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Relationship of *Haemophilus influenzae* Type b Pilus Structure and Adherence to Human Erythrocytes

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Six strains of *Haemophilus influenzae* type b, some expressing immunologically different pili, showed identical patterns of binding to erythrocytes that were characterized for 38 blood group antigens. All six strains appeared to bind to the Anton antigen, as they agglutinated all erythrocytes tested except cord erythrocytes and those characterized as Lu(a−b−), dominant type, including Anton-negative cells.

Haemophilus influenzae type b (Hib), like many other gram-negative bacteria, possesses surface proteins (pili) that are associated with the ability of the bacteria to adhere to respiratory epithelial cells (2, 8, 11, 14) and to agglutinate human erythrocytes (2, 9, 11, 14). We and others have documented that Hib pili differ immunologically from strain to strain and that the pilin subunits of pili from different Hib strains may differ slightly in electrophoretic mobility (1, 6, 14).

Studies by van Alphen (12–14) have documented that the erythrocyte receptor for Hib pili is associated with the Anton blood group antigen, a prevalent erythrocyte antigen whose structure is unknown (10). In the studies by van Alphen, only one Hib strain, which had been enriched by hemagglutination to select for hemagglutinating (piliated) variants, was used to identify the erythrocyte receptor. In order to extend the observation by van Alphen to include pili of multiple Hib strains, we investigated the erythrocyte-binding patterns of six Hib strains whose pili differ immunologically.

The Hib strains used in this study are listed in Table 1. M43(p+) and M42(p−) were isolated from the nasopharynx and cerebrospinal fluid, respectively, of the same patient and have been previously described (2, 6). Strain C54(p+) was obtained from Michael Pichichero, University of Rochester, Rochester, N.Y., and has been shown to possess pili [p(+)] (9, 10). Both M43(p+) and C54(p+) are stably piliated and do not undergo rapid phase variation to p(−).

Strains AA14(p+), AAr108(p+), and AA61(p+) have been previously described (6; J. R. Gilsdorf, K. W. McCrea, and L. J. Forney, Pediatr. Res. **25**:179A, 1989) and differ in their reactivities to antisera directed against pili of Hib strain M43(p+) or in the sizes of their pilin subunits. These patient isolates were originally p(−) and required erythrocyte enrichment (2) to obtain p(+) phase variants. Hemagglutination was measured in a semiquantitative assay as previously described (6). In brief, bacteria were grown to late log phase, washed in phosphate-buffered saline with 0.1% gelatin, and suspended in phosphate-buffered saline with 0.1% gelatin to an optical density at 610 nm of 0.26 with a path length of 13 mm. Erythrocytes from a single donor were washed in phosphate-buffered saline and suspended to a dilution of 1:80. Equal volumes of twofold serial dilutions of bacteria and erythrocytes were incubated at room temperature in round-bottomed microdilution wells for 2 h and observed for agglutination.

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To investigate the possibility that the erythrocyte receptor is a protein, we preincubated the erythrocytes for 15 min with 1% ficin (Sigma Chemical Co., Milwaukee, Wis.) prior to hemagglutination (4). After being treated with the ficin and neuraminidase preparations used in our experiments, control erythrocytes gave positive reactions with *Glycine soya* lectin, which verifies the activities of these enzymes (4). To investigate the presence of sialic acid on the erythrocyte receptor, we preincubated 1 ml of washed erythrocytes with 0.1 IU of neuraminidase (Calbiochem-Behring, La Jolla, Calif.) for 15 min (4). When each of the six Hib(p+) strains was used, neither neuraminidase- nor ficin-treated erythrocytes showed a difference in hemagglutination titers when compared with erythrocytes treated with buffer (Table 2). These results suggest that the erythrocyte receptor does not contain neuraminic acid. Although the receptor is resistant to ficin, additional proteases need to be tested to ensure that the receptor is not a protein.

We attempted to identify possible carbohydrate analogs of the erythrocyte receptor by testing the ability of several carbohydrates to inhibit the adherence of all six Hib strains to erythrocytes. Preincubation of the erythrocytes with 100 mM sucrose, lactose, glucose, galactose, melbiose, *N*-acetylglucosamine, methyl β-D-galactopyranoside, methyl α-D-galactopyranoside, or *N*-acetylgalactosamine had no effect on hemagglutination titer when compared with preincubation of control erythrocytes with buffer containing no carbohydrates.

In a further attempt to localize the erythrocyte receptor(s) that binds to pili on the six Hib(p+) strains, we tested the bacteria in hemagglutination assays by using 65 different erythrocyte samples characterized for 38 different erythro-

TABLE 1. Hib strains used in hemagglutination studies

Strain	Pilus expression	Approx pilin size (kDa)[a]	Homology with M43(p+) strain[b]
M43(p+)	Stable	24	4+
C54(p+)	Stable	24	4+
AA14(p+)	Required enrichment	24	2+
AAr108(p+)	Required enrichment	24.5	1+
AA61(p+)	Required enrichment	24.5	1+
AAr106(p+)	Required enrichment	23.5	4+
M42(p−)	None		0

[a] From reference 6 and J. R. Gilsdorf, K. W. McCrea, and L. J. Forney, manuscript in preparation. kDa, Kilodaltons.
[b] Scale ranged from 0 (no homology) to 4+ (identity) (6; Gilsdorf et al., in preparation).

TABLE 2. Effect of protease (ficin) or neuraminidase treatment of erythrocytes on hemagglutination by Hib

Hib strain	Hemagglutination titer (1/n) for erythrocytes			
	Ficin		Neuraminidase	
	+	−	+	−
M43(p+)	16	8	4	4
C54(p+)	16	16	2	2
AA14(p+)	2	Undiluted	2	Undiluted
AAr108(p+)	8	2	2	2
AA61(p+)	16	8	4	4
AA106(p+)	8	8	16	16
M42(p−)	0	0	0	0

cyte antigens (Table 3). All six Hib(p+) strains showed hemagglutination patterns identical to those of the 65 samples of erythrocytes tested. All six Hib(p+) strains agglutinated the three samples of Lu(a−b−) recessive-inheritance erythrocytes, but none of them agglutinated the Lu(a−b−) dominant-inheritance erythrocytes (Table 3). This pattern suggests that the erythrocyte receptor is under the control of the inhibitor gene *In(Lu)*, which inhibits the expression of Lutheran as well as some non-Lutheran erythrocyte antigens, including the Anton antigen (10). None of the six Hib(p+) strains agglutinated Anton-negative erythrocytes (Table 3). In addition, none of the six Hib(p+) strains agglutinated any of the seven samples of cord cells, which also do not express Anton antigen (13).

Our data corroborate the observation of van Alphen that the Anton antigen is the erythrocyte receptor for Hib pili (13). Although the structure of the Anton antigen is unknown, it is reported to be resistant to ficin (10), as was seen in our study.

TABLE 3. Patterns of erythrocyte agglutination by six Hib(p+) strains

Erythrocyte type	No. of samples	Agglutination[a]	Erythrocyte type	No. of samples	Agglutination[a]
A_1	2	++	K −	2	++
B	2	++	K_o	1	++
O	4	++	Kp(b−)	1	++
At(a−)	2	++	Lan−	1	++
Bg(a+)	7	++	Fy(a−b−)	1	++
Bombay	1	++	Mc(a−)	1	++
Ch(a−)	3	++	p	1	++
Co(b+)	2	++	Rh mod	1	++
Co(a−b−)	1	++	Vel−	1	++
Co(a−)	1	++	Yt(a−)	1	++
Cs(a−)	1	++	Yt(a−b+)	1	++
D − −	1	++			
Di(b−)	2	++	Lu:−8	3	++
Ge−	1	++	Lu(a+b−)	2	+
Gy(a−)	1	++	Lu(a+b−)	5	++
I −	3	++	Lu:−13	2	+
U −	1	++			
Jk(a−b−)	1	++	Lu(a−b−)rec	3	++
Jr(a−)	1	++	Lu(a−b−)dom	15	0
Js(b−)	1	++			
Jo(a−)	1	++	Cord erythrocytes	7	0

[a] ++, Agglutination equal to that of control erythrocytes; +, agglutination less than that of control erythrocytes; 0, no agglutination.

Our study is evidence that the pili of six strains of Hib appear to recognize the same erythrocyte receptor, the Anton antigen, even though these pili differ structurally and immunologically. In addition, the Anton antigen is recognized by Hib that stably express pili as well as by those that require erythrocyte enrichment for pilus expression. These results support the concept that a single ligand on Hib pili binds to a single receptor on the erythrocyte. This single ligand could be among conserved epitopes that may exist on the structural protein (pilin) of pili, even though the pili exhibit immunologic differences; or it could be a separate, conserved adhesin molecule that is independent of the structural protein of pili, as has been seen with adhesins on pili of some other bacterial species (3, 5, 7).

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