The Surface Properties of *Neisseria gonorrhoeae*: Determinants of Susceptibility to Antibody Complement Killing

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Monovalent rabbit antisera were prepared to highly purified gonococcal lipopolysaccharide (LPS), to pili and to two major purified outer envelope proteins. All these antisera were free from significant specific IgM antibody and were standardized to 4 μ g specific IgG antibody per test, permitting accurate comparisons between the different gonococcal surface antigens as triggers of the complement-dependent bactericidal reaction. LPS was the most effective antigen at inducing a bactericidal response to homologous and heterologous gonococci, followed by the two individual outer envelope proteins. Pili were relatively ineffective. Strain p9 gonococci grown *in vivo* or which possessed a 'capsule' *in vitro* were more resistant to serum killing than the non-capsulated parent strain. One highly susceptible strain, F62, which was killed by complement in the absence of any LPS antibody, was able to directly activate complement by the alternative pathway.

INTRODUCTION

Patients with gonorrhoea produce antibodies which activate complement in a bactericidal reaction against the infecting gonococcus *in vitro* (Ward *et al.*, 1970; Kasper *et al.*, 1977). Bactericidal antibodies may be important in preventing blood-borne dissemination of gonococci from the mucosal surface because some, but not all, patients with disseminated gonococcal infections have low levels of bactericidal antibodies to their infecting strains (Brookes *et al.*, 1976; Schoolnik *et al.*, 1976) and, as a corollary, gonococcal strains isolated from patients with disseminated infection are commonly resistant to the bactericidal action of normal human sera (Schoolnik *et al.*, 1976). Thus, if the surface antigens concerned in activating antibody-complement killing of gonococci could be identified, these components would be candidates for vaccines giving protection against disseminated disease.

Glynn & Ward (1970) showed that antibodies reacting with the lipopolysaccharide (LPS) present in the gonococcal outer envelope are bactericidal for gonococci in the presence of complement. Core LPS may act independently of antibody by direct activation of the alternative complement pathway (Götze & Müller-Eberhard, 1976). Comparatively little attention has been paid to other antigens accessible on the gonococcal outer surface.

Recently some of the major proteins of the gonococcal surface have been isolated in this laboratory (Heckels, 1977; Robertson *et al.*, 1977). Rabbit antisera to these defined antigens have been raised and purified by solid phase immunoabsorption. These antisera, standardized to equivalent concentrations of specific IgG antibody by solid phase radioimmunoassay (Lambden & Watt, 1978), have been used to probe the importance of the major components of the gonococcal surface in the serum bactericidal reaction.

METHODS

Bacterial strains. Strains 24585, 24175, 24740, 24764 and 24396 of *Neisseria gonorrhoeae* were isolated locally and frozen in liquid nitrogen after the minimal number of subcultures necessary to ensure purity (Ward & Watt, 1971). Strain p9 had been maintained in this laboratory for some years; strain p9(ps) was the parent strain of p9 which had been subjected to minimal subculture *in vitro* and has caused a laboratory eye infection; strain p9(GP3) was derived from p9(ps) by three passages through guinea-pig subcutaneous chambers as described by Veale *et al.* (1975). This guinea-pig strain when grown on the medium of Swanson (1977) was found to be capsulated when stained by the method of Butt *et al.* (1936). Strain FA5 was kindly provided by Dr P. F. Sparling, University of North Carolina, Chapel Hill, U.S.A., strain 201 by Dr K. K. Holmes, University of Washington School of Medicine, Seattle, U.S.A., and strain r62 by Dr D. Kellogg, Jr, Communicable Disease Center, Atlanta, Georgia, U.S.A. All the strains tested were of the pilated colony types 1 or 2 (Kellogg *et al.*, 1963).

Antigens. Strain p9 gonococci were grown in bulk on Difco GC agar base and purified pili were prepared from them as previously described (Robertson *et al.*, 1977). Outer envelope was prepared from the residual depilated organisms by lithium acetate extraction, and the two major outer envelope proteins with molecular weights of 36000 (protein I) and 24500 (protein II) were purified as previously described (Heckels, 1977). Gonococcal LPS was extracted by the phenol/water technique and purified by enzyme treatment as described by Stead *et al.* (1975).

Antisera. New Zealand White rabbits were injected subcutaneously with 50 µg of either purified p9 pili, LPS, or outer envelope protein in Freund's incomplete adjuvant (Difco) at 0, 2, 4, 6 and 8 weeks. In some cases, the immunization schedule was repeated after a rest period of 6 weeks. Sera showing acceptable levels of antibody activity taken at various times from individual rabbits were pooled and stored at -70 °C.

Antibody estimations. Purified p9 pili, LPS, outer envelope, and each of the two outer envelope proteins were coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) and used as antigens in a solid phase radioimmunoassay specific for IgG antibodies (Lambden & Watt, 1978). Pilus antibody was also assessed by dark ground pilus agglutination (Robertson *et al.*, 1977).

Immunoadsorption. Antisera (5 ml) to purified pili and to the individual outer envelope proteins were made monospecific by immunoadsorption on an 18 × 1.5 cm column of Sepharose 4B to which strain p9 LPS had been coupled by cyanogen bromide activation. The adsorbed antisera in Dulbecco AB (Oxoid) were concentrated to the original volume by negative pressure dialysis. The column was regenerated without detectable loss of binding capacity using 3 M-sodium trichloroacetate (Porath & Kristiansen, 1975). The specificity of these antisera was confirmed by solid phase radioimmunoassay to purified pili, LPS, outer envelope and the outer envelope proteins.

Column chromatography. The IgG and IgM fractions from 2 ml of rabbit anti-LPS serum were separated by chromatography on a 1.5 × 80 cm column of Ultrogel ACA34 (LKB) in 0.1 M-Tris buffer pH 7.5. The fractions were concentrated to the starting volume of the serum by negative pressure dialysis, then dialysed overnight at 4 °C against Dulbecco AB and immediately tested for their bactericidal activity.

Immunofluorescence. Gamma globulin was isolated from 1 ml of sheep anti-rabbit IgM serum (Nordic Immunological, Tillberg, Netherlands) by precipitation with ammonium sulphate. The dialysed globulin was conjugated with fluorescein isothiocyanate isomer 1 (BDH) to a fluorescein:protein ratio of 3.6:1. Strain p9 gonococci were air-dried on to the wells of black Teflon-coated microscope slides (Hendley, Buckhurst Hill, Essex), fixed for 10 min in acetone at -20 °C and dried. The bacteria were incubated with each of the test antisera diluted to contain 4 µg specific IgG antibody. The slides were washed with Dulbecco AB and the remaining specifically bound antibody was tested for the presence of rabbit µ chains using the prepared conjugate. The slides were examined for fluorescence with an Ortholux II microscope equipped with a Ploem I incident light epi-illuminator (Leitz, Wetzlar, W. Germany).

Complement source. Fresh normal human serum from one of the authors (MEW) was used as a source of complement. Natural antibodies cross-reactive with gonococcal core LPS were removed by adsorption of the serum at 4 °C on a 18 × 1.5 cm column of gonococcal LPS coupled to Sepharose 4B equilibrated in Dulbecco AB (Oxoid). Fractions were pooled, sterilized by filtration, aliquoted and stored at -70 °C.

Bactericidal test. Eighteen hour cultures of gonococci were suspended in Dulbecco AB to give a turbid suspension. The concentration of gonococci was then adjusted so that a 1 in 10 dilution of the organisms in 1 M-NaOH had an A_{260} of 0.4 corresponding to a viable count of 10^8 colony forming units (c.f.u.) ml⁻¹. The bacterial suspension(s) were then diluted 10-fold with either fresh normal human serum, or the same serum previously heated at 56 °C for 30 min to destroy the complement. The bacterial suspensions in serum (50 µl) were then immediately added to the wells of microtitre trays containing either 50 µl Dulbecco AB (controls) or 50 µl of the antiserum diluted in Dulbecco AB so as to contain 4 µg specific IgG antibody.

For viable count determinations, a Micro-Medic diluter (Micro-Medic Systems, Philadelphia, U.S.A.)

was used to dilute serially 10 μ l of sample with 90 μ l of 1 % proteose peptone no. 3 (Difco), and then 50 μ l portions were plated on agar and incubated for 48 h in a moist atmosphere containing 5 % (v/v) CO₂.

Alternative complement pathway. Activation of complement by the alternative pathway requires Mg²⁺ and can be distinguished from classical pathway activation which requires Ca²⁺. The Ca²⁺ and Mg²⁺ were removed from the complement by chelation with 10 mM-EDTA. Selective chelation of Ca²⁺ alone in the presence of 10 mM added Mg²⁺ was done with 10 mM-EGTA (Sigma). In both cases, the pH of the mixture was adjusted to 7.4. In bactericidal tests, the toxicity for gonococci of serum containing chelating agent was checked using controls with heat-inactivated complement. Classical complement activation was assessed by titrating two-fold dilutions of the serum in Dulbecco AB against an equal volume of 1.4 % (v/v) rabbit haemolysin-sensitized sheep red blood cells (r.b.c.) and the alternative pathway was assessed using reduced glutathione-treated human group O Rh⁺ cells (Stålenheim *et al.*, 1973).

RESULTS

Normal human sera are bactericidal for many strains of gonococci (Glynn & Ward, 1970). Using our standard bactericidal test, 10^{4.4} c.f.u. of an inoculum of 10⁶ c.f.u. p9 gonococci survived incubation at 36 °C for 1 h in fresh human serum (MEW). The number surviving was increased to 10^{5.7} c.f.u. by immunoadsorption of the serum at 4 °C on a column containing p9 gonococcal LPS. This adsorbed serum was used as a source of complement to study the bactericidal action of 4 μ g specific antibody directed against the different surface components of the gonococcus.

Despite the use of hyperimmune sera, it was necessary to establish that the sera did not contain IgM antibodies, in particular anti-LPS IgM. When this antiserum was separated by column chromatography, no bactericidal activity was detected in the IgM fraction at a concentration equivalent to undiluted serum (Table 1). In contrast, the IgG fraction diluted to contain 4 μ g specific antibody against LPS allowed only 10³ c.f.u. of an inoculum of 10^{4.8} c.f.u. to survive for 1 h. As a result of these data and the failure to detect anti-gonococcal IgM in the sera by immunofluorescence, further work was undertaken with unfractionated sera thus avoiding the possibility of antibody aggregation with loss of activity and non-specific complement fixation.

Antibodies to LPS, the outer envelope proteins I and II, or to pili permitted 10^{2.5}, 10^{4.7}, 10^{4.9} and 10⁵ c.f.u., respectively, of an inoculum of 10^{6.1} c.f.u. p9 gonococci to survive (Table 2). LPS antibody to p9 was clearly the most active in initiating the serum bactericidal reaction, being active against all of the gonococcal strains which were susceptible to serum killing. Antibodies directed against pili and the outer envelope proteins I and II (from strain p9) were also bactericidal for strains 24585, 24175 and 24764 although differences exist. However, strain 24740 was sensitive to antibody directed against outer envelope protein I but not to protein II or pili. This suggests that the surface proteins contain common immunodeterminants as well as strain specific components.

Clearly, some gonococcal strains (FA5, 24396) were extremely resistant to the action of complement and the various antibodies whereas other strains (24764, F62) were sensitive to the complement source alone even though it had been freed of natural bactericidal antibody (see Table 2). One possibility is that the extreme sensitivity to complement of strains like F62 might result from the direct activation of the alternative complement pathway by LPS in the outer envelope. To distinguish between the Ca²⁺-requiring classical and the Mg²⁺-requiring alternative pathways of complement activation, the Ca²⁺ or the Ca²⁺ plus Mg²⁺ was removed from the complement source by selective chelation with 10 mM-EGTA or EDTA and the resulting serum was tested for its bactericidal activity against F62 (Table 3). In the absence of Ca²⁺ and Mg²⁺, both pathways of complement activation were blocked as shown by the failure of the serum to lyse haemolysin-sensitized sheep r.b.c. or to kill F62. When Ca²⁺ was selectively chelated in the presence of excess Mg²⁺, classical pathway activity was abolished preventing lysis of the sensitized sheep r.b.c. but alternative pathway activation still occurred as shown by lysis of reduced glutathione-treated human r.b.c.

Table 1. *The role of IgG and IgM antibodies in the complement-dependent bactericidal action of lipopolysaccharide (LPS) antibodies for N. gonorrhoeae strain P9*

Reaction components	Number (log ₁₀) of c.f.u. surviving*
Fresh adsorbed serum (MEW) as complement source (C)	4.5
C+ whole anti-LPS serum (4 µg specific IgG antibody)	3.2
C+ anti-LPS serum IgG fraction (4 µg specific antibody)	3.0
C+ whole undiluted anti-LPS serum	0.8
C+ undiluted IgM fraction of anti-LPS serum	4.5

* Mixtures were incubated at 36 °C for 1 h. The inoculum was 10^{4.8} c.f.u. P9 colony type 1 gonococci. The numbers of gonococci given were corrected for antibody-mediated bacterial agglutination using a test control containing antibody but no complement.

Table 2. *Comparison of the ability of 4 µg specific IgG antibody to strain P9 gonococcal pili, LPS or outer envelope proteins to induce complement-dependent bactericidal activity against nine strains of N. gonorrhoeae*

Antiserum in test mixture*	Number (log ₁₀) of c.f.u. surviving† Strain of <i>N. gonorrhoeae</i> (colony type 1):								
	P9	24585	24175	24740	201	24764	FA5	24396	F62
None	5.8	5.8	6.8	5.9	5.2	4.4	5.6	6.8	< 2.0
LPS	2.5	4.3	1.7	3.9	3.2	2.5	5.3	6.8	< 2.0
Protein I	4.7	4.4	1.7	4.9	5.5	3.4	5.5	6.8	< 2.0
Protein II	4.9	5.1	5.9	6.0	4.9	3.9	5.5	6.8	< 2.0
Pili	5.0	5.2	6.0	5.7	5.6	4.8	5.5	6.8	< 2.0
Inoculum c.f.u.	6.1	6.1	6.5	6.0	6.0	6.1	5.7	6.8	6.0

* Bacterial suspensions in adsorbed fresh human serum (MEW) were added to antiserum to components of strain P9 containing 4 µg specific (IgG) antibody, standardized by solid phase radioimmunoassay with ³H-labelled protein A.

† Mixtures were incubated at 36 °C for 1 h. Counts were corrected as in Table 1. In controls lacking complement, viable counts at 1 h were always within ± 10^{0.3} c.f.u. of the inoculum at zero time.

Table 3. *The role of classical and alternative pathway complement activation, as distinguished by selective chelation of Ca²⁺ or Ca²⁺ plus Mg²⁺ ions, in the bactericidal activity of fresh human serum for F62 gonococci*

Modification to fresh serum (MEW)	Number (log ₁₀) of c.f.u. surviving*	Dilution of serum after incubation for 1 h at 36 °C lysing an equal volume of:	
		1.4 % (v/v) haemolysin-sensitized sheep r.b.c. (classical pathway)	1.4 % (v/v) GSH-treated human r.b.c. (alternative pathway)§
None	2.1	1:40	NT
Heated at 56 °C, 30 min	6.1	None	None
Ca ²⁺ + Mg ²⁺ removed†	5.8	None	None
Ca ²⁺ removed, excess Mg ²⁺ ‡	3.9	None	1:10

* Mixtures were incubated at 36 °C for 1 h. The inoculum was 10^{6.1} c.f.u. F62 colony type 1. Counts were corrected for appropriate controls.

† Removed by chelation with 10 mM-EDTA; pH maintained at 7.4.

‡ 10 mM-Mg²⁺ added; Ca²⁺ removed by selective chelation with 10 mM-EGTA; pH maintained at 7.4.

§ Human group O Rh⁺ (MEW) r.b.c. treated at 37 °C for 1 h with 0.15 M-reduced glutathione (GSH) followed by extensive washing in Dulbecco AB. Treated cells were used to measure antibody-independent lysis due to the alternative pathway. NT, Not tested.

Table 4. Susceptibility of P9 gonococci grown *in vivo* and of 'capsulated' P9 gonococci to killing by antibody and complement

Antiserum in test mixture*	Number (log ₁₀) of c.f.u. surviving after incubation of mixtures at 36 °C for 1 h		
	Parent strain [P9(ps)]	P9(ps) grown <i>in vivo</i> † [P9(gp3)]	P9(gp3) after one subculture <i>in vitro</i> ‡ ('capsulated')
None	4.1	4.5	4.4
LPS	2.2	3.4	4.1
Protein I	3.9	4.1	4.1
Protein II	3.9	4.5	4.2
Pili	3.7	4.6	4.5
Inoculum c.f.u.	4.4	4.9	4.6

* Bacterial suspensions in adsorbed fresh human serum (MEW) were added to antiserum to components of strain P9 containing 4 µg specific (IgG) antibody.

† Organisms grown in guinea-pig chambers were sedimented and the pellet was gently rinsed three times with Dulbecco AB.

‡ Strain P9(gp3), grown in guinea-pig chambers, was subcultured once for 18 h on to the medium of Swanson (1977).

(Stålenheim *et al.*, 1973). Under these conditions only 10^{3.9} c.f.u. of an inoculum of 10^{6.1} c.f.u. F62 organisms survived (Table 3).

Gonococci which have grown *in vivo* in the natural host are more resistant to the bactericidal action of antibody and complement than the same organism in artificial culture (Ward *et al.*, 1970). This resistance appears to be a phenotypic response to the altered growth conditions *in vivo* and may reflect the elaboration of different surface antigens such as the 'capsule-like' structure which has recently been reported (Hendley *et al.*, 1977; James & Swanson, 1977). Therefore the susceptibility to serum killing of the parent strain of P9, i.e. P9(ps), was compared with that of P9 which had been maintained *in vivo* in guinea-pig subcutaneous chambers [P9(gp3)]. The results confirmed that P9(gp3), when recovered direct from the chamber fluid, was more resistant than P9(ps) to killing by LPS antibody and complement (Table 4). Gonococci grown in guinea-pig chambers possess capsules (Hormaeche *et al.*, 1978) which might explain this resistance to serum killing. These capsules could be maintained *in vitro* by growth on laboratory medium. The capsulated organism was comparable to the organism grown *in vivo* in its resistance to serum killing and was more resistant than the non-capsulated P9(ps) (Table 4). Little significant difference was found in the susceptibility of P9(ps) and extensively subcultured P9 to serum killing (Tables 2 and 4) despite known differences in their outer envelope structure (Heckels & Everson, 1978).

DISCUSSION

In this study the role of antibodies prepared to purified gonococcal surface components was investigated in preference to inhibition studies with the antigens themselves. This approach was chosen because of the difficulty of excluding the presence of small but immunologically significant amounts of contaminating antigens in the surface components.

Following immunoadsorption, the antisera were monospecific as shown by the lack of antibody to other surface components on radioimmunoassay. Thus the results establish that antibody to the individual outer envelope proteins, and particularly to LPS, are bactericidal for the homologous and some heterologous gonococci. By contrast, pili were only capable of inducing a weak bactericidal response, perhaps because most antibody

binding and complement activation occurs at sites too remote from the target cytoplasmic membrane to be effective in serum killing.

The results show that at the standardized level of 4 μ g specific (IgG) antibody, LPS antibody was clearly the most effective at inducing serum killing. This was not due to the presence of specific IgM antibodies, nor was there evidence in any of the antisera of a pro-zone effect due to blocking antibodies such as non-complement fixing IgA antibodies. The importance of LPS antibody in the bactericidal action of human sera from normal or gonococcal infected subjects (Tramont *et al.*, 1974; Rice & Kasper, 1977) or in rabbit antisera to whole gonococci (Glynn & Ward, 1970) is firmly established. LPS tends to induce an IgM response (Cohen, 1967). These antibodies on a mole for mole basis are more actively bactericidal than IgG. Thus LPS may be the critical antigen for the bactericidal reaction in patients with gonorrhoea.

One explanation for the efficiency of LPS antibodies is that a larger area of gonococcal surface is occupied by LPS than by the outer envelope proteins. Thus calculations based on the mass of LPS in the lithium acetate extracted outer envelope of p9 suggest the ratio of LPS : outer envelope protein to be at least 2 : 1, although not all of this may be surface antigen. In gonococci, LPS is thought to form a continuous back-bone layer in which the protein molecules are embedded (Novotny *et al.*, 1977). A greater availability of LPS would increase the likelihood of two molecules of IgG antibody binding in sufficiently close proximity to activate complement. An alternative suggestion is that the LPS antigen fixes complement at a site (e.g. the core region) which is closer to the vulnerable lipid of the membrane.

Gonococcal LPS from different strains have similar core components (Perry *et al.*, 1975; Stead *et al.*, 1975) although there may be differences in the linkages. The O antigen chains (Perry *et al.*, 1975) are produced inconsistently in response to as yet undefined growth conditions. The failure of antibody to kill strains such as FA5 and 24396 must be due to other factors such as the presence of blocking antigens (Glynn & Ward, 1970) or of permeability barriers to the later activated complement components. Strains FA5, F62 and 201 differ from p9 and other English strains tested in that they lack protein II (Heckels, 1977). Strain p9(ps) differs from the extensively subcultured p9 in possessing lower amounts of protein II and by the retention of a third major protein (protein III, molecular weight 60000) in lithium acetate extracted outer envelope prepared from it (Heckels, 1978). Thus there are important differences in the organization of the surface layers in these strains, but any obvious correlation between them and the susceptibility of these strains to serum killing was not forthcoming (Tables 2 and 4).

Gonococci grown *in vivo* in patients with gonorrhoea (Ward *et al.*, 1970) or in guinea-pig subcutaneous chambers (Penn *et al.*, 1977) were resistant to serum killing but rapidly became sensitive on subculture *in vitro*. The possibility that this *in vivo* resistance is due to adsorbed host material blocking antibody binding sites cannot be totally excluded. Nevertheless, it seems likely that the phenotypic effect of growth *in vivo* induces variations in surface structure, such as the production of O chains on the LPS, which may increase resistance to serum killing. Gonococci freshly isolated from guinea-pig subcutaneous chambers possess an extra 'capsular' surface layer which can be visualized by electron microscopy after fixation in the presence of lanthanum (Hormaeche *et al.*, 1978) and which is morphologically similar to the 'capsules' reported by other workers on gonococci grown under special conditions *in vitro* (Hendley *et al.*, 1977; James & Swanson, 1977). The data presented in Table 4 confirm that gonococci grown in guinea-pig chambers are resistant to serum killing and are capsulated on subculture. Capsulated gonococci grown *in vitro* were less susceptible to killing by LPS antibody and complement than ordinary subcultured p9 or p9(ps). Further work is needed to establish the mechanism of this resistance.

Some strains, notably F62, were highly susceptible to serum killing by complement alone. In the absence of LPS antibody in the complement source, it seemed likely that these strains

were directly activating complement by the alternative pathway (Götze & Müller-Eberhard, 1976), perhaps because the 'rough' LPS core is relatively exposed. This theory was supported by the finding reported here that significant numbers of gonococci were killed by complement in the absence of Ca^{2+} provided that Mg^{2+} was present; under these conditions only the alternative pathway operates. Activation of complement through the alternative pathway by f62 gonococci was confirmed by the lysis of reduced glutathione-treated human r.b.c. (Stålenheim *et al.*, 1973). Interactions of gonococci with the alternative complement pathway have largely been ignored although Scherer & Braun-Falco (1976) using immunofluorescence have suggested that the allergic vasculitis in the skin lesions of benign gonococcal sepsis results from direct activation of the alternative complement pathway by gonococci. This pathway might also be involved in the generation of the acute inflammatory response so characteristic of gonorrhoea.

As patients with disseminated gonococcal infection do not produce bactericidal antibodies to the infecting gonococcus, the serum killing reaction may be protective in uncomplicated gonorrhoea. The susceptibility of patients with complement deficiency to disseminated forms of gonococcal infection (Brooks *et al.*, 1976) can also be explained by the failure to mount an effective bactericidal response. Moreover, in both the guinea-pig and chimpanzee models of gonococcal infection, protective immunity correlates best with the levels of bactericidal antibody (Wong *et al.*, 1976; Arko *et al.*, 1976). LPS antibodies are almost certainly involved in these protective responses. However the results reported here show that protein antigens are also involved in serum killing and the cross-reactivity of the exposed immunodeterminants on these proteins and their protective role needs to be evaluated. The newly described 'capsular' layer on gonococci must be isolated and antibodies raised against it to determine the number of immunotypes and whether antibodies to it are protective. Hopefully this molecular approach may eventually lead to the development of a vaccine.

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