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Enhanced Nasopharyngeal Colonization of Rats by Piliated *Haemophilus influenzae* Type b

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Piliated *Haemophilus influenzae* type b strains display an enhanced adherence to human epithelial cells in vitro. However, clinical isolates, even from mucosal sites, are seldom piliated, although piliated populations can be selected from them. Experiments with rats have led some authors to suggest that piliation does not implement colonization by *H. influenzae* type b. Piliated populations were obtained from 35 strains by selection for adherence to human erythrocytes. One strain, *H. influenzae* H305, simultaneously acquired an increased adherence to rat erythrocytes and buccal epithelial cells. In contrast to other strains, *H. influenzae* H305 in piliated form was more effective than in nonpiliated form in the colonization of rats by intranasal inoculation. After the piliated inoculum, however, the colonies cultured from the nasal washes were negative for erythrocyte adherence. Thus, piliated *H. influenzae* type b strains have an apparent advantage to initiating colonization in the rat model but may give rise to nonpiliated progeny that are more readily cultivable from the mucosal surface.

The pili of certain gram-negative bacteria increase the adherence to particular host tissues and thus have a role in pathogenicity (3). No such relationship has been defined for the pili recently discovered on *Haemophilus influenzae* type b (Hib) (5, 11). Piliated (p^+) Hib cells display enhanced binding to human buccal or pharyngeal epithelial cells (ECs) in vitro. However, the Hib cultured from blood or cerebrospinal fluid (CSF) in systemic infection are consistently nonpiliated (p^-), and most isolates from the nasopharynx (NP) likewise are p^- (6, 10, 11). Most such cultures do contain a small minority of p^+ bacteria (4), and highly p^+ populations can be generated in vitro by selection for adherence to human ECs or erythrocytes (RBCs) (4–6, 10, 11). In most strains the piliation gradually diminishes on nonselective subculture. Thus Hib displays an equilibrium between p^+ and p^- phenotypes, and we have hypothesized that the p^+ phenotype may have an advantage in colonization of the NP but give rise to p^- progeny having selective advantage in systemic invasion (11).

The possible role of pili in Hib pathogenesis has been explored in infant rats. Piliation was reported to confer no advantage in colonization of the NP or invasion of the bloodstream by Hib cells inoculated intranasally (6; T. L. Stull, P. M. Mendelman, J. Hass, M. A. Schoenborn, and A. L. Smith, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B58, p. 27). The significance of such findings is questionable, however, since the p^+ variants under investigation did not display an enhanced adherence to rat buccal or pharyngeal ECs. To address the issue we selected human RBC-adherent populations from a large number of Hib strains and identified one (H305) in which there is simultaneous expression of pili and of increased adherence to rat as well as human RBCs and ECs. The capacity of p^- and p^+ populations of this strain to colonize the NPs of rats was then examined.

MATERIALS AND METHODS

We surveyed 36 strains of Hib, isolated at widely distributed times and places within the last 15 years in the United States: 15 were isolated from CSF, 11 were isolated from blood, and 10 were isolated from the NP or middle ear. Except for strain Eag, stocks were prepared from a third serial subculture on chocolate agar after primary isolation from the patient; the bacteria were suspended in sterile skim milk and frozen at −70°C. Strain Eag underwent about six subcultures before being frozen. Fresh cultures were initiated by melting a sample of the stock, streaking it onto brain heart infusion agar supplemented with RBC lysate and NAD (BHI-XV) (1), and incubating it overnight at 37°C. Except for EC adherence assays, observations were made on suspensions of bacteria from the overnight plate. Bacterial density was adjusted by optical density at 490 nm, assuming that 10^9 bacteria per ml produced an optical density of 0.83 at 490 μm. For the rat colonization studies, dilution and plating were done to determine the actual viable count administered, which was in reasonable agreement with the value estimated from the optical density, for both p^+ and p^- populations. Assay for hemagglutination (HA) of human type O RBCs on slides (4), titering of HA in microtiter wells (11), and examination for pili by electron microscopy after negative staining with phosphotungstate were done as described previously (4). Adherence to ECs was measured with bacteria radiolabeled with ^3H in liquid medium (10). Human RBC-adherent populations of 35 of the strains were selected as described previously (10); passage on RBCs was repeated until the population was clearly HA positive by the slide assay. (With 24 of the strains, RBC-adherent populations had been selected and described in a previous report [10], but newly selected populations were used in the present study.) A previously described isolate from the NP, C54, was HA positive on initial examination from frozen stock (11); an HA-negative population was selected by culturing bacteria from the blood of a rat after intraperitoneal injection of 10^7 bacteria.

Colonization of the NP was studied in 21- to 26-day-old COBS/CD Sprague-Dawley albino rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Bacteria suspended in phosphate-buffered saline containing Ca^{2+}, Mg^{2+}, and bovine serum albumin (PCMA) were atraumatically administered to the anterior nares (8). Each litter (reduced to 10 pups) received only one kind of inoculum. Litters were

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TABLE 1. Properties of hHA$^-$ and the respective hHA$^+$ populations of Hib

Hib strain	Source	Piliation[a]		Binding of ^{3}H-labeled bacteria to human buccal ECs (no. of bacteria per cell)	
		hHA$^-$	hHA$^+$	hHA$^-$	hHA$^+$
H305[b]	CSF	0/100	100/100	1.1	4.4
Eag	CSF	0/100	100/100	0.44	7.1
S112	Blood	0/100	100/100	0.77	4.0
C194	Blood	0/100	100/100	1.0	3.5
S109	Blood	0/100	100/100	0.25	1.9
S113	Blood	0/100	100/100	1.2	4.8
C24	NP	0/100	100/100	0.48	2.9
C54[c]	NP	0/100	100/100	1.3	9.8

[a] By electron microscopy, the number of piliated cells in 100 cells examined.
[b] The only studied strain in which the hHA$^+$ population were likewise HA$^+$ with rat RBCs.
[c] A strain in which the stock culture was hHA$^+$. An hHA$^-$ variant was selected.

housed separately in filter-top cages to minimize the risk of cross-infection by aerosol. Nasopharyngeal cultures were obtained by instilling the nares with 20 μl of PCMA and culturing the aspirate on BHI-XV supplemented with bacitracin (10 U/ml).

RESULTS

Thirty-five isolates cultured from frozen stock were examined for hemagglutination of human RBC and found to be negative (hHA$^-$). These cultures underwent selection for adherence to human RBC, and within 1 to 10 passages all 35 gave rise to strongly hemagglutinating (hHA$^+$) populations. From strain C54, which was p$^+$ and hHA$^+$ when cultured from frozen stock (11), an hHA$^-$ population was isolated. The hHA$^-$ and hHA$^+$ populations of all the strains were tested for HA of rat RBCs (rHA). All 36 hHA$^-$ were also rHA$^-$ at $\leq 10^{10}$ bacteria per ml. Thirty-five of the hHA$^+$ isolates were rHA$^-$, and one meningeal strain (H305) was rHA$^+$ at $\geq 10^8$ per ml. The procedure of selection of hHA$^+$ populations from strain H305 and testing with rat RBCs was repeated five times; in all cases the hHA$^+$ population was likewise rHA$^+$. HA was also tested with a panel of additional species: all the strains, whether hHA$^-$ or hHA$^+$, were HA$^-$ with the RBCs of oxen, horses, sheep, guinea pigs, pigeons, and chickens; except for H305 hHA$^+$ (which was slightly positive), all were also negative with pig RBCs.

Strain H305 and seven of the other strains were further studied. The hHA$^-$-hHA$^+$ pairs were examined by electron microscopy and assayed for binding to human buccal ECs in vitro (Table 1). On phosphotungstate staining the individual bacteria displayed either no pili (p$^-$) or ≥40 pili (p$^+$). All eight hHA$^-$ populations had 0 p$^+$ cells per 100 cells examined, whereas all hHA$^+$ isolates, including H305, had 100 p$^+$ per 100. Henceforth, the hHA$^-$ and hHA$^+$ populations of Hib may be referred to as p$^-$ and p$^+$, respectively, although not every hHA$^+$ culture was examined by electron microscopy. Binding of hHA$^-$ bacteria to human buccal ECs was uniformly low (0.4 to 1.3 bacteria per EC). The binding of all the corresponding hHA$^+$ populations significantly increased; in strain H305 the increase was 4-fold, whereas in the others the increases ranged from 3.5- to 16-fold.

Adherence to rat buccal ECs was measured (Table 2). The binding of H305 p$^-$ and p$^+$ populations were 0.61 and 1.30 bacteria per EC, respectively, in the first experiment and 0.44 and 1.1 per EC, respectively, in the second. These differences, although smaller than the differential with human ECs, were highly significant. However, as illustrated with the data on strains C54 and C216, expression of rHA$^-$ pili did not increase adherence to rat buccal ECs.

Hib in p$^-$ or p$^+$ phenotypes was inoculated intranasally into infant rats, and nasopharyngeal colonization was determined after 5 days (Table 3). With strain H305, significantly more rats had positive cultures in nasopharyngeal wash samples after inoculation with p$^+$ than p$^-$ isolates. From each of the 10 rats inoculated with p$^+$ isolates, 10 colonies recovered from the NP were examined for HA by agglutination on slides and found to be negative, both with rat and human RBCs. The colonies recovered after inoculation with p$^-$ isolates were likewise HA$^-$. In contrast to H305, when strains expressing rat HA$^-$ pili were inoculated, the p$^-$ and p$^+$ phenotypes were found to produce similar rates of colonization of the NP (illustrated in Table 3 with strain Eag).

The apparent advantage of the p$^+$ variant of strain H305 in colonization was pursued in an experiment in which larger inocula were given and quantitative cultures of isolates from the NP were done after a shorter interval (Table 4). Under these conditions, positive cultures were produced consistently by the p$^-$ as well as by the p$^+$ inocula, but the numbers

TABLE 2. Adherence to rat buccal ECs of Hib p$^-$ and p$^+$ populations

Expt	Hib population	Binding of ^{3}H-labeled bacteria	
		Avg net cpm per tube[a]	No. of bacteria per EC[b]
1	H305 p$^-$	135	0.61[c]
	H305 p$^+$	435	1.30[c]
1 (control)	C54 p$^-$	194	0.52[d]
	C54 p$^+$	118	0.42[d]
2	H305 p$^-$	768	0.44[c]
	H305 p$^+$	2,010	1.10[c]
2 (control)	C216 p$^-$	515	0.63[d]
	C216 p$^+$	419	0.57[d]

[a] Cpm per tube of 5×10^4 EC incubated with 2.5×10^6 bacteria in a volume of 0.25 ml; average of duplicates, corrected for counts in control tubes incubated without ECs (about 20 cpm).
[b] Based on footnote *a* and the specific activity of the particular bacterial suspensions.
[c] $P < 0.05$ by unpaired *t* test.
[d] $P > 0.05$ by unpaired *t* test.

TABLE 3. Colonization of the NP after intranasal inoculation of infant rats with p$^-$ or p$^+$ Hib populations

Hib inoculated (no.)	No. of rats with positive NP cultures[a]/no. inoculated	No. of HA$^+$ recovered colonies/no. tested[b]
H305 P$^-$ (10^5)	4/9[c]	0/40
H305 p$^+$ (rat HA$^+$) (10^5)	10/10[c]	0/100
Eag p$^-$ (10^9)	7/12[d]	0/70
10^9 Eag p$^+$ (rat HA$^-$) (10^9)	5/12[d]	0/50

[a] Recovery of any viable Hib in the 10 to 15 μl of fluid aspirated.
[b] Ten colonies from each positive rat were tested for HA with human and rat RBG at bacterial densities of 10^{10} to 10^8/ml.
[c] χ^2, with the Yates correction of continuity, 4.94; $P = 0.025$.
[d] $P >> 0.05$ (tested as in footnote *c*).

of organisms recovered after the p^+ inoculum were much higher (ratio of geometric means 5.2; $P < 0.02$). Ten colonies cultured from each of the 10 rats receiving p^+ inocula and 58 total colonies from the 7 rats colonized after receiving p^- inocula were tested and found to be HA^-.

DISCUSSION

The genetic basis for the p^+ phenotype in Hib is unknown, but the reversibility of the pilus expression resembles that in *Neisseria gonorrhoeae*, in which the structural gene is reversibly expressed through chromosomal rearrangement (7). Hib differs from the gonococcus, however, in that mucosal isolates seldom appear to be p^+ (6, 10, 11). If piliation of Hib enhances colonization of the host, it is unclear why so few isolates from the NP include more than a small minority of p^+ bacteria. The observation cannot be accounted for by postulating merely that the Hib cells are p^+ when taken in the nasopharyngeal specimen but that pilus expression is lost on (the routine nonselective) culture or preservation in vitro. Generally we examine the population within four platings (roughly 120 generations) after residence in the body. If the initial specimen were highly p^+, spontaneous loss in culture generally would not by itself result in an HA^- population. (Once a strongly HA^+ population is selected in vitro, the loss of HA in nonselective subculture—although variable among strains—seldom occurs in less than 6 or 7 platings [4].) Thus, if one is to conclude that p^+ Hib strains have an advantage in colonization, their low rate of recovery from human NPs must be explained.

Analysis of the role of pili in pathogenesis would be greatly helped by a suitable animal model. Humans are the only natural hosts of *H. influenzae*, and most other mammalian species are relatively insusceptible when experimentally inoculated. Rats, however, have proved to provide a good model for systemic infection by encapsulated *H. influenzae* strains in several important respects: Hib is much more virulent than the other five capsular serotypes (9), susceptibility is inversely related to age (12), no virulence-enhancing agents such as mucin are required, and infection can be induced by an atraumatic nasal instillation, mimicking the natural route (8). Considering that Hib tends not to express pili under conventional culture conditions (4, 10, 11), it can be inferred that most of the experimental infections described previously have been instituted with predominantly p^- populations. Recently, infant rats have been intranasally inoculated with p^+ Hib strains in several laboratories. In one study the p^+ variants of the three tested strains were no more effective in initiating colonization of the NP than were the corresponding p^- variants; after inoculation with the p^+ variants, many colonies recovered from the NP and all recovered from the blood or CSF appeared to be p^-; in none of the three strains, however, did the p^+ variant significantly exceed the p^- variant in adherence to rat buccal ECs in vitro (6). In a recent abstract it was reported that the p^+ variants of about 30 strains were no more effective in colonization of the NP than were their p^- counterparts; whether the p^+ variants displayed an enhanced attachment of ECs of the rat was not mentioned (T. L. Stull, P. M. Mendelman, J. Haas, M. A. Schoenborn, A. L. Smith, Abstr. Annu. Meet. Am. Soc. Microbiol., 1984, B58, p. 27). Our unpublished observations agree with these reports; however, the pathophysiological validity of such studies has been questioned by Turk (13), a view with which we concur. The question could be meaningfully studied in an experimental species only if the pilus mediated adherence to its various cell types in a similar way to its behavior in human cells.

TABLE 4. Quantitative bacterial cultures in wash samples from the NP 2 days after intranasal inoculation of rats with p^- and p^+ Hib H305

Hib inoculated (no.)	No. of rats with positive cultures/no. inoculated	No. of Hib cultured (colonies/10 μl of wash fluid; geometric mean ± 1 SD)	No. of recovered HA^+ colonies/no. tested.
H305 p^- (10^{10})	7/8	15[b,c] (2.7-83)	0/58
10^{10} H305 p^+ (10^{10})	10/10	78[c] (29-209)	0/100

[a] Ten colonies (or all if <10 were recovered) from each positive rat were tested for HA with human and rat RBCs at bacterial densities of 10^{10} to 10^8/ml.

[b] For calculations of geometric mean and t, the culture having 0 colonies per 10 μl was taken as 0.5.

[c] Unpaired t test performed on logarithms of counts: $t = 2.67$, $P < 0.02$.

It is noteworthy that direct selection for adherence to rat RBCs did not prove a useful way of isolating p^+ populations with increased adherence to rat ECs. By this means, an rHA^+ population was selected from one strain, but this population proved to lack a morphological pilus and to lack enhanced binding to rat buccal ECs. (This result emphasizes the fact that bacteria can express nonfimbrial as well as fimbrial hemagglutinins.) Rather, our strategy was to enrich for piliation by selection for human RBC adherence in a large number of Hib strains in the hope of finding a variant with affinity for rat cells as well as for human cells. Unlike the other 35 strains examined, strain H305 either simultaneously expressed a rat-specific adhesin as well as a pilus when expression of the human cell adhesin was selected or expressed an adhesin with affinity for both human and rat cells. On nonselective culture in vitro, agglutination for both human and rat RBCs disappeared simultaneously with pili from the population. Thus, expression of a multispecific adhesin (perhaps the pilus) seems the more likely alternative. The H305 pilus resembled those found on other Hib strains in morphology, distribution, and multiplicity per bacterium (data not shown), in the correlation of HA and EC binding, and in the degree to which pilus expression was accompanied by enhanced binding to human buccal EC in vitro (Table 1). Enhancement of binding to rat buccal cells was somewhat less than to human (twofold versus fourfold; Table 2). Nevertheless, it seemed that experiments with strain H305 in the rat model would have some validity for Hib pathogenesis in humans.

When modest inocula of p^- and p^+ strain H305 were applied, the incidence of positive cultures from a wash of the NP was higher with the latter (Table 3). With higher inocula, the p^+ strain resulted in quantitatively higher counts in cultures from the NP (Table 4). Thus, p^+ variants were better able to initiate colonization.

Interestingly, however, the colonies recovered after inoculation with the p^+ strain were consistently rHA^- and hHA^-. Although these were not examined for pili by electron microscopy, we assume that there was an accompanying loss of piliation. (In a previous study it was shown that as piliation is lost during nonselective culturing in vitro, HA positivity in the slide test disappears when <30% of the bacteria in a colony are p^+ [4]). The result could be explained by several hypotheses. The simplest is that HA^- bacteria may be selectively recovered by the nasopharyngeal wash, i.e., HA^+ organisms adhere and resist being washed off while giving rise to a certain proportion of HA^- progeny that are more likely to be cultivable by washing. Whatever the mechanism, the model resembles the human

experience, in which (except for rare "pilus-constitutive" strains like C54) the Hib cells cultured from the NP are predominantly p^-. However, the persistence of the capacity within the recovered population suggests that piliation confers some survival value within the human host. It may be that the conventional nasopharyngeal culture tends to selectively sample p^- phase variants "floating" in the mucus layer.

The exact role that pili play may be a subtle one, and its analysis will require insightful further study. Studies in the infant rat model may benefit from the use of strain H305 (available to interested investigators) or similar strains in which adherence to rat cells parallels the adherence to human cells.

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