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# Role of Adherence in the Pathogenesis of *Haemophilus influenzae* Type b Infection in Infant Rats

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We evaluated the role of pili in the pathogenesis of disease due to *Haemophilus influenzae* type b (HiTb), using the infant rat model. Piliated and nonpiliated HiTb strains were isolated from the nasopharynx and cerebrospinal fluid, respectively, of three children. Infant rats inoculated intranasally with nonadherent HiTb developed bacteremia and meningitis more frequently ($P = 0.005$) than animals inoculated with companion adherent HiTb strains. When analyzed separately, only one HiTb pair (884/880) demonstrated significant differences in the incidence of bacteremia and meningitis between the adherent and nonadherent strains. Blood or cerebrospinal isolates recovered from infant rats inoculated with piliated adherent HiTb strains were not piliated and were not adherent in vitro. Adherent and nonadherent HiTb colonized the nasopharynx of infant rats equally. The piliated strains of HiTb were not adherent in vivo or in vitro to rat nasal or buccal epithelial cells, respectively. Piliated strains of HiTb have no apparent advantage over nonpiliated HiTb strains for colonization or invasion of infant rats. Furthermore, the loss of piliation is noted for cerebrospinal fluid, blood, and nasal isolates of HiTb cultured from infant rats inoculated with an adherent piliated HiTb strain. Thus, the loss or suppression of pili may be an important prerequisite for the invasion of the host by *Hi*Tb strains that are highly piliated.

For certain bacteria, the adherence and colonization of epithelial surfaces precede invasion of the host, and pili are important mediators of this attachment (3, 13). The inhibition of adherence by receptor analogs or pilus antibodies may prevent colonization and ultimately, bacterial infection (2, 22).

*Haemophilus influenzae* type b (HiTb), *Streptococcus pneumoniae*, and *Neisseria meningitidis* are the three most common organisms responsible for bacterial meningitis in children over 2 months of age. All have a polysaccharide capsule which is thought to be an important virulence factor; however, the role of adherence of these bacteria to epithelial surfaces has not been demonstrated to be important in either colonization or pathogenesis of disease. Serotypeable strains of *N. meningitidis* and isolates of *N. meningitidis* from patients with systemic illness adhere less to human buccal epithelial cells (BEC) than nongroupable or nasopharyngeal isolates of *N. meningitidis* from carriers (4, 21). Nevertheless, strains of *N. meningitidis* with either high or low adherence to BEC in vitro have pili which are equivalent in size and number (4, 21). Pili have been detected by electron microscopy in the cerebrospinal fluid (CSF) of an infant with *N. meningitidis* meningitis, but the significance of this finding is unknown (20). Anderson et al. (1) noted that isolates of *S. pneumoniae* from the nasopharynx of patients with frequent episodes of otitis media or from healthy carriers adhered to BEC in vitro in greater numbers than did isolates from patients with meningitis or septicemia. Selinger and Reed (18) demonstrated that the polysaccharide capsule of *S. pneumoniae* interfered with adherence to BEC. Thus, for both *N. meningitidis* and *S. pneumoniae*, strains which are isolated from carriers tend to adhere more avidly to BEC than do isolates associated with systemic disease.

HiTb strains from any source generally adhere poorly to human BEC compared with non-encapsulated strains (9). Furthermore, HiTb isolates from the CSF or blood of children do not adhere to BEC of these same children (9). We have isolated three HiTb strains from the nasopharynx of three children with HiTb meningitis on admission which adhere readily to BEC; 90% of these three organisms appear to be piliated by electron microscopy (E. O. Mason, Jr., S. L. Kaplan, E. P. Norrod, W. A. Stenback, and R. D. Feigin, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 648, 1982). The CSF isolates from these same children did not

adhere to BEC, and less than 5% of the organisms had pili. Two groups have shown that piliated subpopulations of HiTb can be demonstrated by enrichment techniques for many strains and suggested that the pili may be important for oropharyngeal colonization of HiTb (5, 15). In this study, we have employed the infant rat model to evaluate the role of pili in vivo in the pathogenesis of HiTb infections.

## MATERIALS AND METHODS

**HiTb strains.** Three strains of HiTb were isolated from the CSF of three children with bacterial meningitis at Texas Children's Hospital. Nasopharyngeal strains of HiTb from these same children were isolated at the time of admission on selective agar containing HiTb antisera. Burro anti-polyribosephosphate (PRP) antibody was kindly provided by J. B. Robbins, Bureau of Biologics, Bethesda, Md. HiTb were identified by halos surrounding characteristic colonies after 48 h and confirmed by standard techniques. All strains were stored at −70°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) with 10% glycerol at pH 7.3 after a single passage from the original culture. For in vitro adherence experiments, the bacteria were grown overnight at 35°C in 10 ml of TSB supplemented with NAD and hemin (10 μg/ml each; Sigma Chemical Co., St. Louis, Mo). (We have demonstrated previously that the in vitro adherence of HiTb to BEC does not vary with the type of broth used for growth.) The in vitro adherence experiments were evaluated by fluorescent-antibody and radioisotope-labeled methods, as previously described, with one modification (9). One percent gelatin was added to the phosphate-buffered saline in the radioisotope method to decrease the nonspecific adherence of bacteria to polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.).

In vitro adherence to infant rat BEC was determined in a manner identical to that used for human BEC. Rat BEC were obtained by swabbing the oropharynx of healthy uninfected infant rats with the soft tip of a flexible-handle nasopharyngeal swab. The BEC from 10 infant rats were pooled and washed before incubation with HiTb. The in vivo adherence of HiTb to nasal mucosal cells was determined by allowing the animals to inhale 20 μl of normal saline 2 days after approximately $10^7$ CFU of HiTb was administered intranasally. The saline was aspirated from the nose. The aspirates were pooled and washed, and the epithelial cells were transferred to glass slides by cytocentrifugation and stained with fluorescent antibody to HiTb.

**Infant rat model.** HiTb strains were grown overnight in 10 ml of brain heart infusion (BHI) broth supplemented with NAD and hemin. Ten milliliters of fresh supplemented BHI broth were inoculated with the overnight culture and incubated at 37°C with shaking for 3 h. The organisms were washed twice before suspension in approximately 4 ml of phosphate-buffered saline (pH 7.4) with 0.1% gelatin to give an optical density of 0.8 at 540 nm, corresponding to $1 \times 10^8$ to $5 \times 10^8$ CFU/ml.

The infant rat model of HiTb meningitis was that of Moxon et al. (12). Pathogen-free outbred albino Sprague-Dawley rats were obtained from the Texas Inbred Mice Co. Houston. Food and water were available ad libitum, and the animals were housed under standard conditions. We could not detect bacteria that cross-reacted with HiTb antisera in the normal nasopharyngeal flora of these infant rats, and the intraperitoneal injection of HiTb resulted in a 100% incidence of bacteremia.

For most experiments, 20 μl (approximately $2 \times 10^6$ to $1 \times 10^7$ CFU) of HiTb suspension was inoculated atraumatically to the nares of 4- to 6-day-old infant rats through a 23-gauge scalp vein needle. This inoculum was delivered three times a day for 3 consecutive days between 10 a.m. and 4 p.m. For other experiments, the same number of organisms were inoculated one to three times per day for 1 to 3 consecutive days to determine the effect of inoculum size on the development of bacteremia and meningitis. After random mixing, each week one group of infant rats received adherent HiTb, and a second group received the companion nonadherent HiTb isolate. The animals were sacrificed 6 days after the initial inoculation. At this time, nasal cultures were obtained by allowing the animals to inhale a drop of sterile phosphate-buffered saline which subsequently was aspirated from the nares and plated onto agar containing HiTb antiserum (6). Five to ten nasopharyngeal colonies were selected from several infant rats for in vitro adherence studies. After the intraperitoneal injection of pentobarbital sodium, blood cultures were obtained by heart puncture, and 0.1 ml was plated onto chocolate agar. CSF was obtained by puncture of the cisterna magnum with a sterile 21-gauge needle after the skin and soft tissues had been dissected free (11). CSF flowed freely into capillary pipettes. Twenty microliters were obtained for culture on chocolate agar and for an erythrocyte/leukocyte count. CSF cultures from bacteremic rats were considered contaminated with blood if fewer than 20 colonies of HiTb were isolated from 10 μl of CSF and the erythrocyte count exceeded 10,000/mm$^3$. Atraumatic nasal instillation of HiTb strain Eagan, as described above, caused bacteremia and meningitis in 23 of 35 (66%) and 21 of 35 (60%) infant rats, respectively, in preliminary experiments.

**Quantitation of PRP.** The PRP capsular material from whole HiTb cultures was determined by an enzyme-linked immunosorbent assay. Briefly, hyperimmune rabbit anti-PRP antisera and normal rabbit sera were adsorbed onto alternating wells of a microtiter plate (Immunlon AUS-coated; Dynatech Laboratories, Inc., Alexandria, Va.) with coating buffer. The wells were washed, and 100 μl of appropriate dilutions of whole cultures of HiTb in the log phase of growth was placed in antibody-coated and normal rabbit serum-coated wells in triplicate. PRP (a gift of Robert Baker, Eli Lilly & Co., Indianapolis, Inc.) solutions in concentrations of 100, 10, 1, and 0.1 ng/ml were run as standards. After incubation and washing, 100 μl of rabbit anti-PRP antisera conjugated to alkaline-phosphatase was added. The wells were incubated and washed three times, and *p*-nitrophenylphosphate (1.0 mg/ml) in diethanolamine buffer (pH 9.6) was added as the substrate. The reaction was stopped with 0.1 N NaOH when the 100 ng/ml of PRP standard reached an optical density of 1.5; the optical density was determined at 405 nm with a MR530 MicroELISA Reader (Dynatech Laboratories). The total PRP content of the HiTb culture (organisms plus supernatant) was adjusted for $10^7$ CFU/ml.

**SDS-PAGE protein analysis.** HiTb cells were grown overnight in 10 ml of supplemented BHI broth. Whole cell lysates were prepared by the centrifugation of the cells, followed by suspension in 1 ml of distilled water. The cells were lysed in an equivalent amount of buffer containing 5% mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 0.0625 M Tris-hydrochloride and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8). Partially purified pilus preparations were prepared by the method of Salit and Gotschlich (16). The cells were harvested from 20 chocolate agar plates into cold 50 mM Tris-hydrochloride buffer (pH 7.2) and homogenized. The cellular material was removed by centrifugation at 10,000 × *g*, and the clear supernatant was dialyzed overnight against 100 mM sodium acetate buffer (pH 4.2). The precipitate (pilus protein and contaminating cell membrane proteins) was collected by centrifugation, suspended in 0.05 M Tris (pH 7.2), and analyzed by SDS-PAGE.

## RESULTS

The characteristics of the HiTb strains used in these experiments are shown in Table 1. PRP production by adherent and nonadherent companion strains was essentially equivalent.

When approximately $10^7$ CFU were administered three times per day for 3 consecutive days, 84 of 226 (39%) infant rats inoculated with nonadherent HiTb (880, 1007, or 1228) developed bacteremia; in contrast, 51 of 208 (26%) animals inoculated with companion adherent HiTb strains (884, 1009, or 1264) were bacteremic ($\chi^2$ = 8.1, $P$ = 0.005). Of 225 infant rats receiving nonadherent HiTb, 62 (28%), versus 34 of 208 [16%] animals given an adherent strain) developed meningitis ($\chi^2$ = 7.9, $P$ = 0.005). Approximately the same percentage of bacteremic animals in each group developed meningitis. In all instances, the bacterial densities in blood cultures were greater than $3 \times 10^3$ CFU/ml, and those in CSF cultures were greater than $3 \times 10^4$ CFU/ml.

A separate analysis of the findings for each adherent-nonadherent HiTb pair revealed differences in the results (Tables 2 and 3). Significant differences in the incidences of bacteremia and meningitis were found in the 880/884 pair only, although meningitis tended to occur more frequently in animals given 1007 compared with strain 1009. Furthermore, at lower inocula (one to three doses for 1 day), we found no differences in the incidence of bacteremia of meningitis between animals administered adherent versus nonadherent HiTb 6 days after inoculation (Table 4). In the experiments using one to three inoculations, four animals receiving nonadherent HiTb and one animal given an adherent HiTb had positive blood cultures 48 h after inoculation.

CSF or blood isolates recovered from infant rats inoculated with adherent HiTb strains 884, 1009, or 1264 were no longer adherent to BEC by in vitro adherence assays. SDS-PAGE analysis of whole cell lysates of HiTb strains 884 and 1009 indicated a protein band at a molecular weight of 25,000 that was not present for strains 880 or 1007. Furthermore, this 25,000-molecular-weight band was qualitatively or quantitatively less apparent for CSF isolates obtained from the infant rats which developed meningitis with strains 884 or 1009 compared with the parent strain (Fig. 1 and 2).

We found no significant difference in the percentage of animals with HiTb isolated from nasal washout cultures at day 6 after an initial inoculation (three doses per day for 3 days) of nonadherent HiTb (63 of 85 [74.1%]) compared with adherent strains (48 of 73 [65.6%]). After one to three intranasal doses of HiTb in 1 day, 39 of 48

TABLE 1. Source, in vitro adherence characteristics, and PRP production of HiTb strains

| Strain no. | Source | In vitro adherence | PRP production (ng/$10^7$ CFU) |
|---|---|---|---|
| 880 | CSF; child no. 1 | No | 187 |
| 884 | Nasopharynx; child no. 1 | Yes | 206 |
| 1007 | CSF; child no. 2 | No | 104 |
| 1009 | Nasopharynx; child no. 2 | Yes | 96 |
| 1228 | CSF; child no. 3 | No | 71 |
| 1264 | Nasopharynx; child no. 3 | Yes | 98 |

TABLE 2. Incidence of bacteremia in infant rats[a]

| Strain no. | No. positive/ no. tested | $\chi^2$ | $P$ |
|---|---|---|---|
| 880 | 28/99 | 16.3 | 0.00005 |
| 884 | 4/80 | | |
| 1007 | 40/76 | 0.5 | 0.5 |
| 1009 | 36/76 | | |
| 1228 | 16/51 | 1.39 | 0.24 |
| 1264 | 11/52 | | |

[a] The rats were inoculated intranasally with nonadherent versus adherent strains of HiTb (after three doses per day for 3 consecutive days).

TABLE 3. Incidence of meningitis in infant rats[a]

| Strain no. | No. positive/ no. tested | $\chi^2$ | $P$ |
|---|---|---|---|
| 880 | 18/98 | 9.0 | 0.003 |
| 884 | 3/80 | | |
| 1007 | 30/76 | 2.58 | 0.11 |
| 1009 | 21/76 | | |
| 1228 | 14/51 | 0.973 | 0.32 |
| 1264 | 10/52 | | |

[a] The rats were inoculated intranasally with nonadherent versus adherent strains of HiTb (after three doses per day for 3 consecutive days).

TABLE 4. Incidence of bacteremia and meningitis in infant rats[a]

| Organism | No. of doses | Blood culture[b] | CSF culture[b] |
|---|---|---|---|
| 1007 | 1 | 0/10 | 0/10 |
| | 3 | 2/10 | 2/10 |
| 1009 | 1 | 0/10 | 0/10 |
| | 3 | 1/9 | 1/9 |
| 1228 | 1 | 4/18 | 3/18 |
| | 3 | 1/11 | 1/11 |
| 1264 | 1 | 4/16 | 3/16 |
| | 3 | 2/11 | 2/11 |

[a] At 6 days after inoculation once or three times with adherent or nonadherent strains of HiTb.
[b] Number positive/number tested.

(81%) of infant rats given nonadherent HiTb and 41 of 46 (89%) inoculated with adherent HiTb had positive nasal cultures 6 days later. Furthermore, HiTb isolated from the nasal washout cultures of 50% of the infant rats inoculated with strains 884, 1009, or 1264 were no longer adherent in vitro. However, nonadherent isolates were not cultured more often than adherent isolates from animals which were bacteremic. The nonadherent CSF or nasal washout strains of HiTb that were cultured from animals inoculated with strains 1009 or 1264 did not become adherent in vitro after multiple passages on chocolate agar. HiTb isolates from the nasopharyngeal washout of infant rats inoculated with strain 1007 were not adherent in vitro.

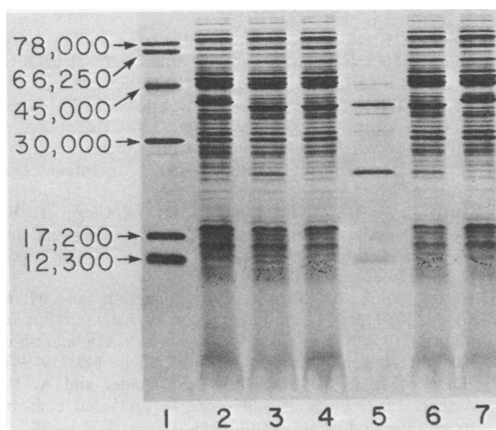


FIG. 1. SDS-PAGE whole cell lysate analysis. Lane 2, HiTb 880 nonadherent isolate from CSF of child no. 1; lane 3, *Hi*Tb no. 884 adherent isolate from nasopharynx of child no. 1; lane 4, isolate no. 884 recovered from the CSF of infant rat (now nonadherent); lane 5, partially purified pilus preparation derived from no. 884; lane 6, isolate no. 884 (same as lane 3); lane 7, same as lane 2. The conditions for PAGE were: 30:0.8 acrylamide- bisacrylamide ratio, 15% acrylamide stained with Coomassie blue R (17). Lane 1, Molecular weight standards (ovatransferrin, bovine serum albumin, ovalbumins, carbonic anhydrase, myoglobin, and cytochrome *c*).

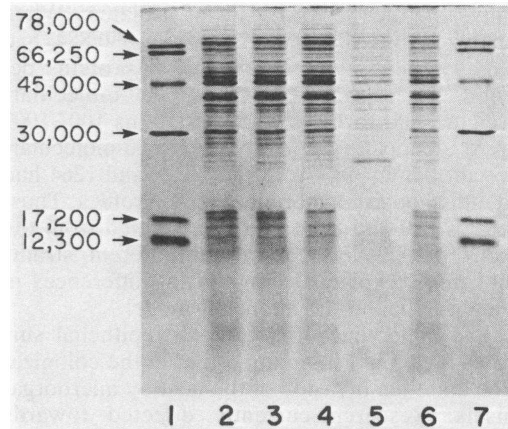


FIG. 2. SDS-PAGE whole cell lysate analysis. Lane 2, HiTb no. 1007 nonadherent isolate from CSF of child no. 2; lane 3, HiTb no. 1009 adherent isolate from nasopharynx of child no. 2; lane 4, isolate no. 1009 recovered from the CSF of infant rat (now nonadherent); lane 5, partially purified pilus preparation derived from no. 1009; lane 6, HiTb isolate no. 1009 (same as lane 3). The conditions for PAGE were the same as those described in the legend to Fig. 1.

Strains 884, 1009, and 1264 did not adhere well (<1 bacterium per cell) to rat BEC as determined by in vitro adherence assays. Furthermore, in vivo we only rarely detected HiTb adhering to nasal epithelial cells obtained by the nasal washout technique. Although some nasal epithelial cells were heavily covered with HiTb, the vast majority had no HiTb adhering to them.

## DISCUSSION

We previously have described two strains of HiTb which were isolated from the nasopharynx of two children with HiTb meningitis and were highly adherent in vitro to human BEC. In contrast, the two CSF isolates from these same two children were nonadherent in vitro, as are all HiTb isolates from systemic illnesses in our experience. The adherence of HiTb to human BEC appears to be mediated by pili and can be partially blocked by the preincubation of bacteria with glucose but not mannose (22nd ICAAC, abstr. 648).

In the infant rat model, three nonadherent and nonpiliated systemic HiTb strains caused bacteremia ($P = 0.005$) and meningitis ($P = 0.005$) significantly more frequently than did the three adherent piliated HiTb strains after atraumatic intranasal inoculation. However, when analyzed separately, significant differences in the incidence of bacteremia and meningitis were noted

only for the 880/884 pair of isolates. When analyzed by SDS-PAGE, strains 880/884 had slightly different outer membrane proteins beyond the absence of the 25,000-molecular-weight band for 880, whereas strains 1007/1009 were identical except for the 25,000-molecular-weight band (Fig. 1). Strains 1228 and 1264 had identical outer membrane protein profiles. Thus, strains 880 and 884, although isolated from the same patient, may represent different strains and, thus, explain the significant differences in the virulence in the infant rat model.

The adherence of bacteria to epithelial surfaces is thought to be important in the colonization and then invasion of the host by microorganisms. Research currently directed towards inhibiting the attachment of certain bacteria to epithelial surfaces in the hope of preventing disease is based on this assumption. The gonococcal pilus vaccine is an example of this approach to the prevention of infection (22). Other investigators have suggested that pili also may be important for HiTb nasopharyngeal colonization (5, 15). In our clinical studies, we have isolated HiTb from the nasopharynx of 50 children with HiTb meningitis at admission. Only three of these nasopharyngeal strains were adherent in vitro and are piliated as determined by electron microscopy; in addition, all systemic HiTb isolates have low levels of piliation and adhere poorly in vitro. In the data presented here, piliated strains of HiTb had no advantage over nonpiliated strains of HiTb in colonizing the nasopharynx of infant rats after intranasal inoculation. Pichichero and co-workers noted that although piliated strains of HiTb adhere to human BEC, they do not adhere to the BEC of infant rats. (M. E. Pichichero, E. M. Connor, and P. W. Anderson, Annu. Meet. Am. Ped. Soc. and Soc. Ped. Res. 1983, Washington, D.C., abstract 1154, p. 279A). Our piliated HiTb strains also did not adhere in vitro or in vivo to infant rat BEC or nasal epithelial cells. This may account, in part, for the similarity in colonization rates between adherent and nonadherent HiTb strains in our studies.

In the animals receiving adherent HiTb strains, nonadherent, nonpiliated HiTb isolates were recovered from blood and CSF cultures. In some animals, the loss of adherence was noted for nasopharyngeal isolates as well. These findings correspond to our observations of children with HiTb meningitis. Thus, the loss of pili may be an important prerequisite for the invasion of the host by HiTb strains that are highly piliated. Examples of this phenomenon have been described for other microorganisms. The suppression of pili formation has been noted for fresh *Escherichia coli* isolated from the urine of patients with urinary tract infections (7, 14). Silverblatt and co-workers have demonstrated that for a strain of *E. coli*, type I pili enhance phagocytosis, possibly by establishing multiple points of attachment with the leukocyte surface (19). *Salmonella typhimurium* with type I pili are cleared more rapidly from the bloodstream than are nonpiliated variants after intravenous infusion in mice (10). The data suggested that the type I pilus is an important factor in the hepatic clearance of the *S. typhimurium*. When (or if) piliated HiTb lose or suppress their pili before or during the invasion of the human host can only be speculated. A pilus vaccine may prevent the attachment and adherence of piliated HiTb strains to mucosal surfaces, but whether invasive disease would be prevented by such an approach requires further study.

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