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Variable Adherence of Fimbriated *Haemophilus influenzae* Type b to Human Cells

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The attachment of isogenic fimbriated and nonfimbriated *Haemophilus influenzae* type b variants to human cells was studied by using a radioactive assay and an indirect immunofluorescent assay. As described previously, fimbriated *H. influenzae* variants adhered to a greater extent than nonfimbriated variants to human buccal epithelial cells (2.1 and 0.29 bacteria per cell, respectively, as determined by the radioactive assay [$P < 0.05$]; 7.6 and 1.6 bacteria per cell, respectively, as determined by the immunofluorescent assay [$P < 0.01$]). As the concentration of fimbriated bacteria was increased, so were the numbers of adherent bacteria; in contrast, increasing the bacterial concentration had a much smaller effect on adherence of nonfimbriated *H. influenzae* type b. The distribution of bacteria on the buccal cells also differed. Whereas 37% of the buccal cells failed to bind nonfimbriated *H. influenzae* type b, failure to bind was observed for only 4% of the buccal cells exposed to fimbriated *H. influenzae*. In contrast, adherence to human foreskin fibroblasts was low regardless of the presence of fimbriae. On the other hand, fimbriated *H. influenzae* type b adhered less well than nonfimbriated variants to HEp-2 cells (1.6 and 3.8 bacteria per cell, respectively, as determined by the radioactive assay [$P < 0.05$]; 1.3 and 4.8 bacteria per cell, respectively, as determined by the immunofluorescent assay [$P < 0.02$]). Whereas adherence to HEp-2 cells increased considerably as the concentration of nonfimbriated bacteria was increased, there was only a small enhancement of adherence with an increase in the concentration of fimbriated *H. influenzae* type b. Furthermore, only 16% of the HEp-2 cells failed to bind nonfimbriated *H. influenzae* type b, whereas 50% failed to bind fimbriated *H. influenzae* type b. These data indicate that *H. influenzae* type b may contain two adhesins. One is associated with fimbriae and enables adherence to buccal cells, whereas the other is nonfimbrial and is associated with adherence to HEp-2 cells. It is not known whether either of these adhesins plays a role in pathogenesis.

Fimbriae (pili) are hairlike surface extensions which can be expressed by several gram-negative bacteria (2). Although fimbriae are not always associated with pathogenesis, evidence from several laboratories indicates that the fimbriae of many bacteria function as adherence factors and thereby enable bacterial colonization of mucosal surfaces (2).

Guerina et al, recently described the presence of fimbriae on *Haemophilus influenzae* type b (N. G. Guerina, S. Langerman, H. W. Clegg, T. W. Kessler, and D. A. Goldmann, Pediatr. Res. **16**:242A, 1982). The work of these authors and subsequent work in other laboratories have revealed a modest body of data regarding *H. influenzae* type b fimbriae, which is summarized below. (i) Cultures of most *H. influenzae* type b isolates from humans contain predominantly nonfimbriated variants, although occasionally an isolate that constitutively expresses fimbriae (at least phenotypically) is obtained; such strains have been isolated only from the nasopharynx (5, 11). However, cultures of predominantly fimbriated forms can be obtained by hemadsorption techniques (3, 5, 11); such cultures continue to express fimbriae if they are subcultured on solid medium (3). (ii) Nonfimbriated variants can switch to fimbriated forms, but at a low rate (10^{-4} bacteria per generation, as measured for one strain) (3). (iii) Compared with nonfimbriated bacteria, fimbriated variants display increased adherence to human cells (e.g., buccal epithelial cells [BEC] [11], pharyngeal cells [5], and erythrocytes [5, 11]), but not to the analogous rat cells (6; M. Pichichero, personal communication). (iv) When tested in the infant rat model of *H. influenzae* type b disease, fimbriated and nonfimbriated bacteria are equally virulent, whether they are given by intranasal or intraperitoneal routes. However, regardless of the state of fimbriation of the challenge, only nonfimbriated bacteria are isolated from the blood or cerebrospinal fluid (6; M. E. Pichichero, E. M. Connor, and P. W. Anderson, Pediatr. Res. **17**:279A, 1983), indicating a switch from the fimbriated state to the nonfimbriated state at some point in the process of pathogenesis.

An important question is whether *H. influenzae* type b fimbriae are virulence factors and, if so, at which stage in the development of systemic disease they function. The data from the infant rat experiments do not clarify this issue. Although the negative results obtained could be ascribed to a lack of influence of fimbriae on virulence, it is also possible, as indicated by the in vitro adherence data, that fimbriated *H. influenzae* type b variants differ from nonfimbriated forms only in their interaction with tissue from humans, their natural host.

Therefore, the adherence studies described in this report were done with human cells primarily. Although previous work had shown that *H. influenzae* type b fimbriae are associated with increased adherence to human BEC, these experiments made use of a radioactive assay. To overcome the drawbacks inherent in this assay (see below), we developed an indirect immunofluorescent adherence assay. We compared the results of the two methods not only with human BEC but also with two human-derived tissue culture cell lines, namely HEp-2 cells, which are epithelial in origin, and human foreskin fibroblasts. Most of our studies were

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done by using isogenic fimbriated and nonfimbriated variants. Pure cultures of either type were selected by the nitrocellulose hemadsorption method (3).

The two methods yielded similar data, with three different types of results obtained. Fimbriated *H. influenzae* type b variants adhered better than isogenic nonfimbriated variants to human BEC, to an equal but minimal degree to human foreskin fibroblasts, and less well to HEp-2 cells. In addition, adherence to two nonhuman cell lines (Buffalo green monkey kidney and Madin-Darby canine kidney cell lines) was not affected by the presence of fimbriae.

MATERIALS AND METHODS

Bacteria. *H. influenzae* strains were isolated from clinical specimens, serologically typed, and stored as skim milk stock cultures as previously described (8). Strain C47 (type b) was isolated from the cerebrospinal fluid of an 8-month-old infant. Strain C54 (also type b) was isolated from the nasopharynx of this infant upon recurrence of systemic disease 6 weeks later. Strain C54 is unusual in that it expresses fimbriae constitutively (however, see below). Except for the presence of a 23,000-dalton protein in strain C54, the outer membrane protein compositions of strains C47 and C54 are identical, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7). Strain 173 (untypeable) was isolated from a culture of upper respiratory tract epithelial cells (obtained by the nasal wash method) from an otherwise healthy 9-month-old child admitted for stridor.

Bacteria were grown in brain heart infusion broth or on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with nicotinamide adenine dinucleotide and hemin (8).

Isolation of isogenic cultures of fimbriated and nonfimbriated variants. Pure isogenic cultures of fimbriated and nonfimbriated strain C47 were obtained by the nitrocellulose hemadsorption technique (3). Briefly, bacteria were suspended in brain heart infusion broth after overnight growth on solid media and then plated to allow for growth of individual colonies. After overnight incubation, the colonies were partially transferred to a piece of nitrocellulose, which was then incubated in a suspension of human erythrocytes. Colonies containing fimbriated cells appeared as red dots on the nitrocellulose. A fimbriated colony and a nonfimbriated colony were then selected, cultured on solid medium, and used in experiments after overnight growth. Fimbriation was maintained for at least six passages on solid medium. These cultures were relatively pure; e.g., about 0.1% of the cells in a culture of nonfimbriated strain C47 were fimbriated (3). Strain C54 is constitutively fimbriated and was used without selection. Isogenic nonfimbriated strain C54 was obtained from the blood of infant rats infected intranasally with fimbriated strain C54 (Pichichero et al., Pediatr. Res. 17:279A, 1983). In most of the experiments described below the presence or absence of fimbriae was corroborated by electron microscopy of negatively stained bacteria, as described previously (3). Also, extensive studies of strains C47 and C54 have shown that the proportion of fimbriated bacteria as determined by the nitrocellulose hemadsorption method correlates closely with the proportion as determined by electron microscopy (3). An electron micrograph of fimbriated strain C54 showing peritrichous fimbriae has been published previously (11).

It should be noted that in early experiments, prior to the development of the selection methods described above, cultures of strains C47 and C54 were used as examples of nonfimbriated and fimbriated forms, respectively. Although probably not isogenic, these strains appear to be closely related.

Tissue culture target cells. Buffalo green monkey cells (obtained from Marilyn A. Menegus), HEp-2 cells, and human foreskin fibroblasts were maintained and grown on Autopow minimal essential medium containing 2% fetal calf serum. Madin-Darby canine kidney cells were maintained on minimal essential medium supplemented with (per 500 ml) 5 ml of 10% gelatin, 0.15 ml of trypsin (Worthington Diagnostics, Freehold, N.J.), 50,000 U of penicillin, 1.25 mg of amphotericin, and 146 mg of glutamine.

Adherence Assays. (i) Radioactive assay. *H. influenzae* type b cells were radioactively labeled by growth in [6-^{3}H]glucose, as described previously (11). Usually, the level of incorporation was 0.01 cpm/bacterium. Adherence to BEC was measured as described previously (11) by using 0.25 ml of a suspension containing 2×10^5 BEC per ml and 0.025 ml of a suspension containing 1×10^8 bacterial cells per ml. Adherence to tissue culture cells was determined as follows: 1-ml portions of cells (4×10^5 cells per ml) in minimal essential medium-2% fetal calf serum were placed into wells (diameter, 2.5 cm) of 12-well sterile tissue cultures dishes (Costar, Cambridge, Mass.) and incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. During this time the cells grew to confluency. The cells were washed twice with 8.1 mM Na_2HPO_4–1.5 mM KH_2PO_4–2.7 mM KCl–137 mM NaCl, overlaid with 0.5 ml of *H. influenzae* type b suspended in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.2) containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ at a concentration of 10^8 cells per ml, incubated at 37°C for 1 h in 5% CO_2, washed three times with PBS, and overlaid with 0.5 ml of trypsin–10 mM EDTA for 0.5 h at 37°C. The trypsinized cells were transferred to 10 ml of Scintiverse II (Fisher Scientific Co., Pittsburgh, Pa.), and adherent radioactivity was determined with a model 3375 scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

(ii) Immunofluorescent assay. Fimbriated or nonfimbriated *H. influenzae* type b cells were incubated overnight at 37°C on brain heart infusion agar. The cells were suspended in PBS to a density of 5×10^9 cells per ml and diluted with PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ to a density of 1×10^8 to 2×10^9 cells per ml; 0.25 ml of BEC (2×10^5 cells per ml) was mixed with 0.025 ml of bacteria, and the preparation was incubated at 37°C for 1 h. The BEC were washed free of nonadherent bacteria with PBS, suspended in 1 ml of PBS, placed onto ethanol-washed cover slips (1.2 cm), and allowed to dry overnight. Prior to staining the cells were fixed in cold 95% ethanol at 4°C for 30 min, rinsed with PBS, and overlaid with 0.4 ml of a 1:100 dilution (in PBS) of rabbit antiserum to Formalin-killed *H. influenzae* type b strain Eag. After incubation at 37°C for 1 h, the cover slips were washed three times with PBS and then incubated at 37°C for 1 h with 0.2 ml of a 1:80 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). (Prior to use the conjugate was cleared of microaggregates by centrifugation at 100,000 × *g* for 3 h.) The cover slips were rinsed three times with PBS, mounted on microscope slides with a 1:1 solution of 0.5 M $NaHCO_3$–glycerol, and kept refrigerated until they were examined (usually overnight). Adherence of *H. influenzae* type b to BEC was determined by observation with a fluorescence microscope, using a ×100 oil immersion lens. Adherence to cells as opposed to other material (e.g., mucus) was determined by examination of the preparation

by phase-contrast microscopy at ×100 magnification; 50 to 75 BEC were examined.

Adherence to tissue culture cells was determined as described above, except that the cells were grown as follows on cover slips for 24 h prior to the adherence assay: cover slips (diameter, 1.2 cm) were dipped in 95% ethanol, flame-sterilized, placed into wells of dishes (Costar), and overlaid with 1 ml of tissue culture cells suspended in growth medium (4×10^5 cells per ml). A total of 75 to 100 cells were examined as described above.

Antisera. Formalin-killed *H. influenzae* type b strain Eag was inoculated intravenously into a New Zealand white rabbit at intervals of 1 to 10 days for a total of 25 times. The serum was collected and stored at −70°C. The immunoglobulin fraction was partially purified by precipitation with 50% saturated ammonium sulfate and extensive dialysis of the resuspended precipitate against PBS. This material was stored in 0.1-ml portions at −20°C.

Statistics. Adherence data were analyzed by the *t*-test.

RESULTS

Adherence to BEC. In agreement with previous results (11), fimbriated *H. influenzae* type b strain C54 adhered in greater numbers than nonfimbriated bacteria (strain C47) to BEC, as measured by the radioactive assay ($P < 0.05$) (Table 1). Similar results were obtained with the immunofluorescent assay (Table 1), in which fimbriated variants of strains C47 and C54 adhered to greater extents than their nonfimbriated counterparts ($P < 0.01$).

The immunofluorescent assay enabled us to obtain additional data about the nature of adherence of *H. influenzae* type b. The distribution of bacteria on the individual BEC is shown in Fig. 1A, in which the cumulative percentage of observed BEC is graphed as a function of the number of adherent bacteria per BEC. In the cumulative data from three experiments, 60% of the BEC with adherent fimbriated strain C54 cells had four or more bacteria per cell, whereas only 10% of the BEC with adherent nonfimbriated strain C54 cells had more than four bacteria per cell. Furthermore, only 4% of the BEC were without adherent fimbriated strain C54 cells, in contrast to 37% of the BEC having no adherent nonfimbriated strain C54 cells. Also, although the human BEC contained some normal flora despite vigorous washing, it was clear from the immunofluorescent assay that *H. influenzae* type b adhered to the BEC and not to the indigenous bacteria. Some *H. influenzae* type b cells were seen adhering to mucous material; these bacteria were not counted. Finally, as the concentration of *H. influenzae* type b increased, so did the number of adherent bacteria per cell

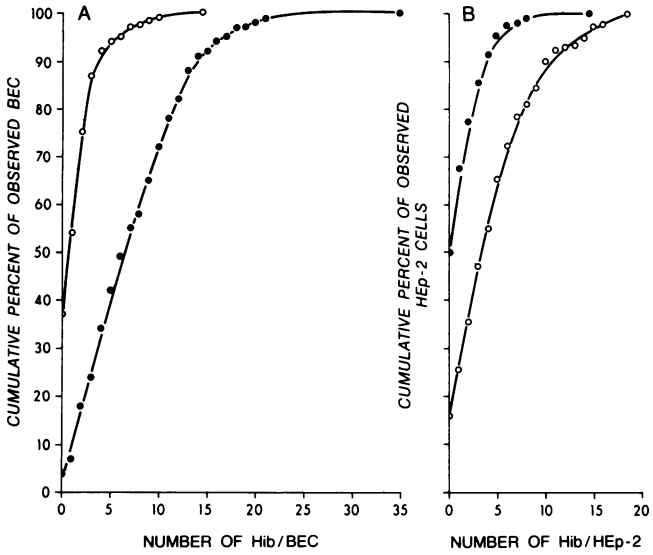

FIG. 1. Adherence of fimbriated and nonfimbriated *H. influenzae* type b strain C54 to human BEC and HEp-2 cells. BEC (A) and HEp-2 cells (B) were incubated with fimbriated (●) or nonfimbriated (○) strain C54, and the number of adherent strain C54 cells per target cell was determined by the immunofluorescent assay. Hib, *H. influenzae* type b.

(Table 2). However, the increase was far more striking for the fimbriated variant, where, at the highest concentration of *H. influenzae* type b tested, the large number of adherent bacteria made it difficult to visually determine the actual number of adherent bacteria per cell.

Tissue culture cells. In contrast to the data obtained with human BEC, fimbriated and nonfimbriated *H. influenzae* type b cells adhered in equal numbers to human foreskin fibroblasts, canine kidney cells, and Buffalo green monkey kidney cells. The general level of adherence was low (0.15 to 0.50 bacteria per cell), as measured by the radioactive assay. As with BEC, incubation of the cell cultures with increasing numbers of *H. influenzae* cells (up to 10×) increased the number of adherent bacteria per cell (up to 2×), but again no difference was observed between fimbriated and nonfimbriated bacteria (data not shown).

However, our results with HEp-2 cells were strikingly different. Nonfimbriated bacteria demonstrated greater adherence than fimbriated bacteria (Table 3). This result was obtained with both assays ($P < 0.05$ with the radioactive assay; $P < 0.02$ with the immunofluorescent assay). Of interest is the finding that strain S173, a nonfimbriated

TABLE 1. Adherence of fimbriated and nonfimbriated *H. influenzae* variants to human BEC

Strain	Fimbriae	No. of adherent bacteria per cell[a]						
		Radioactive assay				Immunofluorescent assay		
		Expt 1	Expt 2	Expt 3	Expt 4	Expt 1	Expt 2	Expt 3
C47	−	0.08	0.35	0.47	0.27	3.7	1.0	
C54	−				0.21	2.6	0.9	1.4
C47	+					4.0	5.9	
C54	+	0.30	2.14	2.11	2.80	9.3	5.8	7.6
S173	−				0.28			

[a] Bacterial concentration, 10^8 cells per ml.

TABLE 2. Effect of concentration of fimbriated and nonfimbriated *H. influenzae* variants on adherence to human cells

Bacterial concn (cells per ml)	No. of adherent bacteria per cell			
	BEC		HEp-2 cells	
	Nonfimbriated strain C54	Fimbriated strain C54	Nonfimbriated strain C54	Fimbriated strain C54
1×10^8	0.9	6.1	3.7	1.7
5×10^8	1.7	31	12	2.1
1×10^9	2.5	32	TNTC[a]	4.2
2×10^9	2.9	TNTC	TNTC	4.4

[a] TNTC, Too numerous to count (more than 40 *H. influenzae* type b cells per cell).

TABLE 3. Adherence of fimbriated and nonfimbriated *H. influenzae* variants to HEp-2 cells

Strain	Fimbriae	No. of adherent bacteria per cell[a]						
		Radioactive assay				Immunofluorescent assay		
		Expt 1	Expt 2	Expt 3	Expt 4	Expt 1	Expt 2	Expt 3
C47	−	1.7	6.0	3.6	3.2			4.9
C54	−				4.0	3.7	6.1	4.6
C47	+							0.4
C54	+	0.44	0.9	1.2	1.5	1.7	0.3	1.9
S173	−				6.8			11.9

[a] Bacterial concentration, 10^8 cells per ml.

untypeable *H. influenzae* strain, showed even greater adherence than the type b nonfimbriated strains; this strain had low adherence to BEC (Table 1). Also, as the concentration of nonfimbriated *H. influenzae* type b cells increased, so did the number of adherent bacteria; in contrast, the fimbriated bacteria showed a less striking increase (Table 2). Finally, (Fig. 1B) the distribution of nonfimbriated bacteria on the HEp-2 cells differed from the distribution of the fimbriated variants. Only 16% of the HEp-2 cells had no adherent nonfimbriated strain C54 cells, whereas 50% lacked fimbriated strain C54 cells. Furthermore, only 10% of the HEp-2 cells with adherent fimbriated bacteria had more than four bacteria per cell, whereas this was true for 55% of the HEp-2 cells exposed to adherent nonfimbriated bacteria.

DISCUSSION

The goal of our investigation was to determine whether the fimbriae of *H. influenzae* type b are associated with greater adherence of *H. influenzae* type b to host cells. As discussed above, recent experiments with infant rats, an excellent animal model of *H. influenzae* type b disease (9), demonstrated the need to use human tissue as the target cells.

Previous studies in which a radioactive assay was used had shown that fimbriated *H. influenzae* type b cells adhere in greater numbers than nonfimbriated *H. influenzae* type b cells to human BEC (11). Similar results were obtained in this study. However, the radioactive assay, although convenient, has the following shortcomings: (i) because of high background levels of radioactivity, only low concentrations of bacteria can be used; (ii) the method does not distinguish between the adherence of whole bacteria and the adherence of radioactive components from the bacteria; and (iii) the distribution of the bacteria on the target cells cannot be determined. By developing an immunofluorescent assay, we were able to show that indeed whole bacteria adhered to the BEC, that some adherence to mucus occurred, that adherence to indigenous bacteria did not occur, and that the number of adhering *H. influenzae* type b cells per BEC was related to the concentration of *H. influenzae* type b cells. Also, we found that the distribution of adherent *H. influenzae* type b cells was such that with nonfimbriated *H. influenzae* type b, 37% of the BEC contained no bacteria and only 8% had more than four *H. influenzae* type b cells, whereas with fimbriated *H. influenzae* type b, only 4% of the BEC had no *H. influenzae* type b cells and 66% had more than four BEC.

In contrast to the results obtained with BEC, two different types of results were obtained with tissue culture cell lines derived from humans. In one case, fimbriated and nonfimbriated *H. influenzae* type b cells adhered to equally low degrees to human foreskin fibroblasts. Similar results were also obtained with two nonhuman cell lines, Madin-Darby canine kidney cells and Buffalo green monkey kidney cells. Regarding the latter it is of interest that type 1 fimbriated (piliated) *Escherichia coli* cells adhere to a greater extent than nonfimbriated isogenic variants to monkey kidney cells (12).

In the second case, nonfimbriated *H. influenzae* type b showed strikingly greater adherence than fimbriated *H. influenzae* type b to HEp-2 cells, a cell line derived from a human nasopharyngeal carcinoma. Only 50% of the HEp-2 cells had adherent fimbriated *H. influenzae* type b cells, and only 8% had more than four fimbriated *H. influenzae* type b cells. In contrast 84% of the HEp-2 cells had adherent nonfimbriated *H. influenzae* type b cells, and 46% had more than four *H. influenzae* type b cells.

In studies by other workers involving HEp-2 cells, different results were obtained. (i) Fimbriated *Salmonella typhimurium* variants, especially if motile, adhered in greater numbers than isogenic nonfimbriated motile variants. This adherence was mannose sensitive (13). (ii) A total of 14% of enterotoxigenic *E. coli* isolates and 80% of enteropathogenic *E. coli* isolates adhered to HEp-2 cells, suggesting that the latter *E. coli* isolates are enriched for an adhesin that recognizes HEp-2 cells. However, no association of fimbriae with adherence (or failure to adhere) was found (4). (iii) Similar results showing no association of fimbriae with adherence were obtained with isolates of *Yersinia enterocolitica* (10). In the two latter studies, isogenic variants were not compared.

In summary, using isogenic variants, we demonstrated that fimbriated *H. influenzae* type b variants adhered better than nonfimbriated variants to human BEC but less well to HEp-2 cells and also that neither type of variant adhered well to human foreskin fibroblasts or to two nonhuman cell lines. For some bacteria adherence has been shown to involve specific recognition of a bacterial surface component by a host surface component (2). Based on our results, we postulate that human BEC have a receptor for *H. influenzae* type b fimbriae, that HEp-2 cells lack this receptor but have a receptor for a nonfimbrial surface component, and that human foreskin fibroblasts lack receptors for either fimbriated or nonfimbriated *H. influenzae* type b. Thus, our data suggest that *H. influenzae* has at least two adhesins for human cells, one of which is associated with fimbriae. It should be emphasized, however, that these conclusions are based on data obtained under only one set of conditions of temperature, pH, etc.

The failure of fimbriated variants of *H. influenzae* type b to adhere to HEp-2 cells indicates either that these variants fail to express an adhesin present on isogenic nonfimbriated cells or that the fimbriae occlude access of this other *H. influenzae* type b adhesin to HEp-2 cells. Since nonfimbrial forms of both unencapsulated (strain S173) and type b encapsulated *H. influenzae* adhere to HEp-2 cells, this other adhesin must be accessible even in the presence of capsule. Although only one set of experiments was performed with strain S173, the data did show that this strain adhered better than nonfimbriated encapsulated *H. influenzae* type b, suggesting that capsule may partially block accessibility to this other adhesin.

If *H. influenzae* type b possesses two adhesins, it will be interesting to determine whether these adhesins function in pathogenesis and at which stage. The first step in acquisition of *H. influenzae* type b disease is considered to be adherence

to and colonization of the nasopharyngeal epithelium (1, 9). In our experiments, human BEC and tissue culture cells were selected as target cells because they are readily obtainable. However, the divergent data which we have collected indicate that to determine whether *H. influenzae* type b has an adhesin that actually enhances colonization of the human upper respiratory tract, human target cells obtained from the natural sites of *H. influenzae* type b colonization should be studied. Hence, experiments to analyze the attachment of *H. influenzae* type b to human nasopharyngeal cells are currently in progress.

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