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# Evidence for Functionally Distinct Pili Expressed by *Neisseria meningitidis*

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**In order to investigate possible functional consequences of phase and antigenic variation of meningococci, the attachment of 15 strains of *Neisseria meningitidis* to human erythrocytes was studied by a nitrocellulose hemadsorption assay. This assay allows the study of individual meningococcal colonies with respect to erythrocyte attachment. Of the 15 strains studied, 7 demonstrated binding of human erythrocytes ($HA^+$). Among these seven strains, the percentage of colonies that were $HA^+$ ranged from 0.2 to 97%. Meningococcal colonies that did not produce pilin (the major structural subunit of pili) did not demonstrate erythrocyte binding ($HA^-$). The $HA^+$ colony phenotype was correlated with assembly of pilin into pili and expression of pili on the meningococcal surface. However, only some piliated colonies bound human erythrocytes. This could not be explained by differences between piliated $HA^+$ and $HA^-$ colonies in the amount of pilin produced or by differences in number of pili expressed per diplococcus. Pili of five of the meningococcal strains with $HA^+$ colonies were antigenically related to gonococcal pili (class I meningococcal pili), but $HA^+$ colonies were also seen in two meningococcal strains expressing class II meningococcal pili. Changes from $HA^+$ to $HA^-$ and from $HA^-$ to $HA^+$, in the presence of continuing pilin production and pilus assembly, occurred at frequencies of up to $10^{-2}$/CFU per generation. Such frequencies resemble those of phase and antigenic variation described previously for *Neisseria* species pilin. These studies indicate that phase variation influences the ability of meningococci to attach to human cells and suggest that meningococci may express functionally different pili.**

Colonization of human mucosal surfaces by *Neisseria meningitidis* involves attachment of meningococci to host cell surfaces (8, 27, 30–32). Initial attachment of meningococci to human cells is mediated by pili (8, 27, 31). Like pili of *Neisseria gonorrhoeae*, meningococcal pili undergo biochemical and antigenic variation during meningococcal infections (1, 35). At least two distinct antigenic classes of pili have been found on *N. meningitidis* (14), and it has been suggested that their antigenic repertoire may be equal to or even greater than that of gonococci (14). Variation in pilus structure and other surface components may allow evasion of the host immune response. This variation may also have functional consequences that may be of even greater importance in meningococcal pathogenesis.

Meningococcal pili have been proposed as mediators of hemagglutination (12, 26, 36), a phenomenon frequently assayed to study mechanisms of attachment. Further, hemagglutination has been proposed as a marker for piliation of meningococci (26). Others, however, have shown that not all piliated meningococci cause hemagglutination (12, 36). Similarly, in gonococci, purified pilus preparations can be shown to differ in their power to agglutinate erythrocytes (28).

To address these issues, and to better understand the mechanisms of meningococcal attachment to human cells, we adapted a hemadsorption assay used for *Haemophilus influenzae* (7). This assay allows study of the characteristics of meningococcal colonies which bind human erythrocytes.

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## MATERIALS AND METHODS

**Microorganisms.** Fifteen strains of *N. meningitidis* were used in these studies. The characteristics of these strains have previously been reported (2, 34) and are summarized in Table 1. All strains were encapsulated, as determined by serogrouping (31), and piliated. Characteristics of pili and pilins produced by these strains have been previously reported (31, 33, 34). Pili were identified by negative-staining electron microscopy (EM) (20, 31, 33). These negatively stained preparations were used to determine the percentage of meningococci with pili and the mean number of pili per diplococcus (20). Briefly, preparations were made by inverting a Formvar carbon-coated grid (Ernest F. Fullam Co., Schenectady, N.Y.) and floating it successively on the surface of a drop each of Eagle minimal essential medium containing Earle salts and L-glutamine (GIBCO Laboratories, Grand Island, N.Y.) and buffered with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.45; Sigma, St. Louis, Mo.) (HEPES-MEM) containing meningococci, 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), distilled $H_2O$, and 1% sodium phosphotungstate (pH 6.5). Grids were examined with an electron microscope (EMU-4; RCA, Camden, N.J.). After we selected areas of the grid with sufficient dispersion of organisms to examine them individually, the first 20 diplococci encountered were examined at a magnification of ~×9,000 to determine the percentage with one or more pili. After determination of the percentage of pili, five or more diplococci were examined to determine the mean number of individual pili seeming to emanate from the surface of each diplococcus. The width, length, and morphology of pili were determined as previously described (20, 33).

**Media.** Solid media for cultivating microorganisms consisted of gonococcal agar base (Difco Laboratories, Detroit, Mich.) plus 2% (vol/vol) IsoVitaleX (BBL Microbiology

TABLE 1. Binding of human erythrocytes by colonies of *N. meningitidis*

| Strain | Type (serogroup: serotype: subtype [11]) | Source[a] | Pilin characteristics[b] | | | | Hemadsorption | |
|---|---|---|---|---|---|---|---|---|
| | | | Molecular mass (kDa) | Western immunoblot reactivity with: | | | No. of colonies screened | % $HA^+$ |
| | | | | CNBr-2 | MAb 2-1-FC | MAb SM1[c] | | |
| 269B | B:4:P1.1 | CSF | 18.5 | + | + | + (class I) | 897 | 97 |
| 1438 | B:NT:P1.15 | CSF | 19.0 | + | + | + (class I) | 61 | 80 |
| 6083 | W:NT:P1.3 | NP | 19.0 | + | + | + (class I) | 263 | 59 |
| RNP | B:NT:NT | NP | 19.5 | + | + | + (class I) | 158 | 23 |
| KB | B:NT:P1.1 | CSF | 18.25 | + | + | + (class I) | 182 | 6 |
| H355 | B:15:P1.15 | CSF | 20.6 | + | + | + (class I) | 150 | 0 |
| 3418 | C:2a:P1.2 | NP | 17.5 | + | − | ND | 93 | 0 |
| 1636 | A:NT:NT | CSF | 18.0 | + | − | ND | 91 | 0 |
| 4682 | C:2a:P1.1 | CSF | 19.0 | + | + | − (class II) | 93 | 0 |
| 2070 | C:2a:P1.2 | CSF | 17.5 | + | − | − (class II) | 603 | 0 |
| HL | B:2b:P1.2 | Blood | 18.0 | + | − | − (class II) | 146 | 0 |
| 3006 | B:2b:P1.2 | CSF | 18.0 | + | − | − (class II) | 62 | 0 |
| 2996 | B:2b:P1.2 | CSF | 17.25 | + | − | − (class II) | 38 | 0 |
| 1643 | B:2b:NT | CSF | 19.0 | + | − | − (class II) | 459 | 0.2 |
| FAM18 | C:2a | Blood | 14.5 | + | − | − (class II) | 442 | 51 |

[a] CSF, cerebrospinal fluid; NP, nasopharynx.
[b] +, reaction in Western immunoblots at 1:50 or higher dilution; ND, not determined; MAb, monoclonal antibody.
[c] Class I pili defined by reactivity of pilin with monoclonal antibody SM1 (14); class II pili defined by nonreactivity of pilin with monoclonal antibody SM1.

Systems, Cockeysville, Md.) (designated GcIso agar) and chocolate agar plus 1% (vol/vol) IsoVitaleX (designated ChocIso agar). Gonococcal broth with 2% IsoVitaleX was used for growth of meningococci in liquid medium. The medium used for suspending and diluting microorganisms was HEPES-MEM or phosphate-buffered saline (PBS; 0.01 M, pH 7.3). *N. meningitidis* strains were stored on GcIso agar slants, ChocIso agar slants, or defibrinated sheep blood (Carr Scarborough Microbiologicals, Decatur, Ga.) at −70°C.

**Preparation of human erythrocytes.** Human O $Rh^+$ whole blood was obtained in a heparinized syringe from one of two volunteers, or human O $Rh^-$ whole blood was obtained from the blood bank of the Atlanta VA Medical Center. The blood was separated into plasma and erythrocytes by centrifugation at 300 × *g* for 5 min. After the plasma was discarded, the erythrocytes were washed three times by resuspension in 0.01 M PBS (pH 7.3) and centrifugation at 300 × *g*. The erythrocytes were then diluted with 0.01 M PBS (pH 7.3) to a 5% (vol/vol) suspension. This suspension was then stored at 4°C and used within 7 days.

**Nitrocellulose hemadsorption assay.** This assay is a modification of the one described by Connor and Loeb for *H. influenzae* type b (7). Meningococci grown on GcIso agar overnight in a humid atmosphere with 3% $CO_2$ at 37°C were suspended (31) in HEPES-MEM or in 0.01 M PBS (pH 7.3) and diluted to an optical density of 0.4. Tenfold dilutions of this suspension were prepared, and 25 μl each of the $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions was plated onto GcIso agar with a sterile bent glass rod. With this procedure, plates with about 30 to 100 isolated colonies were obtained after incubation in 3% $CO_2$ at 37°C for 18 to 24 h.

A transparency map was made of the location of colonies on each plate. This map facilitated recording of results of the nitrocellulose hemadsorption assay and subsequent immunodot experiments. The map also allowed the selection of individual colonies on the original agar plates for preparation for electron microscopy or passage. Nitrocellulose discs (pore size, 45 μm; Schleicher & Schuell, Inc., Keene, N.H.) were wetted in 0.01 M PBS (pH 7.3), blotted with Whatman filters, and placed on the surface of the agar. To ensure complete contact between the disc and the plate, the disc was lightly pressed against the plate with a sterile bent glass rod. The plate was then incubated at 37°C in 3% $CO_2$ for 60 min.

The discs were then carefully removed from the agar plates, and 10 ml of 3% bovine serum albumin (BSA) (fraction V powder; Sigma) in 0.01 M PBS (pH 7.3) was added to block nonspecific attachment of erythrocytes to the nitrocellulose filters. The plates were then gently agitated at room temperature with a Red Rocker (Hoefer Scientific Instruments, San Francisco, Calif.) for 30 min. The BSA solution was discarded, and the discs were washed twice in 10 ml of 0.01 M PBS (pH 7.3) with agitation for 10 min at room temperature. Ten milliliters of the 5% erythrocyte solution was then added to each disc and incubated with gentle rocking in a Red Rocker at room temperature for 60 min. Each disc was then removed and gently washed by lifting the nitrocellulose at one edge, dipping the disc three times into a container filled with 0.01 M PBS (pH 7.3), and blotting the edge of the disc with filter paper. Red dots present on the nitrocellulose were labeled as hemadsorption positive ($HA^+$) and recorded on the map. Colonies that did not have corresponding red dots were labeled hemadsorption negative ($HA^-$).

Key elements in standardizing the assay included (i) allowing the meningococcal colonies to grow for 18 to 24 h, (ii) using inocula that yielded ~30 to 100 colonies, and (iii) strict adherence to the protocol for removal of unattached erythrocytes. Within each strain, all $HA^+$ colonies had a similar appearance. There were strain-to-strain differences, however, in the appearance of $HA^+$ colonies on the nitrocellulose discs. For example, the $HA^+$ colonies of strain 6083, after drying overnight, were glossy and indelible. In contrast, the $HA^+$ colonies of 269B, though they were equally prominent immediately after the procedure was performed, faded overnight. Differences in stability of binding among meningococcal strains or in lysis of erythrocytes after binding may explain this variation. To minimize these differences, hemadsorption was determined for each colony immediately after the final washing step.

**Approximation of the rate of phase variation of hemadsorp-**

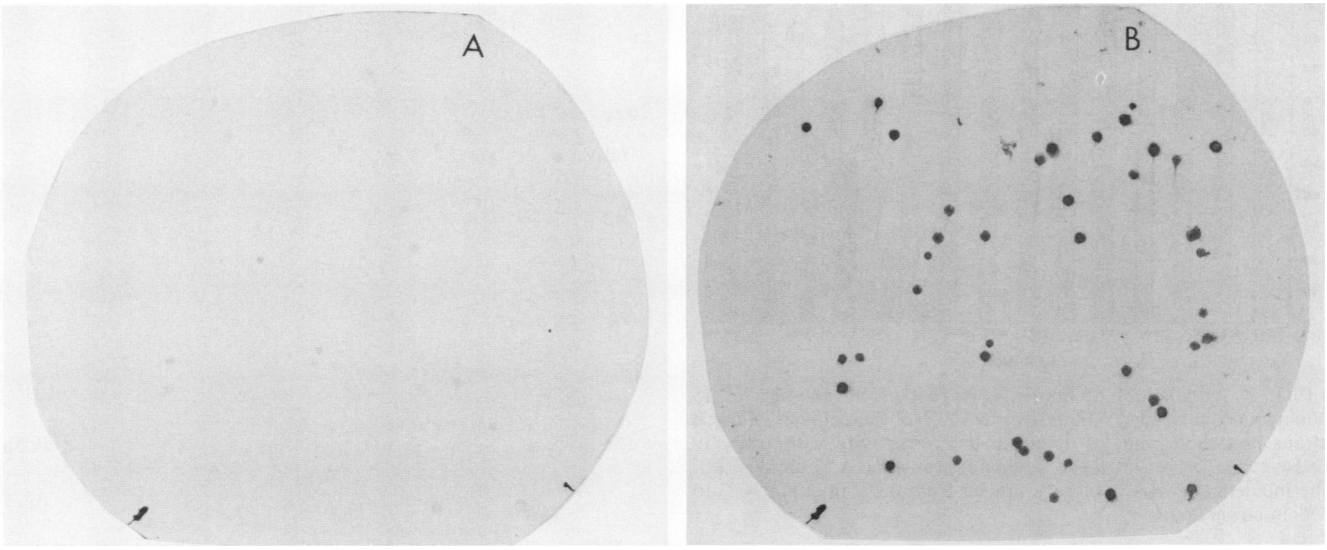


FIG. 1. Hemadsorption assay of meningococcal strain 6083. Forty-two colonies grown on GcIso agar were transferred to nitrocellulose. (A) Fifty-two percent (22 of 42) of meningococcal colonies demonstrated hemadsorption ($HA^+$). (B) Immunoblot of the same nitrocellulose disc using antipilus monoclonal antibody 2-1-FC (33). All of the colonies expressed pili recognized by this antibody.

**tion.** Phase variation rates were determined by a modification of the method of Enomoto and Stocker (10). $HA^+$ or $HA^-$ colonies were selected by the hemadsorption assay, cut from the agar, transferred to gonococcal broth, and vigorously agitated. Colony counts were performed at 0, 3, 12, 18, and 24 h of incubation of these cultures. Samples (0.01 ml) were also withdrawn at these intervals, and the hemadsorption assay was repeated. Rates of change from $HA^+$ to $HA^-$ and $HA^-$ to $HA^+$ were calculated by the formula $M/N/g$, where $M/N$ is the ratio of the number of either $HA^+$ CFU or $HA^-$ CFU to total CFU and $g$ is the number of generations of growth (the natural log, ln, of the viable-colony count) (10). To determine whether a change from $HA^+$ to $HA^-$ in a particular colony reflected phase variation of hemadsorption alone or change from pilin expression to the absence of pilin expression, the same nitrocellulose discs on which the hemadsorption assay was performed were subsequently stained in the immunodot procedure described below. In some experiments, whole-cell lysates of $HA^+$ and $HA^-$ colonies were examined for pilin expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting.

**SDS-PAGE, Western immunoblotting, and immunodot assays.** SDS-PAGE (12.5% acrylamide gels, silver stained) of whole-cell lysates or outer membrane preparations was performed as previously described (33). Samples were electrophoresed after boiling at 100°C for 3 min. Antiserum to cyanogen bromide fragment 2 (CNBr-2) of gonococcal pilin and murine monoclonal antibodies 2-1-FC (30) and SM1 (14) (kindly provided by John Heckels, Southampton, England) were used as probes in Western immunoblots (33, 34). Laser densitometry (34) was used to quantitate antigen-antibody reactions on Western immunoblots. Immunodot assays of colonies transferred to nitrocellulose were performed by techniques identical to those used for Western immunoblotting.

**Analysis of data.** The Fisher exact test with a two-tailed hypothesis was used for analysis of significant differences in frequency of qualitative data. Student's $t$ test with unpaired values and a two-tailed hypothesis was used to test the significance of differences between the means of two variables.

## RESULTS

**Hemadsorption phenotype of *N. meningitidis*.** We studied 15 strains of *N. meningitidis* by using our hemadsorption assay (Table 1). These strains were encapsulated but of various serogroups, serotypes (11), and sources. These strains were also piliated by electron microscopy (29a, 31, 33, 34). Three to 40 pili were seen per diplococcus, and 70 to 95% of the diplococci were piliated. They produced pilins of 14.5 to 20.6 kDa recognized in Western immunoblots by a polyclonal antiserum to the CNBr-2 fragment of gonococcal pilin (designated antiserum CNBr-2) (33, 34). Pilins of these strains were further characterized on the basis of their reactivity in Western immunoblots with monoclonal antibodies 2-1-FC and SM1 (Table 1).

Of the 15 meningococcal strains, we observed $HA^+$ colonies in seven (Table 1). The incidence of $HA^+$ colonies varied among the seven strains, ranging from 0.2% in strain 1643 to 97% in strain 269B. Figure 1 shows the results of a typical hemadsorption assay for meningococcal strain 6083. No relationship (Fisher exact test) was noted between serogroup, serotype, or site of isolation and the HA phenotype. An $HA^+$ phenotype in >5% of the colonies of a strain was associated ($P = 0.03$, Fisher exact test) with reactivity of pilin in Western immunoblots with SM1, a monoclonal antibody that has been used to define class I meningococcal pili (14). The $HA^+$ phenotype in >5% of the colonies of a strain was also associated ($P = 0.04$) with reactivity with 2-1-FC, a monoclonal antibody which recognizes a common epitope on pilin and pili of some strains of *N. meningitidis* and *N. gonorrhoeae* (34).

**Relationship of hemadsorption to piliation.** To further study the association of $HA^+$ colonies with pilin and pili, we performed colony immunodot assays on the same nitrocel-

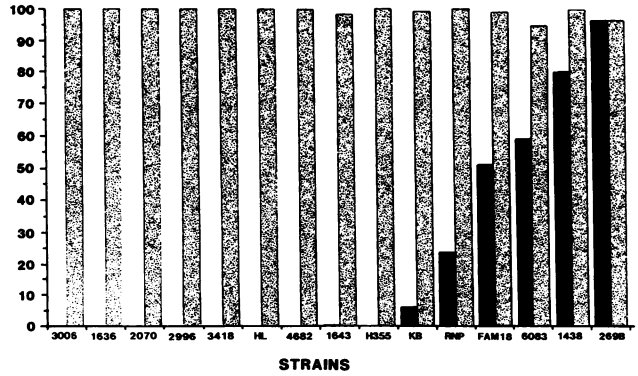


FIG. 2. Attachment of human erythrocytes by colonies of 15 meningococcal strains. Ninety-five to 100% of the colonies of these strains produced pilin, as determined by reactivity with antibody CNBr-2 (▨). Seven of these strains expressed $HA^+$ colonies (■). The incidence of $HA^+$ colonies ranged from 0.2% in strain 1643 to 97% in strain 269B.

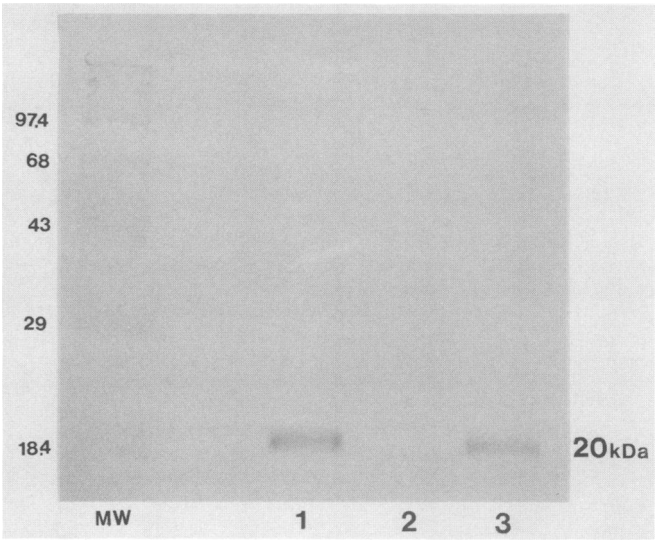


FIG. 3. Western immunoblot of outer membrane preparations (OMP) of erythrocyte-attaching and non-erythrocyte-attaching phenotypes of strain 269B. OMP were separated by SDS-PAGE (12.5% [wt/vol] acrylamide gel) and probed with pilin antiserum CNBr-2. Lanes 1 and 3 contain OMP of two different hemadsorption-positive colonies of strain 269B and show reactivity of CNBr-2 with a 20-kDa pilin. Lane 2 contains OMP of a hemadsorption-negative colony of strain 269B and does not react with this antiserum. Prestained molecular weight markers (high-molecular-weight standards; BRL) are indicated in thousands (MW).

lulose discs that were used to identify $HA^+$ colonies, and in some experiments we performed EM on the portion of these colonies remaining on the agar plate after the HA assay.

**(i) Pilin and hemadsorption. (a) Pilin$^-$ HA$^-$ phenotype.** Meningococcal colonies that did not express pilin did not bind human erythrocytes. Ninety-five to 100% of the colonies of each of the 15 strains expressed pilin, as shown by reactivity with antiserum CNBr-2 (Fig. 2). In strain 269B, 97% of the colonies expressed pilin and were $HA^+$. The $HA^-$ colonies (3%) of this strain did not react with antiserum CNBr-2, which suggests that they did not express pilin. To confirm this hypothesis, $HA^+$ and $HA^-$ colonies of strain 269B were selected and passaged. Comparison of whole-cell lysates and outer membrane preparations of colonies of strain 269B with the $HA^+$ and $HA^-$ phenotypes by SDS-PAGE revealed no differences. In Western blots of these preparations, $HA^+$ colonies of strain 269B contained a 20-kDa pilin reactive with antiserum CNBr-2, while $HA^-$ colonies did not (Fig. 3). $HA^-$ colonies of strain 269B, thus, demonstrated a pilin-negative (pilin$^-$) $HA^-$ phenotype. In each of the other strains, pilin-negative colonies were $HA^-$.

**(b) Pilin$^+$ HA$^+$ and pilin$^+$ HA$^-$ phenotypes.** Meningococcal colonies that bound human erythrocytes expressed pilin, but many pilin-expressing colonies did not bind erythrocytes. $HA^+$ meningococcal colonies reacted with antiserum CNBr-2, demonstrating a pilin-expressing (pilin$^+$) $HA^+$ phenotype. However, as shown in Fig. 2, many pilin-expressing colonies were $HA^-$. Differences in the amount of pilin expressed by colonies or differences in transfer of bacteria to nitrocellulose were considered as possible explanations for the different phenotypes. The amount of pilin, as determined by laser densitometry of Western immunoblots, did not differ between $HA^+$ and $HA^-$ phenotypes of strain 6083 (data not shown). In addition, no differences between $HA^+$ and $HA^-$ colonies, in intensity of antibody reactivity were noted in immunodot assays, suggesting that transfer of $HA^+$ and $HA^-$ colonies expressing pilin to nitrocellulose did not differ. Thus, the capacity of meningococci to bind human erythrocytes and the expression of pilin by meningococci are related but not identical properties.

**(ii) Piliation and hemadsorption (piliated HA$^+$ and piliated HA$^-$ phenotypes).** Assembled pili were necessary for erythrocyte binding, but not all piliated meningococcal colonies bound erythrocytes. The relationship between pilin, the presence of assembled pili, and $HA^+$ colonies was investigated with strain FAM18. Meningococci of strain FAM18 were piliated (mean, 6.6 pili per diplococcus; 88% of diplococci were piliated), as determined by EM, and expressed a 14.5-kDa pilin in Western blots probed with antiserum CNBr-2. In these experiments, 51% of the colonies of strain FAM18 were $HA^+$. A subclone of the strain, FAM18A, was found to produce approximately equal amounts of the 14.5-kDa pilin, but colonies of this clone were nonpiliated (mean, 0.2 pili per diplococcus; <1% of diplococci were piliated), as determined by EM. More than 95% of the colonies of FAM18A were $HA^-$. Thus, assembly of pilin into pili appears to be necessary for FAM18 binding of human erythrocytes.

EM of individual colonies of other $HA^+$ and $HA^-$ strains was performed to further assess the relationship between morphology and number of pili with the HA phenotype. Pili were seen by EM on meningococci from both $HA^+$ and $HA^-$ colonies of strain 6083 and from $HA^-$ colonies of strains 1643 and 1636. In strain 6083, no significant difference (Student's $t$ test) between $HA^+$ (mean, 8.1 pili per diplococcus; 78% of diplococci were piliated) and $HA^-$ (mean, 8.2 pili per diplococcus; 70% of diplococci were piliated) colonies was noted in the number of pili or percentage of meningococci that were piliated. The morphology of pili as assessed by EM appeared similar for $HA^+$ and $HA^-$ colonies. Pilus width (~5 nm) and length (1,000 to 8,000 nm) were similar and bundles of pili (12, 33) were seen in preparations from $HA^+$ and $HA^-$ colonies. Thus, pilin production and assembly into pili did not consistently result in the $HA^+$ phenotype. In fact, the majority of piliated colonies of some strains were $HA^-$. Ninety-eight percent of the $HA^-$ colonies of strain 1643, for example, expressed

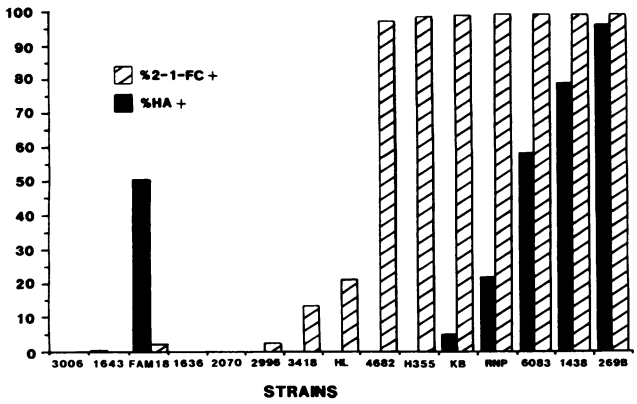


FIG. 4. Relationship of meningococcal colonies reactive with antipilus monoclonal antibody 2-1-FC (2-1-FC$^+$) and HA$^+$ meningococcal colonies. Eleven of 15 piliated meningococcal strains expressed 2-1-FC$^+$ colonies. In four of these strains, 2-1-FC$^+$ colonies were a minority of the colonies. Colonies of five of six strains that expressed >5% HA$^+$ colonies were >99% 2-1-FC$^+$. However, among these strains there were many 2-1-FC$^+$ colonies that were HA$^-$, and in the case of strain FAM18 most HA$^+$ colonies were not reactive with 2-1-FC.

pilin. Assembled pili were demonstrated by EM on meningococci from these colonies (mean, 9.4 pili per diplococcus; 80% of diplococci were piliated), but the colonies were HA$^-$ (Fig. 2). These data suggest that while assembly of pilin into pili is essential for the HA$^+$ phenotype, only certain types of pili may function as a hemagglutinin. Pilin expression and pilus assembly appeared to be necessary but not sufficient conditions for attachment of meningococci to human erythrocytes.

**Hemadsorption and pilus type.** Colony immunodot assays were also used to further evaluate the immunologic type of pili expressed by individual HA$^+$ colonies. In experiments separate from those whose results are shown in Fig. 2, colonies of 11 of the 15 strains showed reactivity with monoclonal antibody 2-1-FC (Fig. 4). Among strains that showed reactivity, the incidence of 2-1-FC-positive colonies ranged from 2.6 to 100%. Four strains (2996, 3418, HL, and FAM18) produced both 2-1-FC-reactive and non-2-1-FC-reactive piliated colonies. For example, 2-1-FC reacted with 26% of the colonies of strain HL, yet >99% of colonies of this strain expressed pilin, as shown by their reactivity with antiserum CNBr-2. The colony immunodot assay was more sensitive than the Western immunoblot for indicating strains with a minority of meningococci expressing the 2-1-FC pilus epitope.

Five of six strains that expressed >5% HA$^+$ colonies expressed >99% 2-1-FC-reactive colonies. However, many 2-1-FC-reactive colonies of these five strains were HA$^-$, and in the case of strain FAM18, >90% of HA$^+$ colonies were not 2-1-FC reactive (Fig. 4). No difference between HA$^+$ and HA$^-$ colonies in the intensity of 2-1-FC reactivity was noted (Fig. 1B), again indicating that transfer to nitrocellulose and the number of 2-1-FC-reactive pili did not differ between the two phenotypes. In other experiments, HA$^-$ colonies of strain 6083 were found to be SM1 reactive and HA$^+$ colonies of strain FAM18 were not SM1 reactive. These data show that meningococcal pili within a strain undergo antigenic variation and indicate that the expression of 2-1-FC- and SM1-reactive pili and erythrocyte binding by meningococci, while they may be related, are not identical properties.

TABLE 2. Rate of phase variation of hemadsorption phenotype

| Strain | Phenotype[a] | | Mean rate of phase variation[b] (range) |
|---|---|---|---|
| | Original | New | |
| 269B | HA$^+$ | HA$^-$ | $<4.50 \times 10^{-5}$ |
| FAM18 | HA$^+$ | HA$^-$ | $1.6 \times 10^{-4}$ |
| | HA$^-$ | HA$^+$ | $2.8 \times 10^{-2}$ ($2.7 \times 10^{-2}$–$2.9 \times 10^{-2}$) |
| 2070 | HA$^-$ | HA$^+$ | $<1.9 \times 10^{-4}$ |
| 6083 | HA$^+$ | HA$^-$ | $3.1 \times 10^{-2}$ ($2.8 \times 10^{-2}$–$3.72 \times 10^{-2}$) |
| | HA$^-$ | HA$^+$ | $5.7 \times 10^{-2}$ ($1.1 \times 10^{-2}$–$1.6 \times 10^{-3}$) |

[a] HA$^+$, binding of human erythrocytes; HA$^-$, no binding of human erythrocytes.
[b] Expressed as number of changes per CFU per generation. Data are from two separate experiments, with rates determined at one or more time points in each experiment.

**Rate of phase variation of hemadsorption.** The HA phenotype of colonies was observed to switch during passage of some meningococcal strains. Rates of phase variation of hemadsorption phenotypes were determined for four strains (Table 2). For strain 269B, switching from a HA$^+$ to a HA$^-$ phenotype was not detected. We calculate the rate of HA$^+$-to-HA$^-$ change for this strain to be less than $10^{-5}$/CFU per generation. Similarly, strain 2070 was not observed to switch from a HA$^-$ to a HA$^+$ phenotype. However, strains 6083 and FAM18 undergo phase variation between HA$^+$ and HA$^-$ phenotypes at high frequencies ($10^{-2}$ to $10^{-4}$/CFU per generation). The phase variation rates of these strains did not change significantly during 24 h of growth in broth. HA$^+$ and HA$^-$ colonies were reactive with pilin antibody 2-1-FC, and selected HA$^-$ pilin expressing colonies were piliated as determined by EM. These data indicate that phase variation in hemadsorption can occur at a high frequency.

## DISCUSSION

Pathogenic *Neisseria* species have evolved a complex genetic system to generate variation in the pili and other surface components that they express (20). Evasion of the host immune response is one consequence of this variation, but the ability to bind to different host cells may be an even more significant result. We used a hemadsorption assay to make several observations about the mechanisms of meningococcal attachment to human erythrocytes. The hemadsorption assay allows determination of the percentage of CFU in a culture that produce hemagglutination and the rates of change of the HA phenotype and other phenotypic characteristics of the colonies (e.g., pilin antigenicity). Recombinants or genetically defined mutants can also be screened for erythrocyte attachment by this assay.

We found that erythrocyte attachment by meningococci occurred only in the presence of pilin production and piliation. Nonpilus surface components have been shown to cause hemagglutination in other bacterial species (3, 29). For example, the hemagglutinin of *Bordetella pertussis* is a 210- to 220-kDa outer membrane protein (5). Also, lipopolysaccharides (LPS) of *N. meningitidis* and *N. gonorrhoeae* have components that are immunologically similar to precursors of human blood group antigens (19). However, unless pili were present, we did not observe meningococcal binding of human erythrocytes in the 15 strains studied. Greenblatt et al. (12) and Salit (26) also noted that nonpiliated meningococci do not attach to human erythrocytes. These data

indicate that pili are necessary for erythrocyte binding but do not exclude the possibility that outer membrane proteins and LPS contribute to the steps required for erythrocyte attachment.

In addition, our data indicate that pilin production alone does not result in erythrocyte attachment. Pilin assembly into pili appears necessary for binding of meningococci to human erythrocytes. Assembled pili have been proposed as necessary for cross-linking of erythrocytes in hemagglutination assays (24).

Previous studies suggested that pili were responsible for the attachment of meningococci and gonococci to erythrocytes (6, 16, 26, 37). However, we found strain-to-strain variation among piliated meningococci with respect to erythrocyte attachment. Fewer than half of the piliated meningococcal strains we studied attached to human O $Rh^+$ erythrocytes, and among these strains the percentage of colonies that exhibited attachment ranged from 0.2 to 97. This could not be explained by detected differences between piliated $HA^+$ and $HA^-$ colonies in the amount of pilin produced (as demonstrated by Western immunoblots) or the degree of piliation and was not due to differences in transfer of colonies to nitrocellulose. Further, within some strains phase variation between piliated meningococcal colonies that exhibited erythrocyte binding and those that did not occurred at a frequency of up to $10^{-2}$/CFU per generation. These data suggest that not all pili mediate attachment of meningococci to human erythrocytes and that the meningococcal hemagglutinin may undergo phase variation, often at a high frequency. Trust et al. (36) also found differences in hemagglutinating power among piliated meningococcal strains. Further, Greenblatt et al. (12) noted that meningococcal strains expressing pili which appeared as single filaments (beta pili) were associated with the ability to hemagglutinate, whereas strains expressing pili which appeared as aggregated bundles (alpha pili) did not. Although we did not observe morphologic differences in pili from $HA^+$ and $HA^-$ colonies, taken together the data suggest that pili of a specialized type function as the meningococcal hemagglutinin.

A minor pilus component functioning as a hemagglutinin and undergoing phase variation is one explanation for our findings. Pap and type 1 pili of *Escherichia coli* are regulated by a gene cluster which encodes the major structural subunit (pilin) and separate gene products that are responsible for adhesion (18, 23, 25). However, in pathogenic neisseriae, multiple gene products forming a pilus apparatus have not thus far been identified. Unlike Pap and type 1 pili of *E. coli* (17, 22), the pilin monomer of the *N*-methyl phenylalanine family of pilins, which includes the pilins of meningococci and gonococci, may function as the adhesin (15). Schoolnik et al. (28) described what they call the "human erythrocyte binding domain of gonococcal pili." They suggested that the CNBr-2 fragment of gonococcal pilin (amino acids 8 to 96) contained the amino acid sequence for the erythrocyte-binding site of gonococcal pili. The CNBr-2 fragment (a region that contains both constant and variable amino acid segments) but not the CNBr-3 fragment (a region that contains predominantly hypervariable segments) inhibited hemagglutination. The frequency of HA variation in some of our piliated meningococcal strains resembles frequencies of variation of pilin structure in gonococci and meningococci (4, 13, 21). High-frequency pilin variation results in antigenically altered pilins or nonpiliated phenotypes. Our data suggest that variation of pilin structure may also result in functionally different meningococcal pili. However, we do not exclude a two-component model requiring both pili and a second meningococcal surface component which together function as the hemagglutinin.

In summary, production of pilin and assembly into pili appear necessary for meningococcal binding to human erythrocytes but are not sufficient. Phase variation influences the ability of meningococci to attach to human cells. Variation in meningococcal pilin structure may result in meningococci that are functionally different.

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