Attachment of *Yersinia pestis* to human respiratory cell lines is inhibited by certain oligosaccharides

Richard Thomas¹ and Tim Brooks²

Correspondence

Richard Thomas
rjthomas@dstl.gov.uk

Defence, Science & Technology Laboratories¹ and Health Protection Agency²,
Porton Down, Salisbury SP4 0JQ, UK

Pneumonic plague is an aggressive disease that is clinically difficult to treat. Inhibition of attachment using oligosaccharide receptor mimics may provide an alternative to antibiotics. The virulent *Yersinia pestis* strain GB was demonstrated to attach to the murine monocyte cell line (J774A.1) and a range of human respiratory epithelial cell lines: nasal (RPMI-2650), bronchial (BEAS2-B) and alveolar (A549). Attachment was greatest to the A549 and BEAS2-B cell lines. Pre-treatment of the cell lines with tunicamycin reduced attachment by 55–65 %, indicating the importance of cell-surface carbohydrates in adhesion. The cell lines displayed differences in the oligosaccharides that inhibited attachment. *p*-Nitrophenol was the best inhibitor for each cell line. Disaccharides such as GalNAc β 1-3Gal and GalNAc β 1-4Gal were also good inhibitors, particularly for the RPMI-2650 cell line. This demonstrates the potential of oligosaccharides as potential anti-adhesion therapeutics.

Received 24 March 2005

Accepted 17 October 2005

INTRODUCTION

Yersinia pestis is the aetiological agent of 'black death', a pandemic that swept across Asia and Europe between the 14th and 17th centuries causing millions of deaths (Perry & Fetherston, 1997). Plague presents as two main clinical manifestations – bubonic and pneumonic plague. Bubonic plague is transmitted to humans via regurgitation into the bloodstream following feeding by fleas originating from infected rodents. Bacteria travel from the bite to the local lymph nodes where they multiply forming characteristic 'buboes'. Multiplication of bacteria in the bloodstream results in septicaemia, allowing plague bacilli to reach lung tissues, causing secondary pneumonia. Such patients can transmit plague by aerosol, resulting in primary pneumonic plague (Perry & Fetherston, 1997; Putzker *et al.*, 2001; Krishna & Chitkara, 2003).

Untreated bubonic plague is fatal in 30–40 % of cases, whilst pneumonic plague reaches a fatality rate approaching 100 %. Septicaemia can result from both clinical presentations, invariably resulting in death within 1–2 days (Inglesby *et al.*, 2000; Putzker *et al.*, 2001). Treatment is usually with tetracycline, streptomycin, doxycycline or chloramphenicol (Putzker *et al.*, 2001). Doxycycline or ciprofloxacin administration would be better in the case of mass casualties, due to superior pharmacokinetics (Inglesby *et al.*, 2000). Indeed, ciprofloxacin, doxycycline and the newer fluoroquinolones gatifloxacin and moxifloxacin increase survival in mice presenting with pneumonic plague (Byrne *et al.*, 1998; Russell *et al.*, 1998; Steward *et al.*, 2004). However, recently there has

been a worrying increase in multidrug-resistant strains (Galimand *et al.*, 1997; Wong *et al.*, 2000).

The infectious nature and high mortality rate of pneumonic plague means that, even with today's medical advances, it remains a disease to be feared. High aerosol transmissibility makes plague a high-priority agent for potential bioterrorists (Inglesby *et al.*, 2000). A novel preventative mechanism of plague infection may be via inhibiting adhesion of *Y. pestis* to the respiratory tract. Bacteria generally attach to mammalian cells using proteins, glycoproteins and glycolipids located on the mammalian cell membrane. In *Y. pestis*, identified putative adhesins include the pH6 antigen, plasminogen activator, LPS and the capsular F1 antigen (Lindler *et al.*, 1990; Kienle *et al.*, 1992; Lindler & Tall, 1993; Straley, 1993; Lähteenmaki *et al.*, 1998; Payne *et al.*, 1998). The potential of short-chain oligosaccharide receptor mimics to inhibit attachment has been investigated with varying success in many bacterial species, both *in vitro* (Krivan *et al.*, 1988; Hambrook *et al.*, 2004; Thomas & Brooks, 2004a, b) and *in vivo* (Idänpään-Heikkilä *et al.*, 1997; Bryan *et al.*, 1999). This study investigated the attachment of *Y. pestis* strain GB to a range of human respiratory epithelial cell lines. The ability of a range of oligosaccharides to prevent attachment was determined.

METHODS

Materials. Tissue culture reagents, *p*-nitrophenol and tunicamycin were purchased from Sigma-Aldrich. Saccharides were purchased as powders from Dextra Laboratories or Sigma-Aldrich. Anti-rabbit IgG-FITC conjugate was purchased from Sigma-Aldrich. Rabbit anti-ganglioside antibodies were purchased from Calbiochem.

Abbreviations: Pla, plasminogen activator; PsaA, pH6 antigen.

Bacterial growth conditions. *Y. pestis* strain GB was part of the site culture collection. It was originally isolated from a fatal laboratory exposure resulting in pneumonic plague. The median lethal dose in BALB/c mice by the subcutaneous route was determined to be 1 c.f.u. (Russell *et al.*, 1995). The bacteria were routinely cultured on agar plates prior to broth inoculation. *Y. pestis* was cultured on blood agar base (BAB) at 28 °C for 48 h. To obtain cell densities of 10^8 c.f.u. ml⁻¹ for the adhesion studies, single colonies were inoculated into broth cultures. *Y. pestis* was grown in BAB broth at 28 °C for 16 h. All broth cultures were incubated statically. Stocks were stored at -80 °C in the respective base broth supplemented with 10% (v/v) glycerol.

Culture of cell lines. The cell lines used were obtained from the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Wiltshire, UK. These were three human respiratory cell lines, alveolar epithelial (A549), bronchial epithelial (BEAS2-B) and nasal epithelial (RPMI-2650), plus the murine monocyte cell line J774A.1. Adherent A549, BEAS2-B and J774A.1 cells were cultured in tissue culture flasks containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine. Similarly, RPMI-2650 cells were cultured in Eagle's modified essential medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine and 1% (v/v) non-essential amino acids. Cells were incubated at 37 °C in an atmosphere of 5% (v/v) CO₂ and split 1:3 every 3–4 days. For adhesion assays, cells were rinsed twice with pre-warmed PBS before incubation in the presence of trypsin/0.05% (w/v) EDTA in PBS. After removal of the trypsin solution, cells were resuspended in warm fresh medium, seeded in 96-well microtitre plates to a density of 50 000 cells per well in 100 µl medium and incubated overnight as previously described to produce confluent monolayers for the adhesion assay. In experiments that required reduced expression of surface oligosaccharides, cells were cultured in the presence of 0.55 µg tunicamycin ml⁻¹ for 96 h prior to completion of the adhesion assay.

Adhesion assay. Confluent monolayers in 96-well microtitre plates were washed five times with 150 µl PBS pre-warmed to 37 °C. Non-specific binding was blocked by incubation for 1 h at 37 °C with 0.5% (w/v) BSA before rinsing twice with pre-warmed PBS. The relevant saccharide concentrations were prepared in tissue culture medium pre-warmed to 37 °C. Bacteria at a density of

10^7 c.f.u. ml⁻¹ were incubated for 1 h at room temperature in a 1:1 ratio with the saccharide solution to provide the final saccharide solution (Table 1). Controls were always performed with no saccharide. Wells containing no eukaryotic cells were used to determine background binding to plates. A 100 µl volume of the bacterial/saccharide solution was added to the cell monolayer and incubated for 1 h at 37 °C. Non-adherent bacteria were removed from the monolayers by rinsing five times with pre-warmed PBS. Cells were lysed by incubation for 30 min at 37 °C with 0.1% (v/v) Triton X-100 in PBS. Serial dilutions were performed in PBS and 100 µl vols of the lysate were plated in duplicate on to BAB plates and incubated at 28 °C for 48 h. Percentage inhibition was calculated using the equation [(control-test)/control] × 100%.

Cytotoxicity assay. The CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) was used for all cytotoxicity assays in accordance with the manufacturer's instructions as described previously (Thomas & Brooks, 2004a).

Antibody labelling of cell lines. Cell monolayers were cultured in 96-well microtitre plates as described previously. The monolayer was washed three times with PBS and bound to the well by a 30 min incubation with 4% (v/v) formaldehyde in PBS. Cells were washed twice with PBS, which was then replaced with 100 µl diluted anti-ganglioside antibody: 1/100 anti-GM1, 1/100 anti-GM2, 1/100 asialo-GM2 or 1/400 asialo-GM2. Cells were incubated for 1 h at 37 °C. Excess antibody was removed by washing three times with PBS before addition of 100 µl FITC-conjugated anti-IgG for 1 h at 37 °C. Cells were washed three times with PBS before a final addition of 100 µl PBS to each well. Controls included wells incubated in the absence of antibody to determine background fluorescence and wells incubated with FITC-conjugated antibody only. Fluorescence was measured by spectrofluorimetry (excitation wavelength 488 nm, emission wavelength 520 nm). Data were acquired and assimilated using SoftMax Pro software (Molecular Devices).

Statistical analysis. All experiments were performed as three or five independent assays. Assays were performed in triplicate for each experimental run. Results were expressed as the mean value and standard deviations are provided where appropriate. *P* values were calculated using paired *t*-tests.

Table 1. Structure and concentrations of anti-adhesion compounds used in this study

Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GlcA, glucuronic acid; IduA, iduronic acid.

Chemical name of anti-adhesion compound	Structure	Concn (mM)
Heparin	(-IduASO ₄ α1-4GlcNAcSO ₄ ⁻) _n (-GlcAα1-4GlcNAc ⁻) _n	0.5
Dextran	(-Glcα1-6Glc ⁻) _n	0.5
Dextran sulphate	(-Glcα1-6GlcSO ₄ ⁻) _n	0.5
Mannose	—	140
Lacto- <i>N</i> -biose I	Galβ1-3GlcNAc	1.5
<i>N</i> -Acetylglucosamine	Galβ1-4GlcNAc	1.5
β1-3 Galactosyl <i>N</i> -acetylgalactoside (terminal disaccharide of globotriose)	GalNAcβ1-3Gal	1.5
β1-4 Galactosyl <i>N</i> -acetylgalactoside (receptor for pili of <i>P. aeruginosa</i>)	GalNAcβ1-4Gal	1.5
<i>p</i> -Nitrophenol	—	40

RESULTS AND DISCUSSION

Kinetics of *Y. pestis* attachment to human respiratory cell lines

Initially, the effects of incubation time and bacterial cell density on attachment of *Y. pestis* to each cell line were ascertained. To determine optimal conditions for *Y. pestis* adhesion, attachment was monitored at two different bacterial densities, 10^6 and 10^7 c.f.u. ml⁻¹, over a 1 h period (Fig. 1a). In each cell line examined, increasing the inoculum increased the number of adherent bacteria. Similar results have been reported for attachment of *Streptococcus pneumoniae* and *Legionella pneumophila* to the A549 cell line (Cundell & Tuomanen, 1994; Thomas & Brooks, 2004a). At an initial inoculum of 10^7 c.f.u. ml⁻¹, attachment from highest to lowest was in the order: alveolar (A549) > bronchial (BEAS2-B) > nasal (RPMI-2650) > monocyte (J774A.1). When using an inoculum of 10^6 c.f.u. ml⁻¹, the order was very similar: alveolar (A549) > bronchial (BEAS2-B) = nasal (RPMI-2650) > monocyte (J774A.1). The temporal effects of attachment using an inoculum of 10^7 c.f.u. ml⁻¹ were determined for each cell line (Fig. 1b). *Y. pestis* adhered more rapidly to the alveolar (A549) and bronchial (BEAS2-B) cell lines compared with the nasal (RPMI-2650) and monocyte (J774A.1) cell lines. Interestingly, this models the descent of the respiratory tract from lowest adherence to the nasal epithelium (RPMI-2650) to the highest adherence to the alveolar epithelium (A549). The main difference between the cell lines occurred in the number of bacteria that attached over the first 30 min, which was approximately 10-fold greater for the A549 and BEAS2-B cell lines. These differences in *Y. pestis* attachment probably reflect differences in the surface expression of receptors in these cell lines. Similarly, differences in *Pseudomonas aeruginosa* PAK strain adhesion to these cell lines have been reported (Hambrook *et al.*, 2004); however, no differences were observed in *L. pneumophila* attachment to these cell lines (Thomas & Brooks, 2004a). No doubt

variation in the expression of host-cell oligosaccharides and proteins would lead to differences in attachment *in vivo*.

Y. pestis attaches to oligosaccharides on the surface of human respiratory cell lines

A reduction in cell-surface oligosaccharides significantly decreases *S. pneumoniae* and *L. pneumophila* attachment to the alveolar (A549) cell line (Cundell & Tuomanen, 1994; Thomas & Brooks, 2004a). The requirement for oligosaccharides in the process of attachment to the respiratory cell lines was investigated by culture in the presence of tunicamycin to reduce the level of oligosaccharide expression at the cell surface (Fig. 2). At the concentration used, tunicamycin was not cytotoxic to any of the cell lines investigated (data not shown). Tunicamycin inhibits the

Fig. 2. Effect of tunicamycin treatment on the attachment of *Y. pestis* strain GB to the human respiratory epithelial (A549, BEAS2-B and RPMI-2650) and murine monocyte (J774A.1) cell lines. Bacteria were allowed to attach for 1 h. Results are the mean of five separate experiments ($\pm$ SD).

Fig. 1. Effect of cell density (a) and time (b) on the attachment of *Y. pestis* strain GB to human respiratory cell lines (A549, BEAS2-B and RPMI-2650) and a murine monocyte cell line (J774A.1).

biosynthesis of high-mannose and complex-type glycoproteins (Cundell & Tuomanen, 1994) and it has been demonstrated that *Y. pestis* adhesion to the alveolar (A549) cell line is reduced after tunicamycin treatment (Thomas & Brooks, 2004b). This study determined that tunicamycin treatment also reduced *Y. pestis* attachment to bronchial (BEAS2-B), nasal (RPMI-2650) and monocyte (J774A.1) cell lines, implicating the involvement of glycoconjugates in adhesion. In the three respiratory epithelial cell lines, the levels of attachment were reduced by similar amounts (~ 63 – 65%). Tunicamycin had a reduced effect in the J774A.1 murine monocyte cell line ($\sim 56\%$). However, this difference was not significant ($P=0.73$). Interestingly, *Y. pestis* attachment was not completely abolished, indicating that cell-surface structures other than those inhibited by tunicamycin are involved in attachment. This may indicate attachment to oligosaccharides that are not inhibited by tunicamycin or by protein–protein interactions.

Gangliosides are present on the surface of human respiratory epithelial cells

The surfaces of mammalian cells are covered with oligosaccharide structures in the form of glycolipids or glycoproteins. One of the major types of glycolipid present is gangliosides. In particular, it is known that cells of the respiratory tract are rich in the sialylated gangliosides GM1 and GM2 and their derivatives asialo-GM1 and asialo-GM2 (de Bentzmann *et al.*, 1996). The distribution of these gangliosides on the cell lines used in this study was investigated (Fig. 3). Each ganglioside was present to varying degrees on the surface of the cell lines under the growth conditions used with the exception of asialo-GM1, which was absent from the bronchial (BEAS2-B) cell line. The major ganglioside present on each cell line was GM1, although it was present in lower amounts on the bronchial (BEAS2-B) cell line. The

alveolar (A549) cell line had more than twice as much asialo-GM1 as the nasal (RPMI-2650) or monocyte (J774A.1) cell line. Plastic wells coated with pure gangliosides produced high fluorescence (170–200 fluorescence units) when bound by their corresponding antibodies. However, fluorescence was diminished to background levels (0.3–1.9 fluorescence units) when the non-specific anti-ganglioside antibodies were applied (data not shown). This indicated that the anti-ganglioside antibodies were not cross-reacting with the other gangliosides used in this study. However, it is possible that they may react against unknown glycolipids on the cell surfaces. These results highlighted the differential expression of oligosaccharide structures on cell lines mimicking descent of the respiratory tract.

Oligosaccharide inhibition of attachment to human respiratory epithelial cell lines

The increase in bacterial resistance over the past couple of decades has increased interest in novel antimicrobial therapies. The use of short-chain oligosaccharides to inhibit attachment has been proposed as a potential therapeutic strategy (Zopf & Roth, 1996; Sharon & Ofek, 2002; Ofek *et al.*, 2003). Pathogenic bacteria may bind to many different oligosaccharide structures located on a diverse range of tissues within the body. Previously, a range of oligosaccharides was demonstrated to inhibit attachment of *Y. pestis* and other respiratory pathogens to the alveolar (A549) cell line (Thomas & Brooks, 2004b). In this study, these oligosaccharides were assessed for their ability to inhibit *Y. pestis* attachment to other human respiratory cell lines and the J774A.1 murine monocyte cell line (Table 2). None of the compounds tested was cytotoxic towards the cell lines at the concentrations used in the assay, nor did they support *Y. pestis* growth over the 1 h of attachment during the assays (data not shown). Across the four cell lines, differences and similarities were observed in inhibition of attachment by the oligosaccharides. The polymeric saccharides (heparin, dextran and dextran sulphate) displayed similarities but also major differences in their ability to inhibit *Y. pestis* attachment to the cell lines. Heparin inhibited attachment to each cell line by ~ 40 – 50% . The generic nature of inhibition probably reflects the presence of the structural analogue heparan sulphate as a common constituent of the extracellular matrix in many tissues and cell lines. Thus, it is not unexpected that *Y. pestis* would express adhesins that attached to such moieties. Dextran and dextran sulphate displayed lower levels of inhibition in the nasal (RPMI-2650) and alveolar (A549) cell lines compared with the bronchial (BEAS2-B) and monocyte (J774A.1) cell lines. The difference observed between dextran and dextran sulphate inhibition indicates a role of charge in attachment to these cell lines. It has been postulated that polymeric saccharides act by coating the bacterial cells, blocking interaction of the adhesins with their receptors (Barghouthi *et al.*, 1996; Bryan *et al.*, 1999). The sulphated polysaccharides may offer additional therapeutic benefits; both heparin and dextran sulphate inhibit LcrG protein binding to HeLa

Fig. 3. Antibody detection of ganglioside expression on the surface of human respiratory cell lines. Results are the mean of three separate experiments ($\pm$ SD). Values were obtained by deducting the natural background fluorescence of the cells.

Table 2. Comparative inhibition of *Y. pestis* strain GB attachment to human respiratory epithelial cell lines by nine anti-adhesion compounds

Results are the mean of three separate experiments ($\pm$ SD). 0–20 %, No inhibition; 21–40 %, low inhibition; 41–59 %, moderate inhibition; 61–74 %, good inhibition; 75–100 %, high inhibition.

Anti-adhesion compound	Inhibition of adhesion to cell line (%)				
	RPMI-2650	BEAS2-B	A549	J774A.1	Mean
Heparin	40.9 $\pm$ 2.7	51.9 $\pm$ 5.4	43.3 $\pm$ 4.7	49.5 $\pm$ 2.7	46.4
Dextran	28.7 $\pm$ 2.6	56.0 $\pm$ 3.3	34.1 $\pm$ 2.2	47.2 $\pm$ 2.4	41.5
Dextran sulphate	25.7 $\pm$ 6.5	25.5 $\pm$ 4.5	30.3 $\pm$ 5.0	67.9 $\pm$ 2.8	37.4
Mannose	68.9 $\pm$ 5.2	50.4 $\pm$ 1.7	26.4 $\pm$ 3.2	60.5 $\pm$ 3.3	51.6
GalNAc β 1-3Gal	74.3 $\pm$ 9.4	44.8 $\pm$ 2.4	55.1 $\pm$ 2.1	44.1 $\pm$ 2.0	54.6
GalNAc β 1-4Gal	83.7 $\pm$ 2.3	46.7 $\pm$ 4.6	54.9 $\pm$ 1.3	53.0 $\pm$ 3.6	59.6
Gal β 1-3GlcNAc	74.7 $\pm$ 0.9	26.0 $\pm$ 12.7	39.9 $\pm$ 1.3	8.6 $\pm$ 6.2	37.3
Gal β 1-4GlcNAc	39.7 $\pm$ 16.1	43.3 $\pm$ 11.1	43.2 $\pm$ 3.7	19.5 $\pm$ 2.3	36.4
<i>p</i> -Nitrophenol	92.9 $\pm$ 2.5	94.0 $\pm$ 1.7	73.0 $\pm$ 3.8	73.9 $\pm$ 3.8	83.5

cells, effectively preventing the translocation of *Yersinia* outer proteins or Yops (Boyd *et al.*, 1998).

Mannose was a good inhibitor across each cell line with the exception of the alveolar (A549) cell line (26 %). The highest inhibition was observed with the nasal (RPMI-2650) and monocyte (J774A.1) cell lines (69 and 60.5 %, respectively), perhaps indicating a larger amount of mannosylated oligosaccharides on these cell lines. Compared with the other compounds, mannose was used at a much higher concentration (140 mM) in order to achieve comparable levels of inhibition. This indicates that mannosylated oligosaccharides are not used as a major receptor for *Y. pestis* attachment and therefore the potential of mannose as an anti-adhesion therapeutic was considered to be reduced. Hydrophobic interactions have been postulated as a strategy for initial non-specific attachment to host tissues before colonization by specific receptor–ligand interactions (Falkowski *et al.*, 1986; Rosenberg *et al.*, 1996; Doyle, 2000). The best inhibitor of attachment to each cell line was the hydrophobic compound *p*-nitrophenol, which probably acts by inhibition of hydrophobic interactions between the receptor and adhesin. Interference with these weak hydrophobic interactions could explain the observed generic inhibition of attachment. Particularly strong inhibition of attachment was observed with the nasal (RPMI-2650) and bronchial (BEAS2-B) cell lines. Indeed, hydrophobic interactions have been identified in adhesion to the ileal brush border membrane in the related bacterium *Yersinia enterocolitica* (Pærregaard *et al.*, 1991).

The β -galactosylated disaccharides Gal β 1-3GlcNAc and Gal β 1-4GlcNAc were fairly good attachment inhibitors in the alveolar (A549) cell line (40–43 %), but poor inhibitors of attachment to the monocyte (J774A.1) cell line (<20 %). The disaccharides acted differently with respect to the nasal (RPMI-2650) and bronchial (BEAS2-B) cell lines, with Gal β 1-3GlcNAc being a far better inhibitor in the nasal (RPMI-2650) cell line and Gal β 1-4GlcNAc being a better

inhibitor in the bronchial (BEAS2-B) cell line. This highlights the expression of different carbohydrate moieties on the surface of these cell lines. In contrast, the disaccharides GalNAc β 1-3Gal and GalNAc β 1-4Gal were good inhibitors for all four cell lines. Previous studies have demonstrated that these disaccharides inhibit the adhesion of *Y. pestis* and other respiratory pathogens (Krivan *et al.*, 1988; Thomas & Brooks, 2004b). The greatest inhibition of adhesion was noted in the nasal (RPMI-2650) cell line, correlating with antibody detection of asialo-GM1 and asialo-GM2, which may act as receptors for attachment (Fig. 3).

Relationship of oligosaccharide inhibition to *Y. pestis* adhesins

The differences in the oligosaccharide inhibitory profiles probably reflect differential expression of oligosaccharide receptors on the eukaryotic cell surfaces and perhaps modulation of adhesin expression on the bacterial surface in response to receptors expressed on a particular cell type or tissue. This will obviously affect the usefulness of oligosaccharides as therapeutics for *Y. pestis* attachment during pneumonic disease. It has been demonstrated that attachment could occur throughout the respiratory tract; however, the pathology of pneumonic disease generally occurs in the deep lung, involving the alveolar epithelium, alveolar macrophages and other leukocytes (Finegold, 1969). The surface-bound plasminogen activator (Pla) binds to glycolipids isolated from CaCo-2 and A498 cells rich in globoseries glycolipids such as globoside (GalNAc β 1-4Gal α 1-4Gal β 1-4Glc β 1-1 ceramide) (Kienle *et al.*, 1992). The ability of Pla to bind surface oligosaccharide structures and components of the extracellular matrix could localize and aid the spread of *Y. pestis* to certain pathogenic locations (Lähteenmaki *et al.*, 2001). The pH6 antigen (PsaA) has been shown to bind Gal β 1 structures present in asialo-GM1 and asialo-GM2. Interestingly, the asialo-GM1 and asialo-GM2 gangliosides contain the GalNAc β 1-4Gal and GalNAc β 1-3Gal moieties (Payne *et al.*, 1998). Given the presence of

GalNAc β 1-4Gal moieties on the respiratory epithelial cells (Fig. 3), it is possible that these proteins may be important in the pathogenesis of pneumonic plague. Both PsaA and Pla are possible candidate adhesins promoting attachment to the respiratory epithelium. However, it is evident from the data that no single compound completely prevents *Y. pestis* attachment to the respiratory epithelium. Only *p*-nitrophenol (84 %), GalNAc β 1-3Gal (55 %), GalNAc β 1-4Gal (60 %) and heparin (46 %) displayed appreciable anti-adhesion activity in each cell line at fairly low concentrations. This is not unexpected; bacteria are known to express multiple adhesins involved in the colonization of host tissues (Sharon & Ofek, 2002; Ofek *et al.*, 2003). *Y. pestis* is unlikely to be an exception given the range of hosts (flea, rat, marmot, man, etc.) and tissues infected. A number of cell-wall components have been implicated as adhesins, including LPS, capsule, Pla and PsaA (Kienle *et al.*, 1992; Straley, 1993; Lahteenmaki *et al.*, 1998; Payne *et al.*, 1998). Indeed, low binding of *Y. pestis* was noted for collagen (I, IV, V), fibronectin and fetuin, but could not be linked to Pla (Lahteenmaki *et al.*, 1998). Two putative adhesins with high homology to the HecA adhesin of *Erwinia* spp. and the filamentous haemagglutinin of *Bordetella pertussis* have been identified in the genome (Rojas *et al.*, 2002). This confirms suspicions of the presence of multiple adhesins in *Y. pestis* mediating attachment to tissues and raises the likelihood of receptor redundancy. However, the specific roles in the pathogenesis of pneumonic disease are uncertain.

This study is the first to our knowledge that investigates the attachment of *Y. pestis* to different epithelial cell types of the respiratory tract and the ability of oligosaccharides to inhibit adhesion. It is evident that for such an approach to be successful, detailed knowledge of the adhesin(s) with which the saccharide interacts is required. The adhesins chosen would need to be important for colonization of the respiratory tissues during the progression of pneumonic plague. Candidates investigated in this study that showed promise included *p*-nitrophenol, GalNAc β 1-3Gal, GalNAc β 1-4Gal and heparin. These compounds are also known to inhibit a wide range of other respiratory pathogens (Krivan *et al.*, 1988; Thomas & Brooks, 2004b).

ACKNOWLEDGEMENTS

R. T. and T. B. recognize the contribution of Ministry of Defence funding for this work. Thank you to Professor Rick Titball and Dr Andrew Simpson for critical review of the manuscript.

REFERENCES

- Barghouthi, S., Guerdoud, L. M. & Speert, D. P. (1996).** Inhibition by dextran of *Pseudomonas aeruginosa* adherence to epithelial cells. *Am J Respir Crit Care Med* **154**, 1788–1793.
- Boyd, A. P., Sory, M.-P., Iriarte, M. & Cornelis, G. R. (1998).** Heparin interferes with translocation of Yop proteins into HeLa cells and binds LcrG, a regulatory component of the *Yersinia* Yop apparatus. *Mol Microbiol* **27**, 425–436.

- Bryan, R., Feldman, M., Jawetz, S. C., Rajan, S., DiMango, E., Tang, H. B., Scheffler, L., Speert, D. P. & Prince, A. (1999). The effects of aerosolized dextran in a mouse model of *Pseudomonas aeruginosa* pulmonary infection. *J Infect Dis* **179**, 1449–1458.
- Byrne, W. R., Welkos, S. L., Pitt, M. L. & 7 other authors (1998). Antibiotic treatment of experimental pneumonic plague in mice. *Antimicrob Agents Chem* **42**, 675–681.
- Cundell, D. R. & Tuomanen, E. I. (1994). Receptor specificity of adherence of *Streptococcus pneumoniae* to human type-II pneumocytes and vascular endothelial cells *in vitro*. *Microb Pathog* **17**, 361–374.
- de Bentzmann, S., Plotkowski, C. & Puchelle, E. (1996). Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. *Am J Respir Crit Care Med* **154**, S155–S162.
- Doyle, R. J. (2000). Contribution of the hydrophobic effect to microbial infection. *Microbes Infect* **2**, 391–400.
- Falkowski, W., Edwards, M. & Schaeffer, A. J. (1986). Inhibitory effect of substituted aromatic hydrocarbons on adherence of *Escherichia coli* to human epithelial cells. *Infect Immun* **52**, 863–866.
- Finegold, M. J. (1969). Pneumonic plague in monkeys. An electron microscopic study. *Am J Pathol* **54**, 167–185.
- Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Carniel, E. & Courvalin, P. (1997). Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. *N Engl J Med* **337**, 677–680.
- Hambrook, J., Titball, R. & Lindsay, C. (2004). The interaction of *Pseudomonas aeruginosa* PAK with human and animal respiratory tract cell lines. *FEMS Microbiol Lett* **238**, 49–55.
- Idänpää-Heikkilä, I., Simon, P. M., Zopf, M., Vullo, T., Cahill, P., Sokol, K. & Tuomanen, E. (1997). Oligosaccharides interfere with the establishment and progression of experimental pneumococcal pneumonia. *J Infect Dis* **176**, 704–712.
- Inglesby, T. V., Dennis, D. T., Henderson, D. A. & 16 other authors (2000). Plague as a biological weapon: medical and public health management. *JAMA* **283**, 2281–2290.
- Kienle, Z., Emody, L., Svanborg, C. & O'Toole, P. W. (1992). Adhesive properties conferred by the plasminogen activator of *Yersinia pestis*. *J Gen Microbiol* **138**, 1679–1687.
- Krishna, K. & Chitkara, R. K. (2003). Pneumonic plague. *Semin Respir Infect* **18**, 159–167.
- Krivan, H. C., Roberts, D. D. & Ginsburg, V. (1988). Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc β 1-4Gal found in some glycolipids. *Proc Natl Acad Sci U S A* **85**, 6157–6161.
- Lähteenmäki, K., Virkola, R., Sarén, A., Emödy, L. & Korhonen, T. K. (1998). Expression of plasminogen activator Pla of *Yersinia pestis* enhances bacterial attachment to the mammalian extracellular matrix. *Infect Immun* **66**, 5755–5762.
- Lähteenmäki, K., Kuusela, P. & Korhonen, T. K. (2001). Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* **25**, 531–552.
- Lindler, L. E. & Tall, B. D. (1993). *Yersinia pestis* pH6 antigen forms fimbriae and is induced by intracellular association with macrophages. *Mol Microbiol* **8**, 311–324.
- Lindler, L. E., Klempner, M. S. & Straley, S. C. (1990). *Yersinia pestis* pH6 antigen: genetic, biochemical, and virulence characterization of a protein involved in the pathogenesis of bubonic plague. *Infect Immun* **58**, 2569–2577.
- Ofek, I., Hasty, D. L. & Doyle, R. J. (2003). Antiadhesion therapy. In *Bacterial Adhesion to Animal Cells and Tissues*, pp. 157–177. Edited by I. Ofek, D. L. Hasty & R. J. Doyle. Washington, DC: American Society for Microbiology.

- Pærregaard, A., Espersen, F., Jensen, O. M. & Skurnik, M. (1991).** Interactions between *Yersinia enterocolitica* and rabbit ileal mucus: growth, adhesion, penetration, and subsequent changes in surface hydrophobicity and ability to adhere to ileal brush border membrane vesicles. *Infect Immun* **59**, 253–260.
- Payne, D., Tatham, D., Williamson, E. D. & Titball, R. W. (1998).** The pH6 antigen of *Yersinia pestis* binds to β 1-linked galactosyl residues in glycosphingolipids. *Infect Immun* **66**, 4545–4548.
- Perry, R. D. & Fetherston, J. D. (1997).** *Yersinia pestis* – etiologic agent of plague. *Clin Microbiol Rev* **10**, 35–66.
- Putzker, M., Sauer, H. & Sobe, D. (2001).** Plague and other human infections caused by *Yersinia* species. *Clin Lab* **47**, 453–466.
- Rojas, C. M., Ham, J. H., Deng, W.-L., Doyle, J. J. & Collmer, A. (2002).** HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana clevelandii* seedlings. *Proc Natl Acad Sci U S A* **99**, 13142–13147.
- Rosenberg, M., Greenstein, R., Barki, M. & Goldberg, S. (1996).** Hydrophobic interactions as a basis for interfering in microbial adhesion. In *Toward Anti-adhesion Therapy for Microbial Disease*, pp. 241–247. Edited by I. Kahane & I. Ofek. New York: Plenum Press.
- Russell, P., Eley, S. M., Hibbs, S. E., Manchee, R. J., Stagg, A. J. & Titball, R. W. (1995).** A comparison of plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model. *Vaccine* **13**, 1551–1556.
- Russell, P., Eley, S. M., Green, M. & 8 other authors (1998).** Efficacy of doxycycline and ciprofloxacin against experimental *Yersinia pestis* infection. *J Antimicrob Chemother* **41**, 301–305.
- Sharon, N. & Ofek, I. (2002).** Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. *Crit Rev Food Sci Nutr* **42** (Suppl.), 267–272.
- Steward, J., Lever, M. S., Russell, P., Beedham, R. J., Stagg, A. J., Taylor, R. R. & Brooks, T. J. G. (2004).** Efficacy of the latest fluoroquinolones against experimental *Yersinia pestis*. *Int J Antimicrob Agents* **24**, 609–612.
- Straley, S. C. (1993).** Adhesins in *Yersinia pestis*. *Trends Microbiol* **1**, 285–286.
- Thomas, R. J. & Brooks, T. J. (2004a).** Oligosaccharide receptor mimics inhibit *Legionella pneumophila* attachment to human respiratory epithelial cells. *Microb Pathog* **36**, 83–92.
- Thomas, R. & Brooks, T. (2004b).** Common oligosaccharide moieties inhibit the adherence of typical and atypical respiratory pathogens. *J Med Microbiol* **53**, 833–840.
- Wong, J. D., Barash, J. R., Sandfort, R. F. & Janda, J. M. (2000).** Susceptibilities of *Yersinia pestis* strains to 12 antimicrobial agents. *Antimicrob Agents Chemother* **44**, 1995–1996.
- Zopf, D. & Roth, S. (1996).** Oligosaccharide anti-infective agents. *Lancet* **347**, 1017–1021.