Effect of sialylation of lipopolysaccharide of Neisseria gonorrhoeae on recognition and complement-mediated killing by monoclonal antibodies directed against different outer-membrane antigens

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Growth of gonococci in the presence of CMP-N-acetylneuraminic acid (CMP-NANA) has previously been shown to induce resistance to the bactericidal effect of normal human serum and is accompanied by sialylation of the gonococcal lipopolysaccharide (LPS). We have used monoclonal antibodies (mAbs) to compare the effect of LPS sialylation on recognition of gonococci and complement-mediated killing by antibodies directed either against LPS or against defined epitopes on outer-membrane protein PI. Despite differences in binding to sialylated LPS on Western blots, all three mAbs directed against LPS showed considerably reduced binding to gonococci grown in the presence of CMP-NANA and a concomitant reduction in ability to promote complement-mediated killing. In contrast, mAbs directed against previously defined epitopes on a surface exposed loop of PI showed little difference in binding between sialylated and non-sialylated gonococci and promoted killing of the sialylated gonococci. Similarly a mAb directed against an epitope on a loop of the outer-membrane Rmp protein, which had previously been shown to block killing by antibodies directed against other surface antigens, also exerted a blocking effect with sialylated gonococci. Thus in the present study the continued biological effect of mAbs was correlated with the ability of the antibody to recognize surface-exposed epitopes on sialylated gonococci. Despite the presence of the sialylation which is likely to occur in vivo, it should be possible to induce complement-mediated killing by focusing the immune response to those surface-exposed epitopes which are least susceptible to the potential inhibitory effect of LPS sialylation.

Keywords: Neisseria gonorrhoeae, sialylation, outer-membrane protein, porin, LPS

INTRODUCTION

Neisseria gonorrhoeae is a human pathogen which is endowed with a range of mechanisms that facilitate immune avoidance (for a review see Heckels, 1993), including antigenic shift in the expression of surface antigens. Thus while pili and the outer-membrane Opa protein (protein II) participate in adhesion to epithelial cells, and antibodies directed against these proteins inhibit adhesion (Virji & Heckels, 1984) and promote phagocytosis (Virji & Heckels, 1985, 1986), antigenic variation in expression of these proteins occurs during natural infection enabling gonococci to avoid the consequences of the host immune response (Zak et al., 1984). This antigenic shift has also frustrated attempts to develop an effective vaccine against gonorrhoea.

In addition to the antigenic shift in protein expression, modifications to the structure of LPS contribute to immune avoidance, promoting the resistance of gonococci to the bactericidal effect of normal human serum.
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(GNS). Gonococci grown in vitro may be killed by NHS, an effect which is thought to be largely due to the presence in human serum of antibodies which cross react with LPS (Apicella et al., 1986) and hence promote complement-mediated killing. However, freshly isolated gonococci are generally resistant to NHS but become sensitive on subsequent laboratory subculture (Ward et al., 1970). The basis of this unstable serum resistance has been explained by the studies of Smith and colleagues, who have demonstrated the presence of a low M factor in human serum (Martin et al., 1981), genital secretions (Martin et al., 1982), leukocytes (Patel et al., 1988) and erythrocytes (Patel et al., 1984) which renders in-vitro-grown gonococci resistant to the bactericidal effect of NHS. This factor was subsequently identified as CMP-N-acetylneuraminic acid (CMP-NANA) (Nairn et al., 1988; Parsons et al., 1993), and structural studies have revealed that the induction of resistance is accompanied by sialylation of LPS, with transfer of the NANA moiety from CMP-NANA to a terminal Galβ1-4GlcNAc residue (Mandrell et al., 1990).

Protein I (PI), the major protein present on the surface of gonococci, does not undergo such antigenic variation (Zak et al., 1984) and is also a major target for potential immune attack. Monoclonal antibodies (mAbs) directed against PI have been shown to promote complement-mediated killing, to opsonize for phagocytosis by polymorphonuclear leukocytes and to inhibit gonococcal interaction with epithelial cells (Virji et al., 1986, 1987). Although PI is stable within a strain, differences occur between strains. Two major classes of PI exist (PIA and PIB) and these can be further subdivided into a number of different serovars on the basis of reactivity with mAbs (Knapp et al., 1984). Considerable insight into the immunobiology of PI has been obtained by the sequencing of the encoding por genes (Gotschlich et al., 1987b; Carbonetti & Sparling, 1987) and by use of the sequence information in mapping experiments to identify the epitopes recognized by mAbs (Burt et al., 1990; Mee et al., 1993). Such information has also been used to synthesize peptides for immunization studies and the resulting antisera have been shown to promote complement-mediated killing (Heckels et al., 1990; Elkins et al., 1992).

By virtue of its stability and immunobiological properties PI appears to be an important potential component of a gonococcal vaccine. This conclusion is reinforced by evidence which suggests that anti-PI antibodies induced during infection provide protection against gonococcal salpingitis (Buchanan et al., 1980) and partial immunity to uncomplicated infection (Plummer et al., 1989). Such immunity is likely to involve complement-mediated killing. The discovery of the effect of LPS sialylation on killing by NHS has therefore prompted further studies to investigate the effect of sialylation on killing by immune sera directed against both LPS and other surface antigens. In this paper we have therefore used mAbs to compare the effect of sialylation of LPS on both recognition of gonococci and complement-mediated killing by mAbs directed either against LPS or against defined epitopes on PI.

METHODS

Bacterial strains and growth conditions. Neisseria gonorrhoeae strain P9 used in these studies expresses PIB serovar IB-26 (Butt et al., 1990) and has previously been shown to lose resistance to NHS following laboratory subculture (Ward et al., 1970). The PII 'Opa' variant P9-1 was grown on proteose peptone agar as previously described (Fletcher et al., 1986). For growth in the presence of CMP-NANA, a solution of CMP-NANA (Sigma) was spread onto the surface of proteose peptone agar and allowed to diffuse into the agar to give a final concentration of 50 μg ml⁻¹ (Elkins et al., 1992). The inoculum used was from plates without CMP-NANA and growth was for 18-20 h at 37°C in an atmosphere of 5% (v/v) CO₂.

Outer membranes were prepared by lithium acetate extraction of gonococci followed by differential centrifugation (Fletcher et al., 1986).

mAbs. The mAbs directed against gonococcal LPS which were used were SM82 (IgM) and SM187 (IgM), which have been described previously (Virji & Heckels, 1988), and SM181 (IgM), which was produced and characterized by similar methods. The mAbs SM22 (IgG3) and SM24 (IgG2a) have been characterized previously and shown to be directed against PIB (Fletcher et al., 1986; Butt et al., 1990) and mAb SM50 is directed against the outer-membrane Rmp protein (Virji & Heckels, 1989).

Bactericidal effect. The bactericidal effect of the mAbs was determined using a micro assay based on a previously described method (Virji & Heckels, 1985). Brieﬂy, gonococci were harvested from agar plates and resuspended in complete Dulbecco phosphate buffered saline (Oxoid, PBSB) containing 0.2% (v/v) heat-inactivated foetal calf serum (Flow) to a concentration of approx. 6 × 10⁴ ml⁻¹. The complement source was human serum, from a donor with no history of gonorrhoea, and was stored in aliquots at −70°C until required. The gonococcal suspension (25 μl) was mixed with an appropriate dilution of mAb and freshly thawed complement (10 μl), in a total volume of 100 μl, and incubated in 5% (v/v) CO₂ for 30 min at 37°C. Samples were plated on proteose peptone agar for determination of surviving bacteria, with each estimation performed in triplicate. In control wells antibody or complement were replaced with PBSB or heat-inactivated complement source, respectively.

SDS-PAGE and Western blotting. Whole cell lysates of gonococci grown in either the presence or the absence of CMP-NANA were prepared by mixing samples (1 mg protein ml⁻¹) with an equal volume of sample buffer containing 20% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.2% (w/v) Brilliant Blue G (Sigma) in 50 mM Tris/HCl, pH 6.8. The mixtures were heated at 40°C for 30 min, then 1/5 volume of a 2.5 mg ml⁻¹ solution of proteinase K (Sigma) in sample buffer was added and the incubation was continued at 60°C for 1 h. The samples were then subjected to SDS-PAGE using a discontinuous Tris/Tricine buffer system described by Schagger & von Jagow (1987). The total acrylamide concentrations of the stacking, spacer and separating gels were 4%, 10% and 16.5% (w/v), respectively, of which 3% (w/w) comprised bis-acrylamide. Electrophoresis was carried out at constant voltage at 30 V for 1 h followed by 90 V for 10 h. LPS was directly detected by silver staining using the LPS-specific staining method of Hitchcock & Brown (1983).

For immunological detection, separated LPS was transferred to nitrocellulose paper (Schleicher and Schuell BA85, 0.45 μm) in a Trans-Blot Semi-Dry transfer cell (Bio-Rad) as described previously (McGuinness et al., 1993) but with the omission of SDS from the transfer buffer. Immunological reactivity with
anti-LPS mAbs was detected with alkaline phosphatase-conjugated goat anti-mouse IgM (Bio-Rad) as described previously (Christodoulides et al., 1993).

**ELISA inhibition experiments.** ELISA was carried out as described previously (Fletcher et al., 1986) in polystyrene microtitre plates (Sterilin) which had been coated with outer membranes prepared from *N. gonorrhoeae* strain P9-1 grown in the absence of CMP-NANA. For inhibition experiments a concentration of each mAb giving 50% of the maximum absorbance in ELISA was used. Gonococci were suspended in PBSB to a concentration of approx. 1 x 10^6 cells ml^-1 and 200 μl of the suspension added to the first well of each row of a U-well microtitre plate (Sterilin). The suspension was subjected to twofold dilution across two plates and 100 μl of the appropriate antibody dilution was added to each row. After mixing, the plates were incubated at room temperature for 1 h and then centrifuged at 700 g for 10 min. Supernatant solution (100 μl) was removed from each well and transferred to the corresponding well of an outer-membrane-coated plate and the procedure for standard ELISA was carried out (Fletcher et al., 1986).

**RESULTS**

**Reaction of mAbs with sialylated and non-sialylated LPS**

The effect of growth in the presence of CMP-NANA was examined following SDS-PAGE of gonococcal lysates which had been digested with protease K. Detection of LPS by silver staining (Hitchcock & Brown, 1983) revealed that growth in the presence of CMP-NANA caused a shift to higher apparent M_r (Fig. 1a) which was also usually accompanied by a change in colour from dark to light brown. Preliminary experiments carried out with the addition of [14C]CMP-NANA revealed the incorporation of ^14C into this higher M_r band, confirming the sialylation of the LPS.

The reactivity of the anti-LPS mAbs with sialylated and non-sialylated LPS was determined by Western blot analysis. Blotting with a range of mAbs which had previously been shown to react with LPS from strain P9 produced three different patterns of reactivity, according to whether or not the gonococci were grown in the presence or absence of CMP-NANA. One mAb with each pattern of reactivity was chosen for further studies. Antibody SM82 reacted with non-sialylated LPS but not with sialylated LPS, mAb SM187 reacted with both forms but usually more strongly with the higher M_r band of the sialylated LPS, while SM181 reacted equally well recognizing a band which did not show a M_r shift between sialylated and non-sialylated gonococci (Fig. 1b–d).

**Reaction of mAbs with gonococci containing sialylated or non-sialylated LPS**

ELISA inhibition experiments were performed in order to examine the effect of sialylation of LPS on the reaction of mAbs with native antigens present on the gonococcal surface. Each of the mAbs used reacted in ELISA with outer membranes from gonococci grown in the absence of CMP-NANA. The mAbs were therefore incubated with increasing concentrations of gonococci grown in the presence or absence of CMP-NANA. After allowing reaction of the mAbs with surface-exposed epitopes, the gonococci were removed by centrifugation and the supernatant solutions were used in ELISA with outer-membrane-coated plates. No loss of LPS sialylation, as monitored by SDS-PAGE, could be detected under the conditions of the incubation.

With each mAb, incubation with an increasing concentration of gonococci grown in the absence of CMP-NANA caused increasing inhibition of the outer membrane ELISA, until the reaction was completely inhibited (Fig. 2). The effect of sialylation of LPS on reaction of the LPS and PI mAbs with gonococci varied markedly, although in each case 100% inhibition could be achieved when sufficiently high concentrations of gonococci were used. As might be expected the greatest difference in ELISA inhibition by gonococci grown in the presence of CMP-NANA was seen with mAb SM82 (Fig. 2a), which had failed to react with sialylated LPS on Western blots. The concentration of sialylated gonococci required to produce 50% inhibition of binding of this mAb was approximately 60-fold greater than with non-sialylated bacteria, suggesting that the epitope recognized by SM82 was expressed on the surface of gonococci grown with CMP-NANA at about 1.5% of the level present during growth in its absence. This residual reactivity presumably reflects either failure to sialylate low levels of LPS or some subsequent desialylation. In contrast to SM82, mAbs SM181 and SM187 both reacted on Western blots with LPS components from sialylated gonococci, but ELISA inhibition experiments with these mAbs (Fig. 2b, c) also

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**Fig. 1.** SDS-PAGE and Western blotting of lysates of gonococcal strain P9-1 grown in the presence (+) and absence (−) of CMP-NANA. Whole cell lysates were digested with protease K, separated on Tris-Tricine gels by the method of Schagger & von Jagow (1987) and subjected to silver blotting with mAbs SM82 (b), SM181 (c) and SM187 (d).
revealed a substantial difference in the antibody bound by gonococci grown with CMP-NANA. This suggests that less than 10% of the epitopes recognized by mAbs on conventionally grown gonococci were available for binding on the surface of the gonococci grown with CMP-NANA and that there is steric inhibition of binding.

In contrast to the differences seen with the mAbs directed against LPS, ELISA inhibition experiments with mAbs directed against PI showed little difference whether or not the adsorbing gonococci were grown in the presence of CMP-NANA. Analysis of the inhibition curves failed to detect any difference in the exposure of the epitope recognized by mAb SM24 and only a slight reduction in that recognized by mAb SM22 (Fig. 2d, e). Inhibition experiments were also carried out with the anti-Rmp mAb SM50; although the ELISA inhibition was considerably less than with the other mAbs, there was little difference between sialylated and non-sialylated organisms (Fig. 2f).

**Complement-mediated killing of gonococci grown in the presence or absence of CMP-NANA**

The effect of sialylation of LPS on complement-mediated killing by mAbs directed against LPS and PI was examined. Gonococci grown in the presence or the absence of CMP-NANA were incubated with different dilutions of each of the mAbs in the presence of complement. With the mAbs directed against LPS, a substantial difference was seen between gonococci grown under the different conditions. For example SM82 was highly effective at killing conventionally grown gonococci, with less than 50% survivors at dilutions down to 1:2500, but failed to kill 50% of organisms grown with CMP-NANA even at the highest concentration tested (1:100) (Fig. 3). Similar results were also obtained with mAbs SM181 and SM187.

The mAbs directed against PI were less effective at high concentrations than were the LPS mAbs at killing conventionally grown gonococci but were much less affected by sialylation of the LPS. Thus, both SM22 and SM24 killed 50% of conventionally grown gonococci down to a dilution of 1:2500 and at the same concentration also killed a similar percentage of the bacteria grown with CMP-NANA (Fig. 3).

**Potential effect of blocking antibodies on sialylated gonococci**

Antibodies directed against gonococcal Rmp protein may block the bactericidal effect of antibodies directed against
Effect of sialylation on killing of gonococci

As was previously reported with non-sialylated gonococci (Virji & Heckels, 1988), almost complete inhibition of the bactericidal activity of SM24 was observed at the highest concentration of SM50 tested (Fig. 4).

DISCUSSION

The details of the mechanisms of gonococcal resistance to NHS produced by sialylation of LPS are not yet fully established but must include masking of LPS target sites for bactericidal antibodies, particularly IgM. The use of IgM mAbs directed against LPS in the current study confirms these conclusions and permits more detailed studies which reveal that such epitope masking could occur by more than one mechanism. Western blotting with mAb SM82 revealed that sialylation of LPS results in loss of antibody binding, suggesting that the terminal Galβ1-4GlcNAc receptor for NANA (Mandrell et al., 1990) plays a critical role in determining the epitope recognized by this mAb. Thus sialylation of the LPS results in destruction of the epitope, hence the failure of the antibody to bind to gonococci and, as a consequence, inhibition of bactericidal activity. In contrast mAbs SM181 and SM187 both react on Western blots with LPS from sialylated and non-sialylated gonococci but fail to bind to gonococci grown in the presence of CMP-NANA. This suggests that the epitopes per se are unaffected by sialylation but that the conformation of LPS on the gonococcal surface is such that the addition of NANA causes steric hindrance in the access of the antibodies. Despite differences in the mechanism involved, the failure of the anti-LPS mAbs to bind to the surface of gonococci with sialylated LPS would result in the lack of activation of complement and hence failure to promote killing.

The sialylation of LPS in gonococci implies that at most stages of the infection gonococci are likely to be resistant to killing by anti-LPS antibodies in NHS. This is likely to be particularly applicable to gonococci which enter the circulation. However, despite this resistance disseminated infections are relatively uncommon, except in patients with defects in the later stages of the complement pathway, indicating a protective role for complement-mediated killing (Ross & Densen, 1984). This suggests either that at some point gonococci may lose the protective sialylation or that antibodies directed against other surface antigens are bactericidal even in the presence of LPS sialylation. A likely target antigen for such immune attack is outer-membrane protein PI. Antibodies to PI are found in sera of patients with gonorrhoea (Zak et al., 1984; Ison et al., 1986) and the presence of antibodies against PI has been correlated with serovar-specific protection against gonococcal salpingitis (Buchanan et al., 1980) and also with partial immunity to uncomplicated infection (Plummer et al., 1989).

Other workers have recently studied the effect of LPS sialylation on the bactericidal effect of polyclonal sera raised against PI or fragments derived from it. Wetzler et al. (1992a), using polyclonal antiserum which had been raised by immunization with liposomes containing puri-
fied PIB, observed that sialylation caused no reduction in antibody binding to intact gonococci, but did result in almost complete inhibition of the bactericidal effect of the same serum. They therefore suggested that inhibition of the bactericidal effect was not due to prevention of antibody binding but might be caused by attenuation of the complement cascade by the presence of sialic acid. In contrast, Elkins et al. (1992), who observed that sialylation resulted in a large decrease in the bactericidal effect of antisera raised against a synthetic peptide derived from the N-terminus of PIB, reported a concomitant inhibition binding of anti-PI mAbs and suggested that such epitope masking could account for the inhibition of bactericidal activity. In addition, the same study also showed that sialylation did not inhibit binding of mAbs directed against two different outer-membrane Opa proteins and correspondingly had only a limited effect on bactericidal activity. These observations therefore suggested that the influence of LPS sialylation on bactericidal activity could be largely correlated with the observed effect on antibody binding. The results obtained with the mAbs in the current work are in accord with this conclusion since the binding of the anti-LPS mAbs is inhibited by sialylation and bactericidal activity is inhibited, while sialylation causes no significant difference in binding of the anti-PI mAbs and is accompanied by only a slight reduction in bactericidal activity.

The reasons for the discrepancies in the effect of anti-PI antibodies between each of the three studies may be methodological but are more likely to result from differences in the nature and accessibility of the epitopes recognized by the antibodies. A model for the organization of PIB predicts that a series of conserved regions form trans-membrane β-sheets, generating eight surface exposed hydrophilic loops (van der Ley et al., 1991). Sequence variation between strains is largely confined to two variable regions Var1 and Var2 located at the apices of predicted loops 5 and 6, respectively (Butt et al., 1990). These regions appear to be the most immuno-accessible regions of the protein and are hence responsible for serovar specificity. Epitope mapping studies have shown that the mAbs used in the present study react with peptides corresponding to the Var1 variable region; SM22 recognizes the sequence ^184EYEH^197 at the apex of loop 5 in strain P9 while SM24 recognizes the adjacent sequence ^185SIPS^199 (Butt et al., 1990). It is therefore possible that sialylation of LPS might hinder access to other less accessible regions of the protein but not exert such an effect on these mAbs directed against the most exposed region of the protein. In the studies of Elkins et al. (1992), the antisera which showed inhibition of killing of sialylated gonococci was raised against a synthetic peptide corresponding to residues 6–32, which includes the predicted loop 1. This region is conserved between different serovars of PIB, appearing to be less subject to immune surveillance and hence less surface exposed (Butt et al., 1990; van der Ley et al., 1991). Thus the difference in the effect of LPS sialylation on recognition and killing by the different antibodies may be explained by the fact that the mAbs used in the current study recognize the most exposed region of the protein and are thus less susceptible to any steric hindrance to binding caused by sialylation of LPS.

It is also interesting to note the effect of LPS sialylation on the biological properties of the anti-Rmp mAb SM50. Antibodies directed against Rmp, present in the sera of patients after gonococcal infection, block the normal bactericidal effect of NHS on non-sialylated gonococci (Rice et al., 1986). Similarly mAb SM50 has been shown to block the bactericidal effect of mAbs directed against both LPS and PI (Virji & Heckels, 1988). Although binding of SM50 to whole cells in the current study was considerably less than observed with the other mAbs, presumably because the protein is present on the surface in lower quantities than PI (Judd, 1982), there was little difference between sialylated and non-sialylated organisms. Thus the continued blocking effect of mAb SM50 on the bactericidal effect of the anti-PI mAb, seen with sialylated gonococci in the current study, is consistent with the observed antibody binding properties. Indeed the observation that the epitope recognized by SM50 lies in the region of Rmp which is believed to form a surface exposed loop on the gonococcal surface (Gotschlich et al., 1987a; Virji & Heckels, 1989) again suggests the importance of surface accessibility of epitopes for continued biological activity in the presence of sialylation of LPS.

Because of its stability and immunological properties, previous studies have identified PI as a potential candidate antigen for immunization against gonococcal infection (Virji et al., 1986; Elkins et al., 1992; Wetzler et al., 1992b). The most frequent serious sequelae of gonococcal infection are locally invasive complications, such as salpingitis and prostatitis, which are therefore the most important targets for a potential gonococcal vaccine. The strains responsible for these complications are typical of urogenital isolates (Brunham et al., 1985) which display the unstable serum resistance (Ward et al., 1970) shown by strain P9 used in the current study. Thus despite the potentially inhibitory presence of the LPS sialylation which is likely to be found in vivo, the current study shows that PI remains a potential vaccine target. Conversely the presence of Rmp in a potential vaccine may be antagonistic to the development of effective immunity because of its capacity to elicit antibodies which block anti-PI bactericidal activity (Wetzler et al., 1992b), even in the presence of LPS sialylation. These conclusions are also supported by recent studies which have demonstrated that the presence of anti-PI antibodies in individuals exposed to infection can be correlated with resistance to infection while the presence of anti-Rmp antibodies predisposes to infection (Donegan et al., 1994). It is likely that an effective potential immunization strategy would need to focus those epitopes which are least susceptible to the potential inhibitory effect of LPS sialylation.

ACKNOWLEDGEMENTS

This work was supported by a Medical Research Council Project Grant.
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Received 11 July 1994; revised 25 October 1994; accepted 21 December 1994.