Antigenic Variation of Outer Membrane Protein II in Colonial Variants of *Neisseria gonorrhoeae* P9

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Antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) in sera from rabbits immunized with outer membranes from colonial opacity variants of *Neisseria gonorrhoeae* P9. ELISA-inhibition experiments with purified antigens revealed approximately equal proportions of antibodies directed against each of the three major surface antigens, lipopolysaccharide, the major outer membrane protein (protein I) and protein II, the variable protein associated with colonial opacity. Inhibition experiments with intact gonococci showed considerable surface antigenic diversity which could be correlated with differences between the protein II species present. Despite their considerable structural homology, different protein II species from colonial variants of the same strain showed little cross-reactivity with specific anti-protein II sera, thus demonstrating the considerable variation in that part of the antigen which is exposed on the surface of the gonococcus and is closely involved in pathogenic mechanisms.

INTRODUCTION

The surface protein antigens of *Neisseria gonorrhoeae* are of great interest in current studies of the molecular basis of gonococcal virulence. Recent work has shown that even in a single strain surface antigens are capable of considerable variations *in vitro* (Swanson, 1978; Lambden & Heckels, 1979) and probably *in vivo* (James & Swanson, 1977; McBride *et al.*, 1981). Colonial variants of the same strain have been shown to produce pili with altered morphology and subunit molecular weight (Penn *et al.*, 1980; Salit *et al.*, 1980), which show differences in their attachment to epithelial cells (Lambden *et al.*, 1981*b*). However, the proteins present in the outer membrane appear capable of even greater variations. Variants which produce opaque colonial forms contain, in addition to the major outer membrane protein (protein I), one or two additional proteins (proteins II) in the molecular weight range 24K to 30K (Swanson, 1978). Previous studies with one particular strain, P9, revealed a family of at least six such protein species (Lambden & Heckels, 1979). Variations in the protein II profile can be correlated with differences in crucial biological properties, such as attachment to epithelial cells and resistance to host defence systems (Lambden *et al.*, 1979), suggesting that they play an important role in the ability of the gonococcus to adapt to the range of environments in which it is found during the course of the natural infection.

Although protein I has been shown to be antigenically distinct in different strains (Johnston, 1980), the antigenic relationship between protein II species derived from the same strain has not yet been reported. A previous study (Heckels, 1981) showed that the protein II species had considerable structural homology but suggested that major differences lay in that part of the molecule exposed on the gonococcal surface. Such surface differences could account for the alteration in biological properties of colonial variants and suggest that protein II variations could make a significant contribution to the antigenic diversity of the gonococcal surface. We have now found that colonial variants of strain P9 indeed show considerable antigenic diversity which can be attributed to differences between protein II species.

Table 1. *Variants of N. gonorrhoeae P9 used and their OM protein composition*

To conform to the recent recommendation for standardized nomenclature of gonococcal outer membrane proteins (*Proceedings of 1980 EMBO Symposium on Genetics and Immunobiology of Pathogenic Neisseria, University of Umea*) this and subsequent publications will denote protein II species by their subunit molecular weight rather than as previously. (Molecular weight $\times 10^{-3} = K$.)

Variant	Previous protein II nomenclature	Protein II molecular weight
P9-1	—	—
P9-9	IIa + IIe	28.5K + 27.8K
P9-11	IIa + IId	28.5K + 28.85K
P9-13	IIa	28.5K
P9-16	IIb	28K
P9-32	IIf	28.3K
P9-39	IIe	27.8K
P9-40	IId	28.85K

METHODS

Bacterial strains and growth conditions. *Neisseria gonorrhoeae* P9 opacity variants were selected and purified by single colony isolation and stored in liquid nitrogen (Lambden *et al.*, 1979). The appropriate variants (Table 1) were grown for 16 h at 37 °C in 5% CO₂ on a clear typing medium containing, per litre, 10 g agar (Oxoid no. 1), 10 g Proteose Peptone no. 3 (Difco), 1 g soluble starch (BDH), 4 g K₂HPO₄, 1 g KH₂PO₄ and 5 g NaCl, and enriched with a supplement similar in composition to commercial IsoVitaleX (BBL) except that L-cystine was omitted. For the large-scale isolation of outer membranes, bacteria were grown under the same conditions on trays (27 × 38 cm) of GC Base (Difco) enriched with the same supplement.

Preparation of gonococcal antigens. Outer membrane complexes (OM) were prepared by extraction into 0.2 M-lithium acetate followed by extraction with 6 M-urea to remove loosely associated proteins (Heckels, 1981). The protein profile of OM preparations was monitored by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, using the discontinuous buffer system of Laemmli (1970) and incorporating a linear concentration gradient of 10–25% (w/v) acrylamide as described previously (Heckels, 1981).

Lipopolysaccharide (LPS) was prepared by aqueous phenol extraction of intact P9-1 bacteria (Stead *et al.*, 1975) and converted to the soluble triethylamine salt by electrodialysis (Galanos & Luderitz, 1975). The LPS present in the other variants used showed identical behaviour on SDS-polyacrylamide gel electrophoresis and in serological cross-reactivity.

Production of antisera. Antisera to OM were raised in Half-lop rabbits (Froxfield Rabbit Co., Froxfield, Hants, U.K.). OM suspension (50 µg protein in 1 ml) was mixed with an equal volume of Freund's complete adjuvant (Difco) and injected subcutaneously into eight sites on the back and hind legs. Booster doses were given in a similar manner on days 14, 35 and 70 except that Freund's incomplete adjuvant (Difco) was substituted. On day 80, blood (25 ml) was taken from a marginal ear vein and the serum was stored at –20 °C.

Enzyme-linked immunosorbent assay (ELISA) procedure. Antibodies to outer membrane components were detected by ELISA based on a method previously used for gonococcal pili (Buchanan, 1978). Outer membranes were suspended in 0.1 M-NaHCO₃ pH 9.6 at the optimal concentration of 1 µg protein ml⁻¹ established by preliminary experiments. Individual wells of flat-bottomed polystyrene microtitre trays (Dynatech, Billingshurst, Kent, U.K.) were coated with the suspension (200 µl) at 37 °C for 16 h. The antigen was then discarded and the wells were washed three times with phosphate-buffered saline pH 7.4 containing 0.05% (v/v) Tween 20 (PBST). Multiple dilutions of the test sera in PBST were added in 200 µl portions to the antigen-coated wells and incubated at 37 °C for 1 h. The wells were then emptied and washed three times with PBST. Control wells containing no antigen were also filled with antiserum. Goat anti-rabbit IgG-peroxidase conjugate (Miles, Stoke Poges, U.K.) was added 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 [*o*-phenylenediamine dihydrochloride, 0.1 mg ml⁻¹, in 3 mM-H₂O₂] was added to each well and incubated at room temperature for 30 min. The reaction was terminated by the addition of 1 M-H₂SO₄ (50 µl) and the A₄₉₀ was determined in a Dynatech MR580 Micro Elisa Auto Reader.

In some experiments ELISA was performed with sera which had been pre-absorbed with soluble gonococcal antigens (LPS, OM suspension) by incubation in an uncoated tray at 37 °C for 30 min. The sera were then transferred to the corresponding well of a coated tray and ELISA was performed as above.

Absorption of anti-OM antibodies by intact gonococcal variants. The antigenic cross-reactivity of gonococcal variants was measured by the ability of the bacteria to inhibit the ELISA reaction of an antiserum with its homologous OM. Antiserum (100 µl) was added to each well of a 96-well polystyrene microtitre tray (Dynatech;

U-shaped wells) and a suspension (100 μ l) of one of the gonococcal variants in Dulbecco complete phosphate-buffered saline (PBS; Oxoid) was added in increasing concentration across each row (from 0 to 1×10^8 colony-forming units (c.f.u.) ml^{-1} , final concentration). The tray was incubated with gentle shaking at 37 °C for 30 min. It was then centrifuged at 1000 g for 15 min in a centrifuge equipped with Dynatech Micro Elisa plate carriers in order to pellet the bacteria. A sample (100 μ l) of the supernatant solution was transferred from each well to the corresponding well in a microtitre tray pre-coated with outer membrane antigen and already containing 100 μ l PBST. The contents of the plate were then assayed in the ELISA system. The final concentration of serum in each well was chosen so that it corresponded to the linear portion of the standard ELISA graph (Fig. 1). Results were calculated from two trays treated identically and expressed as the percentage inhibition in A_{490} compared with control wells containing no added bacteria.

Preparation of antisera specific for protein II species. Antiserum directed against P9-13 outer membranes (OM-13) containing antibodies to protein I, protein II (28.5K) and LPS was absorbed with whole cells of P9-1 (containing protein I and LPS). Serum was heated at 56 °C for 30 min to inactivate complement, diluted 1:200 with PBS, and mixed with an equal volume of a suspension of P9-1 containing 2×10^9 c.f.u. ml^{-1} and incubated at 37 °C for 3 h. Cells were removed by centrifugation at 10000 g for 5 min then a second absorption was carried out in a similar manner. The serum was stored at -20 °C and diluted to the final working concentration before use. The specificity of the absorbed serum for protein II (28.5K) was demonstrated by ELISA which showed its reactivity with OM-13 but not with OM-1. Antiserum specific for protein II (28K) was prepared in a similar manner from anti-OM-16 sera.

RESULTS

Detection of antibodies to specific outer membrane components by ELISA

Initial experiments showed that antibodies present in rabbits immunized with intact outer membranes containing different protein II species could be readily detected in an ELISA system using wells coated with the homologous membrane preparation. A logarithmic plot of A_{490} against serum dilution (Fig. 1) showed an initial absorption plateau with a linear relationship on further dilution. In subsequent experiments involving inhibition of ELISA by specific antigens, antisera were utilized at a dilution which gave an A_{490} of approximately 75% of the maximum value (typically $1:10^4$ dilution).

The relative proportions of antibodies directed against individual outer membrane components (LPS, protein I, protein II) were determined from experiments in which the antisera was pre-incubated with increasing concentrations of either LPS, OM-1 (containing LPS and protein I) or the homologous OM (LPS, protein I and protein II). With antiserum to OM-13, 25% of the total antibodies were absorbed with LPS alone, 65% with OM-1 and 97% with OM-13 (Fig. 2a). Thus the proportions of antibodies directed against each individual antigen were approximately LPS 25%, protein I 40% and protein II (28.5K) 35%. Similar proportions were found with antiserum raised against OM-16 (Fig. 2b).

Absorption of anti-OM antibodies by intact gonococci

The degree of surface antigen cross-reactivity of different variants was measured by the ability of intact gonococci to absorb antibodies reactive in the homologous OM ELISA system (Fig. 3). Cells of P9-13 were capable of absorbing all activity from anti-OM-13 serum showing that the antigenic determinants reactive in the system are those which are expressed on the surface of the gonococcus and not those which would be exposed only on the surface of 'inside-out' OM vesicles. In addition, pre-incubation with cells of P9-1 reduced the activity by only 65%, again reflecting the contribution of antibodies directed against the 28.5K protein II to the system. Pre-incubation with P9-16 (II 28K), P9-39 (II 27.8K) and P9-40 (II 28.85K) showed only marginally greater inhibition than P9-1. In contrast, P9-9 (II 28.5K + II 27.8K) and P9-11 (II 28.5K + II 28.85K) completely inhibited the reaction. Thus, only those variants with the 28.5K protein II were unable to completely inhibit the ELISA, suggesting there is little cross-reactivity between this antigen and the other protein II species.

Fig. 1. Detection of anti-OM antibodies by ELISA. Increasing dilutions of anti-OM-13 (O) and anti-OM-16 (●) sera were tested in the standard ELISA system using wells coated with the homologous OM preparation.

Fig. 2. Inhibition of ELISA by defined antigens. Anti-OM-13 (a) and anti-OM-16 (b) sera were incubated with soluble antigens before reaction with homologous OM. Antigens used were LPS (1), OM-1 (2), OM-13 (3) and OM-16 (4).

Cross-reactivity of specific anti-protein II sera

One possible complication of comparative absorption experiments is that undetected variations in LPS or protein I structure could mask partial cross-reactivities of the protein II species. In order to examine the contribution made by the protein II species alone to the antigenic cross-reactivity of the gonococcal surface, anti-OM serum was first made specific for the relevant protein II by absorption with whole cells of P9-1.

ELISA tests with such purified serum revealed activity with wells coated with the homologous OM but not with wells coated with OM-1, demonstrating specificity for the protein II. The ability of the gonococcal variants to inhibit the ELISA reaction of anti-protein II serum with the homologous OM is shown in Fig. 4. The activity of specific anti-protein II (28.5K) serum was completely inhibited by absorption with whole cells of P9-9, P9-11 and P9-13, each containing that protein II species, whereas P9-16, P9-39 and P9-40, with heterologous protein II species, only inhibited ELISA activity by 12%, 3% and 9%, respectively (Fig. 4a). Similarly, specific anti-protein II (28K) serum was completely inhibited by the homologous P9-16 but not by P9-9, P9-11, P9-13, P9-39 or P9-40 (Fig. 4b).

Fig. 3. Inhibition of ELISA by intact gonococci. Anti-OM-13 serum was incubated with increasing concentrations of *N. gonorrhoeae* P9 colonial opacity variants, then bacteria were removed and the serum was reacted with wells coated with OM-13. Variants used were P9-1 (1), P9-9 (2), P9-11 (3), P9-13 (4), P9-16 (5), P9-39 (6) and P9-40 (7).

Fig. 4. Cross-reaction with specific anti-protein II serum. Anti-protein II (28.5K) (a) and anti-protein II (28K) (b) sera were incubated with increasing concentrations of colonial variants before ELISA with homologous OM. Variants used were as in Fig. 3.

Table 2. Cross-reactivity of specific anti-protein II sera with OM from variants of strain P9

Cross-reactivity was determined from ELISA tests using specific anti-protein II serum and wells coated with OM from one of a range of variants. Percentage cross-reactivity was defined as: $100 \times [A_{490} (\text{heterologous OM}) - A_{490} (\text{OM-1})] / [A_{490} (\text{homologous OM}) - A_{490} (\text{OM-1})]$, calculated from three to five separate experiments (figures in parentheses show standard deviations).

OM from P9 variant	Protein II	Percentage cross-reactivity	
		anti-II (28.5K)	anti-II (28K)
1	—	0	0
9	28.5K + 27.8K	59.3 (± 3.7)	3.4 (± 1.6)
11	28.5K + 28.85K	60.0 (± 10.1)	5.5 (± 1.5)
13	28.5K	100	2.6 (± 0.8)
16	28K	5.3 (± 2.9)	100
32	28.3K	0.03 (± 0.02)	3.5 (± 1.8)
39	27.8K	2.5 (± 1.0)	1.5 (± 1.2)
40	28.85K	3.1 (± 0.2)	3.3 (± 0.9)

The degree of cross-reactivity of the heterologous protein II species was further quantified by use of the specific anti-protein II sera in ELISA with wells coated with OM from each of the P9 variants. The results shown in Table 2 for each specific antiserum are the A_{490} readings of the test OM expressed as a percentage of the A_{490} for the homologous OM. In all cases cross-reactivity of specific anti-protein II serum with membranes containing only heterologous protein II species was 5 % or less.

DISCUSSION

Antigenic diversity between gonococci of different strains may be due to differences either in outer membrane protein I (Johnston, 1980) or LPS (Maeland, 1968). This study has shown that antigenic diversity can occur even between variants of the same strain and is a consequence of alterations in surface structure. Previous studies have suggested a model of the gonococcal surface in which protein I spans the outer membrane (Heckels, 1979) forming a hydrophilic diffusion pore (Heasley, 1980). In addition, protein II species, when present, are located nearer to the outer surface of the membrane. The different protein II species produced by colonial opacity variants of *N. gonorrhoeae* P9 have considerable structural homology as revealed by tryptic ^{125}I -fingerprinting (Heckels, 1981). It was also found that the major differences between the tryptic digests of different protein II species lay in most hydrophilic peptides. Spots corresponding to these peptides were the most intense obtained on tryptic fingerprints of protein II species isolated from cells which had first been surface labelled by ^{125}I -lactoperoxidase. Thus, it was suggested that the protein II species form a family with a common region embedded in the membrane and that modifications to the polypeptide occurring on the surface are responsible for the variations between protein II species.

The current study strengthens this view. The antigenic differences between opacity variants of the same strain are due to almost complete lack of cross-reactivity between the regions of different protein II species which are exposed on the surface of gonococci. This surface variation is also in accord with the observation that the variants show significant differences in biological properties which involve surface interactions. For example, the variant which differs only in possession of the 28K protein II shows a sevenfold decrease in leukocyte association compared with the variant which contains the 28.5K protein II (Lambden *et al.*, 1979). Thus, a small change in surface structure may have a considerable influence on the antigenic and biological properties of the organism.

Although the variants show considerable antigenic cross-reactivity due to the common LPS and protein I components, there is evidence to suggest that antigenic variation of the protein II species may play a part in resistance of the gonococcus to host defences. In an attempt to study *in vivo* selection pressures strain P9 variants have been used to infect chambers subcutaneously implanted into guinea-pigs (McBride *et al.*, 1981). Initially only transparent variants survived, but they were eventually replaced by opaque-pilated forms, which in the majority of cases contained protein II species not present in the original inoculum, suggesting a selection pressure generated by a specific immune response. In addition, the pilus types of the *in vivo*-selected variants were found to be different from those initially present (Lambden *et al.*, 1981*a*). Subsequent studies have shown that they too show considerable structural homology but little antigenic cross-reactivity (P. Lambden, personal communication).

The surface of a single strain of gonococcus is thus capable of considerable antigenic variations which affect both the potential virulence of the organism and its resistance to host defences. It is likely that potential for such variation plays a crucial role in its ability to adapt to the changing environments of the natural history of the disease and is perhaps responsible for its considerable success as a pathogen.

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