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Inability To Express Fimbriae Results in Impaired Ability of *Haemophilus influenzae* b To Colonize the Nasopharynx

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We cloned into the structural fimbrial subunit gene from a fimbriated *Haemophilus influenzae* b a 1.5-kb kanamycin resistance gene. The resultant strain (RKAW5) was tested by Southern analysis, hemagglutination, and electron-micrographic examination to confirm gene inactivation. In comparison with the parent, RKAW5 exhibited a significant decrease in adherence to human buccal epithelial cells and in nasal colonization of yearling rhesus monkeys.

Surface fimbriae may be important for *Haemophilus influenzae* b (Hib) adherence to the nasopharynx. In vitro, fimbriated Hib has increased adherence in comparison with nonfimbriated strains to buccal epithelial cells (BEC) (10, 20, 22) and nasopharyngeal cultures (8, 16). However, the majority of Hib isolates from nasopharynges of children with invasive disease are nonfimbriated, and all Hib strains from blood and cerebrospinal fluid lack detectable fimbriae (11, 16, 17, 21).

Nonprimate models used to study fimbriation have been less than satisfactory because rat erythrocytes do not agglutinate fimbriated Hib and these bacteria are not more adherent to rat BEC. In addition, fimbriated Hib isolates are less virulent in infant rats (2, 4, 25). Primates are the only species that naturally suffer invasive Hib disease. Monkey erythrocytes agglutinate fimbriated Hib, as do human erythrocytes.

We constructed an isogenic strain from Eagan (E1a) (1). E1a was repeatedly absorbed by washed human type O erythrocytes to derive a fimbriated (f^+) strain, R1369 (4, 27). RKAW5, which is nonfimbriated (f^-) and nonhemagglutinating, was constructed from R1369. The b serotype was verified by slide agglutination with type b antiserum, and all strains were verified as Hib by their requirement for β-NAD and heme for aerobic growth on brain heart infusion agar.

Construction of RKAW5. DNA from R1369 was cloned as previously described by Forney et al. (9) by using the amino acid sequence of the fimbrial subunit as previously described (3). A 6.3-kb *Pst*I fragment containing the fimbrial subunit gene (*hifA*) was subcloned into pUC19 (28), transformed into competent DH5α (4), and designated pCD1.

Purified DNA from pCD1 was digested by *Bgl*II (there is only one *Bgl*II site in pCD1 which is located within *hifA*). The 1.5-kb *Bam*HI fragment from pUC4K containing the gene encoding kanamycin resistance (aminoglycoside acetyl-

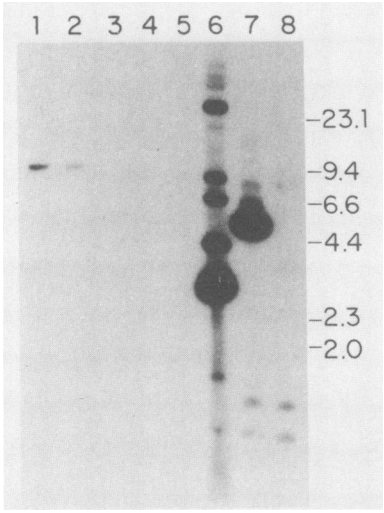

FIG. 1. Southern analysis of whole-cell DNA isolated from *H. influenzae* (lanes 1 to 5) and *E. coli* plasmids pUC4K (lane 6), pCDKAW3 (lane 7), and pCD1 (lane 8). Lanes contain DNA from strains as follows: 1, RKAW5; 2, RKAW14; 3, R906; 4, R1369; 5, Ela. All DNA preparations were restricted with *Bgl*I and electrophoresed in 0.7% agarose. The probe was the 1.5-kb kanamycin resistance gene from pUC4K.

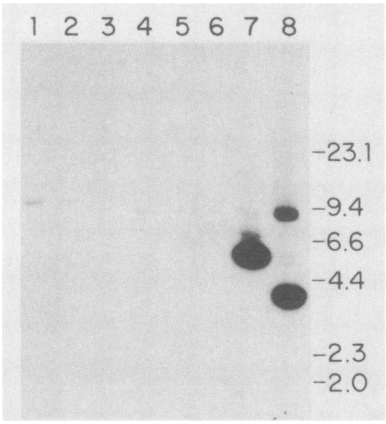

FIG. 2. Southern analysis of whole-cell DNA isolated from *H. influenzae* (lanes 1 to 5) and *E. coli* plasmids pUC4K (lane 6), pCDKAW3 (lane 7) and pCD1 (lane 8). Lanes contain DNA from strains as follows: 1, RKAW5; 2, RKAW14; 3, R906; 4, R1369; 5, Ela. All DNA preparations were restricted with *Bgl*I and electrophoresed in a 0.7% agarose gel. The probe was the 4.3-kb *Pst*I-*Eco*RI fragment from pCD1.

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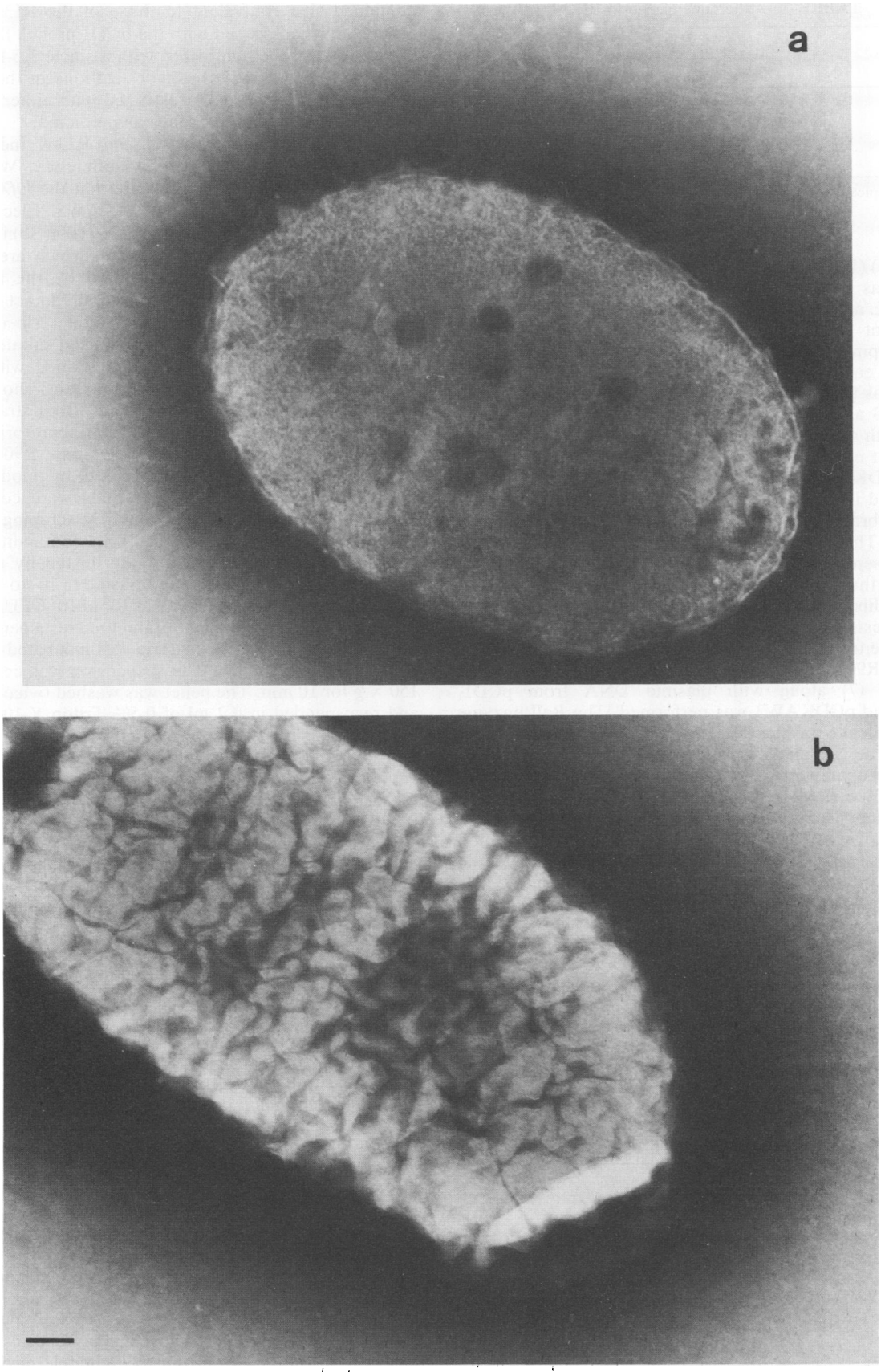

FIG. 3. Negatively stained electron micrographs of *H. influenzae* R1369 (a) and RKAW5 (b). Bar = 0.1 μm.

TABLE 1. Adherence of Hib to BEC

Strain	No. of bacteria per[a]:	
	Viable BEC	Total BEC
R906	5.6 ± 3.4	0.7 ± 0.4
R1369	159.4 ± 87.4	18.5 ± 10.2
E1a	2.6 ± 0.6	0.3 ± 0.3
RKAW5	1.8 ± 1.0	0.2 ± 0.1

[a] Values depicted are the means (n = 3) ± standard deviations.

transferase) (18) was ligated into the *Bgl*II site of pCD1. This plasmid was transformed into competent DH5α and then grown on L agar containing ampicillin and kanamycin. One recombinant was designated pCDKAW3. Restriction enzyme mapping of this strain verified the position of the kanamycin cassette and that a single copy of the kanamycin cassette was inserted. Western blotting (immunoblotting) of pCDKAW3 and pCD1 indicated that the fimbrial antibody reacted with a 23-kDa protein (the fimbrial subunit) in DH5 α/pCD1 but not DH5α/pCDKAW3.

The pCDKAW3 plasmid was purified, linearized, and transformed into competent R1369 (12) and grown on supplemented brain heart infusion agar containing kanamycin at 25 μg/ml. The resulting transformants were screened for ampicillin sensitivity and for hemagglutination with type O human erythrocytes (washed four times with phosphate-buffered saline with 0.1% gelatin [PBSG]). Southern analysis of *Bgl*I digests of whole-cell DNA (13) from two f⁻ ampicillin-sensitive transformants (RKAW5 and RKAW14), R1369, E1a, and R906 (a nonfimbriated nontypeable control *H. influenzae*) (7) along with plasmid DNA from pCD1, pUC4K, and pCDKAW3 was performed. The *Bgl*I enzyme does not cleave inside the *hifA* gene or the kanamycin cassette. One ^{32}P-labelled probe was a 1.5-kb *Bam*HI fragment, the kanamycin cassette from pUC4K, and another was the 4.3-kb *Eco*RI-*Pst*I fragment from pCD1 containing the *hifA* gene. The autoradiographs of these two hybridizations are shown in Fig. 1 and 2. We do not know the location of the *Bgl*I site 3′ to *hifA*; therefore, we could not predict the size of the fragment containing *hifA* in chromosomal DNA. A single band of an equal size is seen in the RKAW lanes with both probes. The bands in the RKAW lanes with the *hifA* probe are 1.5 kb larger than those in the R1369 and E1a lanes. Southern analysis of genomic DNA of the same Hib strains digested with *Pst*I (which cuts out the kanamycin cassette when present) and probed with the previously described probes (pCD1 and kanamycin cassette) produced the predicted fragments. Two fragments from RKAW have a combined size equivalent to those of the R1369 and E1a fragments when probed with the pCD1 probe. The kanamycin cassette probe hybridized with a single 1.5-kb fragment in RKAW digests with no hybridizations in the other Hib digests. All plasmid DNA restricted with either endonuclease hybridized with the probes as predicted.

Studies comparing RKAW5 and R1369 indicated that absense of fimbriae is the only difference. Western blot analysis of the Hib strains indicate that the *hifA* product is present in R1369 but absent in RKAW5. Electron micrographs of RKAW5 (Fig. 3a) and R1369 (Fig. 3b) indicate that RKAW5 lacks peritrichous fimbriae, which are present in R1369. Lipooligosaccharide prepared by the method described by Darveau and Hancock (6) and intact outer membrane proteins prepared by the method described by Stull et al. (26) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and stained with silver or Coomassie blue, respectively; these strains showed no difference between RKAW5 and R1369. Both strains reacted with the capsule "b" antibody (Difco Laboratories, Detroit, Mich.), in contrast to a nontypeable strain, R906.

Adherence to BEC was measured by modification of published methods (18, 22, 24). Cells were collected and pooled from 8 to 10 lab personnel by scraping the buccal surface and suspending cells in PBSG containing 250 μg of streptomycin per ml. Viability was tested by trypan blue exclusion, and the density was adjusted to 10^5 to 10^6 cells per ml. Hib was grown to a density of 10^8 to 10^9 CFU/ml, and the bacteria were washed in PBSG and then resuspended in their initial volume: 0.2 ml of bacteria was incubated with 0.2 ml of BEC at 37°C for 1 h. The suspension was centrifuged at 150 × *g* for 10 min. The pellet was washed twice with PBSG and resuspended in 0.2 ml of 0.5% Triton X-100 in PBSG. This suspension was diluted and plated on brain heart infusion agar. The data shown in Table 1 indicate that adherence, expressed as CFU per viable BEC and total BEC for R1369, is 10- to 100-fold greater than the adherence of the f⁻ strains.

We tested the capacity of strains R1369 and RKAW5 to colonize *Macaca mulatta* yearlings. Scheifele et al. (23) reported that intranasal instillation of strain E1a (10^8 CFU) into neonatal *Macaca mulatta* yearlings resulted in nasopharyngeal colonization and inflammation with subsequent bacteremia and meningitis. After culturing nasal swabs from six male monkeys by the technique of Wong (29) to ensure the absence of *Haemophilus* strains, we introduced a small inoculum (10^3) into each nostril. Four monkeys received RKAW5, and two received R1369. We chose a small inoculum to mimic the possible transference volume that occurs in nature and to minimize the cytotoxicity seen in vitro when respiratory epithelial cells are exposed to 10^6 CFU of Hib (24).

Daily nasal swabs cultures from all inoculated monkeys were suspended in PBSG, diluted, and plated on brain heart infusion agar with (RKAW5) and without (R1369) kanamycin. Table 2 depicts the daily recovery of Hib from each monkey. The colonization density was 10-fold less with the nonfimbriated strain (RKAW5) than with the fimbriated isogenic parent (R1369). Neither strain evoked an inflammatory response, as indicated by a lack of polymorphonuclear leukocytes in the nasal cavity as assessed by a Wright-Giemsa stain of 25 μl of the suspension of nasal swabs. This observation differed from that of Scheifele et al. (23) with newborn monkeys in which local inflammation was indi-

TABLE 2. Number of *H. influenzae* recovered per nasal swab after intranasal inoculation

Animal no.	Strain inoculated	CFU at day[a]:						
		1	2	3	4	5	6	7
89-122	R1369	ND	4	553	335	120	114	3,000
89-169	R1369	ND	11	2,150	2,140	897	395	10,333
89-088	RKAW5	ND	ND	ND	2	180	82	550
89-168	RKAW5	1	ND	123	6	1	5	1
89-167	RKAW5	ND	ND	165	105	110	140	365
89-051	RKAW5	6	ND	170	8	13	24	145

[a] ND, none detected.

cated. This difference may be due to age differences among the monkeys or to sampling technique.

Southern analysis of whole-cell DNA from Hib isolates that were kanamycin resistant and were from the animals inoculated with RKAW5 hybridized with a ^{32}P-labelled *Bam*HI fragment identical to that found in RKAW5. All colonies (maximum of 100 per monkey per day) did not hemagglutinate with human erythrocytes. This suggests that environmental factors in the nasopharynx favor selective growth of nonfimbriated strains, that local environmental factors favor phase reversion to f^-, or that there is selective clearance of f^+ Hib.

The decreased adherence to BEC and the decreased ability to colonize yearling monkey nasopharynges of a nonfimbriated but otherwise isogenic Hib strain suggest that surface fimbriae are advantageous in adherence. We constructed the isogenic f^- strain RKAW5 and inoculated 10^3 CFU to avoid the problem of phase reversion during colonization, which occurs in vitro at a frequency of 10^{-4} to 10^{-5}. We found that RKAW5 colonized the monkey's nasopharynx, which suggests that surface fimbriae are not necessary for colonization and that adherence may be mediated by nonfimbriated adhesins as suggested by Sable et al. (22) and Loeb et al. (16).

The phase reversion occurs in 95% of all Hib isolates, as indicated by hemagglutination (27). This ability must confer favorable properties on the bacterium. One possibility is that fimbriated organisms are "seeded" onto squamous cells in the anterior oropharynx. The movement of seeded bacteria by the ciliated pseudostratified columnar epithelium to the posterior nasopharynx results in inoculation of the respiratory epithelium. Colonization of the nasopharyngeal respiratory mucosa is mediated by a fimbria-independent method, but seeding enhances that capability. Invasion then follows colonization in susceptible primates. This concept is similar to one proposed by Orndorf and Bloch (19) for type I pili in *Escherichia coli*.

We are in the process of testing the hypothesis that fimbriae are not necessary for invasion by determining whether RKAW5 can cause bacteremia and meningitis after intranasal inoculation into newborn monkeys, compared with wild-type Hib.

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