Monoclonal Antibodies to Gonococcal Pili: Studies on Antigenic Determinants on Pili from Variants of Strain P9

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Several hybrid cell lines producing monoclonal antibodies against gonococcal pili have been derived. The reactivity of each antibody against variant pili of strain P9 was determined by ELISA. Using type-specific as well as broadly cross-reacting antibodies labelled with $^{125}$I, the capacity of unlabelled hybridoma antibodies to inhibit their attachment to various pilus types was determined. Some antibodies competed significantly and were judged to react at the same or closely positioned antigenic sites, whereas others bound non-competitively to the same pilus. These studies suggest the existence of distinct antigenic sites on gonococcal pili: a common antigenic region shared by all P9 pilus types and a type-specific region. Both antigenic sites contain more than one epitope.

INTRODUCTION

Gonococcal pili play an important role in the pathogenesis of gonorrhoea, mediating both adhesion to mucosal surfaces (Mårdh & Weström, 1976) and resistance to host defences (Dilworth et al., 1975; Blake & Swanson, 1975). Using in vitro assay systems, anti-pilus antibodies have been shown to inhibit gonococcal attachment to host epithelial cells (Tramont, 1976), to promote phagocytosis by macrophages (Jones et al., 1980) and to protect tissue culture cells from the cytotoxic effect of the organisms (Virji, 1981). Pili are, therefore, attractive candidates in the search for a possible gonococcal vaccine. One obstacle to the use of pili in a vaccine is the considerable antigenic diversity seen not only between strains (Buchanan, 1978) but also within a strain. Four variants of one laboratory strain, P9, have been isolated which produce different pilus types with subunit molecular weights in the range 18000–21000 (Lambden et al., 1981). The variant pili are antigenically distinct and specific antibodies protect against homologous but not heterologous variants in the cell cytotoxicity assay (Virji et al., 1982). Similar pilus antigenic variation occurs during the course of the natural infection. Different isolates of the same strain of gonococcus grown from partners and from different sites in the same patient may possess pili with different subunit molecular weights (Heckels, 1982). Although pilus variation presents a substantial challenge to the development of an effective gonococcal vaccine, gonococcal pili possess shared antigens which are normally less immunogenic than the variable regions (Schoolnik et al., 1982). A strategy for vaccination would be to promote an antibody response to these conserved regions.

An understanding of the immunobiology and genetics of pilus variation would be facilitated by a means of probing the distribution of different antigenic determinants in pili from various strains. This paper reports the production of monoclonal antibodies with varying pilus specificities ranging from type-specific to broadly cross-reacting. These antibodies have been utilized to gain insight into the topographical distribution of antigenic determinants on the variant pili of Neisseria gonorrhoeae strain P9.
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METHODS

Mice. BALB/C mice were used for immunization and the production of ascitic fluid. Peritoneal macrophages and spleen cells used as feeder cells in cultures were derived from normal mice.

Bacterial strains. Variants of strain P9 possessing α, β, γ or δ pili were cultured on solid medium as previously described (Lambden et al., 1981) and harvested after 18 h growth.

Pili. Pili were purified from colonial variants of N. gonorrhoeae strain P9 by extraction into 0-15 M-ethanolamine pH 10 as described previously, and purity established by SDS-PAGE (Lambden et al., 1981). In the current studies α pili (mol. wt 19500), β pili (mol. wt 20500), γ pili (mol. wt 21000) and δ pili (mol. wt 18500) were employed.

Immunization schedule. BALB/C mice were immunized intraperitoneally with purified α pili (100 μg protein) in 100 μl Freund's complete adjuvant (Difco). Three and five weeks later, 10⁶ gonococci in 50 μl PBS (comprising 0-15 M-phosphate buffer pH 7-2 plus 0-15 M-NaCl) were injected intravenously in a tail vein of the mice. For these injections, a mixture of α, γ and δ pilated variants was used. Mice were killed and spleens were removed 3–4 d after the booster for isolation of immune lymphocytes and fusion with NS-1 myeloma cells.

Production of hybrid cell lines. The murine myeloma cell line BALB/C NS1/1 (NS-1) was cultured in TCM [RPMI 1640 medium (Flow Laboratories), buffered with sodium bicarbonate, containing 150 μg penicillin ml⁻¹, 15 μg gentamicin ml⁻¹, 1 mM-sodium pyruvate, 2 mM-λ-glutamine, and 15% (v/v) foetal calf serum]. Incubations were done in the presence of macrophage feeder layers prepared the day before the fusion. Each well contained 0-5–1 × 10⁴ peritoneal macrophages from normal mice. After 6 d 50 μl of fresh HAT medium was added to each of the wells. At 9–12 d after the fusion, supernatants were tested for antibody activity by ELISA using polystyrene microtitre trays coated with purified pili (1 μg ml⁻¹) or outer membrane (1 μg ml⁻¹) as described previously (Lambden & Heckels, 1982).

Antibody binding was detected using rabbit anti-mouse Ig-peroxidase conjugate (Miles, Stoke Poges, U.K.) and o-phenylenediamine as substrate. In order to determine the specificity of the secreted antibodies, Hybrids that produced the required antibody were cloned by limiting dilution in 96-well trays with macrophage or spleen cells as feeder layers. They were considered to produce monoclonal antibody only when the pattern of cross-reactivity against several pilus antigens remained the same upon further cloning. Phenotypically stable hybrids were obtained in most cases after cloning twice. The procedure was repeated, if required, and hybrids were considered stable when >95% of the progeny clones produced the antibody. These stable clones did not require further cloning for the maximum time employed of eight weeks' growth in culture.

Antibody detection. Antibody production was monitored by ELISA using polystyrene microtitre trays coated with purified pili (1 μg ml⁻¹) or outer membrane (1 μg ml⁻¹) as described previously (Lambden & Heckels, 1982). Antibody binding was detected using rabbit anti-mouse Ig-peroxidase conjugate (Miles, Stoke Poges, U.K.) and o-phenylenediamine as substrate. In order to determine the Ig sub-class of the monoclonal antibody, pilus-coated wells were incubated sequentially with monoclonal antibody, specific rabbit anti-mouse Ig sub-class antisera (Litton Bionetics, Kensington, Md., U.S.A.) then goat anti-rabbit Ig-peroxidase conjugate (Miles) and finally the substrate o-phenylenediamine.

Production of ascitic fluid containing monoclonal antibody. Cloned hybridoma cells were expanded by growth in TCM. Hybridoma cells (5 × 10⁶ to 1 × 10⁷) were injected intraperitoneally into BALB/C mice which had been primed 2–4 weeks earlier with a 0-5 ml injection of pristane (2,6,10,14-tetramethylpentadecane; Sigma) (Potter et al., 1972). The intraperitoneal tumours produced ascitic fluid within 10–14 d in most cases and contained high titres of monoclonal antibodies. Peritoneal fluid was aspirated at weekly intervals. The total amount of fluid from a mouse varied from 2 to 15 ml for different hybrid clones. Ascitic fluids were centrifuged at 1500 g for 10 min and stored at –70°C.

Purification of antibodies. Monoclonal antibodies of IgG class were purified using Protein A affinity chromatography (Watanabe et al., 1981). Ascitic fluids were dialysed overnight at 4°C against 0-14 M-phosphate buffer pH 8-2 and centrifuged at 10000 g for 10 min before application to a column (3 × 0-7 cm) of Protein A-Sepharose (Sigma). After washing thoroughly with buffer, bound IgG was eluted with 0-1 M-sodium citrate buffer pH 4. Fractions containing protein were immediately neutralized using 1 M-Tris and dialysed against PBS. The amount of antibody was quantified by measurement of the A₂₈₀.

Inoculation of purified monoclonal immunoglobulins. Flat-bottomed glass scintillation vials were coated with a 1 mg ml⁻¹ solution of 1,3,4,6-tetrachloro-3,6-diphenylglycouril (Iodogen; Pierce, Rockford, Ill., U.S.A.) in chloroform. The chloroform was allowed to evaporate and the vials stored under vacuum until used. Purified Ig
from ascitic fluid was dialysed against PBS, and 0.5 ml containing 100–500 µg protein was added to a coated tube. Carrier-free Na$^{125}$I (Amersham) was added to give a final ratio 2 µCi (µg protein)$^{-1}$ [74 kBq (µg protein)$^{-1}$]. The reaction was allowed to proceed for 10 min at 16 °C when the mixture was removed from the tube and applied to a column (1.4 × 10 cm) of Sephadex G-25. The column was eluted with PBS and fractions were collected and monitored for radioactivity and protein. The iodinated Ig eluted in a peak at 2–4 ml, well separated from unreacted $^{125}$I. The product, which had a specific activity of 1 mCi mg$^{-1}$ (37 MBq mg$^{-1}$) was stored at 4 °C in PBS containing 0.05% (w/v) sodium azide until required.

Radioimmunoassay and competitive binding experiments. Polystyrene microtitre plates were coated with pili under the conditions used for ELISA. Serial dilutions of $^{125}$I-labelled antibody were added and the plates were incubated for 16 h at 4 °C. After washing with PBST [PBS containing 0.05% (v/v) Tween 20], the bound radioactivity was solubilized by incubation with 1% (w/v) SDS in 0.1 M-NaOH and counted in a γ-counter. The minimum concentration of purified monoclonal antibody required for saturation was determined in each case. Serial 10-fold dilutions of ascitic fluid in PBS were mixed with an equal volume of PBS containing the predetermined saturating amount of $^{125}$I-labelled antibody. Antigen-coated plates were washed four times with PBST, drained, and 100 µl of the reaction mixture was added. After 16 h incubation at 4 °C, the plates were washed four times with PBST. The amount of $^{125}$I-labelled antibody bound was determined by γ-counting after solubilization with 1% (w/v) SDS in 0.1 M-NaOH. The unlabelled homologous antibody was included as a positive control for competitive inhibition, and non-specific binding was assayed on uncoated wells under identical conditions. All assays were performed in triplicate. The results are expressed as percentage inhibition of binding of $^{125}$I-labelled antibody. [Percentage inhibition = 100 × $\frac{[1 - (c.p.m. bound in the presence of unlabelled antibody)/(c.p.m. bound in the absence of unlabelled antibody)]}{\text{[percentage inhibition]}}$]

Immunoblotting of SDS-PAGE gels. Purified α, β, γ and δ pili were subjected to SDS-PAGE on linear gradients of 10–25% (w/v) acrylamide as described previously (Heckels, 1981). The separated proteins were electro-phoretically transferred to nitrocellulose sheets (BA85; Schleicher & Schull, Dassel, F.R.G.) according to the method of Towbin et al. (1979). The excess protein-binding capacity of the nitrocellulose was blocked by incubation for 1 h at 37 °C with 3% (w/v) BSA in 10 mM-Tris/HC1 pH 7.4 containing 0.9% (w/v) NaCl. The sheets were rinsed with 50 mM-Tris/HC1 pH 7.4 containing 150 mM-NaCl, 5 mM-EDTA, 0.25% (w/v) gelatin and 0.05% (v/v) Nonidet P-40 (Sigma) and then incubated with radiolabelled monoclonal antibody diluted in the same buffer to give a final activity of 10$^6$ c.p.m. ml$^{-1}$. After 2 h at room temperature the sheets were extensively washed with 50 mM-Tris/HC1 pH 7.4 containing 1 M-NaCl, 5 mM-EDTA, 0.25% gelatin and 0.4% (w/v) sodium lauryl sarcosinate (Sigma), and finally rinsed with water and air-dried. Immunological reactivity was detected following overnight autoradiography using Kodak X-Omat AR film and an X-Omatic regular intensifying screen.

RESULTS

Characterization of monoclonal antibodies

After fusion of parental myeloma NS-1 with spleen cells from BALB/C mice immunized against P9 pili and surface antigens, the hybrid cell lines were screened for antibody production by ELISA using a panel of pilus types including α, β, γ and δ pili. None of the pilus-directed antibodies reacted with outer-membrane proteins or LPS. Cells from selected clones were injected into pristane-primed BALB/C mice to obtain ascitic fluid containing high-titre monoclonal antibody. The ascitic fluids were used in ELISA with α, β, γ and δ as the solid phase. Different patterns of specificity were observed (Fig. 1). Antibody SM1 (Fig. 1a) showed identical reactivity with each pilus at all the dilutions tested. On the other hand antibody SM2 (Fig. 1b), although cross-reacting, showed reduced activity against δ pili. The other monoclonal antibodies exhibited greater specificity; two (Fig. 1f, g) reacted identically with γ and δ pili but not with α or β pili and the remainder reacted with a single pilus type (Fig. 1c, d, e, h). The relative amount of antibody present was determined from the end point of the ELISA curves (Table 1). Differences in the avidity of binding of the monoclonal antibodies to their epitopes were also suggested from the shape of the ELISA curves. When antibody was present at levels greater than required for saturation, the ELISA showed differences in the maximum absorbance achieved. It is likely that this reflects the avidity (Stone & Nowinsky, 1980) since although saturating amounts of antibody would be originally bound, subsequent washing procedures would tend to remove greater amounts of the less-avid antibody to give a lower maximum absorbance. The immunoglobulin sub-class of the hybridoma antibodies was also determined by ELISA using specific antisera (Table 1).
Fig. 1. ELISA of ascitic fluid derived from hybridoma clones. Ascitic fluid was sequentially diluted into PBS and assayed against $\alpha$ (○), $\beta$ (●), $\gamma$ (□) and $\delta$ (■) pilus-coated polystyrene microtitre trays. The clones tested were (a) SM1, (b) SM2, (c) SM3, (d) SM4, (e) SM5, (f) SM6, (g) SM7 and (h) SM8. ELISA titres were calculated as the dilution which gave an increase in $A_{492}$ of 0.1 h$^{-1}$.

Table 1. Cross-reactivity of monoclonal antibodies

Monoclonal antibodies raised against gonococcal pili were screened against P9 pilus types $\alpha$, $\beta$, $\gamma$ and $\delta$ using ELISA. Mean titres from three assays are presented. The Ig sub-class of each antibody was also determined using ELISA, as described under Methods.

<table>
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<tr>
<th>Clone</th>
<th>Ig sub-class</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\gamma$</th>
<th>$\delta$</th>
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</tr>
<tr>
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<td>IgG(3)</td>
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<td>850</td>
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<td>—</td>
<td>—</td>
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<tr>
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<td>IgG(2a)</td>
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<td>—</td>
<td>200</td>
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—, Titre < 0.05.
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Competitive radioimmunoassay

To investigate the distribution of antigenic sites on a pilus, five monoclonal antibodies of IgG class but of different specificities were labelled with $^{125}\text{I}$ and were used in competitive radioimmunoassay. A $\gamma$-specific antibody (SM8) lost activity on iodination and hence could not be used in the labelled form in the competitive radioimmunoassay. The purity of the labelled product was demonstrated by SDS–PAGE (Fig. 2). Preliminary experiments were carried out to determine the amount of antibody required for maximum binding to each of the pilus types. Ascitic fluids were then serially diluted into PBS and mixed with the pre-determined amount of $^{125}\text{I}$ antibody required for saturation binding to the pilus under investigation. ELISA (Fig. 1) had shown that each ascitic fluid contained greater than saturating levels of antibody at dilutions of $<1/500$. Controls showed that under the conditions used the excess of unlabelled antibody present in ascitic fluid at a dilution of 1/50 displaced 80–95% of the homologous $^{125}\text{I}$-labelled antibody (Fig. 3).

Considerable differences were seen in the ability of heterologous antibodies to inhibit binding. The results in Fig. 3(a–d) show that neither the cross-reacting antibody SM2 nor the specific anti-pilus antibodies were able to inhibit the broadly cross-reacting SM1 on $\alpha$, $\beta$, $\gamma$ or $\delta$ pili, although a small amount of competitive inhibition was shown by SM2 when using $\alpha$ pili as the antigen. However, when SM2 was the iodinated antibody, SM1 exhibited significant inhibitory activity with all pilus antigens (Fig. 3e–h). By contrast, the type-specific antibodies were without effect on the binding of the cross-reacting antibody. However, the $\alpha$-specific SM3 was inhibited by SM4 as effectively as by homologous antibody but was unaffected by the addition of another $\alpha$-specific monoclonal, SM5 (Fig. 3i). Similarly, monoclonal SM6 which reacted with $\gamma$ and $\delta$ pili was inhibited by SM7 which was also $\gamma$, $\delta$-reactive (Fig. 3j, k), but not by the $\gamma$-specific antibody SM8 (Fig. 3j).

Immunoblotting

The $^{125}\text{I}$-labelled monoclonal antibodies were also utilized in immunoblotting with pili separated on SDS–PAGE. After transfer to nitrocellulose, cross-reacting clone SM1 reacted with $\alpha$, $\beta$, $\gamma$ and $\delta$ pili (Fig. 4a), as did clone SM2, although as would be expected from ELISA, the band seen with $\delta$ pili was considerably less intense (Fig. 4b). In contrast, the type-specific clones SM3 and SM6 showed no reactivity after overnight autoradiography, and only after a 3–4 d exposure could weak reactivity be observed.
Fig. 3. Competitive radioimmunoassay. 125I-labelled monoclonal antibody preparations were added simultaneously with serial 10-fold dilutions of ascitic fluid to pilus-coated wells as shown. Dilution 1 represents a 1/50 dilution of ascitic fluids. Labeled antibodies were: (a–d) SM1; (e–h) SM2; (i) SM3; and (j–k) SM6. Hybridoma ascites used in competition were: broadly cross-reactive SM1 (○) and SM2 (●); α pili-specific SM3 (■); SM4 (▲) and SM5 (■); γ pili-specific SM8 (▲); and γ and δ pili-cross-reactive SM6 (▼) and SM7 (▽). Broken lines indicate homologous competition in each case. Controls containing ascitic fluid without anti-pilus activity showed less than 15% inhibition of binding.

DISCUSSION

When rabbits are immunized with gonococcal pili, the antibodies raised show less than 10% cross-reactivity with other strains (Buchanan, 1978). However, antibodies formed during the course of the natural infection or produced by vaccination of human volunteers show a greater degree of cross-reactivity (Brinton et al., 1978) suggesting that the human immune response may be more directed to a shared antigen. Recent studies with two fragments derived by cyanogen bromide cleavage of pili suggest that pili contain a common structural region, which contains the epithelial cell receptor, and a variable region (Buchanan et al., 1982; Schoolnik et al., 1982).
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The variable region is immunodominant in intact pili so that antibodies raised do not react with other strains. The site of inter-strain pilus variation is not known but peptide mapping of α, β, γ and δ pili from strain P9 also suggest common and variable regions (Lambden, 1982).

The monoclonal antibodies described here have been used to investigate the spatial distribution of antigenic sites on the four pilus types produced by the strain P9. As shown by ELISA two clones were broadly cross-reacting, with clone SM1 recognizing a determinant which is equally present not only on each of the P9 pilus types (Fig. 1 a) but also on all other gonococci and meningococci so far tested (unpublished observations). In contrast, other clones specifically recognize determinants present on only one of the pilus types. A third type of reactivity is seen with the two clones which react equally with either γ or δ pili but not with α or β pili. Competitive radioimmunoassay was employed to analyse the topography of the epitopes with which the antibodies reacted. This experimental approach was based on the rationale that when epitopes recognized by two monoclonal antibodies are the same, overlapping or physically close, the binding of one antibody sterically blocks the binding of the second; conversely, no blocking occurs when the epitopes are sufficiently distant.

Of the cross-reacting antibodies SM2 partially inhibited the binding of the more avid $^{125}$I-labelled SM1 to α pili but was without effect when the other pilus types were used (Fig. 3 a–d). In contrast, unlabelled SM1 significantly inhibited the binding of labelled SM2 on all pilus types, although it was less effective than the homologous antibody (Fig. 3 e–h). These results imply that these antibodies bind to epitopes which are either partly overlapping or positioned close enough to cause some steric hindrance in binding to pili. The greater efficiency of SM1 in inhibiting $^{125}$I-labelled SM2 can be explained by the greater avidity as shown by ELISA (Fig. 1 a, b). Preferential binding of highly avid antibodies to a single epitope in a mixture of antibodies of varying avidities has been demonstrated by Stone & Nowinski (1980).

On γ pili, two distinct type-specific antigenic sites were observed: one recognized by the iodinated antibody SM6 (γ, δ-cross-reacting) and a second distinct site recognized by SM8 (γ-specific). Clearly γ and δ pili also share a common antigenic site as shown by their equal reactivity to the γ, δ-cross-reacting monoclonals SM6 and SM7 (Fig. 1 f, g).

Using α pili, the α-specific monoclonal antibody SM4 effectively inhibited $^{125}$I-labelled SM3 showing that these antibodies share an epitope or are directed against two overlapping or closely

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Fig. 4. Immunoblotting of $^{125}$I-labelled monoclonal Ig with pili after SDS–PAGE. Antibodies tested were (a) SM1 and (b) SM2, with 1, β; 2, γ; 3, α; and 4, δ pili.
positioned epitopes. But, in addition, α pili may possess another distinct epitope recognized by the third α-specific antibody employed (SM5) which failed to block the binding of SM3. Since SM5 is a less avid antibody, an experiment was carried out to allow SM5 to bind first to α pili before the addition of iodinated SM3. Again no inhibition of binding was observed, suggesting that the binding sites of the two antibodies are distinct.

The type-specific and cross-reacting antibodies showed a difference in their reactivity with pili when subjected to SDS as revealed by immunoblotting. Cross-reacting antibodies SM1 and SM2 bound efficiently to the SDS-unfolded pilin from α, β, γ or δ pili, whereas the specific antibodies bound significantly less to the appropriate pilus types under identical experimental conditions. This suggests that the cross-reacting antibodies recognize the conserved primary sequence in unfolded pilin whereas the specific antibodies may recognize part of the tertiary structure of the variable region.

Results presented in this paper show clearly that the variants from a single strain (P9) possess conserved regions on pili (also shared by other gonococci) and these elicit an antibody response in mice which recognize the primary sequence. The highly specific antigenic regions expressed on pili from different variants within the strain are unique to that pilus type and tertiary structure may be important for their antigenicity. The ability of monoclonal antibodies to recognize epitopes common to all gonococcal pili emphasizes the potential use of pili as candidate antigens for a future gonococcal vaccine; current research is directed towards the enhancing of the antibody response to the shared determinant by immunochemical manipulation of pili. The availability of the panel of monoclonal antibodies of different specificities should prove invaluable in the investigations regarding the role of the common and variable regions in the pathogenesis of gonorrhoea. The biological functions of these antibodies are currently under investigation.

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REFERENCES


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1973


