INFECTION AND IMMUNITY, Dec. 1986, p. 905–908
0019-9567/86/120905-04$02.00/0

Vol. 54, No. 3

Piracy of Adhesins: Attachment of Superinfecting Pathogens to Respiratory Cilia by Secreted Adhesins of *Bordetella pertussis*

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Received 16 June 1986/Accepted 6 September 1986

Two proteins secreted by *Bordetella pertussis* are known to mediate adherence of these bacteria to mammalian respiratory cilia. When either ciliated cells or other pathogenic bacteria were pretreated with these adhesins, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* acquired the ability to adhere to cilia in vitro and in vivo. Such piracy of adhesins may contribute to superinfection in mucosal diseases such as whooping cough.

Bordetella pertussis, the causative agent of whooping cough, is unique in its ability to adhere to human respiratory cilia (7, 10). Two bacterial proteins which are secreted into the surrounding medium during growth, filamentous hemagglutinin (FHA) and pertussis toxin (TOX), are important to the adherence process (8). Both secreted adhesins appear to mediate attachment in vitro by binding to cilia and binding back onto the bacterial surface, thereby creating an adherence bridge (8, 10). We sought to investigate if the adhesins bound to the surface of the cilia also enhance the adherence of other pathogenic bacteria which do not normally adhere to this site. Such activity might facilitate the superinfections which are common in complicated cases of whooping cough.

In 58% of pertussis cases, the nasopharynx has been shown to be colonized with species of pathogenic bacteria other than *B. pertussis* (6). Most secondary respiratory infections result from *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*. We compared the following: (i) the ability of these bacteria in an in vitro assay to adhere to normal ciliated cells versus cells which had been preincubated with one or both *B. pertussis* adhesins, (ii) the ability of *B. pertussis* adhesins to bind directly to encapsulated and nonencapsulated surfaces of heterologous bacteria, and (iii) the fate of pneumococci pretreated with *B. pertussis* adhesins upon intratracheal challenge in rabbits.

(Part of this research was presented earlier [E. Tuomanen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-87, p. 38].)

S. pneumoniae strains S_{III} (type III, encapsulated clinical isolate), A_{II} (type II, encapsulated laboratory strain), and R_6 (unencapsulated derivative of A_{II}) were grown in a casein hydrolysate medium (4). Three clinical isolates of *S. aureus*, strains Cr, 789, and WANYH/N1300 (obtained from Barry Hartman of the Cornell University-New York Hospital Medical Center), were grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.). Four strains of *H. influenzae* (strains Garret and Eagen [encapsulated, type b], Whittier and 37561 [nontypable]), obtained from G. Noel (Cornell University-New York Hospital Medical Center), were grown in supplemented brain heart infusion (2). *B. pertussis* 338 (phase I) was grown in modified Stainer-Scholte medium (10). All bacterial strains were grown to mid-exponential phase (10^8 CFU/ml), centrifuged, washed in medium 199S (M. A. Bioproducts, Walkersville, Md.), and suspended to 2×10^9 CFU/ml in medium 199S for use in the in vitro adherence assay.

The in vitro adherence assay was adapted from the method described for *B. pertussis* (10). Briefly, ciliated cells were harvested from New Zealand White rabbits (Hare-Marland, Hewitt, N.J.), washed, and suspended at a density of 2×10^5 ciliated cells per ml of medium 199S. To test the adherence of each strain, half the ciliated cells were incubated with 5 μg of FHA per ml and 5 μg of TOX per ml (adhesins prepared by J. Cowell, National Institutes of Health, Bethesda, Md. [8]) for 30 min at 37°C. Control cells were incubated without exogenous adhesins. Cells were washed by centrifugation, and 0.5 ml of treated and untreated cells was then incubated with 0.5 ml of each bacterial strain for 1 h at 37°C. Cells were washed free of nonadherent bacteria over a polycarbonate membrane (8-μm pore size; Nuclepore Corp., Pleasanton, Calif.), spread on a slide, and Gram stained. The mean (±standard deviation) number of bacteria adherent to ciliary tufts of 25 cells was determined for each preparation by light microscopy. Each condition was tested a minimum of three times, and significant differences were calculated as described before (11).

Table 1 shows the difference in the ability of the three secondary pathogens to adhere to normal cilia versus cilia which had been pretreated with *B. pertussis* adhesins. *S. pneumoniae* and *S. aureus* did not adhere to normal ciliated cells, while *H. influenzae* showed a variable, low level of adherence to ciliary tufts. In contrast, several strains adhered remarkably well to cilia pretreated with *B. pertussis* adhesins. Enhanced adherence was most notable for the unencapsulated strains of pneumococci and *H. influenzae*. One strain of *S. aureus* showed enhanced adherence, while the encapsulated *H. influenzae* showed only a moderate increase in adherence in the presence of *B. pertussis* adhesins. When each adhesin was tested alone for the ability to facilitate adherence, differences were noted between TOX and FHA. TOX enhanced adherence (>five *B. pertussis* per cell) for all strains of *H. influenzae*, *S. pneumoniae* R_6, and *S. aureus* Cr. In contrast, FHA enhanced adherence only of *H. influenzae* Eagen, 37561, and Whittier. Interestingly, when TOX and FHA were incubated with the heterologous bacteria, washed, and then exposed to adhesin-free ciliated cells, the same pattern of facilitated adherence was found. An example of the enhancement of adherence is depicted for each of the three pathogens (Fig. 1). It is apparent that, as has been shown for *B. pertussis*, the site of adherence of heterologous pathogens was also restricted to the ciliary tuft; no bacteria adherent to the cell bodies were detected.

These results suggested that bacteria of heterologous species can bind to *B. pertussis* adhesins. To demonstrate this, we tested the adhesins for their ability to bind directly

TABLE 1. Enhancement of adherence of pathogens by *B. pertussis* adhesins

Strain	Capsule	Mean no. of organisms/ciliated cell (±SD)		Binding of *B. pertussis* adhesins to heterologous bacterial surfaces	
		Untreated	Pretreated with *B. pertussis* adhesins	FHA	TOX
S. pneumoniae					
S_{III}	+	0	0	−	−
A_{II}	+	0	0.4 ± 0.08	−	−
R_6	−	0.2 ± 0.06	15.7 ± 0.3[a,b]	−	+
S. aureus					
789	+	0	0	−	−
N1300	+	0	0	−	−
Cr	+	0	>20[a,b]	−	+
H. influenzae					
Garret	+	0.8 ± 0.7	3.6 ± 0.2[a]	−	+
Eagen	+	1.1 ± 0.8	5.4 ± 0.6[a]	+	+
Whittier	−	5.1 ± 0.1	>20[a,b]	+	+
37561	−	1.8 ± 0.6	10.7 ± 0.7[a]	+/−	+
B. pertussis 338		2.4 ± 0.5	>20[a]	+	+

[a] $P < 0.01$.
[b] Illustrated in Fig. 1.

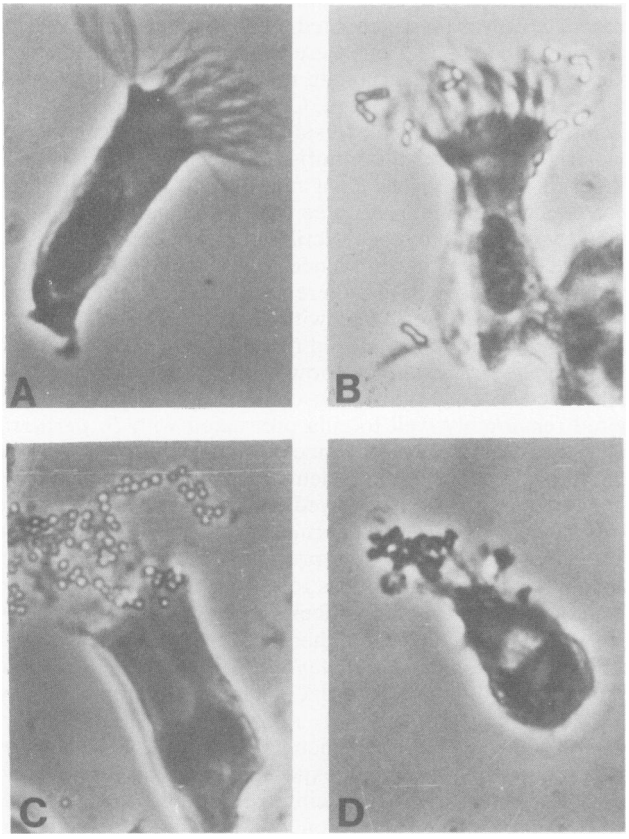

FIG. 1. Adherence of pathogens to cilia treated with *B. pertussis* adhesins. Ciliated cells (A) were incubated with *B. pertussis* adhesins and then exposed to *S. pneumoniae* R_6 (B), *S. aureus* Cr (C), and *H. influenzae* Whittier (D). The bacteria adherent to ciliary tufts were visualized by Gram stain.

to the surface of each of the bacterial strains. Each strain (10^6 CFU/ml) was incubated with 0.5 μg of each adhesin per ml of medium 199S for 30 min at 37°C. The samples were washed by centrifugation and incubated for 60 min with 10 μl of goat antibody to the adhesin (8) (courtesy of J. Cowell). The cells were again washed and incubated for a second 60 min with 10 μl of fluorescein-labeled anti-goat immunoglobulin G (Calbiochem-Behring, La Jolla, Calif.). After washing, cells were spread on a slide and viewed by phase and fluorescent microscopy. As shown in Table 1 and Fig. 2, the indirect immunofluorescence assay demonstrated binding of the TOX adhesin to the surfaces of each of the strains which showed enhanced adherence in the ciliated cell assay. Similarly, binding of FHA was restricted to the two strains of *H. influenzae* (Eagen and Whittier), which showed increased binding to the ciliated cells by FHA. Thus, the ability of *B. pertussis* adhesins to attach to the surface of heterologous bacteria correlated with the enhancement of binding of these same strains to ciliary tufts.

To determine if enhanced adherence in the in vitro assay correlated with the ability to adhere to cilia in vivo, the survival of pneumococci instilled intratracheally into rabbits was assessed in the presence and absence of *B. pertussis* adhesins. Pneumococcal strains S_{III}, A_{II}, and R_6 (10^7 CFU/ml each) were incubated for 30 min with and without FHA (5 μg/ml) and TOX (5 μg/ml). Each preparation was then washed, and the bacteria (10^8 CFU/0.2 ml of pyrogen-free saline per dose) were instilled into the right mainstem bronchus of 12 anesthetized rabbits through a tracheostomy incision (9). Four animals were sacrificed from each group at 0, 4, and 24 h, and the lungs were excised. One pair of lungs from each time point was fixed for histologic sectioning; the remaining three pairs of lungs per time point were homogenized, and the number of viable pneumococci per milliliter of homogenate was determined by plating on Trypticase soy agar (Difco) supplemented with 5% sheep blood. Each experiment was done three times. Strain R_6, but not strains A_{II} and S_{III}, demonstrated prolonged survival in lungs in the presence of *B. pertussis* adhesins (Fig. 3). Normally, the R_6 strain was cleared from the lungs in 24 h. In the presence of the adhesins, however, >90% of the initial inoculum re-

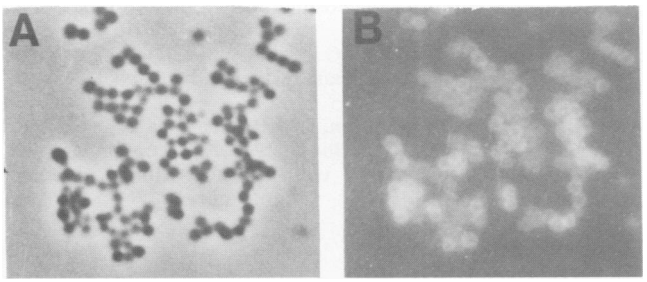

FIG. 2. Binding of *B. pertussis* TOX adhesin to *S. aureus* strain Cr. (A) Phase-contrast micrograph of *S. aureus*. (B) Fluorescence micrograph of same field as in panel A demonstrating TOX binding to bacterial surface by fluorescein-conjugated antibody staining technique.

mained viable in the lungs for 24 h. This is the same strain that showed increased adherence in the in vitro assay in response to the adhesins, and this strain would be comparable to the untypable pneumococci found to represent a significant fraction of the nasopharyngeal flora (1). Histological sections prepared from the lungs infected with the adhesin-coated pneumococci showed a significant number of the bacteria associated with the ciliated epithelium. No control pneumococci (uncoated with adhesins) were detectable at this site. The clinical isolate S_{III} survived equally well in lungs with or without exogenous adhesins. The laboratory strain A_{II} was cleared quickly from lungs despite the presence of adhesins.

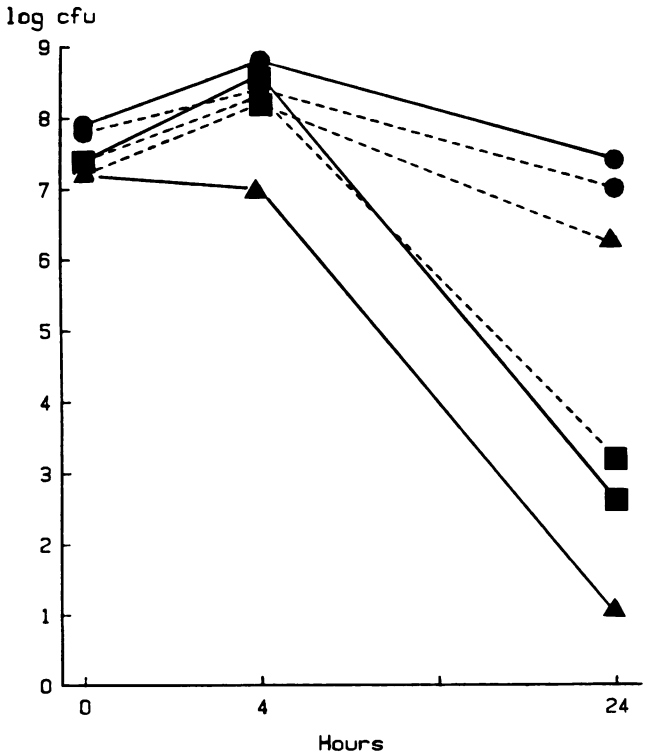

FIG. 3. Effect of *B. pertussis* adhesins on survival of pneumococci inoculated into rabbit lungs. Rabbits were challenged with three strains of pneumococci: encapsulated S_{III} (●), encapsulated A_{II} (■), and unencapsulated R_6 (▲). Survival of bacteria treated with adhesins (. . . .) was compared with untreated bacteria (——). Each point is the mean CFU per milliliter of lung homogenate of three rabbits.

In summary, it appears that *B. pertussis* adhesins can bind to the surfaces of several pathogenic bacteria, including encapsulated *H. influenzae* and *S. aureus*. The TOX adhesin also bound particularly well to nonencapsulated (nontypable) strains of pneumococci and *H. influenzae*. These nonencapsulated bacteria frequently colonize the upper respiratory tract (1, 5), and variation between encapsulated and nonencapsulated forms in vitro and in vivo has been described (2). Binding of adhesins was found for several species but not all strains in a given species. From these data it is not possible to determine if the interaction between heterologous bacteria and the *B. pertussis* adhesins is receptor mediated or is nonspecific (i.e., involves electrostatic or hydrophobic interactions that differ in degree from one capsular type or cell wall to another). The potential pathophysiological importance of this interaction is suggested by the finding that strains binding the adhesins acquire the ability to adhere to a new site in the respiratory tree—the cilia. The adhesin-ciliary interaction is known to be specific (7, 8) and affords these bacteria with an anchor to the respiratory epithelium which they normally do not possess. It is reasonable to suggest that as *B. pertussis* grows on the ciliated epithelium during clinical whooping cough, the adhesins it secretes bind to neighboring ciliated cells. These ligands may then serve to enhance adherence of either *B. pertussis* daughter cells or, through the piracy of these adhesins, secondary pathogens such as pneumococci, staphylococci, and *H. influenzae*, prolonging their resident time in the lungs. Thus, the adherence system of the primary pathogen could potentially contribute to secondary pneumonias during whooping cough. Such a mechanism may also contribute to the association of pertussis and adenovirus infection (3). It is possible that piracy of adhesins may be a common general mechanism of superinfection complicating mucosal diseases.

This work was supported in part by a Parker B. Francis Fellowship, the Irma T. Hirschl Research Award, and by grant no. RO1-23459 from the National Institutes of Allergy and Infectious Diseases.

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