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Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*

Jos P.M.van Putten

Max-Planck-Institut für Biologie, Abt. Infektionsbiologie, Spemannstrasse 34, D-72076 Tübingen, Germany

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Phase variation of *Neisseria gonorrhoeae* lipopolysaccharide (LPS) controls both bacterial entry into human mucosal cells, and bacterial susceptibility to killing by antibodies and complement. The basis for this function is a differential sialylation of the variable oligosaccharide moiety of the LPS. LPS variants that incorporate low amounts of sialic acid enter human mucosal epithelial cells very efficiently, but are susceptible to complement-mediated killing. Phase transition to a highly sialylated LPS phenotype results in equally adhesive but entry deficient bacteria which, however, resist killing by antibodies and complement because of dysfunctional complement activation. Phase variation of *N.gonorrhoeae* LPS thus functions as an adaptive mechanism enabling bacterial translocation across the mucosal barrier, and, at a later stage of infection, escape from the host immune defence.

Key words: complement-mediated killing/virulence/LPS variation/*Neisseria gonorrhoeae*/sialic acid

Introduction

Many bacterial pathogens, including *Neisseria gonorrhoeae*, are endowed with sophisticated mechanisms to adapt to a rapidly changing microenvironment in the host. One such system is the reversible switching of the expression of cell surface components (phase variation) (Meyer *et al.*, 1990). It is increasingly recognized that this phenotypic variation not only acts as a means of evading the host immune response but also enables the bacteria to go through different stages of the infectious process (Meyer and van Putten, 1989; Robertson and Meyer, 1992). A good example of the functional implications of phase variation are the gonococcal opacity proteins. The gonococcus can express a repertoire of 11 different opacity proteins independently of each other, but only one renders them able to invade mucosal epithelial cells (Makino *et al.*, 1991; Weel *et al.*, 1991a), whereas the others preferentially interact with different cell types (Virji and Heckels, 1986; Kupsch *et al.*, 1993). This switching in opacity protein expression correlates with the course of a natural infection (Swanson *et al.*, 1988).

Another major gonococcal surface component that shows molecular and antigenic heterogeneity within a single bacterial strain is the lipopolysaccharide (LPS) (Apicella *et al.*, 1987; Schneider *et al.*, 1988; Weel *et al.*, 1989). This principal bacterial glycolipid consists of a hydrophobic lipid backbone (lipid A) and variable hydrophilic core oligosaccharide moieties, some of which mimic human cell membrane glycosphingolipids (Mandrell, 1992). A single bacterium can produce a limited repertoire of different LPS molecules, and the degree to which each of the LPS molecules are produced varies and ultimately determines the LPS phenotype (Schneider *et al.*, 1988). Phenotypic variation of LPS thus reflects interconversion of the LPS that is predominantly expressed. This phase variation of LPS molecules, which has also been found in other mucosal pathogens like *Neisseria meningitidis* (Tsai *et al.*, 1983), *Bordetella pertussis* (Ray *et al.*, 1991) and *Haemophilus influenzae* (Moxon and Maskell, 1992), occurs spontaneously at a frequency of 0.2–0.02% (Schneider *et al.*, 1988) and can be monitored by an altered antibody reactivity, and different migration of the LPS molecules in SDS–PAGE. Recent evidence indicates that there is an as yet unexplained relationship between the expressed LPS phenotype and the stage of a natural infection (Schneider *et al.*, 1991).

In addition to the intrinsic structural variation of gonococcal LPS which results from changes in LPS biosynthesis, LPS can also be externally modified by the sialylation of terminal sugar residues (for review, see Smith, 1991). This event, which occurs *in vivo* (Apicella *et al.*, 1990; Parsons *et al.*, 1990), renders the bacteria resistant to killing by normal human serum by supposedly masking the LPS target site for 'natural' bactericidal antibodies (Parsons *et al.*, 1989). It also impairs adherence and thus killing by polymorphonuclear phagocytes (Kim *et al.*, 1992; Rest and Frangipane, 1992). The covalent transfer of sialic acid to LPS is mediated by a bacterial sialyltransferase using the natural sialyl donor cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) as a substrate (Mandrell *et al.*, 1990). Data from experimental infections in human volunteers (Schneider *et al.*, 1991) and in animal models (Demarco de Hormaeche *et al.*, 1988) suggest that the sialylation event varies with the quality of the LPS, but the biological significance of this relationship has not been established.

The present study aimed to provide a functional basis for the observed phenotypic variation of LPS in the pathogenesis of gonococcal disease. Using a set of isogenic LPS variants and taking advantage of the ability to sialylate LPS exogenously, a series of *in vitro* infection experiments was designed to determine the involvement and possible functional role of LPS phase variation and sialylation in the infection. The results provide evidence that sialylation of LPS controls the entry of the bacteria into human mucosal cells and their bactericidal killing by antibodies and complement in a LPS phase variation dependent fashion, indicating that LPS phase variation functions as an adaptive strategy of bacteria enabling passage across the mucosal barrier and, at a later stage of infection, evasion of the human host defence.

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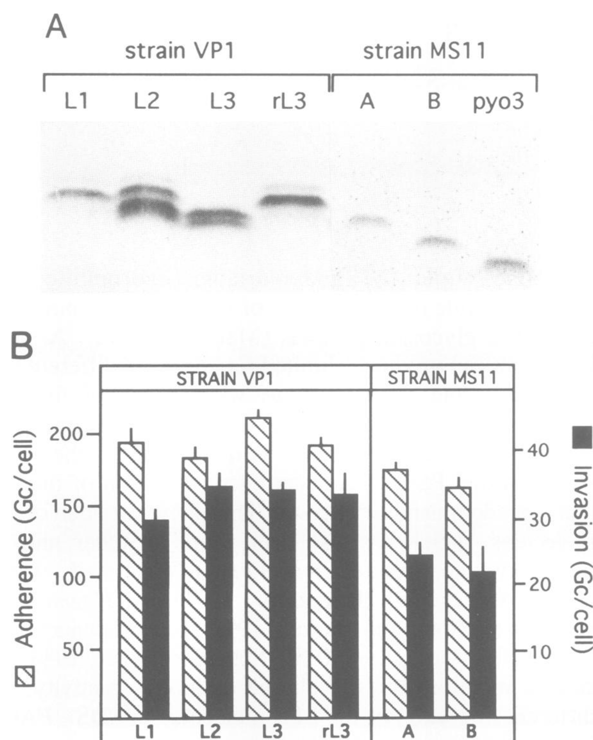

Fig. 1. Intrinsic structural phase variation of gonococcal LPS and its effect on bacterial adherence to and entry into epithelial cells. (A) SDS−PAGE migration patterns of LPS purified from three spontaneous LPS variants (L1, L2 and L3) and one revertant (rL3) of strain VP1, and from two variants of strain MS11 (A and B). MS11 pyo3 was obtained by pyocin selection. LPS was visualized by silver staining. (B) Adherence to and invasion of the isolated LPS variants into cultured Chang human conjunctiva epithelial cells (6 h of infection). The number of adherent and intracellular gonococci (Gc) was determined using light microscopy as described in Materials and methods. Values are mean ± SE of five experiments. Similar results were obtained with ME-180 human cervical cells and PC-3 human prostate cells. HEp-2 larynx cells were barely infected by the bacteria (see also Figure 3).

Table I. Antigenic characteristics of the different isogenic LPS variants as determined by immuno-electron microscopy

	Strain VP1				Strain MS11	
	L1	L2	L3	rL3	A	B
D6A	−	−	+	−	−	+++
8B2E	+	−	−	−	+++	−
7B1E	+++	+++	+++	+++	−	−
4D6B	+++	+++	−	+++	+++	−
2-1-L8	−	−	+	−	−	+++
06B4	−	−	+	−	+++	−
1-1M	+++	+++	−	+++	+++	−

MAbs 1-1M and 06B4 react with the phase variable terminal βGalNAc and βGal residue at the non-reducing end of the lacto-*N*-neotetraose-bearing oligosaccharide moiety, respectively (Kerwood *et al.*, 1992). MAb 2-1-L8 and probably also MAb D6A recognize epitopes within the lacto-*N*-neotetraose-bearing carbohydrate chain (Kim *et al.*, 1988; Kerwood *et al.*, 1992). MAb 7B1E recognizes the terminal Galα1-4Gal carbohydrate moiety (J.P.M.van Putten, not shown). The variable epitopes 8B2E and 4D6B epitopes have not been mapped. +++ positive; + weakly positive; − negative.

Results

Effect of intrinsic gonococcal LPS variation on the interaction of gonococci with human mucosal cells

As a first step in establishing the functional significance of LPS phase variation in *N.gonorrhoeae*, a number of spontaneous isogenic LPS variants from the parental strains VP1 and MS11 was selected using colony immunoblotting with monoclonal antibodies of different LPS specificity. In this way, three different variants of strain VP1 and two variants of strain MS11 were obtained. Further propagation of individual colonies and repetition of the selection procedure resulted in some bacterial progeny that had regained the original immunoreactivity (revertants). The frequency of spontaneous switching between the different phenotypes was estimated to be ~0.05−1%. The structural phase variation of LPS was reflected in heterogeneity in the LPS migration pattern of the different isogenic variants in SDS−PAGE (Figure 1A), and confirmed at the single cell level by immuno-electron microscopy using LPS phenotype-specific monoclonal antibodies. Each of the selected variants consisted of >95% bacteria of a single LPS phenotype (not shown). The antigenic characteristics of the variants are summarized in Table I.

The possible functional implications of LPS phase variation in the early interaction of the bacteria with host cells was evaluated in a number of *in vitro* infection experiments. Human epithelial cells of different origins (conjunctiva, cervix, prostate and larynx) were inoculated with the variants and the adhesion and entry of the bacteria into the host cells were assessed with time. All LPS variants expressing the invasiveness conferring opacity protein (Makino *et al.*, 1991; Weel *et al.*, 1991a) showed similar adherence and invasive properties, resulting in a mean number of intracellular bacteria of 20−30 per cell after 6 h of infection, except for the HEp-2 cells which were barely susceptible to gonococcal infection (Figure 1B). These data indicate that the intrinsic spontaneous LPS phase variation has no direct influence on the bacteria−host cell interaction.

External modification of gonococcal LPS

Addition of CMP-[^{14}C]NANA, a substrate for the gonococcal sialyltransferase, to the parent strains VP1 and MS11 resulted in a rapid incorporation of radiolabel into the bacteria. After 10 min gonococci contained up to 10^6 sialic acid residues per bacterium. This reaction could be specifically inhibited by the addition of CMP (5 mM), a competitive inhibitor of sialyltransferases (Scudder and Chantler, 1981), and the radioactivity could be removed by neuraminidase treatment of the cells, indicating that it was [^{14}C]NANA and not CMP-[^{14}C]NANA that was transferred on to the bacteria (Gottschalk, 1972) (data not shown). SDS−PAGE analysis of LPS purified from the different LPS variants grown in the absence and presence of CMP-NANA demonstrated that the covalent transfer of NANA resulted in a reduced migration of one or more LPS bands in all of the LPS variants (Figure 2).

Inhibitory effect of CMP-NANA on bacterial entry into cultured epithelial cells

Infection of cultured Chang epithelial cells with gonococcal parent strain VP1 (LPS type L1) in the presence of CMP-NANA (40 nmol) resulted in a dramatic reduction in the number of intracellular bacteria, but only an insignificant decrease in adherence (Figure 3A). This inhibition was

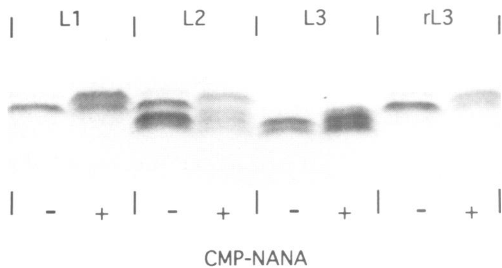

Fig. 2. External LPS modification of LPS variants by sialylation as demonstrated by electrophoretic analysis of purified LPS derived from bacteria grown in the absence and presence of 20 nmol/ml CMP-NANA for a period of 3 h. LPS was separated by tricine SDS–PAGE (15% gel) and stained with silver.

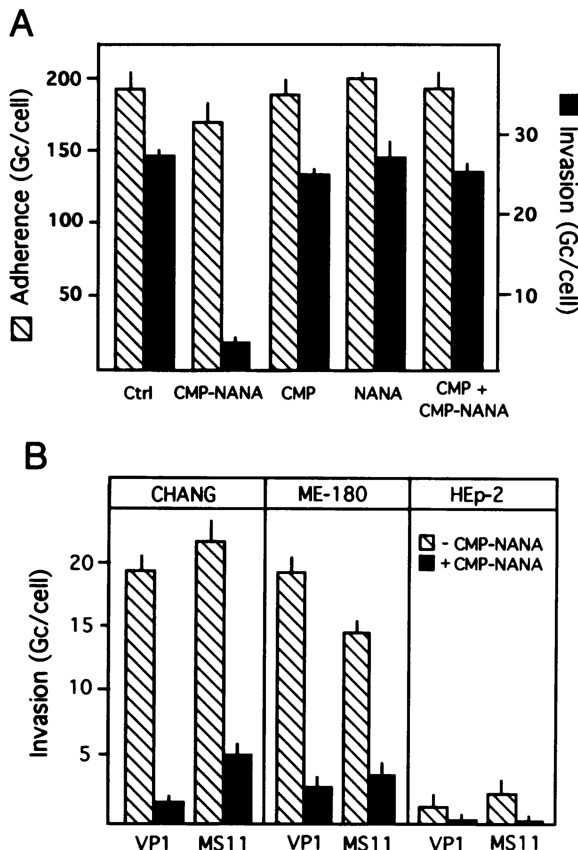

Fig. 3. (**A**) Adherence to and entry of *N.gonorrhoeae* (strain VP1-L1) into Chang epithelial cells in the absence and presence of CMP-NANA (40 nmol), CMP (5 mM), NANA (100 nmol) or a mixture of CMP-NANA and CMP (6 h of infection). Adherence and entry of the bacteria were scored by light microscopy. Data are from one of three experiments. (**B**) Effect of CMP-NANA on the entry of two gonococcal strains (VP1-L1 and MS11-A) into three mucosal cell types of different origin (6 h of infection). Efficient blocking of bacterial entry was obtained in two of the three cell lines ($n = 5$; $P < 0.005$). HEp2 cells were only barely infected by gonococci, even in the absence of CMP-NANA.

absent when either CMP (5 mM) or NANA (0.1 mM) were added. Neither of these are substrates for the sialyltransferase. The inhibitory effect of CMP-NANA was almost totally abolished by the addition of a high concentration of CMP (5 mM), suggesting that (bacterial or host cell-derived) sialyltransferase activity was indeed involved in the specific inhibition of bacterial entry (Figure 3A). The inhibitory effect of CMP-NANA was

Table II. [^{14}C]NANA incorporation-deficient gonococci exhibit unaltered invasiveness in the presence of CMP-NANA

	MS11 parent	MS11 pyo3	*P*-value
[^{14}C]NANA bound (c.p.m.)	4630 ± 514	272 ± 51	$P < 0.001$
Invasion (Gc/cell)			
without CMP-NANA	23.6 ± 2.3	19.0 ± 0.2	NS
with CMP-NANA	3.8 ± 0.9	15.2 ± 1.7	$P < 0.005$

The pyocin-sensitive MS11 parent (LPS type A) and the resistant variant MS11 pyo3 were tested for incorporation of radiolabelled NANA (10 min, 37°C) and at the same time for their invasiveness into cultured Chang epithelial cells (6 h of infection) in the absence and presence of CMP-NANA (40 nmol). The lack of NANA incorporation in MS11 pyo3 is associated with a loss of the inhibitory effect of CMP-NANA on the bacterial entry, indicating that it is the sialylation of LPS that blocks bacterial entry ($n = 6$).

concentration dependent, with half-maximal inhibition at ~80 pmol/ml and maximum inhibition at 400 pmol/ml, which is slightly above the supposed concentration of CMP-NANA in normal human blood (70 pmol/ml) (Nairn *et al.*, 1988). At a lower bacterial inoculum (10^5 instead of 10^6 bacteria) only 40 pmol of CMP-NANA sufficed to block bacterial entry completely (data not shown). The general validity of the effect was demonstrated when identical results were obtained with strain MS11 (LPS type A) and with epithelial cells of different origin (Figure 3B).

Evidence that sialylation of LPS is responsible for the inhibition of gonococcal entry

The nature of the inhibitory effect of CMP-NANA was further analysed by pre-exposing either the host cells or the bacteria to the compound before the start of the infection experiment. Preincubation of the host cells (Chang epithelial cells) for a period of 1 h had no effect on the subsequent bacteria–host cell interaction. In contrast, preincubation of the bacteria (1 h, 37°C) resulted in a marked inhibition of the bacterial entry with 9.0 ± 1.5 and 30.4 ± 0.8 intracellular bacteria with and without bacterial preincubation, respectively (6 h of infection; $n = 4$; $P < 0.01$). Control experiments in which epithelial cells were exposed to radiolabelled CMP-NANA (0.4 nmol, 6 h, 37°C) confirmed that the compound was not incorporated into host cell components (data not shown).

To ascertain that sialylation of LPS was indeed responsible for the inhibition of bacterial entry, LPS variants were selected that lacked the NANA acceptor site using pyocin selection (Morse *et al.*, 1976). This procedure is based on the recognition by pyocin of specific carbohydrate structures in the LPS, resulting in killing of bacteria that express such an epitope, thus selecting for variants expressing spontaneous truncated LPS structures lacking the recognition site (John *et al.*, 1991). By this method, totally NANA incorporation deficient phenotypes lacking the sialylatable LPS band visible in SDS–PAGE, were obtained for strain MS11 (Table II). Infection experiments with these pyocin resistant bacteria, which still exhibited sialyltransferase activity (not shown), in the absence and presence of CMP-NANA showed that the loss of sialylation in these bacteria was accompanied by a loss of the inhibitory effect of CMP-NANA on the entry process, indicating that sialylation of LPS is responsible for this effect (Table II). Experiments with a genetically defined

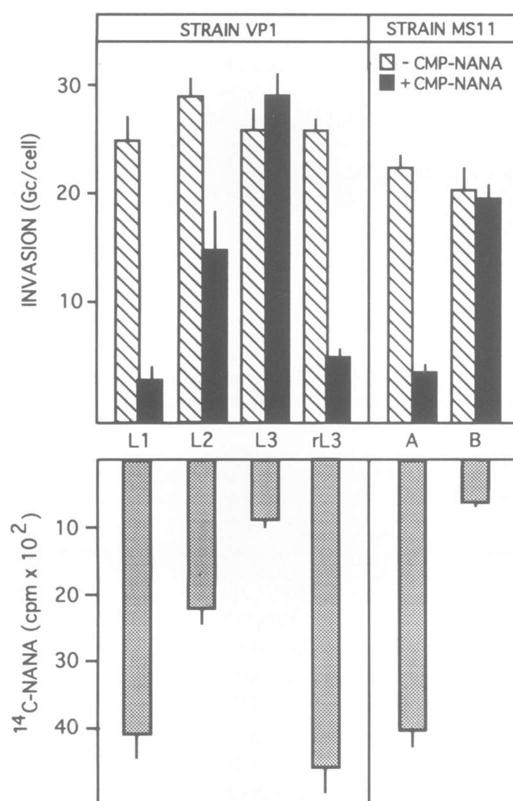

Fig. 4. Phase variation-associated external modification of gonococcal LPS directs the invasiveness of LPS variants in the presence of CMP-NANA. **Upper panel**: adherence to and entry of isogenic LPS variants of strain VP1 and MS11 into Chang epithelial cells in the absence and presence of CMP-NANA (40 nmol) (6 h of infection). A marked LPS phenotype dependent differential inhibition of bacterial entry was observed with both strains ($P < 0.001$ for L1 and A; $P < 0.05$ for L2). Adherence was not significantly affected (not shown). Values are mean ± SE of six experiments. **Lower panel**: incorporation of [^{14}C]sialic acid into the LPS of the different LPS variants. The amount of radioactivity incorporated (10 min, 10^7 bacteria) was inversely related to the invasiveness of the bacteria. Values are the mean ± SE of eight experiments. *P*-values: $P < 0.001$ for L3 compared with L1; $P < 0.005$ for L2 and B compared with L1 and A, respectively.

LPS mutant (*galE* mutant) lacking the NANA acceptor site (Robertson *et al.*, 1993), confirm these results (not shown).

Differential sialylation of LPS phase variants controls the bacterial entry process

The observation that sialylation of LPS, an event which also occurs *in vivo* during the natural infection (Apicella *et al.*, 1990; Parsons *et al.*, 1990), inhibits bacterial entry, seems to be at variance with the proposed definitive role that opacity protein phase variation plays in controlling gonococcal entry into epithelial cells (Makino *et al.*, 1991; Weel *et al.*, 1991a). This problem was solved when each of the isolated LPS variants was evaluated for its invasiveness in the presence of CMP-NANA. Surprisingly, although all VP1 variants could be sialylated (Figure 2), a considerable difference in the inhibitory effect of CMP-NANA on bacterial invasion was observed among the variants with entry of variant L1 being 95% inhibited, variant L2 ~45% and variant L3 not inhibited by sialylation (Figure 4). Similar results were obtained with the isogenic LPS variants from strain MS11 with 90% and 8% inhibition of entry for variants A and B, respectively (Figure 4). Quantitation of the

Table III. LPS variation as an adaptive mechanism enabling gonococcal entry into epithelial cells in the presence of CMP-NANA

	Invasion (Gc/cell)	LPS phenotype (%)	
		VP1-L1	VP1-L3
VP1-L1			
without CMP-NANA	36.5	99	1
with CMP-NANA	1.7	78	22
VP1-L3			
without CMP-NANA	34.6	0	100
with CMP-NANA	38.1	0	100
VP1-L1 + L3			
without CMP-NANA	35.0	48	52
with CMP-NANA	18.2	4	96

Chang epithelial cells were infected with VP1-L1, VP1-L3 or a 1:1 mixture of both LPS variants in the absence or presence of CMP-NANA (40 nmol). After 6 h the cells were washed, exposed to gentamicin (100 μg/ml) for 2 h, lysed using saponin (1% in PBS for 15 min), and the intracellular bacteria were plated and, after overnight growth, analysed by colony-immunoblotting using the LPS-L1 and LPS-L3-specific antibodies 4D6B and D6A, respectively. In parallel experiments, invasion of the bacteria into Chang epithelial cells (6 h of infection) was quantitated using light microscopy. Values are from one of four experiments.

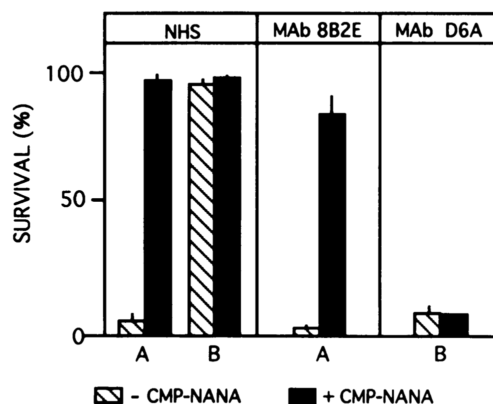

Fig. 5. LPS phenotype dependent complement-mediated killing of *N.gonorrhoeae* strain MS11 in the absence or presence of CMP-NANA (20 nmol/ml). CMP-NANA was added to the bacteria 3 h before the addition of either 10% fresh human serum (NHS) or monoclonal antibody 8B2E (reactive with variant A) or D6A (reactive with variant B) + 10% human gamma globulin-free serum to allow sialylation of the LPS to occur. Note that CMP-NANA prevents bactericidal killing of the MS11 phenotype A but not B. Values are the mean ± SE of four experiments.

incorporated sialic acid in the individual isogenic LPS variants using CMP-[^{14}C]NANA as a sialyl donor revealed an LPS variant dependent differential sialylation of the bacteria, with variant L1 showing high incorporation of sialic acid, L2 intermediate incorporation and L3 only minor incorporation (Figure 4, lower panel). Thus, the number of sialic acid residues on the bacterial surface rather than the sialylation event itself, appear to control directly the invasiveness of the bacteria. In strain MS11 the phase variation resulted in an almost total loss of NANA acceptor sites in variant B (Figure 4, lower panel), thus explaining the high invasiveness of this variant even in the presence of CMP-NANA (Figure 4, upper panel).

To confirm that LPS phase variation directs the invasiveness of *N.gonorrhoeae*, epithelial cells were infected

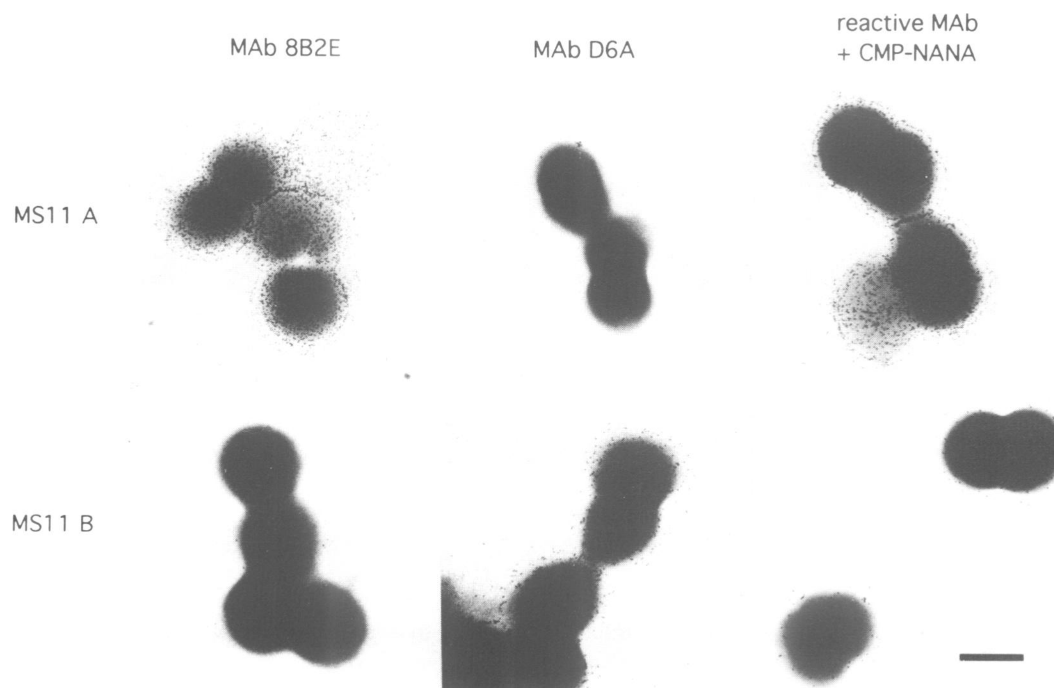

Fig. 6. Immunoelectron micrographs illustrating the accessibility of the gonococcal LPS epitopes 8B2E and D6A in bacteria grown in the absence and the presence of CMP-NANA (20 nmol/ml, 3 h, 37°C). The antibodies were visualized using protein A−gold (10 nm particles). Bar: 0.5 μm.

with a heterogeneous population of LPS variants of strain VP1 in the absence and presence of CMP-NANA and the intracellular bacteria were re-isolated using the gentamicin killing assay (Makino *et al.*, 1991). Colony immunoblotting, incorporation of [^{14}C]NANA, and SDS−PAGE analysis of the surviving, and thus invasive bacteria demonstrated that in the presence of CMP-NANA only variants that were barely sialylated had entered the host cells, whereas in the absence of CMP-NANA the same distribution of LPS variants was found as in the inoculum (Table III). Infection experiments using inocula containing different ratios of high and low sialylatable variants confirmed the preferential entry of low sialylatable variants, and in addition demonstrated that the number of intracellular bacteria recovered decreased as the number of inhibitable LPS variants increased, which is consistent with their equal and apparently competitive adherence capabilities. Together these data indicate that phase variation of LPS functions as an adaptative mechanism allowing only certain variants to enter epithelial cells in the presence of the natural sialyl donor CMP-NANA.

Adverse effects of LPS variation on complement-mediated killing of N.gonorrhoeae

The selective entry of low-sialylatable LPS phenotypes of gonococci into mucosal cells raises the question of the biological significance of the highly sialylatable variants. This point was addressed by testing the resistance of the isolated LPS phase variants to natural human serum, bearing in mind that sialylation of gonococcal LPS has been demonstrated to confer serum resistance on bacteria by blocking the target site for natural bactericidal antibodies (Parsons *et al.*, 1989; Griffiss *et al.*, 1991). Since VP1 is intrinsically serum resistant (a stable, non sialylation-related form of serum resistance; Rice, 1989), the serum-sensitive strain MS11 was used in these experiments. Exposure of MS11 variant A to fresh human serum for 1 h resulted in the expected CMP-NANA inhibitable complement-mediated killing of the bacteria (Figure 5). The B variant however, survived the serum incubation both in the absence and presence of CMP-NANA (Figure 5), suggesting that the LPS of this variant either lacks the appropriate acceptor site for the antibody or interferes with complement deposition (Rice, 1989). This was further examined using bactericidal monoclonal antibodies differing in LPS specificity. Immuno-electron microscopy in conjunction with bactericidal assays using the MAbs 8B2E and D6A demonstrated that the LPS phase transition from variant A to B resulted in loss of the 8B2E epitope and gain (or unmasking) of the D6A epitope (Figure 6) and that both antibodies conferred complement-mediated killing in the reactive LPS variants (Figure 5), indicating that intrinsic LPS phase variation alters epitopes that are recognized by bactericidal antibodies, but does not interfere with complement-mediated killing. In contrast, in the presence of CMP-NANA, variant A, but not variant B was protected against bactericidal activity of the appropriate antibody (Figure 5), though antibody binding was not influenced by sialylation (Figure 6). These data indicate that the degree of sialylation, which is much higher in variant A than in variant B, and consequently the LPS phenotype expressed, determines the resistance of the bacteria to killing by antibodies and complement, and that this effect results from a non-functional complement deposition rather than from blocking of antibody binding. Together these findings suggest that LPS phase variation-directed transition from a low sialylatable phenotype (variant B) to a high sialylatable phenotype (variant A) may act as a mechanism directing interconversion of an invasive and an immuno resistant phenotype of *N.gonorrhoeae*.

Discussion

Gonococcal infection of the human genital tract involves bacterial colonization of the mucosal surface, invasion and disruption of the mucosal barrier, and, in most cases, the development of a symptomatic exudative inflammatory response (Cohen and Sparling, 1992). In this report evidence is provided that structural phase variation of *N.gonorrhoeae* LPS may act as an adaptive mechanism enabling the pathogen both to enter into mucosal epithelial cells and to resist the initial human immune defence, both prerequisites for establishing infection. The biological significance of the phenotypic variation first became apparent in the presence of the natural sialic acid donor CMP-NANA, which can be used by the gonococci to sialylate LPS. In the presence of this compound, intrinsic LPS variation allows a differential sialylation of the expressed LPS molecules, and thus a reversible switching between an invasive, barely sialylated phenotype that is susceptible to complement-mediated killing and a non-invasive, highly sialylated phenotype that is not killed by antibodies and complement.

The proposed central role for intrinsic LPS variation in gonococcal pathogenesis, conferred by differential sialylation of the LPS, is achieved by the predominant expression of none, one or multiple LPS molecules, some bearing a NANA acceptor site (cf. Figure 2). Sialylation of LPS has been demonstrated to involve the covalent transfer of a single sialic acid to a lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc)-bearing carbohydrate structure at one of the non-reducing termini of the LPS (Mandrell *et al.*, 1990; Robertson *et al.*, 1993). The present identification of both non-sialylated and multiple sialic acid-containing LPS molecules in the different variants (Figure 2) and the antigenic profile of these variants (Table I), indicate that LPS variation in the strains used involves variable expression of the lacto-*N*-neotetraose-bearing moiety, as well as variation elsewhere in the LPS molecule, which leaves the putative NANA acceptor site(s) intact. This finding supports the view that gonococcal LPS phase variation involves a reversible structural variation of mainly the oligosaccharide moieties of the molecule, with the possibility that multiple types of molecules can be expressed by a single bacterium at one time (Apicella *et al.*, 1987; Griffiss *et al.*, 1987; Weel *et al.*, 1989; John *et al.*, 1991). Variable expression of LPS molecules more or less truncated at their non-reducing termini explains the observed heterogeneity in LPS migration pattern in SDS–PAGE, sialylation and antibody reactivity among the isolated variants.

The molecular mechanism behind the structural diversity of LPS, is not known. In *H.influenzae*, which exhibits a comparable type of LPS phase variation, LPS phase switching has been attributed to several distinct, but interactive mechanisms one of which is a translational frame shift caused by changes in the number of tandem (CAAT)-repeats within the open reading frame of the first of several downstream LPS biosynthesis genes (Weisser *et al.*, 1989; Moxon and Maskell, 1992). Evidence for the existence of a similar type of regulation in other mucosal pathogens including the pathogenic *Neisseria* species, has not been reported. In both *H.influenzae* and *N.gonorrhoeae* the LPS phenotype is influenced by environmental conditions and the growth rate of the bacteria (Norrod *et al.*, 1982; Langford and Moxon, 1992). It is possible that alterations in environmental signals present at different stages of an infection lead to the expression or selection of discrete LPS phenotypes. One such signal may be the availability of glucose, which influences LPS biosynthesis of *N.gonorrhoeae in vitro* probably by a shift in the glucose and energy metabolism of the bacteria (Morse *et al.*, 1983).

A major conclusion from this work is that a specific LPS phenotype is required for gonococcal entry into mucosal cells. So far, LPS phase variation has not been implicated as a virulence factor modulating gonococcal entry into epithelial cells. On the contrary, bacterial invasion has been associated with the expression of another phase variable surface component: the opacity proteins. Dependent on the variant opacity protein expressed, gonococci predominantly interact with either epithelial cells or professional phagocytes irrespective of their LPS type. These results were achieved in the absence of CMP-NANA (Makino *et al.*, 1991; Weel *et al.*, 1991a). Demonstration of the key role of LPS in gonococcal invasion requires, as described in this report, more natural infection conditions including the presence of CMP-NANA. The importance of LPS variation in bacterial entry, particularly illustrated by the selective entry of a specific LPS variant from a heterogeneous population (Table III), implies, together with the previously reported influence of opacity protein phase variation (Makino *et al.*, 1991; Weel *et al.*, 1991a), that in the natural infection concurrent phase switching of multiple surface components may be required to create an invasive gonococcal phenotype.

Gonococcal entry into human epithelial cells involves the formation of opacity protein-associated intimate contact between the bacterial and host cell membranes, accompanied by changes in immunoreactivity of the gonococcal membrane pore protein I (P.I), which has been demonstrated to translocate into host cell membrane and to interfere with cell signalling pathways (Lynch *et al.*, 1984; Haines *et al.*, 1991; Weel and van Putten, 1991; Weel *et al.*, 1991a,b). This close contact is followed by microfilament dependent endocytosis of bacteria (Makino *et al.*, 1991). There are several conceivable mechanisms of how sialylation of LPS might interfere with these events. One is that sialylation increases the overall negative charge of the bacteria (Swanson, 1991), thus preventing close contact with the negatively charged host cell surface, as has been proposed for the antiphagocytic effect of bacterial capsules (Smith, 1977). Arguments against this hypothesis are that sialylated gonococci show a typical opacity protein dependent adherence which can compete with that of their non-sialylated counterparts, and that centrifugation of bacteria on to the host cells does not overcome the inhibitory effect of sialylation (data not shown). An alternative explanation is that sialylation allows opacity protein mediated adherence but that it prevents further receptor interactions and/or the insertion of the pore protein P.I into the host cell membrane. The immunoaccessibility of the P.I protein and its meningococcal homologue at the cell surface depends on the quality of the LPS (Poolman *et al.*, 1988; Judd and Shafer, 1989). A mechanism in which the oligosaccharide moiety of the LPS molecule itself is involved in the engulfment of the bacteria, and where sialic acids mask the appropriate structures seem less likely considering the recent finding that a genetically defined mutant lacking the non-reducing termini on the LPS retained its invasive capabilities (Robertson *et al.*, 1993).

Another basic outcome from the present study is that LPS

variation converts bacteria from an invasive phenotype to one that is resistant to host immune defences. This total change in bacterial behavior, which is probably crucial in establishing an infection, is achieved by several mechanisms. First, the phase switch leads to a masking of at least one LPS epitope (D6A) that is a target for bactericidal activity even in the presence of CMP-NANA (Figure 5). Second, the novel, highly sialylatable phenotype exhibits increased expression of a lacto-*N*-neotetraose-bearing moiety that may mimic mammalian glycosphingolipids (Table I), and thus will not be immunogenic. Third, this phenotype resists complement-mediated bactericidal activity of antibodies directed against LPS (Figure 5) and other surface components (Elkins *et al.*, 1992; Wetzler *et al.*, 1992), by a dysfunctional activation of the complement cascade. Moreover, recent studies indicate that sialylation also impairs attachment to and thus killing by polymorphonuclear cells (Kim *et al.*, 1992; Rest and Frangipane, 1992), allowing evasion from this type of host defence as well. The transition to a gonococcal phenotype that resists killing by complement and phagocytes and is poorly immunogenic upon development of an inflammatory response may explain the lack of protective immunity against the bacterium in the natural disease.

The current concept that LPS phase transitions are an essential feature for bacteria to cross the mucosal barrier and resist the host inflammatory response by allowing differential sialylation, requires that the sialylation event occurs *in vivo* during the natural infection. Several lines of evidence suggest that indeed this is the case. First, the sialic acid donating compound CMP-NANA is present in human serum, professional phagocytes and genital secretions at a concentration high enough for LPS sialylation (for review, see Smith 1991). Second, fresh clinical isolates of gonococci are resistant to killing by normal human serum and this resistance can be abolished by treating the bacteria with neuraminidase, which removes sialic acids from the cell surface (Parsons *et al.*, 1990). Third, immuno-electron microscopic analysis of infected tissues from patients with a symptomatic gonococcal infection unequivocally demonstrates a neuraminidase-sensitive masking of the putative NANA acceptor site on the LPS of the bacteria (Apicella *et al.*, 1990). Additional strong support for the biological relevance of the present data is that they provide a rationale for the so far unexplained LPS phase switch observed *in vivo* during experimental gonococcal infection of human volunteers (Schneider *et al.*, 1991). In the initial stage of the infection, in these studies, only non-sialylatable and thus, as demonstrated here, potentially invasive and complement-killing susceptible LPS variants are isolated, whereas after the development of an inflammatory response, other, highly sialylatable LPS phenotypes appear, which represent an immuno resistant gonococcal phenotype.

LPS variation is a common characteristic of many mucosal pathogens, including *N.meningitidis*, *H.influenzae* and *B.pertussis*, and *in vivo* infection data suggests that this variation is linked to virulence. Meningococcal and *H.influenzae* isolates from the human nasopharynx and from systemic sites of infection often differ in LPS phenotype (Mertsola *et al.*, 1991; Jones *et al.*, 1992), and experiments in an animal model using genetically defined LPS mutants of *H.influenzae* lacking phase-variable oligosaccharide structures suggest that LPS is involved in the translocation of *H.influenzae* from the nasopharynx to the bloodstream (Weiser *et al.*, 1990). Furthermore, *H.influenza* and meningococci but not their non-pathogenic counterparts possess a sialyltransferase and are able to sialylate their LPS (Mandrell *et al.*, 1991, 1992). All these findings indicate that the present function of structural phase variation of gonococcal LPS may be only the first example of a more generally applicable virulence mechanism which allows bacteria to adapt to the rapidly changing host environment at the different stages in a natural infection.

Materials and methods

Bacterial strains

N.gonorrhoeae strains VP1 (identical to strain 830563) and MS11 have been previously described (Makino *et al.*, 1991; Weel *et al.*, 1991a). Pyocin producing *Pseudomonas aeruginosa* strains G6 and G9 were provided by L.Visser, Amsterdam Medical Center, The Netherlands. Bacteria were grown on gonococcal agar plates (Difco Labs, Detroit, MI) supplemented with 1% Vitox (Oxoid Ltd, Basingstoke, Hampshire, UK), unless indicated otherwise. In the infection experiments only bacteria expressing the invasion-associated opacity proteins were used (Opa_{50} and Opa_{68} for MS11 and VP1, respectively) (Makino *et al.*, 1991; Weel *et al.*, 1991a; Kupsch *et al.*, 1993).

Cell culture

Chang human conjunctiva epithelial cells (ATCC CCL20.2), ME-180 human cervical cells (ATCC HTB33), HEp-2 human larynx carcinoma cells (ATCC CCL23), and PC-3 human prostate epithelial cells (ATCC CRL 1435) were grown (37°C, 5% CO_2) in RPMI 1640 with 5% fetal bovine serum (FBS) in 25 cm^2 tissue culture flasks as described (Weel *et al.*, 1991a). For infection experiments cells were grown to confluence on 12 mm circular glass coverslips in 24-well tissue culture plates.

Infection experiments

Infection experiments were essentially carried out as described (Weel *et al.*, 1989). Confluent epithelial cells maintained in 1 ml RPMI + 5% FBS were inoculated with gonococci at a m.o.i of 10, and incubated for 0–6 h in a 5% CO_2-rich atmosphere at 37°C. CMP-NANA (40 nmol, unless stated otherwise), or the related compounds CMP (cytidine 5′-monophosphate) and NANA (*N*-acetylneuraminic acid) (all from Sigma Chemical Co., St Louis, MO) were usually added immediately before the start of the infection. If the epithelial cells or the bacteria were preincubated with CMP-NANA (1 h, 37°C), the cells were washed twice with medium before the start of the infection experiment. In initial experiments, the culture medium was replaced by fresh medium after 3 h of infection with fresh CMP-NANA (when appropriate). However, this procedure did not influence the results and was omitted in later experiments. The infection was stopped by rinsing the cells several times with PBS, followed by at least 15 min fixation (1% paraformaldehyde + 0.1% glutaraldehyde in PBS, pH 7.4) at 20°C. Bacterial adherence and invasion were scored after immunogold–silver staining and/or crystal violet staining of the specimen (van Putten *et al.*, 1990, 1993) by microscopic counting of extra- and intracellular bacteria in at least 100 epithelial cells. The validity of this procedure has been confirmed by electron microscopy (van Putten *et al.*, 1990). Each type of experiment was repeated at least five times and gave consistent results, though with a day-to-day variation in absolute values of up to 30%. The values shown are either from typical experiments or represent the mean ± SE of different experiments. Data were analysed by Student's *t*-test for paired comparison.

Selection of isogenic LPS variants

Gonococcal LPS variants were selected by different procedures. Pyocin resistant bacteria were obtained by repeated plating of gonococci on to agar plates containing a gradient of pyocins and selecting for stable survivors within the inhibition zone (Dudas and Apicella, 1988). Pyocins were isolated from *P.aeruginosa* pyocin reference strains G6 and G9 after mitomycin induction, according to Morse *et al.* (1976). Other LPS phenotypes and also revertants to the original phenotype were obtained by colony-immunoblotting using monoclonal antibodies with different LPS specificity (MAbs 4D6B, 8B2E and D6A). Colony-immunoblotting was performed using the immunogold–silver staining method as described (van Putten, 1993). When necessary, invasive variants of the isogenic LPS variants were selected on the basis of the opacity of the colonies under transmitted light, or by gentamicin selection of intraepithelial bacteria (Makino *et al.*, 1991).

Purification and electrophoretic analysis of LPS

Gonococci, grown either in 10 ml of GC broth (Difco) + 1% Vitox (Oxoid) or in tissue culture medium in the absence or presence of CMP-NANA on a gyratory shaker (150 r.p.m., 37°C), were isolated by centrifugation (2 500 g, 10 min, 4°C) and subjected to the rapid LPS isolation procedure of Inzana (1983). Under these conditions, 3 h of growth yielded $\sim 2 \times 10^8$ gonococci per ml, whereby 20 nmol/ml of CMP-NANA was sufficient for total sialylation of the gonococcal LPS, as judged from the complete shift of the sialylatable LPS component in SDS-PAGE. Neuraminidase treatment (*Clostridium perfringens* type V, Sigma; 20 mU added to 5 μg LPS in PBS, pH 6 and incubated for 60 min at 37°C) reversed the original phenotype. CMP-NANA did not affect the growth rate of the bacteria.

LPS molecules were separated by SDS-PAGE using 15% gels with urea (Hitchcock and Brown, 1983) or the tricine SDS-PAGE system (Lesse *et al.*, 1990). LPS bands were silver-stained according to Tsai and Frasch (1982) but with dichromate as oxidizing agent (Merill *et al.*, 1984).

CMP-NANA incorporation assay

The incorporation of [^{14}C]NANA into gonococci was measured by incubating (37°C) a suspension of 10^7 gonococci in 0.2 ml tissue culture medium in the presence of CMP-[^{14}C]NANA (200 pmol/ml, specific activity: 300 mCi/mmol, Dupont-New England Nuclear, Boston, MA) and various concentrations (0.2-200 nmol/ml) of the unlabelled compound to ascertain an excess of substrate. In some experiments, an excess of free CMP (5 mM) was added to determine non-specific CMP-NANA binding. At appropriate times, the reaction was stopped by adding ice-cold PBS, followed by centrifugation (10 000 g, 3 min in Eppendorf centrifuge). The pellets were washed twice with PBS to remove free label, and dissolved in Quickzint 212 (Zinsser Analytic Ltd, Frankfurt, Germany). Radioactivity was counted in a Beckmann liquid scintillation counter (model LS1801, Beckmann Instruments Inc., Palo Alto, CA).

Immuno-electron microscopy

The variation of LPS epitope expression among the isogenic LPS variants at the single cell level was determined by immuno-electron microscopy using different LPS-specific monoclonal antibodies and gold-coupled conjugate, as described (Weel *et al.*, 1989; van Putten *et al.*, 1993). The samples were viewed with a Zeiss M 109 EM at 80 kV.

Monoclonal antibodies

The LPS-specific antibodies 06B4, 1-1M, 2-1-L8, D6A and 7B1E have been characterized (Mandrell, 1986; Kim *et al.*, 1988; Weel *et al.*, 1989; Kerwood *et al.*, 1992). MAbs 8B2E and 4D6B were raised against a variable LPS component of strain F-62 as described (Weel *et al.*, 1989). MAbs 06B4, 1-1M and 2-1-L8 were kindly provided by Dr R.E.Mandrell; MAb D6A was a generous gift from Dr J.T.Poolman.

Serum resistance and complement-mediated killing

The sensitivity of the LPS variants to killing by natural human serum, and by monoclonal antibody and complement was determined using log phase grown bacteria cultured in 10 ml of GC broth (Difco) + 1% Vitox (Oxoid) in the absence and presence of CMP-NANA (20 nmol/ml). The bacteria (10^4 c.f.u.) were incubated in 0.1 ml of RPMI (final volume) containing either 10% fresh human serum or monoclonal antibody + 10% gamma globulin-free human serum. Incubations with heat-inactivated serum, non-reactive LPS-specific monoclonal antibodies, complement and antibody without complement source were used as controls. After 1 h of incubation at 37°C, serial dilutions were plated on agar plates. Colonies were counted and analysed for LPS phenotype by colony immunoblotting, SDS-PAGE and/or immuno-electron microscopy.

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