The Role of Common and Type-specific Pilus Antigenic Domains in Adhesion and Virulence of Gonococci for Human Epithelial Cells

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A panel of monoclonal antibodies with varying pilus specificities ranging from cross-reacting to type-specific has been used to investigate the role of conserved and variable antigenic domains in the adhesion of gonococci to human epithelial cells. The binding of ¹²⁵I-labelled α pili from strain P9 to buccal epithelial cells was inhibited by three type-specific but not by two cross-reacting antibodies. Four type-specific antibodies inhibited the binding of γ pili while the two cross-reacting antibodies were again without effect. The virulence of the variants P9-2 (α pili) and P9-35 (γ pili) for Chang conjunctiva epithelial cells was similarly reduced only in the presence of relevant type-specific antibodies. These results indicate the importance of variable antigenic domains in the adhesion of gonococci to human epithelial cells.

INTRODUCTION

Pili are important virulence factors of gonococci, mediating adhesion to mucosal surfaces during the initial events of pathogenesis which subsequently lead to penetration and multiplication within epithelial cells (Swanson, 1973; Punsalang & Sawyer, 1973; Ward et al., 1974). Thus pili appear to be attractive candidates for a gonococcal vaccine. However, pili show considerable inter- and intra-strain structural and antigenic diversity both under laboratory conditions (Lambden et al., 1981α) and during the course of the natural infection (Duckworth et al., 1983). These variations are associated with alterations in adhesive properties. Variants of strain P9 with α pili (mol. wt 19500) and γ pili (mol. wt 21000) vary in attachment to and virulence for Chang conjunctiva epithelial cells in tissue culture (Virji et al., 1982).

Structural analysis shows that gonococcal pili from different strains possess a region of conserved amino acid sequence and a variable region which determines serological specificity (Schoolnik et al., 1982; Buchanan et al., 1982). Studies with monoclonal antibodies suggest a similar model for intra-strain antigenic variation (Virji & Heckels, 1983). The occurrence of a conserved structural region suggests a common functional role and this region has been implicated in the binding of pili to erythrocytes (Schoolnik et al., 1982). However the role of the conserved and variable regions in adherence to human epithelial cells has not been defined. Indeed, several reports show that anti-pilus antisera inhibit attachment of homologous strains much more efficiently than heterologous strains (Tramont, 1976, 1977; Virji et al., 1982) demonstrating the importance of antibodies directed against the variable determinants in inhibition of epithelial cell binding.

The role of conserved and variable regions in adhesion to epithelial cells can be better studied by the use of monoclonal antibodies. We have recently produced a panel of antibodies with different specificities for pili from strain P9. Two antibodies recognized epitopes in the common region present on all gonococcal pili tested. In addition, several type-specific antibodies were obtained which reacted with a range of distinct epitopes in the variable regions of α and γ pili.

Abbreviation: MM, maintenance medium.

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The current study reports the use of these monoclonal antibodies to probe the role of the variable and conserved regions in the adhesion of pili and virulence of gonococci for human epithelial cells.

METHODS

Bacterial strains and growth conditions. Variants of Neisseria gonorrhoeae strain P9 used have been described previously (Lambden et al., 1981a). P9-2 produces α pili (mol. wt 19500) and P9-35 produces γ pili (mol. wt 21000). Bacteria were grown on a clear typing medium for 16 h at 37 °C in an atmosphere of 5% (v/v) CO₂ (Diaz & Heckels, 1982).

Monoclonal antibodies. The procedure for the production of hybridoma secreting gonococcal pili-reactive monoclonal antibodies has been fully described (Virji et al., 1983). Briefly, spleen cells from BALB/c mice immunized with purified pili or pilated gonococci were fused with NS-1 myeloma cells. The resulting hybridoma were screened for antibody production using ELISA. Ascitic fluid containing high concentrations of monoclonal antibodies were obtained by the growth of cloned hybrids in BALB/c mice previously primed by intraperitoneal injection of 0.5 ml pristane (Sigma). The antibodies employed in the current work together with their reactivities are summarized in Table 1.

Pili purification and ¹²⁵I-labeling. Pili were purified essentially as described previously (Brinton et al., 1978; Lambden et al., 1981b). Gonococci were harvested into 0.15 M-ethanolamine/HCl pH 10-2 and subjected to shearing for 2 min in a Vortex mixer (P. Silver, Hampton, London, UK) with a blade clearance of 0.025 mm. Bacteria were removed by centrifugation and pili precipitated by the addition of (NH₄)₂SO₄ to 10% saturation. Crude pili were purified by two further cycles of disaggregation and precipitation and stored at 4 °C in 1 M-NaCl containing 0.05% (w/v) NaN₃.

Purified pili were labelled using chloramine-T and Na¹²⁵I (Amersham). Pili (1 mg) suspended in 0.05 M-phosphate buffer pH 7.5 (0.5 ml) were reacted with 500 μCi (18.5 MBq) Na¹²⁵I and 50 μl 1% (w/v) chloramine-T solution. After 5 min at room temperature the reaction was terminated by the addition of 0.5 ml 0.3 M-KI, 0.5 ml 0.13 M-N₂S₂O₅ and 1 ml saturated (NH₄)₂SO₄. Labelled pili were recovered by centrifugation at 20000 g for 1 h, washed three times with 50% saturated (NH₄)₂SO₄, resuspended in 1 M-NaCl containing 0.05% (w/v) NaN₃ and stored at 4 °C until required. The specific activity of the ¹²⁵I-labelled pili was 0.3-0.5 μCi (μg protein)⁻¹ [11.1-18.5 kBq (μg protein)⁻¹].

Inhibition of attachment of ¹²⁵I-labelled pili to buccal epithelial cells. The effect of monoclonal antibodies on attachment of pili to buccal epithelial cells was determined in an assay system based on that of Lambden et al. (1981b). Buccal epithelial cells were scraped with wooden spatulas from several volunteers, pooled in PBS (0.15 M-phosphate buffer pH 7.2 plus 0.15 M-NaCl) and centrifuged at 500 g for 5 min. The pellet was washed three times in PBS and finally resuspended at a packed cell volume of 5% in attachment buffer which consisted of 0.05 M-Tris-acetate buffer, pH 7.5, containing 140 mM-NaCl, 5 mM-CaCl₂, 5 mM-KCl, 2 mM-MgCl₂ and 1 mg BSA ml⁻¹.

Attachment experiments were carried out in 2 ml polypropylene screw-capped tubes which had previously been treated for 16 h at 4 °C with 1% BSA in PBS to prevent non-specific binding. The reaction mixture contained 0.4 ml buccal cell suspension, 0.5 μg pili (10⁶ d.p.m.) in 0.1 ml attachment buffer and 0.1 ml ascitic fluid containing monoclonal antibody diluted in attachment buffer. Tubes were incubated with rotation for 2 h at 37 °C.

Table 1. Properties of monoclonal antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IgG subclass</th>
<th>α</th>
<th>β</th>
<th>γ</th>
<th>δ</th>
<th>Other strains</th>
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<tr>
<td>SM1</td>
<td>2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>All tested</td>
</tr>
<tr>
<td>SM2</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SM3</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>None or limited</td>
</tr>
<tr>
<td>SM5</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>SM6</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
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<td>2a</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SM13</td>
<td>2a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Role of gonococcal pilus domains in adhesion

Samples of the suspensions (3 × 0.1 ml) were layered onto 3.5 ml 6% (w/v) dextran in 0.9% (w/v) NaCl solution (Dextragen 110; Fisons) in 0.6 cm diameter glass test-tubes. The tubes were centrifuged at 200 g for 1 min to pellet cells plus attached pili. The dextran layer containing unattached pili was aspirated and the amount of bound pili in the pellet was determined by gamma counting. Controls containing pili but no buccal cells were carried out in parallel. The percentage of pili adhering was determined from:

\[
100 \times \left[ \frac{\text{mean c.p.m. in pellet} - \text{mean c.p.m. in control}}{\text{total c.p.m. applied to tube}} \right]
\]

All experiments were carried out in triplicate. In a typical experiment in the absence of antibody, α pili gave a mean binding of 43.8% with an SD of 3.5%.

**Cytotoxicity assay.** Chang conjunctiva epithelial cells (Flow Laboratories) were grown in medium 199 with Earle salts supplemented with 10% (v/v) calf serum. Monolayers for cytotoxicity assays were prepared as previously described (Virji & Everson, 1981) in 96-well plates by seeding at 10⁶ cells per well in medium 199 supplemented with 2% calf serum (maintenance medium, MM). Cultures of gonococci were suspended in MM and any aggregates were removed by centrifugation prior to estimation of bacterial cell numbers. Host cell monolayers prepared the previous day were challenged with gonococci (2 × 10⁷ per monolayer) and incubated for 3 h. The unattached organisms were removed by washing the wells with MM at the end of this period. The incubation was continued for a further 24 h for estimation of the toxic effect of the attached organisms (Virji & Everson, 1981).

Cell viability was determined by a dye assay. At the end of the 24 h incubation, the target monolayers were washed in Dulbecco's complete medium four times to remove dead cells. Viable cells still attached to the plastic wells were fixed in absolute methanol for 10 min then washed twice with water to remove excess alcohol prior to staining. The working dilution of the dye was freshly prepared from a stock aqueous solution containing 1% (w/v) Safranin-O (Raymond A. Lamb, North Acton, Middlesex, UK) which was filtered and stored at room temperature in the dark. Fixed washed cells were stained with 50 μl of 0.1% (w/v) of the dye in water at room temperature for 20-30 min. The plates were then thoroughly washed with water (five times), drained, and the cells solubilized in 1% (w/v) SDS in 0.1 M NaOH by shaking on a Titertek Plate Shaker (Flow Laboratories) for 10 min. The absorbance was measured at 492 nm in a Titertek Multiskan photometer (Flow Laboratories).

**Protection of host cells against gonococcal challenge using monoclonal antibodies.** Ascitic fluids containing monoclonal antibodies were heated at 56 °C for 30 min and filter-sterilized. Their effect on the viabilities of the host cells as well as the gonococcal variants was monitored prior to use in the cytotoxicity experiments. Antibodies were diluted in Dulbecco's complete medium and 50 μl layered onto the target monolayers. Standard gonococcal suspensions containing 2 × 10⁷ organisms in 50 μl double strength MM were added and the tissue culture plates were briefly shaken on a Titertek Plate Shaker. The plates were then treated as above after 3 h incubation and surviving target cells were quantified after a further 24 h incubation.

**RESULTS**

**Effect of monoclonal antibodies on pilus binding**

The buccal cell attachment system of Lambden et al. (1981 b), using low concentrations of pili and separation of bound from non-bound pili on cushions of dextran, provided a convenient means to determine the effect of monoclonal antibodies on pilus attachment. Control tubes without cells showed that little or no aggregation of pili was caused by the monoclonal antibodies under the test conditions. In contrast, a polyclonal rabbit anti-pilus antisera caused considerable aggregation making results difficult to assess (data not shown).

The effect of monoclonal antibodies on the adhesion of 125I-labelled purified pili to buccal epithelial cells is shown in Fig. 1. With α pili the three type-specific antibodies SM3, SM5 and SM13 showed inhibition of attachment (P < 0.01, Student's t test) at a range of concentrations. In contrast cross-reaction antibody SM1 showed no significant inhibition of attachment and antibody SM2 showed slight inhibition only at the highest concentration tested. Similar results were obtained with purified γ pili. Antibody SM13 which reacts with both α and γ pili also inhibited binding of γ pili to buccal cells as did the three antibodies with specificity for γ pili SM6, SM8 and SM9. Again cross-reacting SM1 failed to inhibit and SM2 showed slight inhibition only at the highest concentration.

**Measurement of cytotoxic effect of gonococci on tissue culture cells**

In the current studies, the measurement of host cell viability was carried out by staining the surviving cells at the end of an experiment using the dye Safranin-O as described in Methods.
Fig. 1. Effect of monoclonal antibodies on the attachment of $^{125}$I-labelled pili to human buccal epithelial cells. Pili were incubated with buccal cells in the presence of antibodies; pili bound were detected by gamma counting. Final dilutions of ascites were: 1/10 (filled boxes); 1/100 (hatched boxes); 1/1000 (stippled boxes). Mean values from one experiment performed in triplicate are shown: SD were < ±5%. The results are typical of those obtained in at least three separate experiments.

This procedure for the estimation of cell viability has considerable advantages in saving both time and resources compared with the previously described method which utilized $^{51}$Cr (Virji & Everson, 1981). The sensitivity of the assay results from the intense staining of the cells compared with the background under the conditions described. The dye assay was quantified by seeding varying numbers of Chang cells ranging from $5 \times 10^3$–$1 \times 10^5$ cells per well in microtitre plates (equivalent to 5–100% viability in cytotoxicity experiments). A linear relationship between the number of cells seeded and absorbance at 492 nm was obtained as described in Methods. All estimations were done in quadruplicate and the SD were usually less than ±5%.

Protection of Chang conjunctiva epithelial cells against gonococcal invasion using monoclonal antipilus antibodies

The virulence of gonococcal variants P9-2 and P9-35 for Chang epithelial cells was reduced in the presence of several monoclonal antibodies. The results (Fig. 2) paralleled those seen in the pilus attachment system. Both in the case of α-piloted P9-2 as well as γ-piloted P9-35, the corresponding type-specific antibodies protected the host cells against tissue damage ($P < 0.01$, Student's $t$ test). Of the two cross-reacting antibodies, SM1 was ineffective, although SM2 gave a low level of inhibition also seen in the pilus attachment system.

It was important to establish that these effects were directly due to inhibition of bacteria–cell interactions and not due to differences in gonococcal growth or survival in the presence of ascitic fluids containing monoclonal antibodies. Experiments carried out under identical conditions to the cytotoxicity experiments but without the host cells showed that gonococci nearly doubled in mass after the first 3 h incubation. The rate of growth was independent of the presence of ascitic
Fig. 2. Protection of Chang conjunctiva epithelial cells with monoclonal antibodies. Epithelial cells were exposed to gonococci in the presence or absence of antibodies. Final dilutions of ascitic fluids were: 1/10 (filled boxes); 1/50 (hatched boxes); 1/250 (stippled boxes). Cytotoxicity was measured as described in Methods. Mean values of quadruplicate estimations from one experiment are shown; SD were < ± 5%. Percentage protection = 100 × [1 - (% killing in the presence of antibody + % killing in the absence of antibody)]. The results are typical of those obtained in three separate experiments.

DISCUSSION

The considerable antigenic heterogeneity between gonococcal pili has stimulated investigations on the relationships between different pili and in particular those determinants which might be involved in the common function of adhesion to epithelial cells. Immunization of rabbits with purified pili induces antibodies which are largely serotype-specific although some strains, designated 'senior', have the ability to induce high levels of cross-reacting antibodies (Brinton et al., 1982). Similarly studies with monoclonal antibodies have shown the presence of common antigenic domains on all gonococcal pili tested (Virji & Heckels, 1983). These immunological studies suggested that pili contain common antigenic determinants as well as the serotype-specific determinants which are normally immunodominant. Structural studies on fragments obtained on cyanogen bromide cleavage of pili from several strains are in accord with this model (Schoolnik et al., 1982; Buchanan et al., 1982). Amino acid sequencing shows the presence of a conserved region in a fragment derived from the amino-terminal end and a variable region in a fragment derived from the carboxy-terminus. Studies with the purified fragments show that a domain with human erythrocyte binding activity is present in the fragment containing the common region (Schoolnik et al., 1982) and that it also binds to Chinese...
hamster ovary cells (Gubish et al., 1982). The role of the variable region in adhesion is more difficult to assess since preparation of this fragment results in destruction of its antigenic specificity (Buchanan et al., 1982). Hence the current investigation of the role of variable pilus domains in human epithelial cell binding has studied the ability of monoclonal antibodies of defined specificity to inhibit model systems of pilus attachment and gonococcal virulence.

The attachment of iodinated purified pili to buccal cells was inhibited by type-specific antibodies whether x pili or y pili were used, whereas the cross-reacting antibodies were without effect. In cytotoxicity assays the virulence of two pilated variants was also reduced only when type-specific antibodies were present. The difference in inhibition is not due to difference in avidities of the antibodies used, since the cross-reacting antibody SM1 exhibits high avidity for gonococcal pili and is without effect, whereas the antibody SM5 with lower avidity (Virji et al., 1983) is inhibitory. The considerable similarity in the inhibition patterns using the two systems suggests that gonococcal virulence is reduced by inhibition of initial pilus adhesion to the epithelial cells. These data show that the variable domains of gonococcal pili play an important role in adhesion to human epithelial cells. Two possible explanations appear likely. Either the variable region itself contains the epithelial cell receptor or the tertiary structure of pili brings the binding site close enough to the variable region for the type-specific antibodies to hinder attachment. The failure of the cross-reacting antibodies to inhibit attachment suggests the former explanation although it is possible that these cross-reacting antibodies bind to determinants in the common region which are spatially removed from the epithelial cell binding site. However, the variation in adhesion of different pilus types (Lambden et al., 1981b) also suggests the direct involvement of the variable region in epithelial cell binding.

Whatever the precise mechanisms the importance of type-specific antibodies in inhibiting adhesion to human epithelial cells is in accord with previous studies. Antibodies present in genital secretions from patients with gonorrhoea are considerably more effective in inhibiting adhesion of the homologous infecting strain (Tramont, 1977) compared with other pilated strains. Similarly studies with Chang conjunctiva epithelial cells showed inhibition of attachment and virulence only in the presence of antibodies to the homologous pilus type (Virji et al., 1982). These studies have a direct consequence on development of a gonococcal vaccine. An attractive approach is to induce high levels of cross-reacting antipilus antibodies, using purified common fragment, which would be expected to protect against all infecting serotypes. Our studies cast some doubt on this strategy since competitive inhibition studies suggest that the epitope recognised by antibody SM1 is located in this common peptide (Virji & Heckels, 1983). However, although type-specific antibodies are protective we cannot eliminate some role for antibodies directed against the common region. Clearly a larger panel of cross-reacting monoclonal antibodies directed against different epitopes in the common region would be required to unequivocally establish this point.

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REFERENCES


Role of gonococcal pilus domains in adhesion


