Effect of Anti-pilus Antisera on Virulence of Variants of *Neisseria gonorrhoeae* for Cultured Epithelial Cells

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Variants of *Neisseria gonorrhoeae* P9 possessing α , γ or δ pili were shown to vary in their toxicity and virulence for human epithelial cells. Studies with antisera raised against purified pili showed that attachment and virulence were reduced to a significant degree in the presence of antisera to homologous pili. Heterologous antisera, while capable of agglutinating whole organisms, were largely ineffective in reducing attachment and cytotoxicity. Using an enzyme-linked immunosorbent assay (ELISA) system, the cross-reactivity between pili and heterologous antisera was estimated to be no more than 10–20%.

INTRODUCTION

Attachment of gonococci and virulence of the organism for certain human epithelial cells are closely linked phenomena (Ward & Watt, 1977). The differences between two different pilated variants (α and β) in their virulence and attachment for Chang conjunctiva epithelial cells have been reported (Virji & Everson, 1981). Moreover, it has been demonstrated that the virulence of the β -pilated variant P9-20 for these host cells can be decreased significantly in the presence of anti-pilus antisera, whereas anti-lipopolysaccharide (LPS) or anti-outer membrane antisera have little effect (Virji, 1981).

Studies with guinea-pig subcutaneous chambers have provided a useful animal model for investigations of antigenic variations which may arise due to host-induced environmental pressure (Penn *et al.*, 1978). Recently, Lambden *et al.* (1981*a*) have described *in vivo*-selected pilated variants of strain P9 possessing γ pili (mol. wt. 21 000) and δ pili (mol. wt. 18 500).

In the present investigations, differences in attachment and virulence for cultured human epithelial cells have been assessed for variants possessing these pili. Further, in order to study the nature of biologically relevant host–pilus interactions and shared antigenicity between pilus types in attachment, antisera raised against purified pili have been used to protect host cells against infection.

METHODS

Gonococcal variants. Transparent colonial variants P9-35 (γ pili) and P9-37 (δ pili) of *N. gonorrhoeae* strain P9 were isolated from the corresponding opaque variants originating from guinea-pig chambers (Lambden *et al.*, 1981*a*). The laboratory isolated variant P9-2 (α pili; mol. wt. 19 500) was included for comparison. For each experiment, pure colonies were re-isolated on clear typing medium (Lambden & Heckels, 1979) from stock cultures stored in liquid nitrogen. Organisms were harvested after 18–20 h growth at 37 °C in an atmosphere of 5% (v/v) CO₂ in air and suspended in maintenance medium (described below). Aggregated organisms were removed from bacterial suspensions by centrifugation at 150 g for 1 min prior to estimation of bacterial numbers (Virji & Everson, 1981). The pilus type was routinely checked using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Lambden *et al.*, 1981*b*).

Tissue culture. The growth medium (GM) for Chang conjunctiva epithelial cells (Flow Laboratories) was medium 199 with Earle's Salts, supplemented with 10% (v/v) donor calf serum. For cytotoxicity experiments, exponentially growing cells (approaching confluency) were used. 10^5 cells were seeded per well in 96-well microtitre trays in medium 199 supplemented with 2% donor calf serum (maintenance medium, MM). After overnight incubation at 37 °C in a CO₂ (5%, v/v, in air) incubator, the cells were challenged with gonococci.

Estimation of LD₅₀ by cytotoxicity assay. Target monolayers challenged with various numbers of gonococci in 100 µl MM were incubated for 24 h prior to estimation of host cell killing (Virji & Everson, 1981).

Determination of the relative virulence of variants by a modified cytotoxicity assay. Host cells were challenged with gonococci suspended in MM and left in contact with the bacteria (4×10^7 per monolayer) for 3 h. The unattached gonococci were then removed by washing the monolayers five times in MM, and the host cells were incubated for a further 24 h in this medium to allow attached organisms to bring about host cell killing. A 3 h initial exposure was found sufficient to obtain maximum killing for the given dose of gonococci (Virji & Everson, 1981).

Estimation of host cell death. A post-labelling method using ⁵¹Cr has been described fully (Virji & Everson, 1981). Briefly, after incubation of host cells in the presence of gonococci, surviving host cells in monolayers were labelled using ⁵¹Cr [sodium chromate; 100–400 mCi (mg Cr)⁻¹, 3.7–15 GBq (mg Cr)⁻¹; Amersham] at 100 µCi ml⁻¹ (3.7 MBq ml⁻¹) in MM. After 1 h incubation, the plates were washed in phosphate-buffered saline (PBS) supplemented with 2% donor calf serum. The cells were then lysed in 0.1 M-NaOH containing 1% (w/v) SDS and counted in a γ-counter. All experiments were done at least in triplicate.

Attachment of ³H-labelled gonococci. D-[1-³H]Glucose (500 mCi mmol⁻¹, 18.5 GBq mmol⁻¹) was used in the solid medium at a final concentration of 25 µCi ml⁻¹ (0.9 MBq ml⁻¹) for the growth of labelled variants. Host cell monolayers were challenged as described above with labelled gonococci in MM at 5×10^6 per monolayer. After 3 h incubation, monolayers were washed five times in MM. Finally, host cells with attached gonococci were solubilized in 0.1 M-NaOH containing 1% (w/v) SDS and counted in a scintillation counter (LKB). Controls were carried out to assess non-specific attachment to plastic wells.

Preparation of pili and anti-pilus antisera. Pili were purified from bulk-grown variants and the purity of the preparation was determined by SDS-PAGE (Lambden *et al.*, 1981b).

Interaction of antisera with whole gonococci. To a standard suspension of gonococci in PBS on a glass slide, an equal volume of antiserum diluted in PBS was added. Controls were incubated with PBS alone. The slides were rocked from side to side and bacterial agglutination was recorded after 5 min at room temperature.

Interference in attachment and toxicity of gonococcal variants in the presence of antisera. Antisera were diluted in PBS and 25 µl layered on to the target monolayers. Standard gonococcal suspensions (100 µl) were added and the microtitre plates were briefly shaken on a Titretek (Flow Laboratories) shaker. The plates were washed after an initial 3 h attachment period, and incubated in fresh MM for a further 24 h, as described above, for the estimation of toxicity due to attached organisms. To estimate the attachment of variants, ³H-labelled organisms were used in a similar experiment and host cells with the attached organisms were solubilized immediately following the 3 h wash and counted in a scintillation counter.

Immunization. Rabbits were injected subcutaneously at several sites on the back with a total of 100 µg of purified pili in Freund's complete adjuvant (Difco). Booster injections in Freund's incomplete adjuvant (Difco) were given at 2, 4, 6 and 8 weeks after the first injection and blood samples were taken 2 weeks after the last booster dose. Sera were stored at -20 °C.

Enzyme-linked immunosorbent assay (ELISA) determination of shared antigenicity between pili. The extent of cross-reaction between the various pili and their antisera was determined by an ELISA procedure (Diaz & Heckels, 1982). Pili were dispersed in 0.1 M-NaHCO₃ (pH 9.6) at a previously determined optimal concentration of 1 µg ml⁻¹. Individual wells of flat-bottomed polystyrene microtitre trays (Dynatech, Billingshurst, Kent, U.K.) were coated with the suspension of pili (250 µl) at 37 °C for 18 h. The antigen was then discarded and the wells were further coated for 2 h at 37 °C with 200 µl of 0.5% (w/v) bovine serum albumin 'filler'. Wells were then washed three times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 (PBST). Appropriate dilutions of antiserum were pre-absorbed with a range of concentrations of pili [0.25–10 µg (ml serum)⁻¹] by incubation in an uncoated tray at 37 °C for 1 h. The absorbed sera (200 µl) were then transferred to the appropriate wells of pilus-coated microtitre trays and incubated at 37 °C for 1 h. Control wells were filled with PBST only. Wells were then washed three times with PBST, filled with 200 µl of goat anti-rabbit IgG-peroxidase conjugate (Miles, Stoke Poges, U.K.) at a dilution of 1/2000 in PBST, and incubated at 37 °C for 1 h. After washing three times with PBST, 200 µl of enzyme substrate (0.1 mg *o*-phenylenediamine dihydrochloride ml⁻¹ in 3 mM-H₂O₂) was added to each well and incubated at room temperature for 30 min. The reaction was stopped by the addition of 50 µl 1 M-H₂SO₄ and the A₄₉₀ was determined in a Dynatech MR580 Micro Elisa Auto Reader. The degree of cross-reactivity was determined by comparing different antisera absorbed with both homologous and heterologous pili. Results were expressed as the percentage inhibition of colour formation produced by absorbed sera when compared with the unabsorbed controls.

No contaminating anti-LPS activity was detectable at 1/20 dilution of antisera by haemagglutination of LPS-coated erythrocytes.

Fig. 1. Cross-absorption of anti-pilus sera with homologous and heterologous pili. Colour formation in the ELISA test (A_{490}) with absorbed serum, expressed as a percentage of the unabsorbed value, was determined after absorption with different amounts of purified pili. (a), (b) and (c) show results for antisera to α , γ and δ pili, respectively, absorbed with α pili (●), γ pili (▲) and δ pili (■) in typical experiments.

RESULTS

Antisera. High titre hyperimmune sera to purified α , γ or δ pili were raised in rabbits. The specificity of the sera and the degree of shared antigenicity between strain P9 pilus variants was determined using an ELISA system. The results of cross-absorptions with homologous and heterologous pili are shown in Fig. 1. Sera were used at a dilution of 1/40 000–1/80 000 and were pre-absorbed with various amounts of pili. Results show that homologous pili removed more than 95% of the antibody at the pilus levels used. The results also showed that there was only 10–20% cross-reactivity between heterologous pili indicating a number of unique antigenic sites on each of the pilus types.

Agglutination of gonococci in the presence of antisera. Slide agglutination tests using anti-pilus antisera at the 1/20 dilutions used in the experiments, showed that the organisms were agglutinated in the presence of homologous as well as heterologous anti-pilus antisera.

Toxicity. Variants producing γ or δ pili showed marked differences in their toxicities for epithelial cells, P9-35 (γ pili) being more cytotoxic than P9-37 (δ pili): the LD_{50} values were 5×10^5 and 2×10^6 , respectively. The LD_{50} of P9-2 (α pili) in these experiments was 6.5×10^5 .

Relative virulence. This was determined in experiments where attached bacteria were subjected to shearing forces by repeated washing to assess their avidity of attachment and subsequent killing of host cells (Table 1). The γ -pilated variant P9-35 was found to be more virulent than the δ -pilated variant P9-37 under these conditions. A two- to five-fold difference

Table 1. *Effect of homologous and heterologous anti-pilus antisera on the virulence of gonococcal variants in a modified cytotoxic assay*

Epithelial cell monolayers were exposed to gonococci at 4×10^7 per monolayer, in the absence or presence of antiserum (final dilution 1/20). Host cell killing was estimated by labelling surviving cells with ^{51}Cr . Mean values of triplicate estimations are shown; standard deviations were $< \pm 15\%$. The percentage protection was calculated as $100 \times [1 - (\% \text{ killing in the presence of antiserum})/(\% \text{ killing in the absence of antiserum})]$.

Strain P9 variant	Pilus type	Antiserum addition	Host cell killing (%)	Protection (%)
P9-2	α	None	32	—
		Anti- α pili	8	75 ($P < 0.001$)
		Anti- γ pili	34	0 ($P > 0.1$)
		Anti- δ pili	36	0 ($P > 0.1$)
P9-35	γ	None	62	—
		Anti- α pili	50	19 ($P < 0.05$)
		Anti- γ pili	9	85 ($P < 0.001$)
		Anti- δ pili	57	8 ($P > 0.1$)
P9-37	δ	None	25	—
		Anti- α pili	19	24 ($P < 0.02$)
		Anti- γ pili	21	16 ($P < 0.05$)
		Anti- δ pili	6	76 ($P < 0.001$)

Table 2. *Attachment of ^3H -labelled gonococci to Chang epithelial cells in the presence of anti-pilus antisera*

Microtitre plates with target monolayers were incubated for 3 h at 37°C with ^3H -labelled gonococci (5×10^6 organisms per monolayer) in the absence or presence of antiserum (final dilution 1/20). After washing with MM, the well contents were solubilized and counted in a scintillation counter. Mean values of triplicate estimations, corrected for non-specific attachment to plastic wells, are shown; standard deviations were $< \pm 20\%$. The percentage decrease in attachment was calculated as $100 \times [1 - (\% \text{ attachment in the presence of antiserum})/(\% \text{ attachment in the absence of antiserum})]$.

Strain P9 variant	Pilus type	Antiserum addition	Attachment (%)	Decrease in attachment (%)
P9-2	α	None	3.5	—
		Anti- α pili	1.5	57 ($P < 0.01$)
		Anti- γ pili	3.4	3 ($P > 0.1$)
		Anti- δ pili	3.8	0 ($P > 0.1$)
P9-35	γ	None	5.6	—
		Anti- α pili	5.5	2 ($P > 0.1$)
		Anti- γ pili	4	29 ($P < 0.01$)
		Anti- δ pili	5.7	0 ($P > 0.1$)
P9-37	δ	None	3.2	—
		Anti- α pili	3.2	0 ($P > 0.1$)
		Anti- γ pili	2.6	19 ($P > 0.1$)
		Anti- δ pili	1.5	53 ($P < 0.001$)

in virulence was observed with all doses of gonococci tested ranging from 5×10^6 – 2×10^8 . Variant P9-2 showed intermediate virulence.

Effect of anti-pilus antibodies on virulence. In these studies, antisera to the three pilus types were compared: the results in Table 1 indicate that antisera to homologous pili interfered with the process of killing and protected the target monolayers. Under the same conditions, antisera to heterologous pili, though able to agglutinate, were largely ineffective and did not prevent target cell killing.

Effect of anti-pilus antisera on attachment of ^3H -labelled gonococci. Table 2 summarizes the results suggesting that the effect of homologous antisera was to reduce attachment of the

variants to the host cell surface. Heterologous antisera showed little or no effect on attachment.

DISCUSSION

The potential for antigenic variation of the gonococcus in the human host, and even in a single patient, necessitates the search for common antigenic characters which may induce broadly cross-reacting antibodies suitable for development of vaccines. Variations in outer membrane protein composition as well as in pili have been reported by McBride *et al.* (1981) and Lambden *et al.* (1981*a*). Other workers have reported serological cross-reactivity between pili from different strains. Novotny & Cownley (1980) reported that antisera raised against strains obtained from one partner did not react with pili from strains obtained from the corresponding partner. Similar antigenic heterogeneity has also been reported by Lind & Reimann (1980).

In order to clarify the role of antisera in gonococcal virulence, a detailed study of the toxicity of purified major outer membrane antigens (LPS, outer membrane complex and pili) as well as of the effect of antisera raised against these antigens was carried out using the β -pilated variant P9-20. These studies suggested that the purified antigens used were not toxic to Chang epithelial cells (Virji, 1981). Further, anti-pilus antisera protected host cells against killing to a significant degree. A small degree of protection was observed using antisera directed towards outer membrane complex but anti-LPS antisera afforded no protection. In the present study to investigate the effect of antigenic diversity of pili on the interaction of gonococci with host cells, transparent colonial variants were isolated from the *in vivo*-grown organisms. The use of transparent variants excluded the possibility that additional outer membrane proteins seen in opaque variants might influence the virulence of the organism.

The pilated variants exhibited different toxicities, in terms of LD₅₀, in the cytotoxicity tests using conjunctiva epithelial cells. Variant P9-35 (γ pili) was more toxic than P9-37 (δ pili). The exact cause of the variation cannot be defined precisely until the true nature of virulence and toxic factors are known. However, the experiments which measured both the avidity of attachment and toxicity indicated that the γ -pilated variant was more virulent for the epithelial cells than the δ -pilated variant. It is probable that increased association with the host cell surface in the case of the γ -pilated variant, for example, increased the local concentration of toxic factors, thus increasing cell damage. Observations on the attachment of ³H-labelled gonococci confirm that increased virulence is associated with increased gonococcal attachment.

Some cross-protection was observed when γ - and δ -pilated strains were challenged with heterologous sera both in attachment and 'virulence' experiments. This probably reflected the observed cross-reactivity between heterologous pili seen in the ELISA experiments. However, no significant cross-protection was seen with strain P9-2 (α pili). Agglutination of gonococci alone does not protect the host cells since anti-LPS antisera, while strongly agglutinating were ineffective in protection of target cells (Virji, 1981). Further, at the dilutions employed, significant agglutination of the gonococci was observed in the presence of antisera to heterologous pili in the present studies. It is apparent, then, that selection pressures operative *in vivo* induce variation in the pilus types on a single strain and that these variations require highly specific antibodies capable of interference with the attachment only of the homologous pilus types. This observation has significance regarding the nature of pilus-host cell interactions that are possible *in vivo* and has important implications in any attempts to develop an antigenococcal vaccine based on pilus antigens.

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