeruginosa binding to IB3-1 and S9 cells was not significant (Fig. 5A, C versus B, D), indicating that Gg3 and Gg4, or indeed any other glucosyl ceramide-based GSLs, do not play a role in the host cell attachment of these organisms. Double labelling of cell-bound organisms and anti-Gg4 (Fig 5E, F) clearly shows that P. aeruginosa bound to membrane regions devoid of Gg4. No cell binding of a T4P-deleted P. aeruginosa mutant strain was seen (Fig. 5G versus H). Preincubation of bacteria with BSA or gelatin had no effect on cell binding (not shown). Pseudomonas host cell binding is not intimate, and frequently comprises a network of T4P tip-to-tip interactions. These networks are seen for host-cell-bound, but not for the infrequent substrate-bound, organisms.

T4P are retractable, and it was therefore considered possible that GSL recognition could be transient, and perhaps not detected in our TLC overlay experiments. In order to amplify any potential receptor role for GSLs, we tested the effect of P4 on the cell binding of a pilT mutant of P. aeruginosa. This mutant is hyperpiliated, and unable to retract its pili (Chiang & Burrows, 2003). The multipiliated nature of this mutant can be clearly seen in host-cell-attached bacteria (Fig. 6). As reported by Comolli et al. (1999a), this strain bound host cells less effectively than the wt organism, and the extensive interbacterial attachment was not evident; however, P4 GSL depletion did not affect the host cell binding of this strain.

**Effect of CFTR overexpression on P. aeruginosa attachment**

CFTR itself has been implicated as a receptor for P. aeruginosa (Pier et al., 1997). We therefore examined the binding of P. aeruginosa to BHK cells stably transfected with wt or ΔF508 CFTR tagged with HA (Haardt et al., 1999). These cells do not make Gg3 or Gg4 (not shown). The location of CFTR in these transfectants was confirmed by anti-HA staining. Surface expression of CFTR was only evident for wt BHKcells. ΔF508 CFTR is not at the cell surface, but found intracellularly, consistent with endoplasmic reticulum (ER) accumulation (Haardt et al., 1999). No difference between the binding to wt-CFTR- or ΔF508-CFTR-transfected BHK cells was seen (Fig. 7A versus B). P4-mediated GSL depletion was similarly without effect on P. aeruginosa BHK cell binding (not shown). As for S9 and IB3-1 cells, T4P-mediated networks of non-intimately bound bacteria were present.

**GSL binding of cell-free T4P**

Several studies have investigated the Gg3 and Gg4 binding of isolated P. aeruginosa T4P, rather than the binding of whole bacteria. We used the RELISA assay to examine the GSL binding of T4P sheared from the PAK strain of P. aeruginosa.

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Fig. 2. Assessment of P. aeruginosa GSL binding by TLC overlay. Clinical and laboratory strains were metabolically radiolabelled, and binding to GSL standards, and GSL extracts from IB3-1 and S9 cells, was determined by overlay of TLC-separated GSLs. The GSL binding of EHEC strain CL56 was assayed in a similar way. (A) Orcinol stain, (B) EHEC strain CL56, (C) P. aeruginosa strain 1123, (D) P. aeruginosa strain PA103. Strains PAK, PAKFliC, PAO1, PAKpilA, 1244, 5196, 1457, PA14, PA13, HSCPs1, C27330 and C1841 were also tested, and showed no GSL binding. Lanes 1–4 are as in Fig. 1.
Fig. 3. Comparison of EHEC and P. aeruginosa GSL binding by RELISA. Bacterial binding to Gg₄ (A) and Gg₃ (B) immobilized in microtitre plates was measured. P. aeruginosa strains: PAK (▲), PAKpilA (+), PAKpilT (○), C27330 (◇). EHEC strain CL56 (■) was used as positive control. PAK binding to Gg₃ was also tested using bovine skin gelatin as a blocking agent (△). Binding was monitored using a rabbit polyclonal anti-PilA antibody. Error bars show SD.

Fig. 4. Effect of P4 on anti-GSL cell staining. (A) Permeabilized untreated IB3-1 cells, with anti-Gg₄ staining; (B) non-permeabilized untreated IB3-1 cells, with anti-Gg₄ staining, overlaid on a phase-contrast image; (C) permeabilized P4-treated IB3-1 cells, with anti-Gg₄ staining; (D) non-permeabilized P4-treated IB3-1 cells, with anti-Gg₄ staining, overlaid on a phase-contrast image; (E) permeabilized untreated S9 cells, with anti-Gg₄ staining; (F) non-permeabilized untreated S9 cells, with anti-Gg₄ staining, overlaid on a phase-contrast image; (G) permeabilized untreated S9 cells, with anti-SGC staining; (H) permeabilized P4-treated S9 cells, with anti-SGC staining. Permeabilized S9 and IB3-1 cells showed no staining with anti-Gg₃ (not shown).
Strong Gg3 and Gg4 binding was seen for the cell-free T4P (Fig. 8). No binding of the T4P to an unrelated glycolipid, globotriaosylceramide (Gb3), was observed.

DISCUSSION

Glycolipids are implicated, but not proven, P. aeruginosa receptors

Pulmonary P. aeruginosa infections are a major cause of morbidity in CF. GSL receptor binding has been implicated as the initial means of T4P-mediated bacterial host cell binding, but rigorous studies establishing this connection have yet to be reported. GSL binding is a common feature of microbial pathogenesis (Lingwood, 1992, 2000). The carbohydrate of GSLs closely apposes the membrane, and is organized in dynamic lipid rafts, which are involved in signalling and internalization, to provide a receptor option commonly selected by bacterial and viral pathogens (Rosenberger et al., 2000). Many organisms have been shown to bind to two GSLs in particular, Gg3 and Gg4, with the common GalNAcβ1-4Gal providing the shared binding epitope (Krivan et al., 1988b; Schweizer et al., 1998; Strömberg et al., 1988). This binding specificity has been ascribed to intact Pseudomonas cells, and isolated T4P responsible for attachment and twitching motility. Gg4 receptor function has often been ascribed indirectly via inhibition by the mAb antiGg4 (Davies et al., 1999; De Bentzmann et al., 1996; Hazlett et al., 1993; Hobden et al., 1996).

GSLs are not P. aeruginosa receptors

We question the Gg4 receptor function for intact organisms, as our investigation of more than 12 P. aeruginosa strains showed no evidence for GSL, let alone Gg3 or Gg4, binding by TLC overlay and, for several strains, by RELISA. The exponential-phase culture conditions used to radiolabel the organisms retain viability and T4P expression. Metabolic labelling provides the least ambiguous means to detect binding of viable organisms. RELISA using stationary-phase organisms and immunodetection supported these findings. However, some other factor involved in GSL binding may be suboptimal under our culture conditions. Irrespective of any overt or subtle GSL-binding phenotype, Pseudomonas
strains attach to human lung epithelial cells in an efficient manner, regardless of the presence of cell surface GSLs (Gg₃ and Gg₄). Immunostaining with anti-Gg₄ showed that Gg₄ is available on the cell surface, but distinct from the sites of bacterial attachment.

**CF cells express more Gg₄**

An accumulation of Gg₄ in CF cells (Bryan et al., 1998; Saiman & Prince, 1993), resulting from hyposialylation (Poschet et al., 2001) due to an altered Golgi pH in CFTR mutant cells, has been questioned (Jiang et al., 1997), but was verified in our work. IB3-1 cells contain at least twice the levels of Gg₄ of S9 cells; intracellular Gg₄, in structures consistent with Golgi/ER, was observed for both cell types. Gg₃ was barely detected in either cell line. The distinction in surface Gg₄ expression, but the lack of differential *P. aeruginosa* binding to IB3-1 and S9 cells, and our finding that bacteria bind to regions of the plasma membrane devoid of anti-Gg₄ binding, argue against Gg₄ receptor function.

**GSL depletion does not affect *P. aeruginosa* T4P-mediated binding**

It is surprising, in light of the many studies reporting Gg₃ and Gg₄ binding by *Pseudomonas* (Bryan et al., 1998; De Bentzmann et al., 1996) (or its T4P) and other bacterial pathogens (Deal & Krivan, 1990; Krivan et al., 1991; Strömberg et al., 1988), that the effect of depletion of cellular GSLs on bacterial binding has not been reported. Competitive inhibition of glucosylceramide synthase with a substrate analogue P4 (Lee et al., 1999), in both S9 and IB3-1 cells (and BHK cells), resulted in the loss of anti-Gg₄ (not present in BHK cells), TLC and cell-surface binding, but the expression of a non-glucosylceramide-based GSL, SGC, was not affected. This attests to the efficacy and specificity of GSL inhibition. It is clear that *Pseudomonas* expressing T4P retain the ability to bind these cell types, either with or without GSL inhibition.

**Fig. 6.** Cell binding of *P. aeruginosa pilT* mutant. The binding of the hyperpiliated pilT mutant, which is unable to retract pili, to untreated and P4-treated IB3-1/S9 cells was compared. (A) Untreated IB3-1 cells, (B) P4-treated IB3-1 cells, (C) untreated S9 cells, (D) P4-treated S9 cells.

**Fig. 7.** Comparison of *P. aeruginosa* PAK binding BHK cells transfected with CFTR. (A) PAK–wt-CFTR-BHK cell binding, (B) PAK–ΔF508-CFTR-BHK cell binding. Bacteria binding to host-cell-bound organisms form extensive networks, irrespective of CFTR expression (or P4 treatment, not shown).

**Fig. 8.** GSL binding of released PAK T4P. T4P were prepared by shearing from *P. aeruginosa* PAK, and analysed for pilin content by SDS-PAGE and Western blotting using anti-pilin antibodies (not shown). Binding to Gg₃ (○) and Gg₄ (■) was monitored by RELISA, using rabbit anti-pilin antibodies. No binding to the unrelated GSL Gb₃ (▲) was observed. Error bars show SD.
The similar binding of *P. aeruginosa* to BHK cells expressing either wt or ΔF508CFTR, in which a clear difference in CFTR expression on the cell surface is seen, is also inconsistent with a receptor function for CFTR (Pier et al., 1997). Under our assay conditions, non-piliated *P. aeruginosa* mutants do not bind cells (Woods et al., 1980), despite the fact that such strains bind mucins (Ramphal et al., 1991b). O-linked mucin carbohydrates have been implicated in *P. aeruginosa* flagellum-mediated binding (Ramphal & Arora, 2001), which may play a later role, particularly within the accumulated mucus of the CF airway. T4P are required for initial host cell attachment, twitching motility, and the subsequent development of a non-cell-attached biofilm (Wall & Kaiser, 1999). Prevention of initial T4P attachment should prevent twitching and biofilm development (Burrows, 2005). Our assay conditions were adjusted to assess T4P-mediated *P. aeruginosa* initial attachment. Other adhesive mechanisms, which might occur later, could nevertheless involve GSLs.

**Cell-free T4P bind Gg₃ and Gg₄**

Our finding that T4P bind both Gg₃ and Gg₄ when sheared from the *P. aeruginosa* organism is unlikely to result from increased sensitivity due to increased ‘pilin concentration’, as compared with the intact organism. Our studies show that the polyclonal anti-pilin antibody binds to the bacterium itself, and thus the bacterium would serve to amplify any signal resulting from GSL-bound pili. Our finding offers an explanation for earlier results indicating bacteria–Gg₃/Gg₄ binding (de Bentzmann et al., 1996; Hazlett et al., 1993; Krivan et al., 1988a; Ramphal et al., 1991a). If *P. aeruginosa* cultures assessed for binding contained cell-free T4P, GSL–bacteria binding could be inferred. Any pathophysiological significance of bacteria-free T4P binding to Gg₃ and Gg₄ remains to be determined. In our cell-binding assays, it is clear that the intact bacterium is visualized bound to the eukaryotic cells using anti-pilin antibody and receptor analog: implications for synthetic vaccine design. The authors thank Dr D. Foster, Ryerson University, for help with CL56 studies.

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