

## Effects of *Bordetella pertussis* Infection on Human Respiratory Epithelium In Vivo and In Vitro

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*Bordetella pertussis* infection probably involves attachment to and destruction of ciliated epithelial cells, but most previous studies have used animal tissue. During an epidemic, nasal epithelial biopsy specimens of 15 children (aged 1 month to 3 1/2 years) with whooping cough were examined for ciliary beat frequency, percent ciliation of the epithelium, and ciliary and epithelial cell ultrastructure. In addition, the in vitro effects of filtrates from a 24-h broth culture and of tracheal cytotoxin derived from *B. pertussis* on human nasal tissue organ culture were measured. *B. pertussis* was cultured from nasal swabs from 12 children. The mean ciliary beat frequency of their nasal biopsy specimens, 11.3 Hz (range, 10.4 to 13.0 Hz) was similar to that found in biopsy specimens from 10 normal children (mean, 12.5 Hz; range, 11.8 to 13.5 Hz). The abnormalities of the epithelium observed in 14 of 15 patients were a reduction in the number of ciliated cells, an increase in the number of cells with sparse ciliation, an increase in the number of dead cells, and extrusion of cells from the epithelial surface. In vitro, neither culture filtrate nor tracheal cytotoxin had any acute effect on ciliary function, but culture filtrate and tracheal cytotoxin (1 and 5  $\mu$ M, respectively) caused extrusion of cells from the epithelial surface of turbinate tissue, loss of ciliated cells, an increased frequency of sparsely ciliated cells, and toxic changes in some cells. These changes were dose dependent and progressive, and between 36 and 90 h ciliary beating ceased. The observations made with patient tissue confirm that *B. pertussis* infection damages ciliated epithelium, and the in vitro experiments suggest that tracheal cytotoxin may be responsible for the abnormalities observed in vivo.

*Bordetella pertussis* is a bacterial respiratory tract pathogen and is the major cause of whooping cough (pertussis) in children. Earlier this century whooping cough was common, but use of the current inactivated whole-cell vaccine significantly reduced its incidence (5). More recently, in the United Kingdom, however, concern about adverse effects associated with the administration of this vaccine led to loss of public confidence in it, and reduced use of the vaccine was associated with several large epidemics. Although the clinical features of pertussis are well recognized (paroxysmal coughing and choking often culminating in vomiting or retching and cyanosis, apneic attacks, and long duration of illness), the pathogenesis of the disease is ill understood.

Almost 80 years ago, Mallory and Hornor (12) histologically characterized pertussis as a noninvasive bacterial colonization of ciliated cells in the respiratory epithelium. *B. pertussis* has been shown to adhere to epithelial cells, and filamentous hemagglutinin (a cell surface protein) has been proposed as the bacterial adhesin (17, 23, 24). However, the precise interaction between *B. pertussis* and the respiratory mucosa has not been described. For example, how the bacterium interacts with mucus, beating cilia (26), and epithelial cells is poorly understood. Organ culture studies using animal tissue suggest that late in the infection epithelial cell injury occurs, resulting in cessation of ciliary beating (2, 14, 15); at this stage the requirements for bacterial colonization may change (11).

*B. pertussis* produces a number of biologically diverse

toxins, including pertussis toxin (also known as lymphocytosis-promoting factor), extracytoplasmic adenylate cyclase, and heat-labile toxin (25). Until recently, none of the toxins described above produced the characteristic changes induced by *B. pertussis* infection in respiratory epithelium (2, 12, 14, 15). Tracheal cytotoxin was first characterized by Goldman and coworkers and is a 1,6-anhydromuramic acid-containing monomeric disaccharide-tetrapeptide of peptidoglycan (3, 6-9, 18) which can be purified from culture supernatants of *B. pertussis*. It causes progressive cytotoxic changes in ciliated cells of hamster tracheal organ cultures.

The aims of the present study were to examine the function and ultrastructure of respiratory epithelial tissue obtained from the upper respiratory tract of children with whooping cough and to investigate for the first time the effects of tracheal cytotoxin on normal human respiratory epithelial tissue in vitro.

### MATERIALS AND METHODS

**Patients.** In 1985 and 1986, during a pertussis epidemic, 15 children (nine boys and six girls aged 1 to 42 months) with whooping cough were investigated. All patients had a prolonged paroxysmal cough associated with whooping, vomiting, and/or apnea. Pertussis was confirmed by isolation of *B. pertussis* from a postnasal swab cultured on charcoal agar supplemented with 10% sheep blood and 40  $\mu$ g of cephalixin per ml (16) and/or by an increase in antibody titers to pertussis antigens (22). Ten normal children without recent history of respiratory infection (five boys and five girls, aged 1 to 28 months) acted as controls for ciliary beat frequency

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(CBF) studies, but sufficient tissue was not available for electron microscopy. Ten nonsmoking normal adults without recent history of respiratory infection acted as controls for ultrastructural studies of cilia and epithelium. Although these controls were not completely appropriate, we did not think it reasonable to repeat the biopsies of normal children.

**Nasal epithelial biopsy.** Upper respiratory tract epithelial tissue was obtained from the inferior turbinate of a nostril with a cytology brush by a technique described previously (19, 21, 27, 28) without local anesthesia. Informed written consent was obtained from a parent of each child, and this investigation was approved by the Ethical Committees of the Brompton Hospital and St. George's Hospital. The inferior turbinate was viewed directly with an auroscope, and a cytology brush was moved backward and then forward along the lateral or inferior border. The procedure was well tolerated, although it did provoke whooping in some patients. The strips of epithelium obtained were dislodged from the brush by brisk agitation in 2 ml of medium 199 cell culture fluid (Flow Laboratories, Irvine, Scotland).

**CBF.** Some of the epithelium (700  $\mu$ l) was transferred to a sealed microscope coverslip-slide preparation with a pipette, and the preparation was placed on an electrically controlled warm stage at 37°C and allowed to equilibrate for 15 min. CBF was measured by a previously described photometric technique (10, 19, 21, 27, 28). A single recording of CBF was made on between 10 and 20 strips of epithelium depending on the quality of the sample. Readings were not taken from small groups of cells or single cells, as previous experience with biopsies from normal persons has shown that such recordings are very variable. A mean CBF was calculated from the readings obtained.

**Percentage of epithelium ciliated.** An estimate of the extent of ciliation of the epithelium was obtained by a method developed to study the effect of viruses on the nasal epithelium (27). At least 20 strips of epithelium were identified, and the number of ciliated strips was counted and expressed as a percentage of the total number of strips identified. No attempt was made to estimate the extent of ciliation on each individual strip, as this was studied by electron microscopy later, but in this way an overall assessment was achieved.

**Transmission electron microscopy.** The remaining tissue was fixed in 2.5% cacodylate-buffered glutaraldehyde and postfixed in 1% osmium tetroxide. The nasal brushings were encapsulated in liquid agar (2%) and processed as previously described (19). Nasal turbinate tissue (see below) was treated similarly but without embedding in agar. This method led to retention of dead and single cells in nasal brushing examinations, but these were lost during nasal turbinate experiments, either during the experiments (into the petri dish) or during processing (no agar to retain unattached cells). The histological appearance of the tissue available under transmission electron microscopy was scored by a morphometric technique similar to that used to assess *in vitro* experiments (see below).

**Broth culture filtrates.** A 30-h shaking Stainer-Scholte (20) broth culture of a clinical isolate of *B. pertussis* (BPH 54) was centrifuged and filtered through a 0.22- $\mu$ m-pore-size filter.

**Tracheal cytotoxin.** Tracheal cytotoxin was purified as previously described (3) and dissolved to the required concentration in either Ringer's solution ( $\text{Na}^+$  [147 mmol/liter],  $\text{Ca}^{2+}$  [2 mmol/liter],  $\text{Cl}^-$  [156 mmol/liter]; Travenol, Thetford, United Kingdom) for short experiments or minimal essential medium (MEM) (GIBCO, Uxbridge, United Kingdom) supplemented with penicillin (50 U/ml), streptomycin

(50  $\mu$ g/ml), and gentamicin (50  $\mu$ g/ml) (MEM-ATB) for longer experiments.

**Normal human respiratory epithelium.** For shorter experiments (4 h), strips of nasal respiratory epithelium were obtained from normal volunteers by the brushing technique described above. The tissue used in longer experiments (up to 90 h) was derived from inferior nasal turbinates. These were resected from patients undergoing operations for relief of nonallergic nasal obstruction (by V. Lund). Tissue was transported to the laboratory in MEM-ATB. The turbinates were checked by light microscopy, and those that had a smooth, fully ciliated surface with normal ciliary beating were then transferred to fresh MEM-ATB and cut aseptically into adjacent 3-mm-thick cross sections.

**Effect of broth culture filtrates and tracheal cytotoxin on human respiratory epithelium *in vitro*.** The effect of culture filtrates (in a 1:1 mixture with medium 199 plus epithelium) was assayed on strips of epithelium obtained by the brushing technique for 4 h. The effect of culture filtrates (in a 1:1 mixture with MEM-ATB plus turbinate tissue) was assayed with epithelium obtained from nasal turbinates and incubated for 60 h. The effect of tracheal cytotoxin (20 and 40  $\mu$ M) in Ringer's solution plus epithelium from nasal brushing was assayed for 4 h, and the effect of tracheal cytotoxin (1 and 5  $\mu$ M) in MEM-ATB plus epithelium from nasal turbinates was assayed for 90 and 36 h, respectively. CBF was measured at hourly intervals from each of 10 strips of nasal epithelium in a sealed microscope coverslip-slide preparation. CBF was measured from turbinate tissue in a petri dish by using a graticule to select 10 evenly spaced points on the epithelial surface which were returned to at each time point. A mean CBF was calculated at each time point, and any changes in the integrity of the epithelium observed by light microscopy were recorded. Control preparations containing broth, medium, or both were included in each experiment. The preparations in each experiment were randomized so that the observer was unaware of their content.

**Examination of ultrastructure by transmission electron microscopy.** The effect of culture filtrates and tracheal cytotoxin on the ultrastructure of epithelial cells and cilia of turbinate tissue was examined by transmission electron microscopy. An ultrathin section through the central portion of each specimen was examined. Sections typically consisted of 150 to 250 cells. Each epithelial cell was scored by an observer unaware of its treatment by using the following criteria: extrusion of a cell from the epithelial surface, loss of cilia (only cells bearing cilia were scored, as it was not possible to determine whether a totally unciliated cell was originally unciliated or had become so during organ culture), numbers of unciliated cells, presence of cytoplasmic blebbing from the cell's luminal surface, and mitochondrial damage. The last two features were scored separately for ciliated and unciliated cells.

**Statistics.** The unpaired Student *t* test was used to compare the CBF of children with pertussis with the CBF of normal children. By comparing the control mean CBF with the test mean CBF at each time point during an experiment, maximum ciliary slowing was detected. The 10 control readings of CBF were compared with the 10 test readings at the time point of maximum ciliary slowing by the unpaired Student *t* test.

## RESULTS

Biopsy specimens were taken from the nasal epithelia of 15 children with pertussis infection (Table 1). *B. pertussis*

TABLE 1. Examination of nasal biopsy specimens taken from 15 children with *B. pertussis* infection

Patient	Age (mo)	Duration of cough (days)	Culture result	CBF (Hz)	% Epithelium ciliated
1	1	13	+	10.4	25
2	21	14	+	10.7	30
3	7	5	-	10.8	20
4	42	9	+	10.8	75
5	3	6	+	10.9	20
6	40	6	+	11.1	80
7	2	12	+	11.4	20
8	1	11	+	11.9	85
9	1	14	-	12.2	85
10	2	22	+	13.0	20
11	3	4	-	0	0
12	1	12	+	0	0
13	7	17	+	0	0
14	2	6	+	0	0
15	3	10	+	11.0	95

was isolated from 12 of 15 children, and significant rises in antibody titers to two or more *B. pertussis* antigens were present in all 15. All patients had nasal symptoms, including blockage, snuffles, and anterior rhinorrhea. The nasal epithelium was usually reddened, but in some patients it was pale and edematous. Excess tenacious mucoid secretions were always seen. Good samples of strips of nasal epithelium were obtained from 11 of the patients, but only cellular debris, mucus, and single epithelial cells were obtained from the other 4 patients. The failure to obtain a good sample is unusual in our experience unless there is an abnormality of the epithelium, and the biopsy was not repeated because of the patients' condition. It is likely that these children had extensive damage to their epithelium in the area that was biopsied. The mean CBF on epithelial strips of the children with pertussis was within the normal range (11.3 Hz; range, 10.4 to 13.0 Hz; standard deviation, 0.78 Hz). Although it

was a little lower, it did not differ significantly from the mean CBF of 10 normal children used as controls (12.5 Hz; range, 11.8 to 13.5 Hz; standard deviation, 0.58 Hz). In all 10 of the normal children, >95% of the epithelial strips were ciliated, but in 10 of 11 of the children with pertussis the percentage of strips of epithelium that were ciliated was reduced, in some cases markedly so (Table 1). For one patient (patient 15), the biopsy specimen obtained appeared completely normal by light microscopy.

The results of ultrastructural studies of patients' nasal epithelial samples are shown in Table 2, and a representative example is shown in Fig. 1. Patient 15 was analyzed separately, as a large sample of epithelium was obtained, probably because the epithelium was relatively normal, which would otherwise bias the results. In the nine specimens examined in which sufficient tissue was available for electron microscopy, there was an increase in the percentage of cells extruding from the epithelial surface, an increase in the number of dead cells, and a decrease in the number of cilia on ciliated cells. However, the most noticeable change was the increased frequency of cells with no cilia. The nasal epithelium of patient 15 was also abnormal when examined by electron microscopy. There was an increased number of dead cells and an increased frequency of poorly ciliated cells, but the percentage of cells with no cilia was similar to that of control subjects. Examination of the ultrastructure of cilia from children infected with pertussis (excluding patient 15) showed no increase in abnormal forms, either of the dynein arms (patients, 304 cilia examined, 98% normal; controls, 560 cilia examined, 98.9% normal) or the microtubular pattern (patients, 790 cilia examined, 97.8% normal; controls, 996 cilia examined, 92.6% normal). Bacteria were not seen associated with the epithelial biopsy specimens taken from *B. pertussis*-infected children, except in one sample. In this sample, from patient 4 (Table 1), rod forms were seen closely associated with cilia (Fig. 2).

The effect of a Stainer-Scholte broth *B. pertussis* culture filtrate on human respiratory epithelial tissue in vitro was assessed by light microscopy measurement of CBF and electron microscopy of epithelial cell and ciliary ultrastruc-

TABLE 2. Results of transmission electron microscopy of nasal biopsy specimens taken from children with *B. pertussis* infection<sup>a</sup>

Patient	No. of:							
	Normal cells	Cells extruding from epithelial surface	Single cells	Dead cells	Cells with normal complement of cilia	Cells showing loss of cilia	Cells showing gross loss of cilia	Cells with no cilia
1	34	2	1	0	3	10	3	21
2	88	13	8	3	0	7	4	101
3	3	5	1	0	0	0	0	9
4	43	5	3	0	19	6	0	26
5	25	0	2	0	1	2	1	23
6	32	5	0	0	0	5	3	29
8	96	5	4	7	24	33	5	50
9	15	2	14	8	10	19	1	9
Total <sup>b</sup>	336 (79.2)	37 (8.7)	33 (7.8)	18 (4.2)	58 (13.6)	82 (19.3)	17 (4.0)	268 (63.1)
15 <sup>b</sup>	174 (81.3)	9 (4.2)	6 (2.8)	25 (11.7)	136 (63.6)	50 (23.4)	4 (1.9)	24 (11.2)
Controls <sup>b</sup>	197 (85.3)	10 (4.3)	22 (9.5)	2 (0.9)	165 (71.4)	29 (12.6)	0 (0)	37 (16.0)

<sup>a</sup> Nine nasal biopsies of patients provided sufficient tissue for examination by electron microscopy. The results for patient 15 are shown separately because a large sample of epithelium was obtained, probably because the epithelium was relatively normal. Nasal biopsies from 10 nonsmoking normal adults acted as controls.

<sup>b</sup> Values in parentheses are the percentages of the total number of cells examined that showed the indicated characteristics. The results are analyzed separately with respect to cell cytopathic effects and the number of cilia on the cell surface.

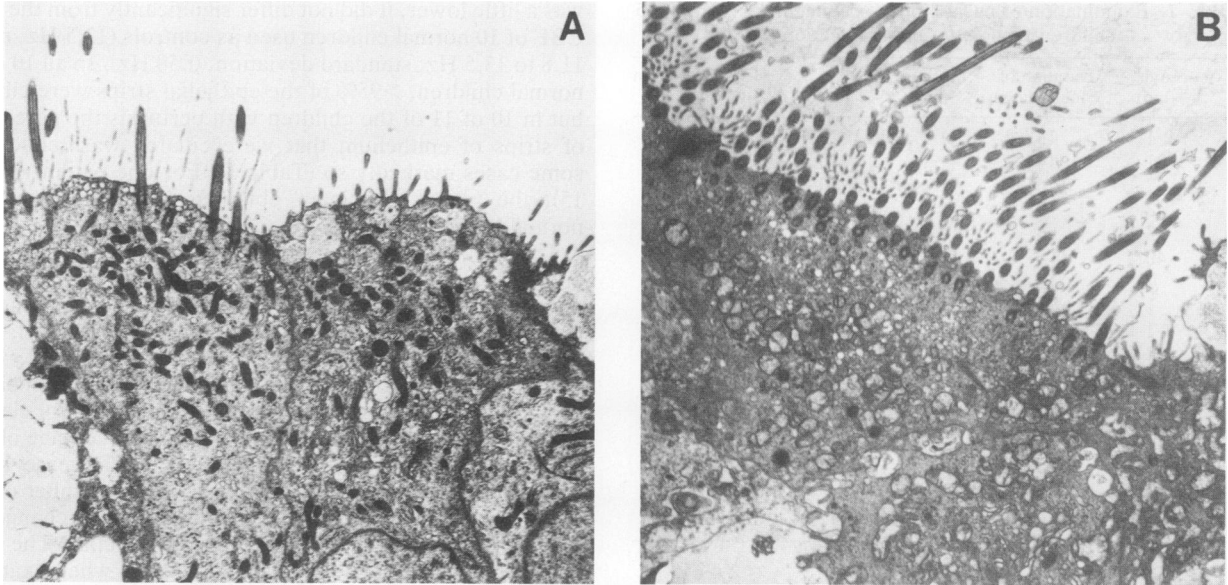


FIG. 1. Nasal epithelial biopsy specimens (magnification,  $\times 5,400$ ) from patients 10 (A) and 5 (B). (A) A sparsely ciliated cell is shown, and an unciliated cell is beginning to extrude from the epithelial surface. (B) Mitochondrial damage is evident.

ture. The culture filtrate had no effect on CBF over 4 h. Two experiments were performed. In the first, the mean CBF of the control sample after 4 h was 14.0 Hz and that of the test sample was 13.9 Hz; in the second, the mean CBFs were 12.7 and 13.4 Hz, respectively, after 4 h. Similarly, the mean CBF measurements at each of the hourly time points up to 4 h were equal. However, over a longer period (Fig. 3), the mean CBF was affected by the culture filtrate. The mean CBF began to decrease after 22 h, and by 60 h there was no ciliary beating present in the organ culture. However, the effect of the culture filtrate did not appear to be a direct effect on ciliary beating but rather a cytotoxic effect, i.e., disruption of the epithelial surface. For example, in an experiment done after incubating samples with culture filtrate for 36 h, 5 of 10 CBF readings yielded values of 12.5, 12.1, 12.5, 10.9, and 11.3 Hz, and 5 other readings, taken from areas where ciliary beating had previously occurred but had ceased, yielded values of 0 Hz. In addition, mucus and cell debris accumulated in the areas in which ciliary beating did not occur, making it difficult to distinguish the surface of the organ culture.

The effect of the *B. pertussis* culture filtrate on the ultrastructure of respiratory epithelial tissue is shown in Table 3. In the presence of culture filtrate for 36 h, there was an increase in the number of cells extruding from the epithelial surface and an increase in the frequency of poorly ciliated cells, and a greater proportion of the epithelial cells became unciliated. An increased number of the epithelial cells showed toxic changes, with increased cytoplasmic blebbing from their luminal surface and mitochondrial damage. All of these changes were more pronounced after 60 h. There seemed to be no difference between ciliated and unciliated cells with respect to their susceptibility to the toxic effects of culture filtrate. No changes were detected in the ultrastructure of cilia after 60 h of exposure to culture filtrate.

The effects of tracheal cytotoxin were examined in a similar way, and the effects of culture filtrate and tracheal cytotoxin on the ultrastructure of epithelial tissue are shown

in Fig. 4. Tracheal cytotoxin did not have an acute effect on the CBF, even when large concentrations were used. After 4 h in one experiment, the mean control CBF was 11.5 Hz and the mean CBF in the presence of 20  $\mu\text{M}$  tracheal cytotoxin was 12.3 Hz; after 4 h in another experiment, the mean control CBF was 12.1 Hz and the mean CBF in the presence of 40  $\mu\text{M}$  tracheal cytotoxin was 13.2 Hz. The experimental CBFs at the other time points were similarly not significantly different from the control CBFs. However, as with culture filtrate, over a prolonged period tracheal cytotoxin caused loss of ciliary beating (Fig. 3). Tracheal cytotoxin at a 1  $\mu\text{M}$  concentration caused a complete loss of ciliary beating in the organ culture after 90 h, and at a 5  $\mu\text{M}$  concentration it caused a complete loss of ciliary beating after 36 h. Again the effects were predominantly cytotoxic, with disruption of the epithelial surface occurring after 36 h at 1  $\mu\text{M}$  and 22 h at 5  $\mu\text{M}$ , but the CBF in those areas where cilia continued to beat was normal.

The effects of tracheal cytotoxin on the ultrastructure of ciliated epithelial cells are shown in Table 4. The effects were very similar to those of the culture filtrate, with tracheal cytotoxin causing extrusion of cells from the epithelial surface, loss of ciliated cells, an increased frequency of sparsely ciliated cells, and toxic changes in some cells. The changes were dose dependent, and ciliated and unciliated cells showed toxic changes with equal frequency. No changes were detected in the ultrastructure of cilia after 24 h of exposure to tracheal cytotoxin (5  $\mu\text{M}$ ). Although no effect of tracheal cytotoxin on CBF was detected at 4 h, transmission electron microscopy did show abnormal changes in the epithelium even after this short exposure to tracheal cytotoxin.

## DISCUSSION

The characteristic pathological phenomenon which has been described as following *B. pertussis* infection is specific colonization of ciliated epithelial cells and destruction of ciliated cells (12). The appearances of the nasal biopsy

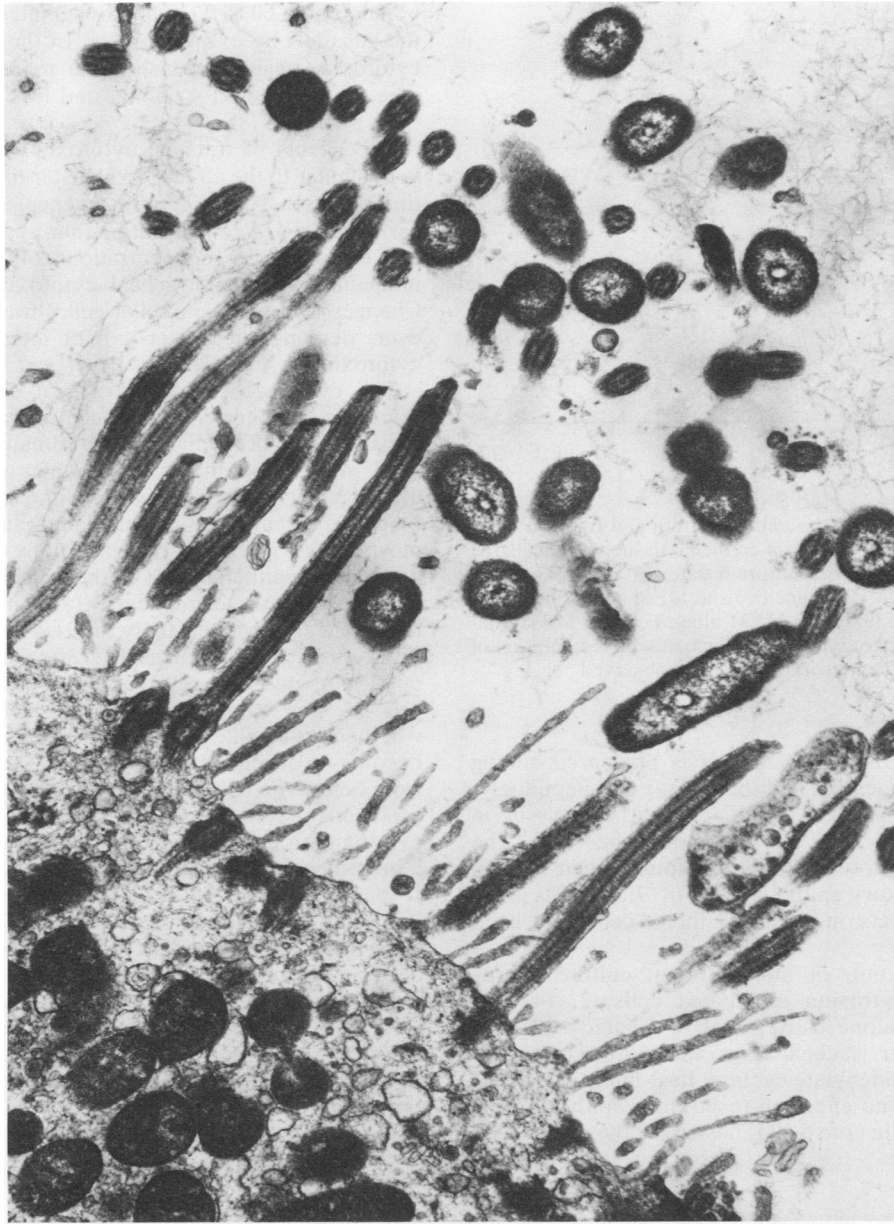


FIG. 2. Nasal epithelial biopsy specimen from patient 4 (magnification,  $\times 18,750$ ). Rod-shaped bacterial forms are shown closely associated with cilia.

samples taken from children with pertussis infection during our study were in keeping with this description, in that there was a marked reduction in the number of ciliated cells. The loss of ciliated cells appeared to be due to extrusion of cells from the epithelial surface and cell death and also due to loss of cilia from cells. However, the beat frequency of those cilia that remained was normal.

Incubation of respiratory epithelial tissue with *B. pertussis* culture filtrate showed that bacterial products did not affect ciliary function directly but that they did have cytotoxic effects on the epithelial cells, producing mitochondrial swelling and cell surface cytoplasmic blebbing. Cells were seen to extrude from the epithelium and were probably replaced by unciliated cells from below the epithelial surface, leading to

a largely unciliated epithelium. In addition, the tight junctions between epithelial cells were broken and poorly ciliated cells were seen, suggesting that cilia were lost. These changes would cause a profound disturbance of the mucociliary clearance system. We previously determined that the concentration of tracheal cytotoxin in a 30-h shaking culture of *B. pertussis* in Stainer-Scholte medium is approximately  $1.5 \mu\text{M}$ . Further experiments showed that the effects of the crude bacterial culture filtrate were reproduced by similar concentrations of purified tracheal cytotoxin from *B. pertussis*.

Tracheal cytotoxin is a single molecule produced by *B. pertussis* and is a 1,6-anhydromuramic acid tetrapeptide of peptidoglycan containing monomeric disaccharide (3, 4).

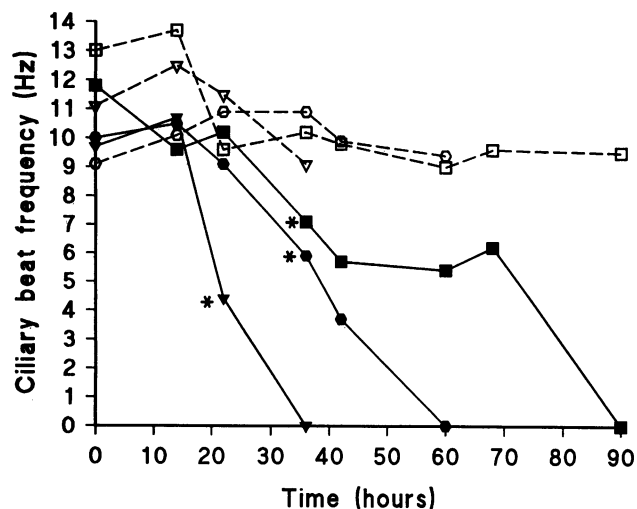


FIG. 3. Effect of *B. pertussis* culture filtrate and tracheal cytotoxin on CBF in vitro. Human nasal epithelial tissue was incubated with broth (○) and *B. pertussis* culture filtrate (●) in a 1:1 mixture with medium 199, with MEM alone (▽) and MEM containing 5 μM tracheal cytotoxin (▼), and with MEM alone (□) and MEM containing 1 μM tracheal cytotoxin (■). \*, Time at which disruption of the integrity of the epithelial surface was first observed.

The production of tracheal cytotoxin is preserved among other *Bordetella* species (7), and the release of a peptidoglycan fragment which is toxic for ciliated epithelial tissue in *Neisseria gonorrhoeae* has also been described (13). The effects of tracheal cytotoxin have previously been studied with hamster respiratory epithelial cells (8, 9), but this is the first time that its effects on human epithelial cells have been described.

*B. pertussis* infection of animal organ culture causes ciliostasis due to extrusion of ciliated cells (2, 14, 15). Tracheal cytotoxin alone causes loss of ciliated cells and ciliostasis in hamster tracheal rings and cultured hamster tracheal cells, while adenylate cyclase, heat-labile toxin, and pertussis toxin have no effect on respiratory epithelial cells (9). Tracheal cytotoxin (1 to 5 μM) destroyed the ciliated cell

population in 60 to 96 h, and nonciliated cells migrated to fill the gaps left by extruded cells. In the presence of tracheal cytotoxin, ciliated cells became more round, and loss of cilia, mitochondrial swelling, and loss of intercellular junctions occurred (8).

The effects of tracheal cytotoxin on human tissue were very similar to the effects on hamster tissue and had a similar time course. The effects of tracheal cytotoxin were seen earlier at a 5 μM concentration than at a 1 μM concentration, but even at high concentrations (40 μM) there was no immediate effect of tracheal cytotoxin on ciliary beating. Changes in human epithelial cell ultrastructure were, however, demonstrated after 4 h of incubation with tracheal cytotoxin.

The cytotoxic effect of tracheal cytotoxin on hamster tracheal epithelial cells was specific for ciliated cells, and the ultrastructure of unciliated cells remained normal (9). However, in the experiments with human nasal tissue described in this paper, mitochondrial swelling and cytoplasmic blebbing were seen equally in unciliated and ciliated cells. This most likely represents a species difference, or possibly the nonciliated epithelial cells in the upper respiratory tract are more responsive to tracheal cytotoxin than are those from the trachea. However, there are two further considerations. Firstly, we have shown that after exposure to tracheal cytotoxin, epithelial cells with poor ciliation are seen. If this process was complete, it would be impossible to assess whether an unciliated cell was originally unciliated or had become so during an experiment. Secondly, unciliated cells from below the epithelial surface probably replace the damaged cells that are shed. We examined the effect of tracheal cytotoxin on epithelial cell ultrastructure after 24 h, i.e., 12 h before complete ciliostasis occurred, by light microscopy. If the response of the unciliated cells to tracheal cytotoxin had been examined later, then the effect of tracheal cytotoxin might have been underestimated because the unciliated cells appearing fresh on the epithelial surface from below would have been exposed to tracheal cytotoxin for less time.

Since *B. pertussis* infection is believed to be limited to the ciliated epithelium of the respiratory tract, an understanding of bacterial interactions with the respiratory mucosa is crucial if the pathogenesis of whooping cough is to be explained. The production of tracheal cytotoxin alone is not

TABLE 3. Results of transmission electron microscopy of human respiratory tissue treated with *B. pertussis* culture filtrates

Treatment	% of cells showing indicated degree of given histological feature <sup>a</sup>																	
	Cells extruding from epithelial surface				Loss of cilia				Blebbing on:						Mitochondrial damage in:			
									Ciliated cells			Unciliated cells			Ciliated cells		Unciliated cells	
	0	+	++	+++	0	+	++	Unciliated cells	0	+	++	0	+	++	Absent	Present	Absent	Present
Control (36 h; n = 371)	88.9	11.1	0	0	60.9	14.8	0.8	23.5	76.5	0	0	21.0	2.2	0.3	75.7	0.8	22.6	0.8
Culture filtrate (36 h; n = 304)	72.4	25.7	2.0	0	33.9	17.1	2.6	46.4	53.0	0.7	0	42.8	2.6	0.9	50.3	3.3	44.4	2.0
Control (60 h; n = 207)	70.5	29.5	0	0	33.3	15.5	1.4	49.8	49.3	1.0	0	47.3	1.9	0.5	49.3	1.0	48.8	1.0
Culture filtrate (60 h; n = 171)	57.3	29.8	10.5	2.3	12.3	17.5	13.5	56.7	26.9	8.8	7.6	53.2	2.9	0.6	32.7	10.5	46.8	9.9

<sup>a</sup> Each cell in a tissue section was examined for extrusion from the epithelial surface (a score of 0 indicates normal positioning in the epithelium), the presence of cilia (0 indicates a full complement of cilia on the cell surface), cell blebbing (cytoplasmic projections from luminal cell surfaces), and mitochondrial damage.



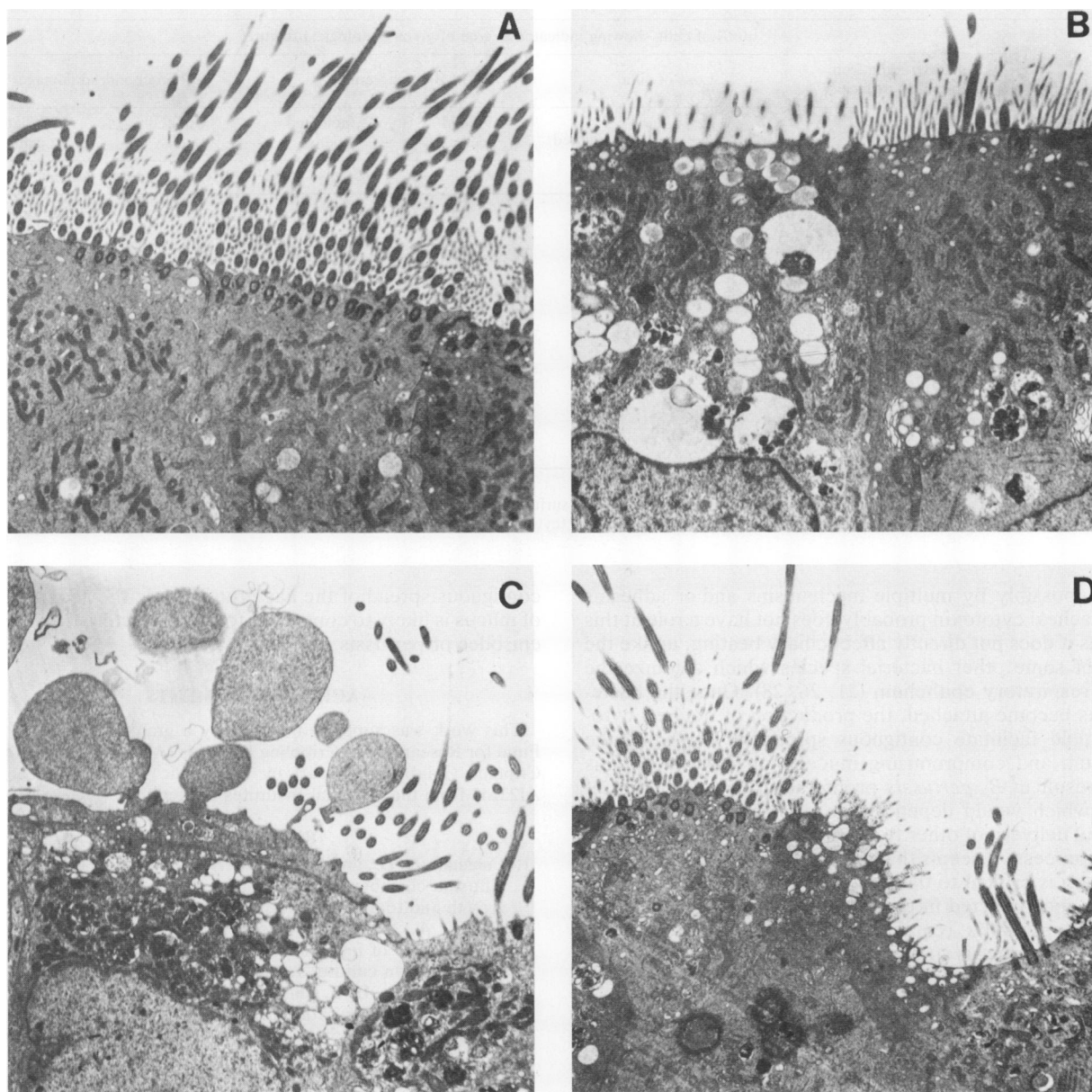


FIG. 4. Nasal turbinate tissue (magnification,  $\times 3,000$ ) incubated with Stainer-Scholte broth (in a 1:1 mixture with MEM) for 60 h (A), with *B. pertussis* culture filtrate (in a 1:1 mixture with MEM) for 36 h (B) and 60 h (C), and with  $5 \mu\text{M}$  tracheal cytotoxin in MEM for 24 h (D). The following features are shown: normal epithelial tissue (A), a sparsely ciliated cell and an unciliated cell with mitochondrial damage and vacuolation (B), cytoplasmic blebbing of the cell surface (C), and cell extrusion and a damaged unciliated cell (D).

sufficient to permit colonization of the respiratory tract. A *B. pertussis* strain (Tohama 111) produced normal amounts of tracheal cytotoxin but had lost its ability to attach to ciliated cells. This strain was unable to colonize the respiratory tract (6).

Filamentous hemagglutinin is a cell surface protein with a rodlike structure which appears to be involved in the adherence of *B. pertussis* to ciliated respiratory cells and unciliated cells (17, 23, 24). However, it may not be the sole determinant of adherence, as pertussis toxin may influence *B. pertussis* attachment to rabbit ciliated respiratory cells (17). Immunization with filamentous hemagglutinin causes a decrease in mouse pulmonary and tracheal colonization by

*B. pertussis* (11). Using a filamentous hemagglutinin-negative strain of *B. pertussis*, Kimura et al. (11) showed that filamentous hemagglutinin was important during colonization of the trachea early in the infective process in the presence of a normal mucociliary barrier, but later in infection, when the epithelium had been damaged, the filamentous hemagglutinin-negative strain was able to colonize the trachea. These results suggest the *B. pertussis* adherence is a critical event during colonization in the presence of normal mucociliary clearance.

Pertussis is a disease in which a wide variety of toxic molecules act in concert. The initial event in the pathogenic process appears to be bacterial adherence to the epithelial

TABLE 4. Results of transmission electron microscopy of human respiratory tissue treated with TCT<sup>a</sup>

Treatment	% of cells showing indicated degree of given histological feature <sup>b</sup>																	
	Cells extruding from epithelial surface				Loss of cilia				Blebbing on:						Mitochondrial damage			
	0	+	++	+++	0	+	++	Unciliated cells	Ciliated cells			Unciliated cells			Ciliated cells		Unciliated cells	
									0	+	++	0	+	++	Absent	Present	Absent	Present
Control (4 h; n = 225)	81.8	18.2	0	0	79.1	10.7	0	10.2	86.2	3.6	0	9.3	0.9	0	84.4	5.3	9.3	0.9
TCT (1 μM; 4 h; n = 263)	73.0	26.2	0.8	0	55.9	23.2	0.8	20.2	74.5	3.8	1.5	17.1	2.3	0.8	70.0	9.9	15.6	4.6
TCT (5 μM; 4 h; n = 165)	52.7	46.7	0.6	0	63.0	17.0	0	20.0	67.9	11.5	0.6	15.8	4.2	0	60.0	20.0	18.2	1.8
Control (24 h; n = 233)	63.5	33.9	1.3	1.3	57.5	17.2	0	25.3	72.5	1.3	0.9	24.0	0.9	0.4	70.0	4.7	21.0	4.3
TCT (1 μM; 24 h; n = 215)	27.9	43.7	14.0	14.4	34.9	15.3	7.2	42.6	55.3	5.3	1.4	28.8	8.2	1.0	51.9	10.1	29.8	8.2
TCT (5 μM; 24 h; n = 218)	51.4	46.8	1.8	0	42.7	17.9	2.8	36.7	57.8	4.6	0.9	29.8	6.9	0	37.6	25.7	25.2	11.5

<sup>a</sup> TCT, Tracheal cytotoxin.

<sup>b</sup> Each cell in a tissue section was examined for extrusion from the epithelial surface (a score of 0 indicates normal positioning in the epithelium), the presence of cilia (0 indicates a full complement of cilia on the cell surface), cell blebbing (cytoplasmic projections from luminal cell surfaces), and mitochondrial damage.

surface, possibly by multiple mechanisms and/or adhesins (17). Tracheal cytotoxin probably does not have a role at this stage, as it does not directly affect ciliary beating, unlike the toxins of some other bacterial species which colonize the human respiratory epithelium (21, 26, 28). Once the bacterium has become attached, the production of tracheal cytotoxin could facilitate contiguous spread by damaging the epithelium and compromising mucociliary clearance. This would result in *B. pertussis* proliferation and a buildup of mucus which would depend upon coughing for clearance. The local delivery of other toxins may then contribute to the disease process. It seems that colonization of the respiratory epithelium is critical to the development of serious disease, because mice infected intraperitoneally with chambers containing *B. pertussis* remained healthy despite developing antibody to pertussis toxin, which suggested that they had been exposed to bacterial toxins diffusing out of the chambers (1).

The precise interaction between *B. pertussis* adhesins and respiratory mucosae remains to be determined. *B. pertussis* appears to adhere to cilia (23). This was also suggested by the sample from patient 4 (Table 1; Fig. 2), although we cannot be sure that the bacteria seen were *B. pertussis*, and it is possible that in the other specimens bacteria were dislodged during processing. However, the observation that pertussis cytopathic effects occur in patient samples in which no bacteria are present suggests that toxic levels of tracheal cytotoxin can be reached outside the immediate *B. pertussis*-epithelial cell microenvironment. How adherence of bacteria to cilia occurs in the presence of mucus and normal ciliary beating is not understood.

These observations made with patient tissue confirm previous ones that *B. pertussis* infection damages human ciliated epithelial tissue (12), and the in vitro experiments with human tissue suggest that tracheal cytotoxin may be responsible for the histological abnormalities observed in vivo. The initial persistence of the bacterium in the respiratory tract probably depends on adherence to respiratory epithelial tissue, which would permit local release of tracheal cytotoxin. The damage that this toxin causes to the epithelial cells and the loss of ciliary activity could in turn facilitate

contiguous spread of the bacterium, while the accumulation of mucus is likely to contribute to the characteristic coughing episodes of pertussis.

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