ORIGINAL ARTICLE

Haemophilus parainfluenzae infection of respiratory mucosa

A.M. MIDDLETON*, R.B. DOWLING*, J.L. MITCHELL†, S. WATANABE*, A. RUTMAN*, K. PRITCHARD*, G. TILLOTSON‡, S.L. HILL§ AND R. WILSON*.

*Host Defence Unit, Royal Brompton Hospital, Sydney Street, London U.K., †Micropathology, University of Warwick Science Park, Coventry, U.K., ‡Public Health Research Institute, New York City, NY, U.S.A. and §Department of Respiratory Medicine, Queen Elizabeth Hospital, Birmingham, U.K.

Abstract

The pathogenicity of *Haemophilus parainfluenzae* (*Hpi*) in the respiratory tract is unclear, in contrast to the accepted pathogenicity of its close relative non-typable *H. influenzae*. We have investigated the interaction of two *Hpi* isolates with the mucosa of adenoid and bronchial tissue organ cultures. The adherence of bacteria to the mucosa of organ cultures, the effect of broth culture filtrates on human nasal epithelium, and interleukin (IL)-8 production by A549 cell cultures was investigated. *Hpi* 4846 adhered infrequently in clusters of pleomorphic cocco-bacilli to areas of epithelial damage, mucus and unciliated cells in adenoid organ culture experiments at 24 h, but not bronchial mucosa. *Hpi* 3698 was seen in only one adenoid and no bronchial organ cultures at 24 h. In separate experiments, *Hpi* 3698 was cleared more rapidly from the centre of the adenoid organ culture and was not cultured at 24 h. Although not adhering to the mucosa at 24 h, *Hpi* 3698, but not *Hpi* 4846, caused an increase in the amount of epithelial damage in both types of organ culture. Broth culture filtrates of both strains caused immediate slowing of ciliary beat frequency that progressed, and disrupted epithelial integrity. Dialysed culture filtrates of both strains stimulated IL-8 production by A549 cells, with the culture filtrate of *Hpi* 3698 being most potent. We conclude that two strains of *Hpi* varied in their adherence to adenoid tissue, and neither adhered to bronchial tissue. These results lead us to speculate that *Hpi* is only likely to be a pathogen in the lower respiratory tract when impaired airway defences delay bacterial clearance.

doi: 10.1053.rmed.2002.1454, available online at http://www.sciencedirect.com

Keywords *Haemophilus parainfluenzae*; bacterial adherence; organ culture; interleukin-8

INTRODUCTION

The role of *Haemophilus parainfluenzae* (*Hpi*) as a respiratory pathogen is unclear, in contrast to the accepted pathogenicity of its close relative non-typable *H. influenzae* (1–3). The potential of *Hpi* to be a pathogen is demonstrated by occasional reports of invasive diseases such as pneumonia, endocarditis, septic arthritis and bacteraemia (4–6). *Hpi* is quite commonly isolated from the sputum of patients with chronic obstructive pulmonary disease (COPD) and bronchiectasis, but there is disagreement about whether isolation of *Hpi* from sputum should be regarded as an infection that requires treatment (7–11). For example, it has been demonstrated that the presence of *Hpi* in sputum of COPD patients does not correlate with a rise in antibody titre (10) and that biotypes of *Hpi* cultured from patients with chronic bronchitis were no different to those isolated from healthy individuals (11). The conclusion from these studies was that *Hpi* represents part of the normal flora. However, more recent studies have suggested that *Hpi* might be regarded as a pathogen. In some cases, *Hpi* was isolated more frequently from patients with moderate-to-severe COPD during an exacerbation compared to their stable state (8) and *Hpi* has occasionally been identified as the causative organism in pneumonia (12).

We have previously studied the effect of *H. influenzae* culture filtrates on respiratory epithelium demonstrating slowed ciliary beat frequency (CBF) and disrupted

Received 19 February 2002, accepted in revised form 27 August 2002
Correspondence should be addressed to: Dr R. Wilson, MD FRCP, Consultant Physician, Royal Brompton Hospital, Sydney Street, London SW3 6NP, U.K.
Fax: +44 (0)207 351 8338; E-mail: r.wilson@rbh.nthames.nhs.uk

epithelial integrity (13). In the same organ culture system used in the present paper, *H. influenzae* adhered in large numbers to the respiratory mucosa, in particular to mucus, areas of epithelial damage and normal unciliated cells. After 24 h, *H. influenzae* caused increased damaged of the epithelium compared to controls (14). It has also been shown that endotoxin purified from *H. influenzae* stimulates the secretion of several pro-inflammatory cytokines including interleukin (IL)-8 by cultured human bronchial epithelial cells (15). IL-8 is an important neutrophil chemoattractant, and it is accepted that damage to normal bystander lung tissue occurs during chronic bacterial infection that is mediated via proteinases and oxidant species released by neutrophils (7, 16,17).

In the present study, we have investigated the pathogenic potential of *Hpi* by studying the interaction of two clinical isolates of *Hpi* with upper and lower respiratory tract tissue organ cultures; and the effect of culture filtrates of the two isolates on CBF of human nasal epithelial cells and IL-8 production by an epithelial cell line.

MATERIALS AND METHODS

Bacteria

Two clinical isolates of *Hpi* were used in experiments. Isolate *Hpi* 3698 was obtained from the sputum of a patient with bronchiectasis at the end of a 2-week course of an oral antibiotic cefuroxime despite symptomatic improvement. Isolate *Hpi* 4846 was isolated from the sputum of an adult patient with cystic fibrosis. *Pseudomonas aeruginosa* was also isolated from this patient. The *Hpi* were identified by colonial morphology, X factor (Haemin) independence, V factor (NAD) dependence, inability to haemolyse blood agar, and fermentation and enzyme reactions included in the API test strip system (BioMerieux UK Ltd, Basingstoke, U.K.). The purity of cultures used in experiments was checked by Gram stain and culture on chocolate agar plates at 37°C in 5%CO_2.

Brain–heart infusion broth (75 ml) supplemented with NAD (10 μg/ml) was inoculated with 3 ml of an overnight broth culture of *Hpi*. The broth was incubated overnight at 37°C with shaking. Serial dilutions on blood agar showed that this provided a pure culture of approximately 10^8 colony-forming units (cfu) per ml. The estimation of bacteria numbers by measuring the optical density of the broth was found to be difficult with these strains because of clumping. Bacteria were harvested by centrifugation, washed ($\times$ 2) in phosphate-buffered saline (PBS), and resuspended in PBS for organ culture experiments. The purity of each strain was checked again. Supernatants of 24 h shaking broth cultures were passed through a 0.2 μm filter to provide a sterile culture filtrate, which was aliquoted and stored at −20°C until required for CBF experiments. For IL-8 experiments, 38 ml of sterile culture filtrate was dialysed overnight against 5 l of PBS at room temperature.

Effect of bacterial culture filtrates on CBF

Strips of normal human nasal epithelium were obtained from the inferior turbinate of volunteers who had been free of symptoms of respiratory infection for at least 4 weeks (18). A single different volunteer provided epithelium for each experiment. Strips were dispersed in 2 ml of medium 199 cell culture fluid (Flow Laboratories Inc., McLean, VA, U.S.A.). The cell suspension was mixed 1:1 with culture filtrate or broth for control experiments and sealed microscope coverslip-slide preparations were constructed. Each culture filtrate was tested on six separate occasions with different epithelium. Six to ten strips of epithelium were identified in the slide during 15 min in which the slides were allowed to equilibrate at 37°C. CBF, pattern of ciliary beat and epithelial integrity were measured at time 0 and at hourly intervals for 4 h as previously described, no more than two recordings at each timepoint being made from a single strip of epithelium (13,16,19).

Organ culture experiments

Construction of the organ cultures has been described previously (20–23). Briefly, human adenoid tissue or bronchial tissue resected from patients undergoing surgery was transported to the laboratory in minimal essential medium (Gibco, Paisley, U.K.) containing antibiotics to remove commensal bacteria. Tissue was used with the patients consent and permission for the work was granted by the Royal Brompton Hospital Ethics Committee. Tissue from one resection specimen was used in each experiment. Dissection was performed to yield squares of tissue approximately 9 mm^2 in area and 2–3 mm thick, followed by immersion in non-antibiotic-containing medium for at least 1 h to remove antibiotics. The absence of residual antibiotics in the tissue was confirmed by placing an organ culture on a chocolate agar plate onto which *Hpi* had been seeded and incubating the plate overnight. No zone of inhibition was observed. The amount of bronchial tissue available was limited, so only one control organ culture was used in this series. Each strain was tested on six separate occasions with different tissue.

In each experiment, 2 μl of washed bacteria suspended in PBS were gently pipetted onto the surface of the organ culture. Control organ cultures were inoculated with 2 μl of PBS alone. Organ cultures were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 24 h. After incubation, the four edges of the organ cultures were touched gently with a sterile loop and plated onto chocolate agar to assess the sterility of the control

and the purity of the *Hpi*. In three separate experiments using strain *Hpi* 3698, samples were taken with a sterile loop from the four edges and centre of adenoid organ cultures at regular intervals to determine the level of infection.

Tissue was fixed and processed for scanning electron microscopy (SEM) as previously described (20–23). All specimens were coded and randomised prior to analysis using a Hitachi S400 SEM (Katsuta-shi, Ibaraki-Ken, Japan) by the same observer. Morphological assessment of the mucosal coverage of mucus, the amount of damaged, ciliated and unciliated epithelium was made for a representative area of the mucosal surface measuring $1.42 \times 10^4\ \mu m^2$. The total number of bacteria adhering to an organ culture was also calculated. Both methods have previously been described (20). Bacterial adherence was defined as physical contact between bacterium and organ culture seen by electron microscopy, which had persisted after washing of the organ culture.

H. parainfluenzae stimulation of Il-8 production by epithelial cells

A549 cells (human respiratory epithelium; European Collection of Animal Cell Cultures) were grown to confluence in 24 well plates in 500 μl media consisting of Dulbecco's Modified Eagles Medium plus 4 mM glutamine mixed 1 : 1 with Ham's F10 nutrient mixture plus 1 mM glutamine (Sigma-Aldrich, Poole, Dorset, U.K.) containing 10% foetal calf serum at 37°C in 5% CO_2. Once confluent, the culture supernatant was aspirated and the cells were washed with PBS, which was aspirated and replaced by serum-free culture medium. The next day, 100 μl of dialysed culture filtrate or dialysed broth alone was added to the cells to make a total volume of 600 μl. The medium was sampled at 24 and 48 h. Each culture filtrate was tested on six separate occasions.

After 48 h, the A549 cell layer was trypsinised (1 × trypsin—EDTA solution, Sigma-Aldrich) and viable counts were performed using the trypan blue exclusion method. Concentrations of IL-8 in cell culture supernatant were measured using a commercially available ELISA kit (R&D systems, Abingdon, Oxon, U.K.). Baseline concentrations were determined by measurement of IL-8 in the aliquot of cell culture supernatant removed at 0 h.

Statistics

Values are described as mean ± standard error. Comparisons of the mean percentage surface area occupied by each of the four mucosal features in the organ culture model were made with the Mann–Whitney *U* test. Comparisons of bacterial densities adherent to each mucosal feature and of total bacterial numbers adherent to the respiratory mucosa were made with Wilcoxon's matched signed-ranks test. CBF and the results of the IL-8 assay were compared using the Student's *t*-test for unpaired data.

RESULTS

Bacteria

Bacterial viable counts (cfu's) used to produce culture filtrates for CBF experiments were: 1.25×10^8 cfu/ml (*Hpi* 4846), 1.20×10^8 cfu/ml (*Hpi* 3698); for organ culture experiments in 2 μl PBS were: adenoid 6.7×10^5 (*Hpi* 4846), 1.6×10^6 (*Hpi* 3698), bronchial 1.3×10^7 (*Hpi* 4846), 2.6×10^7 (*Hpi* 3698); for IL-8 culture filtrate experiments both strains were 1.0×10^8 cfu/ml.

Ciliary beat frequency

CBF results are shown in Table 1. Culture filtrates from both strains caused immediate slowing of CBF ($P < 0.05$). There was further slowing of ciliary beat during the 4 h experiment, and at all timepoints the test sample was significantly slower than control values that remained constant. Ciliary dyskinesia was noted in some epithelial strips for the first time at 1 h. There was stasis of some cilia and disruption of the integrity of the epithelium in some strips at 2 h. These changes became more

Table 1. Effect of *H. parainfluenzae* broth culture filtrates on CBF of nasal epithelium

	Time (h)				
Experiment	0	1	2	3	4
Control	13.3 ± 0.8	13.6 ± 0.8	13.4 ± 0.8	13.3 ± 0.8	13.3 ± 0.8
Strain 3698*	10.4 ± 1.4	10.3 ± 1.4	9.6 ± 1.2	9.2 ± 1.1	9.2 ± 1.0
Control	14.2 ± 0.5	14.5 ± 0.6	14.2 ± 0.7	14.2 ± 0.7	14.1 ± 0.8
Strain 4846*	9.6 ± 0.5	9.5 ± 0.5	9.6 ± 0.5	8.8 ± 0.5	8.3 ± 0.4

Results are CBF (Hz) mean ± standard error (n=6).
*The culture filtrate caused significant ($P < 0.05$) ciliary beat slowing compared to control at every timepoint.

Table 2. Effect of *H. parainfluenzae* infection on the respiratory mucosa of adenoid organ cultures

	Mucosal feature			
Experiment	Mucus	Damaged epithelium	Ciliated cells	Unciliated cells
Control	23.5 ± 9.7	33.1 ± 3.7	5.3 ± 2.2	38.1 ± 8.5
Strain 3698	12.5 ± 1.9	79.6 ± 6.4*	0.1 ± 0.1*	7.8 ± 5.7*
Control	6.0 ± 1.3	9.2 ± 5.1	6.1 ± 2.1	78.7 ± 6.9
Strain 4846	9.3 ± 2.8	24.7 ± 11.7	15.0 ± 6.8*	51.0 ± 9.9*

Results are per cent of the organ culture surface represented by each mucosal feature mean ± standard error (n=6).
*$P < 0.05$ *vs.* control.

prominent at the later timepoints. There was no difference between the effects of the two culture filtrates.

Organ culture experiments

The effect of the two strains on the respiratory mucosa of adenoid tissue is shown in Table 2. There was a difference in the amount of mucosal damage seen in control organ cultures in the two series. The adenoid tissue used in experiments with *Hpi* 4846 was unusually healthy, and that used with *Hpi* 3698 was more in keeping with our usual experience (14). *Hpi* 3698 caused a significant increase in mucosal damage, and a significant reduction in the number of ciliated and unciliated cells. Although there was a trend towards increased mucosal damage with *Hpi* 4846 this was not significant. *Hpi* 4846 caused a significant reduction in the number of unciliated cells, but there were more ciliated cells on the tissue inoculated with bacteria.

Hpi 3698 was only seen in one of six adenoid organ culture experiments at 24 h. There was a single cluster of bacteria overlying an area of epithelial damage. *Hpi* 4846 was seen adhering to epithelial damage, mucus and unciliated cells in all six experiments, but never to ciliated cells. Bacterial density was greatest in areas of epithelial damage (48.1 ± 31.5 bacteria adhering per unit area) and fewer bacteria adhered to unciliated cells (7.7 ± 4.7) and mucus (2.9 ± 2.7). The mean total number of bacteria adhering to the area surveyed in each organ culture was 671 ± 296. *Hpi* 4846 adhered to the mucosa in clusters, and individual or small numbers of bacteria adhering to a mucosal feature were rarely seen (Fig. 1). The clusters were made up of pleomorphic cocco-bacilli, and the majority of bacteria were mainly seen to be adherent to each other rather than to the mucosal feature. Strands were seen between bacteria, but the nature of these are unknown (Fig. 2).

Three further experiments were carried out with *Hpi* 3698 in which sterile loops were touched to the centre and four edges of an adenoid organ culture then streaked onto chocolate agar separately at 15 min intervals during the first hour and then at hourly intervals for 8 h and again at 24 h. In each experiment, a heavy growth (3+) was found in both the centre and edges of the organ culture at 0 h, but by 45 min had reduced to 1+ at both sites. Bacteria could be isolated for longer at the edges, compared to the centre, so that there were no bacteria

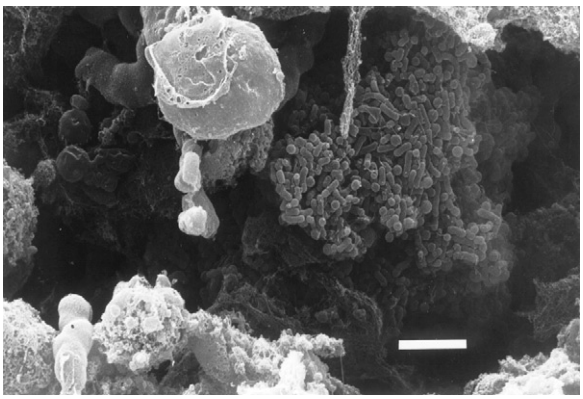

Fig. 1. A cluster of *H. parainfluenzae* strain 4846 is seen in an area of epithelial damage. Magnification × 4,300. Bar = 4 μm.

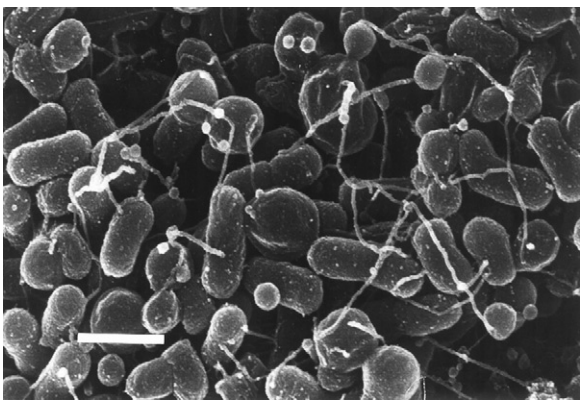

Fig. 2. Higher power of the bacterial cluster shown in Fig. 1. The cluster contains pleomorphic cocco-bacilli. The strands of material seen between bacteria were a consistent finding, but their origin was not determined. Magnification × 21 500. Bar = 1 μm.

cultured from the centre at 6, 6 and 2 h in the three experiments (and at subsequent timepoints), compared to no bacteria cultured at 24, 24 and 6 h, respectively at the edges. These results suggest that bacteria were being cleared to the edges of the organ cultures probably by ciliary beat.

The results of experiments with bronchial tissue are shown in Table 3. In these experiments, neither strain was seen on the mucosa at 24 h, but despite this *Hpi* 3698 caused a significant ($P < 0.05$) increase in mucosal damage. There was a similar trend with *Hpi* 4846 but this was not significant.

The effect of *H. parainfluenzae* on Il-8 production by epithelial cells.

Culture filtrates from both isolates caused increased production of IL-8 by A549 cells (Fig. 3). The culture filtrates from *Hpi* 3698 induced more than three times as much IL-8 production as *Hpi* 4846 at 24 h ($P < 0.01$) and twice as much at 48 h ($P < 0.01$). Neither of the culture filtrates had any effect on viability of A549 cells over 48 h since mean cell viability was more than 95% at both timepoints.

DISCUSSION

Hpi is quite commonly isolated from the sputum of patients with COPD and bronchiectasis (7–11), but has often been regarded as an upper respiratory tract contaminant. Bronchoscopy studies using the protected specimen brush method have shown that *Hpi* can be isolated from the lower respiratory tract in severe COPD (24), and a specific antibody response has been measured in the serum and sputum of patients infected with *H. parainfluenzae* (8,25,26). The presence of a specific antibody response, over and above those observed in healthy individuals, is often used as a marker of current or previous infection by a variety of infectious agents.

Compared to *H. influenzae,* the pathogenic potential of *Hpi* would appear to be less, which may correlate to variability in the composition of virulence factors such

Table 3. Effect of *H. parainfluenzae* infection on the respiratory mucosa of bronchial organ cultures

	Mucosal feature			
Tissue	Mucus	Damaged epithelium	Ciliated epithelium	Unciliated epithelium
Control	43.3 ± 7.8	26.8 ± 7.0	0.2 ± 0.2	29.7 ± 7.2
Strain 3698	25.0 ± 6.9	57.1 ± 7.9*	0.5 ± 0.3	17.4 ± 6.1
Strain 4846	8.0 ± 4.2	38.0 ± 7.7	4.3 ± 1.6	49.7 ± 7.4

Results are per cent of the organ culture surface represented by each mucosal feature mean ± standard error (n=6)
*$P < 0.05$ *vs.* control.

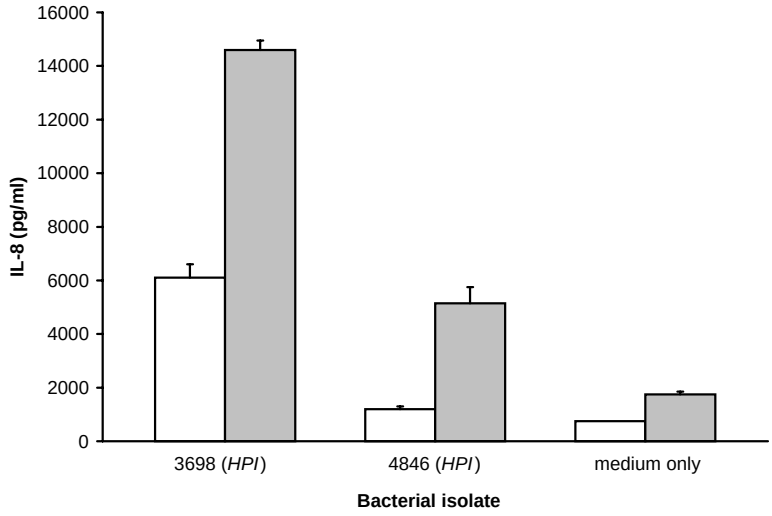

Fig. 3. IL-8 production by A549 cells exposed to dialysed bacterial culture filtrates or medium alone for 24 (clear columns) and 48 (filled columns) hours (n=6). *HPI = Haemophilus parainfluenzae.*

as lipopolysaccharide endotoxins (27–29). Tutau and Sims (28) demonstrated that *Hpi* produces approximately 10 000 times less free endotoxins than *H. influenzae* type b and is significantly less potent in producing experimental infections. However, they also concluded that *Hpi*, despite lacking the attributes of invasiveness and resistance to host defences of *H. influenzae*, was not devoid of pathogenic potential. *Hpi* possesses similar virulence genes to *H. influenzae*, for example the *tonB* gene, but it is not known if *Hpi*, like *H. influenzae*, expresses the protein product (30).

Only *Hpi* 4846 was seen adhering to adenoid organ cultures at 24 h and was associated with areas of epithelial damage more than unciliated epithelial cells and mucus. Most bacteria on the organ culture surface were present in clusters consisting of pleomorphic cocco-bacilli largely adherent to each other rather than to the underlying mucosal feature. *Hpi* 3698, was only seen in one adenoid organ culture out of six experiments, associated with an area of epithelial damage and further experiments showed that it was cleared from the mucosa by 24 h. Clearance from the centre of the organ culture and bacterial persistence at the edges of the organ culture suggests that the bacteria were cleared from the organ culture by ciliary beat rather than being killed by the tissue. The absence of any bacteria (dead or alive) on the mucosal surface assessed by SEM at 24 h confirmed this interpretation. Tissue was incubated with antibiotics prior to construction of the organ culture, but after rinsing the tissue there was no residual antibiotic activity since there was no zone of inhibition around tissue cultured overnight on agar with *Hpi*. Poor bacterial adherence to mucosal features would seem to be the most likely explanation of these results.

Neither of the *Hpi* strains were seen adhering to bronchial tissue organ cultures at 24 h yet there was an increase in the amount of damaged mucosa present at 24 h in both types of organ culture infected with *Hpi* 3698 compared to controls. There was a similar trend with *Hpi* 4846 but the changes were not significant. The type of damage most commonly seen was disruption of the integrity of the epithelial surface due to extrusion of both ciliated and unciliated epithelial cells. We have previously seen similar damage after inoculation of organ cultures by non-typable *H. influenzae* (14,31), but in these experiments bacteria were seen in large numbers. These results show that although bacteria were not seen adhering to the organ culture after 24 h, their presence prior to being cleared stimulated processes that led to the epithelium being damaged.

There was no correlation between the bacterial adherence and epithelial damage, which was greater after *Hpi* 3698 infection of both adenoid and bronchial organ cultures. It is also of interest that *Hpi* 4846 was adherent to only adenoid tissue, including apparently healthy unciliated cells. This result cannot be explained by lack of sites of damaged epithelium nor by efficient mucociliary clearance since the number of ciliated cells on bronchial tissue was low. These results suggest that different strains of *Hpi* vary in their adherence to respiratory tissue and that the availability of receptors for *Hpi* adherence may vary in different parts of the respiratory tract. Variation in epithelial cell glycoconjugates, a potential target for bacterial adherence, has been shown in different levels of the hamster bronchial tree (32). Bacterial adherence to the respiratory mucosa is considered a critical step in the process of infection (33) and our results suggest that both strains would have difficulty establishing infection in the bronchial tree of healthy subjects with intact mucociliary clearance.

The culture filtrates of both strains caused immediate slowing of CBF, which progressed during the experiment and was associated with disruption of epithelial integrity. Similar results with the culture filtrates of *H. influenzae* have previously been obtained (13), although other respiratory tract pathogens such as *Staphylococcus aureus* do not have this property (13). Culture filtrates of both strains stimulated IL-8 production by epithelial cells, although there was a difference between the two strains, which we speculate may be due to variability in the composition of their lipopolysaccharide endotoxins. *H. influenzae* endotoxin has been shown to stimulate IL-8 production by cultured human bronchial epithelial cells (15) and these results show that *Hpi* elicits a similar response.

Our results suggest that the cytokine response of the epithelial cells might be more important with regard to epithelial damage, rather than the direct effect of bacterial factors. The effect of the culture filtrates from the two isolates on CBF and the integrity of nasal epithelium was the same, and one might therefore have expected *Hpi* 4648, the strain that adhered to the adenoid organ culture, to cause more damage to the organ culture epithelium if direct toxicity of bacterial factors was most important. In fact, *Hpi* 4846 did not cause significant epithelial damage at 24 h and the slowing of CBF did not stop clearance of *Hpi* from the centre to the edge of the organ culture. The culture filtrate of *Hpi* 3698, the strain that caused epithelial damage, was more potent in stimulating IL-8 production by epithelial cells. The involvement of a cytokine in epithelial damage by a bacterial pathogen has been demonstrated for IL-1 and *Bordetella pertussis* (34), and a similar mechanism may operate for *Hpi*, and since bacterial factors can stimulate a cytokine response from epithelium, cell damage would not be dependent on bacterial adherence.

We conclude from our results that *Hpi* has the potential to cause disease in the lower respiratory tract. However, only one of the two strains investigated consistently persisted on adenoid organ cultures, and neither strain persisted on bronchial tissue organ cultures. We would speculate from these results that *Hpi* is

more likely to be a pathogen in the lower respiratory tract in patients with more severely impaired host defences, such as in patients with cystic fibrosis, bronchiectasis or severe COPD, in whom mucociliary clearance is impaired. In these circumstances, bacteria aspirated from the nasopharynx may remain in the bronchial tree for sufficient time to provoke an inflammatory response and cause mucosal damage. However, further work is required before this interpretation of our data can be accepted.

Acknowledgements

This study was funded by an educational grant from Bayer plc.

REFERENCES

1. Ball P, Tillotson G, Wilson R. Chemotherapy for chronic bronchitis. *Presse Med* **24:** 189–194.
2. Kilian M, Frederiksen W. Ecology of *Haemophilus*, *Pasteurella* and *Actinobacillus*. In: Kilian M, Frederiksen W, Biberstein EL (eds). *Haemophilus*, *Pasteurella, and Actinobacillus*. New York Academic Press, 1980: 11–37.
3. May JR. The bacteriology of chronic bronchitis. *Lancet* 1953; **ii**: 534–537.
4. Cooney TG, Harwood BR, Meisner DJ. *Haemophilus parainfluenzae* thoracic empyema. *Arch Intern Med* 1981; **141:** 940–941.
5. Oill PA, Chow AW, Guze LB. Adult bacteremic *Haemophilus parainfluenzae* infections. *Arch. Intern Med* 1979; **139:** 985–988.
6. Albritton WL. Infections due to *Haemophilus* species other than *Haemophilus influenzae*. *Ann Rev Microbiol* 1982; **36:** 199–215.
7. Cole PJ. Bronchiectasis. In: Brewis BAL, Corrin B, Geddes DM, Gibson GT (ed). *Respiratory Medicine,* vol 2, 2nd edn. London, United Kingdom: W. B. Saunders Co. Ltd., 1995: 1286–1316.
8. Hill SL, Pye A, Johnson MM, Munday C, Stockley RA. A role for *Haemophilus parainfluenzae* in chronic lung disease. *Am J Respir Crit Care Med* 1997; **155:** A105.
9. Rhind GB, Gould GA, Ahmad F, Croughan MJ, Calder MA. *Haemophilus parainfluenzae* and *H. influenzae* respiratory infections: comparison of clinical features. *Br Med J* 1985; **42:** 772–778.
10. Smith CB, Kanner RE, Golden CA, Renzetti AD. *Haemophilus influenzae* and *Haemophilus parainfluenzae* in chronic obstructive pulmonary disease. *Lancet* 1976; **I:** 1253–1255.
11. Taylor DC, Cripps AW, Clancy RL, Murree-Allen K, Hensley MJ, Saunders NA, Sutherland DC. Biotypes of *Haemophilus parainfluenzae* from the respiratory secretions in chronic bronchitis. *J Med Microbiol* 1992; **36:** 279–282.
12. Pillai A, Mitchell JL, Hill SL, Stockley RA. A case of *Haemophilus parainfluenzae* pneumonia. *Thorax* 2000; 55: 623–624.
13. Wilson R, Roberts D, Cole PJ. Effect of bacterial products on human ciliary function *in vitro*. *Thorax* 1985; **40:** 125–131.
14. Rutman J, Dowling RB, Wills P, Feldman C, Cole PJ, Wilson R. Effect of dirithromycin on *Haemophilus influenzae* infection of the respiratory mucosa. *Antimicrob Agents Chemother* 1998; **42:** 772–778.
15. Khair OA, Devalia JL, Abdelazia MM, Sapsford RJ, Davies RJ. Effect of erythromycin on *Haemophilus influenzae* endotoxin-induced release of IL-6, IL-8 and sICAM-1 by cultured human bronchial epithelial cells. *Eur Respir J* 1995; **8:** 1451–1457.
16. Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D, Stockley RA, Cole PJ. Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol* 1991; **4:** 26–32.
17. Murphy TF, Sethi S. Bacterial infection in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1992; **146:** 1067–1083.
18. Rutland J, Cole PJ. Non-invasive sampling of nasal cilia for measurement of beat frequency and study of ultrastructure. *Lancet* 1980; **2:** 564–565.
19. Steinfort C, Wilson R, Mitchell T, Feldman C, Rutman A, Todd H, Sykes D, Walker J, Saunders K, Andrew PW, Boulnois GJ, Cole PJ. Effect of *Streptococcus pneumoniae* on human respiratory epithelium in vitro. *Infect Immun* 1989; 57: 2006–2013.
20. Dowling RB, Rayner CFJ, Rutman A, Jackson AD, Kanthakumar K, Dewar A, Taylor GW, Cole PJ, Johnson M, Wilson R. Effect of salmeterol on *Pseudomonas aeruginosa* infection of respiratory mucosa. *Am J Respir Crit Care Med* 1997; **155:** 327–336.
21. Jackson AD, Rayner CFJ, Dewar A, Cole PJ, Wilson R. A human respiratory-tissue organ culture incorporating an air-interface. *Am J Respir Crit Care Med* 1996; **153:** 1130–1135.
22. Rayner CFJ, Jackson AD, Rutman A, Dewar A, Mitchell TJ, Andrew PW, Cole PJ, Wilson R. Interaction of Pneumolysin-sufficient and -deficient isogenic variants of *Streptococcus pneumoniae* with human respiratory mucosa. *Infect Immun* 1995; **63:** 442–447.
23. Tsang KWT, Rutman A, Tanaka E, Lund V, Dewar A, Cole PJ, Wilson R. Interaction of *Pseudomonas aeruginosa* with human respiratory mucosa *in vitro*. *Eur Respir J* 1994; **7:** 1746–1753.
24. Fagon JY, Chastre JL, Trouillet J, *et al*. Characterization of distal bronchial microflora during acute exacerbations of chronic bronchitis; use of the protected specimen brush technique in 54 mechanically ventilated patients. *Am Rev Respir Dis* 1990; **142:** 1004–1008.
25. Suzuki S, Nakatoma Y, Odami S, Sato H, Gejyo F, Arakawa M. Circulating IgA, IgG and IgM class antibody against *Haemophilus parainfluenzae* antigens in patients with IgA nephropathy. *Clin Exp Immunol* 1997; **104:** 306–311.
26. Mitchell JL, Hill SL. Immune response to *Haemophilus parainfluenzae* in patients with chronic obstructive lung disease. *Clin Diagn Lab Immunol* 2000; **7:** 25–30.
27. Virji M, Weiser JN, Lindberg AA, Moxon ER. Anitigenic similarities in lipopolysaccharides of *Haemophilus* and *Neisseria* and expression of a digalactoside structure present on human cells. *Microb Pathog* 1990; **9:** 441–450.
28. Tutau JE, Sims W. Aspects of the pathogenicity of some oral and other haemophili. *J Med Microbiol* 1983; **16:** 467–475.
29. Matar GM, Chahwan R, Fuleitan N, Uwaydah M. PCR-based detection, restriction endonuclease analysis, and transcription of *tonB* in *Haemophilus influenzae* and *Haemophilus parainfluenzae* isolates obtained from children undergoing tonsillectomy and adenoidectomy. *Clin Diagn Lab Immunol* 2001; **8:** 221–224.
30. Roberts MC, Mintz CS, Morse SA. Characterization of *Haemophilus parainfluenzae* strains with low-M(r) or ladder-like lipopolysaccharides. *J Gen Microbiol* 1986; **132:** 611–616.
31. Dowling RB, Johnson M, Cole PJ, Wilson R. Effect of salmeterol on *Haemophilus influenzae* infection of respiratory mucosa *in vitro*. *Eur Resp J* 1998; **11:** 86–90.
32. Christensen TG, Breuer R, Lucey EC, Hornstra LJ, Stone PJ, Snider GL. Lectin cytochemistry reveals differences between hamster trachea and bronchus in the composition of epithelial surface glycoconjugates and in the response of secretory cells to neutrophil elastase. *Am J Respir Cell Mol Biol* 1990; **3:** 61–69.
33. Niederman MS. The pathogenesis of airway colonisation: lessons learned from the study of bacterial adherence. *Eur Respir J* 1994; **139:** 985–988.
34. Heiss LN, Moser SA, Unanue ER, Goldman WE. Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. *Infect Immun* 1993; **61:** 3123–3128.