Adherence Kinetics of *Haemophilus influenzae* Type b to Eucaryotic Cells¹

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ABSTRACT. *Haemophilus influenzae* type b (Hib) adhere *in vitro* to buccal epithelial cells (BEC) via pili (fimbriae). *In vivo* studies have conflicting lines of evidence concerning the role of pili in adherence. This study characterizes the kinetics of Hib binding to BEC and compares pilus *versus* nonpilus adherence. Adherence was assessed using radioactive, immunofluorescence, and electron microscopic techniques. Paired Hib strains were obtained from three children with Hib meningitis; pilated Hib were isolated from the nasopharynx and nonpilated isolates from the cerebrospinal fluid. Pilated strains adhered avidly to BEC after a 1-h incubation, whereas nonpilated strains adhered poorly ($p = 0.002$). Kinetic analysis revealed increased adherence to BEC by pilated and nonpilated strains with time, with maximum adherence occurring between 24–36 h. Immunofluorescence and electron microscopic assays visually confirmed the radioactive adherence findings. These results characterize the kinetics of Hib adherence to BEC. BEC incubated with nonpilated Hib for 30 h were found to be coated with pilated strains, suggesting the induction of pilus production or the selection of a pilated subpopulation. (*Pediatr Res* 26:500–503, 1989)

Abbreviations

BEC, buccal epithelial cells
BHI, brain heart infusion
CFU, colony-forming unit
CSF, cerebrospinal fluid
EM, electron microscopy
Hib, *Haemophilus influenzae* type b
³H-Hib, tritium-labeled Hib
IF, immunofluorescent
LOS, lipooligosaccharide
NP, nasopharyngeal
P+, pilated
P–, nonpilated
PRP, polyribosylribitol phosphate

Hib is the leading cause of bacterial meningitis in the United States (1). An infant rat model for Hib meningitis has delineated four separate, sequential steps in the pathogenesis consisting of

1) naso/oropharyngeal colonization, 2) penetration of the mucosa with entry into the bloodstream, 3) development of a bacteremia, and 4) development of meningitis (2, 3).

The colonization of the naso/oropharyngeal area is considered to be the initial step in the pathogenesis of Hib infections (4). The adherence of Hib to naso/oropharyngeal cells for colonization has not been clearly defined. There are conflicting lines of evidence as to the contribution pili or fimbriae have in this process. *In vitro*, P+ Hib have an increased adherence to erythrocytes (5), BEC (6), and pharyngeal cells (7), but the majority of NP isolates of Hib from patients with systemic diseases at the time of hospital admission are P–, and all blood and CSF isolates are P– (8). Additionally, P+ and P– Hib are equally virulent in the infant rat model (9). This study further characterizes the kinetics of Hib adherence for P+ and P– isolates to BEC.

MATERIALS AND METHODS

Bacterial Strains and Media. Hib isolates used in this study have been previously described (8). Three paired isolates were studied, 1007, 1009; 880, 884; 1228, 1264 consisting of a CSF P– isolate and a NP P+ isolate, respectively. EM was used to confirm the presence or absence of pili (8).

Hib were grown in BHI broth supplemented with 10 µg/mL of NAD and Hemin (Sigma Chemical Company, St. Louis, MO) in 5% CO₂ at 37°C. Isolates were not serially passaged but were frozen at –70°C in BHI with 10% glycerol, pH 7.3.

Hib were labeled for radioactive adherence assay by inoculating five colonies of Hib into 10 mL of BHI supplemented with NAD and Hemin with 25 µCi tritium-labeled amino acids (1 µCi/µL; TRK 440 Amersham Corp., Arlington Heights, IL) overnight. The ³H-Hib were harvested by centrifugation at 7000 × *g* for 10 min at 4°C and washed three times in Dulbecco's PBS, pH 7.3. The specific activity was 1.5×10^{-4} to 5.5×10^{-5} cpm/CFU. The organisms were resuspended in Dulbecco's PBS, pH 7.3 with 1% gelatin (Sigma).

Eucaryotic Cells. BEC were obtained from healthy male volunteers and handled in a manner previously described (10). A final suspension of 10⁴ BEC/mL was obtained by counting using a hemocytometer. Cell viability was assessed by adding 0.9 mL trypan blue (GIBCO Laboratories, Grand Island, NY) with 0.1 mL of eucaryotic cells and counting 100 cells with a hemocytometer.

Adherence Assays. Radioactive assay. A total of 0.5 mL of ³H-Hib at 10⁸ CFU/mL was incubated with 0.5 mL of BEC (10⁴ cells/mL) for varying time periods at 37°C in a reciprocal water bath. Colony counts were determined for each experiment. Non-adherent ³H-Hib were removed by washing cells three times with Dulbecco's PBS, pH 7.3 using a Millipore manifold system (Millipore Corporation, Bedford, MA) with 12-µm Nucleopore polycarbonate filters (Nucleopore, Pleasanton, CA). The filters

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were removed from the manifold and placed into scintillation vials with 10 mL of scintillation fluid (Scintiverse II, Fisher Scientific, Orangeburg, NY). Duplicate vials were counted in a scintillation counter for 2 min.

Controls consisted of 1) ^3H -Hib alone without BEC and 2) a control for the spontaneous release of ^3H by ^3H -Hib with subsequent uptake by BEC (radio-release control). Total cpm for ^3H -Hib was obtained by placing 100 μL of washed ^3H -Hib into a scintillation vial, counting as described above, and multiplying by 5 to give total cpm per 0.5 mL.

Briefly, the radio-release control involved incubating the ^3H -Hib (10^8 cfu/mL) without BEC for identical time periods as the adherence assay. The ^3H -Hib were then removed by a filtration using a 0.22- μm syringe filter (Millipore Corporation). The filtrate was then incubated with fresh BEC (10^4 cells/mL) for identical incubation periods as the adherence assay and handled as described above for radioactive counting.

An adherence percentage was calculated using the following formula:

$$\frac{(\text{cpm of BEC} + ^3\text{H-Hib}) - (\text{cpm } ^3\text{H-Hib alone}) - (\text{cpm of radio-release control})}{\text{total cpm of } ^3\text{H-Hib}} \times 100$$

IF adherence assay. The indirect IF adherence assay was identical to the radioactive adherence assay except the Hib were not radiolabeled. After incubation for varying times, the bound Hib to BEC were removed from the unbound Hib using the Millipore manifold system with Nucleopore filters as described. The cells were displaced from the filters with gentle agitation into Dulbecco's PBS, pH 7.3, and subsequently spun onto glass slides using a cytospin centrifuge. Controls consisted of Hib and BEC alone, or BEC plus an Hib strain known to adhere by EM studies. The slides were air-dried and fixed in ethanol. Indirect IF was achieved by incubating the slide for 60 min in a moist chamber at room temperature with an anti-Hib LOS murine MAb kindly provided by Dr. Eric J. Hansen, University of Texas Health Science Center at Dallas as the first step. Anti-Hib LOS was used instead of anti-whole Hib antisera containing predominantly anticapsular PRP antibody because of the difficulty of released capsular PRP during incubation periods as previously described by Anderson *et al.* (11). The released PRP coated the BEC, increasing the background intensity, rendering fluorescent visualization very difficult. This problem was overcome by using an anti-LOS MAb which was known to be specific for the LOS-serotype (12) of the isolates studied. This MAb has been described elsewhere (13). After incubation with anti-LOS MAb the slides were washed and incubated with fluorescein-conjugated goat antimouse IgG (Sigma) for 30 min in a moist chamber. The slides were again washed and counterstained with 0.2% Evans blue dye for 2 min, allowed to air dry in the dark and examined by oil immersion with a fluorescence microscope. The number of adherent bacteria per BEC were quantitated for 25–50 eucaryotic cells.

EM adherence assay. The assay system was identical to the indirect IF adherence assay. The nonadherent Hib were again removed using Nucleopore filters. Adherent Hib to BEC were fixed in 3% phosphate-buffered glutaraldehyde and subjected to critical-point drying with carbon dioxide. The samples were then coated with 150 angstroms (15 nm) of gold palladium in a Denton vacuum evaporator. Specimens were viewed with a Joel 100/cx electron microscope. The number of adherent bacteria per BEC was assessed counting 50 eucaryotic cells.

Statistical Analysis. Student's *t* test analysis was used for statistical analysis of continuous variables. Proportions were analyzed by χ^2 .

RESULTS

Three paired isolates from children with Hib meningitis were used for adherence kinetics using BEC. One set of isolates (1007

and 1009) was studied in depth. All paired isolates consisted of a NP isolate which was predominately (>98%) P+ and a CSF isolate which was predominately (>99%) P-. The presence or absence of pili was assessed using EM (data not shown).

Figure 1 shows a kinetic analysis of the two prototypic isolates, 1007 (P-) and 1009 (P+) adhering to BEC as assessed by percent radioactive binding. The pilated isolate, 1009, had significantly higher percent radioactive binding to BEC than the P- isolate ($p = 0.002$). Minimal binding was noted at 1 h for 1007 (P-) adhering to BEC. Both Hib isolates showed increasing adherence to BEC with time with maximum binding occurring at 36 h. Quantitative studies measuring bacterial CFU obtained during this time study showed a maximum 2-log death rate over 48 h. Bacterial contamination from normal oral flora became apparent at 36–48 h with BEC adherence. No appreciable change in viability of BEC with time was evident using trypan blue studies. Thus, a 30-h incubation was chosen as a prolonged time point to compare to the standard 1-h incubation for time kinetic studies.

Table 1 shows percent radioactive binding of the two prototypic Hib isolates with BEC at 1- and 30-h incubation, 1007 (P-) and 1009 (P+) bound to BEC at 30 h significantly more when compared to 1 h binding values ($p \leq 0.002$). Of note is the moderate degree of adherence by the non-piliated isolate, 1007 to BEC cells at 30 h.

Fig. 1. Kinetic analysis of percent radioactive binding of prototypic P+ (1009) and P- (1007) Hib to BEC.

Table 1. Time kinetic adherence as assessed by radioactive binding assay for prototypic pilated (1009) and nonpilated (1007) Hib strains to BEC

Strain	% Radioactive Binding		<i>p</i>
	1 H	30 H	
1007 (P-)	0.003 ± 0.008* (6)†	0.42 ± 0.1 (9)	<0.001
1009 (P+)	0.16 ± 0.06 (4)	1.83 ± 0.78 (6)	0.002

* Mean ± SD.

† Number of experiments, each in duplicate in parentheses.

Table 2. Time kinetic adherence as assessed by radioactive binding for paired Hib strains to BEC*

Strain	% Radioactive binding		<i>p</i>
	1 H	30 H	
880 (P-)	0	2.03 ± 0.80†	<0.001
884 (P+)	0.94 ± 0.13	2.57 ± 0.70	0.0004
1228 (P-)	0	0.70 ± 0.16	<0.001
1264 (P+)	0.22 ± 0.08	1.13 ± 0.89	0.06

* Results of two experiments, each in duplicate.

† Mean ± SD.

Complementary data using two additional paired isolates are shown in Table 2. No adherence was detected at 1 h except for piliated isolates (884, 1264) binding to BEC cells. All isolates adhered to BEC at 30 h; adherence in all cases but one (1264 to BEC) was significantly increased at 30 h compared to binding at 1 h, regardless of the initial state of piliation.

Indirect IF and EM adherence assays were used to visualize adherence independent of the radioactive adherence data. Table 3 shows the percentage of BEC with different numbers of adherent Hib per eucaryotic cell at 1 and 30 h for the two prototypic

Table 3. Adherence of Hib to BEC: representative indirect immunofluorescent and electron microscopic studies

	No. of adherent bacteria/cell					
	Incubation time					
	1 H			30 H		
Immunofluorescence	0	1-5	>5	0	1-5	>5
1007 (P-)	92*	8	0	52	48	0
1009 (P+)	20	32	48	0	28	72
Electron microscopy						
1007 (P-)	84	12	4	0	16	84
1009 (P+)	8	30	62	2	2	96

* Percentage of buccal epithelial cells with the indicated number of adherent Hib. *p* values for all comparisons (1 versus 30 h) $p < 0.001$.

isolates. Small numbers of 1007 (P-) could be seen adhering to BEC at 1 h. The piliated Hib isolate (1009) adhered well at 1 h both with the indirect IF and EM studies. At 30 h all isolates adhered in significantly greater numbers to BEC compared to 1 h values confirming the radioactive assay.

Figure 2A shows EM photomicrographs of the piliated prototypic Hib isolate (1009) with more than 5 bacteria bound to this BEC at 1 h. Figure 2B shows this same strain, 1009 binding to a BEC cell at 30-h incubation with an appreciable increase in bound bacteria. The P isolate, 1007, had minimal binding to BEC at 1 h (figure not shown), but at 30 h the majority (84%) of BEC had >5 bacteria adhering to them (Fig. 3A). The presence of pili on the adherent bacteria became apparent upon increasing the magnification to 30 000 $\times$ (Fig. 3B).

DISCUSSION

The ability of Hib to adhere to the naso/oropharyngeal epithelia is the presumed initial step in pathogenesis of Hib infections. Pili or fimbriae discovered on Hib in 1982 (6, 7) were originally thought to be the sole mediators of Hib adherence in a manner similar to Enterobacteriaceae adherence (14). Recent evidence has suggested the presence of a second Hib adhesin in addition to pili (15, 16). The significance of these two adhesins is not known. This study was designed to evaluate the role of pili in adherence with time because 1) P- Hib that do not adhere to BEC *in vitro* are the predominant organisms found in the nasopharynx in patients with systemic disease (8), and 2) the mucous

Fig. 2. EM photomicrographs of prototypic Hib isolate 1009 adhering to buccal epithelial cells at 1 h (A, 3000 $\times$) and at 30 h (B, 6000 $\times$) incubations.

Fig. 3. EM photomicrographs of prototypic Hib isolate 1007 adhering to BEC at 30-h incubation (A, 4000 $\times$). B shows higher magnification of Hib isolate 1007 adhering to BEC with apparent pili (30 000 $\times$).

blanket which coats the NP region (17) could keep the Hib in close proximity to eucaryotic cells for prolonged intervals.

We studied P+ and P- Hib adherence to BEC with three paired Hib clinical isolates over a 30-h time period. The conditions of our assay were kept similar to previous studies to compare time-course analysis. A ratio of bacteria to eucaryotic cells of 10⁴:1 was chosen because other adherence model systems (18, 19) and the Hib system (10, 20) used this ratio. The temperature of 37°C and pH of 7.3 were kept constant as previously described (10). Additionally, one percent gelatin was included in our assay system to minimize non-specific binding to Nucleopore filters (9).

Kinetic analyses were evaluated for P+ and P- mediated adherence. Previous work by Lampe *et al.* (10) and Pichichero (20) had shown little change in adherence with epithelial cells after 30 min of incubation, although the longest incubation time was 2 h in the Lampe *et al.* (10) study.

In this study, all three modalities used to assess adherence reconfirmed binding of piliated Hib to BEC at 1 h. Timed adherence experiments showed a significant increase in adherence with time which has not been previously reported. The reason for this increased adherence is not known but could be due to an increase in exposed pili receptors on BEC, an increase in the number of pili on Hib, or an increase in exposure time allowing more Hib to adhere to BEC. The P- strains bound minimally or not at all to BEC at 1 h which is consistent with their being >99% P-; incubation to 30 h produced pronounced adherence of the P- strain to BEC in all three assay systems. EM studies showed pili on the attached Hib which were more than 99% P- when first incubated with the BEC. This increased binding with time by the P- Hib appears to be occurring by the proposed high frequency switching of the P- Hib to a P+ form (5).

We have shown that the adherence of both P+ and P- Hib to BEC increases as the incubation period increases. EM data have shown that the P- Hib have demonstrable pili on the surface when bound to BEC at 30 h indicating that Hib could modify their state of piliation to achieve adherence.

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