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Biological Properties of Two Distinct Pilus Types Produced by Isogenic Variants of *Neisseria gonorrhoeae* P9

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Isogenic variants from a single strain of *Neisseria gonorrhoeae* were shown to produce two distinct types of pili. These pili, designated α and β, differed in both subunit molecular weight and in ability to attach to buccal epithelial cells.

Pili, protein filaments protruding up to 5 μm from the bacterial surface, are critical determinants of gonococcal virulence which facilitate adhesion to human tissues (2) and, together with the leukocyte association protein (13), influence the process of phagocytosis (4). The reported amino acid compositions for gonococcal pili (1, 3, 8) suggest significant structural variation in the pili isolated from different strains, and this is reflected in marked antigenic variation (1, 3). Evidence for the production of different pili by a single strain of gonococcus came from the studies of Robertson et al. (8). These authors showed that a minor pilus component in strain P9 differed in both isoelectric point and buoyant density; however, the possibility that these different pilus fractions represented altered aggregation states rather than two unique types of pili could not be excluded. Salit and Gotschlich (11) have recently noted that transparent and opaque variants of the same strain of gonococcus produce pili with differing physicochemical properties. In this report we confirm this observation of Salit and Gotschlich and present evidence for two different pilus types (designated α and β) produced by isogenic variants of *Neisseria gonorrhoeae* P9.

Gonococcal variants were isolated and purified by single colony isolation on clear typing medium as previously described (6, 12). Strain P9-2 was identified as a P^{2+} transparent clone producing α pili and, strain P9-20 was classified as a P^{2+} intermediate/opaque variant producing β pili. Both variants possessed the same principle outer-membrane protein (36,000 daltons), but P9-20 produced an additional surface protein, protein II*, (29,000 daltons) (6). Gonococcal pili were isolated and purified essentially as described by Brinton et al. (1). Gonococci were grown on trays of clear typing medium at 36°C in an atmosphere of 5% (vol/vol) CO_2. The organisms were harvested into ice-cold ethanolamine buffer (0.15 M, pH 10.5) and subjected to mild shearing in an Ultro-Turrax homogenizer (Vortex Mixers Ltd., Hampton, Middlesex, England) for 2 min. The organisms were removed by centrifugation at 15,000 $\times$ *g* for 30 min, and the pili were precipitated from the resulting supernatant by the addition of saturated ammonium sulfate solution (pH 10) to give a final saturation of 10%. The pili were collected by centrifugation at 13,000 $\times$ *g* for 1 h. At this stage the pili were contaminated with outer-membrane bleb material and were further purified by repeated disaggregation in ethanolamine buffer and precipitation with ammonium sulfate. The final purity of the pilus preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a separating gel composed of a linear 10 to 25% acrylamide gradient (6). Gels were deliberately overloaded with 10 to 20 μg of protein, and no contaminant protein bands were detected on staining with Coomassie brilliant blue R250 (Fig. 1). The apparent molecular weight of the pilin subunit was determined from the polyacrylamide gels and was found to be 19,500 for the α pilin and 20,500 for the β pilin. The stained protein band corresponding to β pili was always accompanied by a minor band of slightly greater molecular weight, but it is not known at this stage whether this minor protein represents a further type of pilin molecule.

Radioiodination of pili was achieved by using chloramine-T and carrier-free $Na^{125}I$ (The Radiochemical Centre, Amersham, England). Pili (2 to 3 mg) in sodium phosphate buffer (0.05 M, pH 7.5) were treated with chloramine-T (0.5 mg) and $Na^{125}I$ (1 mCi) in a final volume of 0.6 ml. After 5 min at room temperature the reaction was stopped by the addition of sodium metabisulfite (1.25 mg) and potassium iodide (2.5 mg). The pili were purified from other reaction products by repeated washing in 20% saturated ammonium sulfate. The specific activity of the radioiodinated pilus preparation was determined by protein estimation and gamma counting (Wilj 2001, Wilj Electronics Ltd., Ashford, Kent, England) and was found to be between 0.3 and 0.4

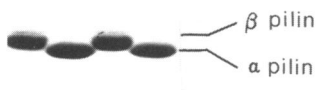

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel showing the difference in apparent subunit molecular weight of α and β pili. Electrophoresis was carried out at a constant 200 V for 24 h at 4°C by using a separating gel with a linear gradient of 10 to 25% acrylamide (6). Subunit molecular weights were determined by using the following standards: bovine serum albumin, ovalbumin, trypsin, and lysozyme.*

μCi per μg of protein. The proportion of radioiodinated protein in the sample was determined by precipitation with 7.5% (wt/vol) trichloroacetic acid. The amount of trichloroacetic acid-precipitated material was always greater than 98%. The final iodination density was calculated to be ca. 1 atom of iodine per 300 pilin subunits. ^{125}I-labeled pili behaved identically to normal pili on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and showed a single protein band by staining with Coomassie brilliant blue R250 and by autoradiography.

Preparations of radioiodinated α and β pili were tested for their ability to attach to human buccal epithelial cells. Striking differences between α and β pili were seen when attachment was measured over a broad pH range (Fig. 2). The attachment of α pili was markedly pH dependent with a maximum binding of 44% at pH 6.5. By contrast, β pili showed a steady decline in binding ability over the pH range tested from a maximum of 13% attachment at pH 4.5 to 4% binding at pH 8.5. These findings for β pili are in close agreement with the results of Pearce and Buchanan (7) for F62 pili, which showed a pH optimum of 4.5 and a three- to fourfold reduction in attachment at pH 7.5.

Further differences in the cohesive properties of α and β pili were demonstrated by using host cells modified by treatment with exoglycosidases (Table 1). Removal of sialic acid residues from buccal cell surface carbohydrates by neuraminidase treatment markedly inhibited the binding of α pili, but had little effect on β pili. Treatment of the neuraminidase-modified buccal cells with a mixture of exoglycosidases further reduced the binding of α pili (6% attachment) to a level comparable to that of β pili (8% attachment). Interestingly, there was no difference in the ability of α and β pili to bind to the human erythrocytes (7% attachment), and this adhesion was unaffected by pH over the range pH 5.0 to 8.5 (data not presented). The ability of erythrocytes to bind both α and β pili was increased by treatment of the cell with neuraminidase or with a neuraminidase-exoglycosidase mixture. A similar phenomenon has been observed with *E. coli* type I pili (9, 10), where protease or neuraminidase treatment of erythrocytes and monkey kidney cells significantly increased type I pilus attachment, probably as a result of removal of charged sialic acid residues from the cell surface.

These results demonstrate clear differences in the ability of human cells to bind gonococcal pili and in the cohesive properties of the α and β pili. A possible explanation is that α pili specifically bind to a receptor, involving sialic acid plus other sugar residues, present on the surface of human buccal cells and that β pili lack such receptor recognition. Binding to this receptor is pH dependent, with an optimum at pH 6.5; this is appropriate if α pilus adhesion is important in natural infection because the average pH of endocervical secretions and the male ejaculate are close to this value (5). The possibility that increased binding of α pili at near neutral pH is simply due to greater pilus-pilus aggregation would seem unlikely because this effect was seen only with buccal epithelial cells and not with erythrocytes. However, if the observed binding curve is in part due to increased pilus interactions, then this may be an important virulence mechanism, resulting in increased adhesion to host cells for gonococci carrying α pili. Indeed, attachment of intact P9-2 (α pili) gonococci to buccal epithelial cells is pH dependent and reaches a maximum at pH 6.5 (unpublished data). The binding of α pili to erythrocytes and to enzyme-treated buccal cells is comparable to β pilus adhesion and may involve a second less-specific attachment mechanism. Given that gonococcal pilin contains some 46% nonpolar amino acids (1, 3, 8), one possibility is binding by hydrophobic interaction. This possibility was not taken into account in recent studies on the nature of the receptor for gonococcal attachment (7).

Gonococcal pili are one candidate for a vaccine to prevent gonorrhea; preliminary results suggest that human volunteers immunized with pili resist challenge with gonococci producing the homologous pilus type (1). Clearly, it is important to establish what type(s) of pili are expressed on gonococci in the natural infection.

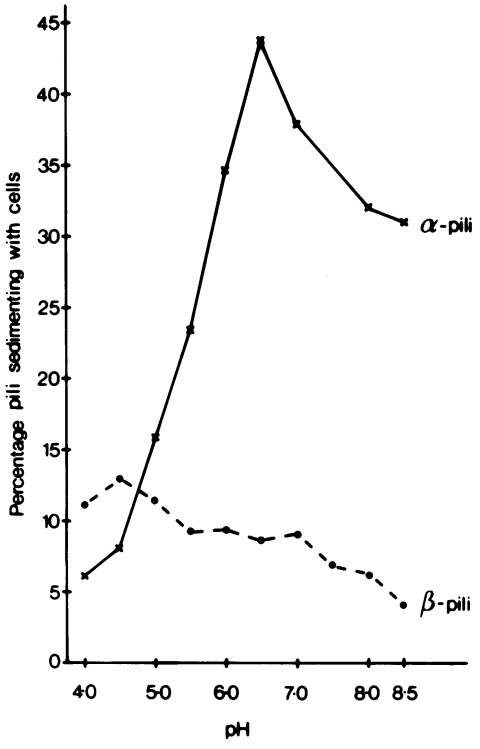

FIG. 2. *Effect of pH on the attachment of ^{125}I-labeled α and β pili to human buccal epithelial cells. Pooled buccal epithelial cells from several volunteers were obtained by scraping the buccal surfaces with wooden spatulas. Cells were collected into phosphate buffered saline (PBS, Oxoid), washed three times in PBS, and finally suspended at a packed cell volume of 5% in attachment buffer: 50 mM Tris-acetate containing 140 mM NaCl, 5 mM $CaCl_2$, 4 mM KCl, 2 mM $MgCl_2$, and bovine serum albumin at a final concentration of 1 mg/ml. The attachment buffer was adjusted to the appropriate pH by addition of 1 M Tris or acetic acid. The incubation mixture contained 400 μl of 5% buccal cells and 1 to 3 μg of ^{125}I-labeled pili (ca. 20,000 cps) for a final volume of 600 μl. Cell suspensions were incubated at 37°C with gentle mixing for 2 h. Cells were separated from unattached pili by layering samples (4 × 100 μl) onto cushions of dextran (Dextraven 110, Fisons, Loughborough, England) in plastic test tubes (75 by 12 mm) and centrifuging at 100 × g for 1 min. Tubes were frozen at −70°C, the bottom of the tube containing buccal cells with attached pili was cut off, and the amount of bound pili was determined by gamma counting. Controls containing pili alone were run for each pH tested, and the number of counts in the control tubes were subtracted from the tests. Actual counts recovered varied from around 1,300 cps for 40% attachment to 50 cps for control counts. Results were expressed as the percentage of pili recovered from the total added. The data presented are the mean values obtained from two separate experiments using two independently prepared batches of ^{125}I-labeled pili. The standard deviation for α and β pilus binding under standard control conditions was ± 1.4% and ± 0.8% attachment, respectively.*

TABLE 1. *Effect of modification of the host cell surface with exoglycosidases*[a]

Determination	^{125}I-labeled pili binding to cells (%)[b]	
	α	β
Buccal epithelial cell treatment		
Control	33	13
Neuraminidase	18	10
Neuraminidase/exoglycosidase	6	8
Erythrocyte treatment		
Control	7	7
Neuraminidase	11	14
Neuraminidase/exoglycosidase	11	9

[a] Buccal epithelial cells and erythrocytes were adjusted to a packed cell volume of 5% in attachment buffer pH 6.0 as described in the legend to Fig. 2. Erythrocytes were collected from one of us and were of blood group type O Rh positive. Neuraminidase (EC 3.2.1.18; Behring) and mixed exoglycosidases (Miles) were added to a final concentration of 0.1 U/ml and 1 mg/ml, respectively. The mixed glycosidases contained the following enzyme activities: α- and β-*N*-acetylhexosaminidase, α- and β-mannosidase, α- and β-glucosidase, α- and β-galactosidase, α-L-fucosidase, and β-xylosidase. Cells were incubated with enzymes for 1 h at 37°C, washed twice, and finally suspended in attachment buffer (pH 6.5). Determination of the attachment of pili was carried out at pH 6.5 as described in the legend to Fig. 2. Controls were cells which had been incubated for 1 h at pH 6 in the absence of enzymes. In addition, buccal epithelial cells and erythrocytes were routinely run in a standard assay procedure as a monitor of the efficiency of the attachment process.

[b] Average values obtained from at least three independent experiments with two separately prepared batches of ^{125}I-labeled pili.

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