Surface Antigens of Gonococci: Correlation with Virulence and Serum Resistance

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(Received 26 April 1982; revised 26 October 1982)

Encapsulated and non-encapsulated variants of one strain of gonococcus were compared for their capacity to produce infection in chambers implanted subcutaneously in mice, for their reactions with specific antibody and for their precipitation with wheat germ agglutinin. Only the encapsulated variant could infect implanted chambers. Specific rabbit antiserum raised against the non-encapsulated variant killed only that variant, whereas antibody raised against the encapsulated variant killed both variants.

Saline extracts and lipopolysaccharide preparations of the encapsulated variant differed from those of the non-encapsulated one in their reactions with wheat germ agglutinin and antibody in diffusion and electrophoresis tests. Preparations from infective encapsulated gonococci reacted with wheat germ agglutinin while those from the non-encapsulated variant did not. Immunodiffusion tests showed that lipopolysaccharides from both variants share a common antigenic determinant, but saline extracts and lipopolysaccharides from encapsulated gonococci possess an additional determinant. The significance of these findings is discussed.

INTRODUCTION

Gonococci in urethral pus are more infective than those subcultured in vitro. The inoculation of human urethral exudates containing gonococci into the urethra of human volunteers (Mahoney et al., 1946) or chimpanzees (Lucas et al., 1971) results in an infection which closely resembles gonorrhoea. Cultured gonococci often fail to produce symptoms of urethritis (Mahoney et al., 1946; Brown et al., 1972), suggesting that gonococci in urethral pus have some characteristics which are different from those of cultured organisms. Animal-adapted gonococci are also more infective in subcutaneous chambers than those grown in vitro and this is unrelated to the presence of pili on their surface (Arko et al., 1976; Penn et al., 1976).

Gonococci recently isolated from patients (Ward et al., 1970) or guinea-pig chambers (Penn et al., 1976) are more resistant to serum killing than those subcultured in the laboratory and it seems possible that gonococci in vivo may possess a virulence factor which renders the bacteria less sensitive to the bactericidal or opsonic action of serum.

Both gonococcal LPS and, to a lesser extent, outer membrane proteins have been shown to be the target for bactericidal antibodies (Ward et al., 1978). Lambden et al. (1979) recently reported changes in surface proteins which may be of importance in serum resistance and Guymon et al. (1978) suggested that resistance might be determined by alteration of the LPS structure. Although such variations in LPS could explain differences in virulence and serum resistance they have not been previously demonstrated since the four colony types of gonococci possess an apparently identical 'rough' type LPS (Perry et al., 1978). It is also possible that encapsulation might determine serum resistance as capsules would block the reaction between bactericidal

Abbreviations: KDO, 2-keto-3-deoxyoctulosonic acid; WgA, wheat germ agglutinin.
antibodies and outer membrane antigens. Capsules could also increase gonococcal virulence by interference with opsonization and phagocytosis and free capsular material or slime might neutralize antibody at a distance from the gonococcal cells. That encapsulation might be involved in resistance to serum killing and phagocytosis has already been suggested (Ward et al., 1978; Richardson & Sadoff, 1977), but the role of capsules in gonococcal virulence has not yet been established.

In this study we have examined encapsulated and non-encapsulated variants of one gonococcal strain. The results indicate that they differ in infectivity, serum resistance and antigenic composition.

METHODS

Gonococci. Strain gc40, originally isolated from the urine of a male with acute urethritis, was used throughout this study. It had been subcultured at least 50 times in vitro when this study was commenced. It did not show capsules by light microscopy using Leishman's stain or did so only occasionally around a few cells and was referred to as variant N. Variant C was obtained by inoculating variant N in a rabbit subcutaneous chamber where it produced a mixed infection together with a corynebacterium sp.; after reisolation from the rabbit chamber (a process involving a total of three in vitro cultures) the organism was grown in a guinea-pig chamber as previously described (Demarco de Hormaeche et al., 1978, 1979). It was confirmed as Neisseria gonorrhoeae by oxidase reaction, sugar fermentations (glucose, maltose, lactose) and nitrate reduction tests. After growth in vivo, in either rabbit or guinea-pig chambers, gonococci appeared different from the parent strain showing clear capsules around all cells (Fig. 1). The possible effect of corynebacteria on gonococcal growth in vivo and in vitro is currently under investigation.

In vitro cultures. These were made on Ge agar (Difco) enriched with 1% (w/v) IsoVitaleX (BBL), referred to as GCIV, and incubated at 36 °C in candle jars. Cultures were not selected for colony type, both variants gave rise predominantly to large colonies, similar to types 3 and 4 of Kellogg et al. (1963). For storage, standard suspensions of gonococci of each variant were made in Greaves' medium (1960), the total volume divided in portions and frozen in liquid nitrogen. A new ampoule was used each time the variants were compared. Variant C was always used after being subcultured three times in guinea-pig chambers.

Infectivity tests. The open subcutaneous chamber model was used for these tests. Chambers were made by cutting 6 to 8 mm long sections of vinyl tubing (12 mm, autoclavable PVC, Baird and Tatlock). C3H/He mice of either sex, aged from 3–4 months, were implanted with two chambers each and were inoculated 3–4 weeks later, by which time the inflammation had subsided. Chambers were inoculated with 100 μl of serial 10-fold saline dilutions of a freshly prepared suspension of organisms grown for 18–20 h in plate culture. In most experiments, mice were given both variants of gc40 simultaneously, one in each chamber. Samples (100 μl) were taken at 2 and 5 d after inoculation and cultured immediately on GCIV medium or chocolate agar. Inoculation and sampling of the chambers were performed with the animals under ether anaesthesia.

Bactericidal test. This was performed in sterile microtitre plates with filter-sterilized complement fixation test (CFT) diluent (Oxoid) containing 0.5% (v/v) IsoVitaleX (BBL), referred to as GCIV, and incubated for 20 h and the colonies counted. The highest serum dilution killing 50% of the gonococci was taken as the titre for bactericidal activity.

Antisera. These were obtained by inoculation of gonococci of strain gc40 into rabbits. Variants N or C were subcultured on GCIV medium covered with dialysis tubing to avoid agar contamination. After 18–20 h incubation, the gonococci were harvested in PBS containing per litre: 8 g NaCl, 0.2 g KCl, 1.15 g Na2HPO4, 0.2 g KH2PO4, pH 7.2 and mixed with equal volume of complete Freund’s adjuvant (Difco). Rabbits were inoculated intramuscularly with a total of 1 ml mixture containing 109 cells. At days 14 and 20 the rabbits were challenged intravenously with 107 gonococci suspended in saline and were bled on day 28. Sera were kept at –20 °C.

Saline extracts. Gonococci were subcultured on GCIV medium covered with dialysis tubing and incubated for 18–20 h at 37 °C, harvested and suspended in saline. Comparable extracts of gc40 N and C were made by growing gonococci of each variant under identical conditions and by making suspensions of equal concentration adjusted to give A600 = 0.3 for 1/100 dilutions. The suspensions were centrifuged in a Beckman microfuge at 8000 g for 5 min, and the supernatants were then termed saline extracts and stored at –20 °C.
**Gonococcal surface antigens and virulence**

**LPS extracts.** Gonococci were subcultured, harvested and centrifuged as described above. Gonococci were sonicated while kept on ice, using an MSE sonicator. The disrupted gonococci were heated to 69 °C and an equal volume of hot phenol (95%, w/w, 69 °C) was added. The extraction was carried out at 69 °C for 15 min with constant stirring. The preparation was then cooled at 4 °C and centrifuged at 1000 g for 10 min. The water phase was collected and kept, while the interface and phenol phase were re-extracted with an equal volume of distilled water.

The aqueous layers of successive extractions were pooled and dialysed against running tap water until no phenol smell was detected. This crude phenol–water extract was freeze-dried, weighed and resuspended in distilled water. Insoluble material was removed by centrifugation at 2000 g for 10 min. Supernatant was treated with DNAase and RNAase (Sigma) final concentration 10 μg ml⁻¹ for 1 h at 37 °C and centrifuged at 100000 g for 18 h. After freeze-drying of the pellet the enzyme treatment and centrifugation were repeated once. The final pellet was resuspended in distilled water, freeze-dried and weighed. Both variants gave similar amounts of LPS; 5% of the total crude phenol–water extract was recovered as judged by dry weight measurements. The preparations were free of contamination by nucleic acids and proteins as determined by carbocyanine dye assay and measurements of A₂₅₀ and A₂₈₀.

Lipid A was determined by the carbocyanine dye method of Janda & Work (1971). The 2-keto-3-deoxyoctulosonic acid (KDO) was determined colorimetrically by the method of Weissbach & Harwitz (1959).

**Gel diffusion techniques.** These were performed in 1% (w/v) agarose (Indubiose A45, I.B.F.) in sodium barbitone buffer 0.043 M pH 8.6 containing 0.01 M-sodium azide. Results were recorded with fresh gels or after washing, drying and staining with Coomassie brilliant blue.

**Double diffusion.** Wells were cut in the gels to contain 18 μl volume samples of antigen or antiserum. Saline extracts of gonococci or LPS extracts were treated with aqueous sodium deoxycholate (0.25% final concentration pH 8.4) before use. The gels were incubated overnight at room temperature.

**Crossed immune electrophoresis (CIE) and crossed affinity electrophoresis.** These were performed on 7.5 × 7.5 cm glass slides. Separation of the first dimension was carried out in 1% (w/v) agarose (3 ml) dispensed on 1/3 of the area of the plate. Wells were cut to contain 18 μl volume of antigen (N and C preparations were always compared simultaneously at equal volumes). Electrophoresis was carried out for 45 min at 40 V measured end to end of the gel. After this 4 ml agarose containing either antisera (4%v,v) or one lectin [wheat germ agglutinin (WgA), concanavalin A or Limulus, Sigma] at a concentration of 0.75 to 20 μg ml⁻¹ was dispensed onto the rest of the plate and the second dimension was run at 20 V overnight using a cooling system.

**Rocket electrophoresis.** Agarose containing WgA (0.75 to 20 μg ml⁻¹) was used for this technique and electrophoresis was run overnight at 20 V.

**RESULTS**

**Infectivity of strain gc40 in mice**

The two variants of strain gc40 showed a marked difference in their infectivity when inoculated into subcutaneous chambers in mice. Non-encapsulated gonococci of variant N did not infect any of the chambers inoculated at doses varying from 10² to 10⁸. On the other hand the encapsulated variant was very efficient, producing infection in chambers inoculated with doses.
Table 1. Infectivity of variants of strain gc40 in mouse subcutaneous chambers

The results indicate no. chambers infected/no. chambers inoculated and this is shown as a percentage in parentheses.

<table>
<thead>
<tr>
<th>Dose (c.f.u.)</th>
<th>Variant C</th>
<th>Variant N</th>
</tr>
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<tbody>
<tr>
<td>$10^6$</td>
<td>NT</td>
<td>0/10</td>
</tr>
<tr>
<td>$10^7$</td>
<td>8/10 (77%)</td>
<td>0/10</td>
</tr>
<tr>
<td>$10^6$</td>
<td>34/45 (76%)</td>
<td>0/35</td>
</tr>
<tr>
<td>$10^5$</td>
<td>11/22 (50%)</td>
<td>0/22</td>
</tr>
<tr>
<td>$10^4$</td>
<td>19/37 (52%)</td>
<td>0/37</td>
</tr>
<tr>
<td>$10^3$</td>
<td>10/18 (55%)</td>
<td>0/18</td>
</tr>
<tr>
<td>$10^2$</td>
<td>5/10 (50%)</td>
<td>0/15</td>
</tr>
<tr>
<td>$10^1$</td>
<td>3/10 (30%)</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested.

as low as $10^1$ gonococci. However, a minimum infectious dose or an infective dose 50 could not be determined for variant C as some of the animals became infected at the lowest dose employed and no differences were seen in the infectivity of doses ranging from $10^3$ to $10^5$ c.f.u. (Table 1).

This clear cut difference in infectivity of the variants was consistently seen in repeated experiments in mice carrying two chambers and inoculated simultaneously with one variant in each chamber.

**Resistance to bactericidal effect of specific antibody and complement**

The resistance of the C and N variants to the killing effect of antibody and complement was tested *in vitro* using antisera raised in rabbits by inoculation of either variant. A clear difference was seen in resistance to killing by anti-N antisera. Several anti-N antisera produced in different rabbits were bactericidal for variant N but not for variant C (Fig. 2), suggesting the presence of a different antigen on the surface of variant C. On the other hand, both variants were killed to a similar titre by anti-C antisera.

**Reactivity of surface antigens with lectins and antibody in gel diffusion**

Saline extracts of equal quantities of variants N and C were tested in CIE against an antiserum to variant C. The two variants were shown to contain different proportions of a number of negatively charged antigens, which gave reactions of identity when tested in tandem. Most of those antigens have been demonstrated to be of cytoplasmic origin (Demarco de Hormaeche, 1979). Extracts of variant N showed one precipitation line near the antigen well indicating the presence of a slow moving antigen in the preparation. Extracts of variant C
Fig. 3. CIE of saline extracts of gonococci of strain gc40 against antiserum to variant C. (a) Variant N, (b) variant C. Though present in different relative amounts, all but one antigen (arrow) were shown to be present in both variants when preparations were tested in tandem.

Fig. 4. Crossed affinity electrophoresis of variant C saline extract with 5 µg WgA ml⁻¹ in the second gel. Only one precipitation line is formed.

showed two precipitation lines near the origin well suggesting the presence of an extra antigen in this variant (Fig. 3a, b), which did not give reactions of identity when the preparations were run in tandem.

Crossed affinity electrophoresis of the saline extracts using WgA in the second dimension showed a difference in the reactivity of variants N and C. Extracts of variant N did not form precipitation lines with WgA at any of the concentrations tested while extracts of variant C gave a distinct line of precipitation at an optimum concentration of 5 µg WgA ml⁻¹ (Fig. 4). This single precipitation line appeared at the location in which an extra antigen was seen in extracts of variant C by CIE (arrow in Fig. 3b). No reactions were detected using concanavalin A or Limulus in the second gel.

Reactivity of LPS extracts with antibodies and WgA

Matching LPS preparations extracted from variant C (C LPS) and variant N (N LPS) were shown to contain equal amounts of lipid A and also very similar proportions of KDO: 6-2% for N LPS and 6-4% for C LPS. However they differed greatly in their reactivity with WgA and specific anti gc40 antisera. Whereas, N LPS did not form precipitation rockets with WgA at any of the concentrations of lectin tested (0-75–20 µg ml⁻¹), C LPS gave rockets at various concentrations with an optimum at that of 2-5 µg WgA ml⁻¹ (Fig. 5). Differences between N and C LPS were also found using the double diffusion technique. C LPS reacted with anti
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Fig. 5. Rocket electrophoresis of LPS preparations into agarose containing 2.5 μg WgA ml⁻¹. LPS from variant C formed neat rockets at all concentrations tested (0.5 to 2 mg LPS ml⁻¹: 1 to 3) while LPS from variant N (shown here at the concentration of 2 mg ml⁻¹, 4) did not.

variant C antiserum forming a single precipitation line. N LPS also produced a precipitation line with this antiserum but gave a reaction of only partial identity with C LPS antigen demonstrating the presence of a different antigenic determinant in the latter (Fig. 6a). The antigens detected in the LPS preparations by the anti variant C antiserum were also found in the saline extracts of the corresponding variants. Absorption of the variant C antiserum with N LPS removed all its reactivity with N LPS but the antibodies reacting with C LPS and antigens of the surface washes remained in the antiserum (Fig. 6b). Absorption with C LPS removed antibodies directed to either LPS, the antiserum reacted only with antigens present in the saline extracts and apparently unrelated to the LPS (Fig. 6c).

DISCUSSION

An encapsulated variant of gonococci of strain gc40 selected by in vivo growth was infective when inoculated into subcutaneous chambers implanted in mice, while the parent strain was not; the encapsulated variant was no longer killed by antibodies directed to the parent strain. An additional antigen determinant was detected in saline extracts and LPS extracted from washed bacteria of the variant C compared with variant N.

Although saline extracts were expected to contain the capsular material, attempts to demonstrate a capsular polysaccharide distinct from the LPS, have not so far succeeded (unpublished observations). It is not known whether some properties of the C LPS may give rise to the morphological appearance of a capsule and this is under investigation at the moment. It is still possible that the observations of differences in encapsulation and differences in antigenic structure of the LPS between the variants N and C may relate to two independent properties.

The resistance to killing by fresh human sera of gonococci in human pus demonstrated by Ward et al. (1970) was lost after one subculture, and similar observations have been made by Penn et al. (1976, 1977) and Rittenberg et al. (1977) on gonococci in subcutaneous chamber fluid. However, the serum resistance reported here for gonococci of variant C was maintained for at least three cultures in vitro, indicating that serum resistance was not determined by phenotypic change, the protective effect of substances present in inflammatory exudate or by the intracellular location of the gonococci.

The morphological appearance of capsules has been revealed by electron microscopy of preparations fixed in the presence of Alcian blue of gonococci of strain gc40 recently grown in vivo (Demarco de Hormaeche et al., 1978) or gonococci grown on a special medium (Hendley et al., 1981). Encapsulation could explain the serum resistance seen in variant C as the capsule would provide a surface layer for which anti variant N antisera would not possess antibody. The capsule might then protect the outer membrane from the lytic effect of antibody and
complement. A similar effect has been demonstrated by Glynn & Howard (1970) in *E. coli*; K antigens confer resistance to the bactericidal effect of antibody and complement which act on the outer membrane antigen and the protection conferred is proportional to the amount of K antigens formed.

In this study an antigen absent in the parent strain was demonstrated in the encapsulated *in vivo* grown variant using gel precipitation techniques. This antigen was found in the saline extracts and also in isolated LPS and it is likely that it is involved in the reaction with WgA. It seems also likely that it corresponds with the antigen of *in vivo* grown gonococci shown to react with WgA by Perera *et al.* (1980). Allen *et al.* (1980) demonstrated the presence of sugars on the
gonococcal surface with terminals corresponding with the specificity of WgA (N-acetyl-D-glucosamine) using an agglutination technique. They showed these sugars in the gonococci bound WgA while to have different antigenic specificity and WgA reactivity.

In this investigation, extracts from our isogenic virulent and avirulent gonococci were shown to have different antigenic specificity and WgA reactivity. LPS extracted from variant C gonococci bound WgA while LPS from variant N did not, demonstrating chemical differences in the sugar terminals of LPS molecules. At the same time, double diffusion technique using anti-virulent gonococci antisera clearly showed an antigenic determinant in C LPS which was absent in the N variant. Changes in the LPS structure are known to influence virulence variation in enterobacteria (Wilson and Miles, 1975). Variants of salmonella possessing S-type LPS (with side chains) are virulent while variants with R-type LPS (without side chains) are not. The presence of side chains in the polysaccharide portion of our LPS could explain the differences seen between the variants, but it should have been reflected in the proportion of KDO in the C and N LPS preparations. Since both LPS preparations had similar lipid A and KDO contents it seems likely that alterations in WgA binding and antigen specificity were caused by alterations in sugar composition or configuration of the oligosaccharide portion of the LPS rather than acquisition of additional polysaccharide side chains. Structural differences in the LPS molecule of isogenic pyocin resistant and sensitive gonococci have been reported (Connelly et al., 1981) and changes in the configuration of gonococcal LPS have also been suggested as determinants of variation in human serum resistance (Schneider et al., 1982). It seems possible that variations on the LPS might be important in gonococcal virulence in as much as a change in the LPS molecular configuration might render the gonococcus resistant to bactericidal or opsonic antibodies.

Finally, although gonococci with C LPS and encapsulated appearance are resistant to the bactericidal effect of antibodies directed to the parent strain, they are killed effectively by antisera raised against themselves. This suggests that the extra surface antigen seen in virulent variant C gonococci acts as a target antigen in the serum bactericidal reaction as long as specific antibodies are present. It seems likely that in order to obtain effective protection to gonococcal infection what should be promoted is the production of antibodies directed to a particular antigen involved in immune lysis. It is possible that the determinant that we describe here in the LPS of virulent gonococci might be one such site.

We are grateful to Professor P. Wildy for his interest and advice. R.D.H. and M.J.T. were supported by Medical Research Council grants.

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