Brief Definitive Report

Variation of Gonococcal Lipooligosaccharide Structure Is Due to Alterations in Poly-G Tracts in *lgt* Genes Encoding Glycosyl Transferases

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Summary

The lipooligosaccharide (LOS) expressed by gonococci spontaneously varies its structure at high frequency, but the underlying genetic mechanism has not been described. We have previously reported that the genes encoding the glycosyl transferases responsible for the biosynthesis of the variable α chain of the LOS of *Neisseria gonorrhoeae* are located in a locus containing five genes, *lgtA*, *lgtB*, *lgtC*, *lgtD*, and *lgtE*. Sequence analysis showed that *lgtA*, *lgtC*, and *lgtD* contained poly-G tracts within the coding frames, leading to the hypothesis that shifts in the number of guanosine residues in the poly-G tracts might be responsible for the high frequency variation in structure of gonococcal LOS. We now provide experimental evidence confirming this hypothesis.

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The lipopolysaccharide of gonococci (GC) is small compared with that of enteric bacteria, and is often referred to as lipooligosaccharide or LOS. The structure of the LOS of a few strains of GC has been published (1–3) and is summarized in Fig. 1. Of particular note is the principal carbohydrate chain on heptose, which is known as the α chain and is subject to phase variation at several points in its structure. Fig. 1 *A* shows the most complete structure that has been reported for strain F62 (1). In the smallest naturally occurring variant, the α chain consists of a lactosyl group (Galβ1-4Glcβ1-). This LOS has an apparent molecular weight of 3,600 and reacts with mAbs 2-1-L8 and 4C4 as indicated in Fig. 1 (4, 5). GC undergo a variation at high frequency (10^{-2}–10^{-3} per generation) between the 3,600 mol wt LOS and a form with an apparent molecular weight of 4,500 that reacts with mAb 3F11. The tetrasaccharide α chain may be further substituted with a GalNAc residue to form the pentasaccharide shown in Fig. 1 *A*. van Putten (6) has reported high frequency variation of the ability of LOS to react with mAb 1-1-M, which is specific for the terminal GalNAc. An alternative substitution of the lactosyl group with an α1-4–linked Gal produces the structures shown in Fig. 1 *B*. This structure has been reported for strain 1291b (3) and reacts with mAb 1-17-L1. The fact that these structures are mimics of human glycolipids has been noted previously (7). The α chain may be the pentasaccharide GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, which is identical to the human red cell X_2 antigen (8). When the terminal GalNAc is not added, the tetrasaccharide is identical to lacto-*N*-neotetraose, the core structure of the blood group substances (7). The Galα1-4Galβ1-4Glc trisaccharide is found on globotriglycosyl ceramide or the p^k blood group antigen (7). Finally, the α chain can be limited to a disaccharide lactosyl unit and is then identical to lactosylceramide.

Recently we have characterized a locus in *Neisseria gonorrhoeae* strain F62 that is responsible for the synthesis of the α chain of LOS. The locus contains five open reading frames coding for glycosyl transferases, and by immunochemical and biochemical analysis of deletion mutants, the function of the genes was determined and is summarized in Fig. 1 (9). Three of the glycosyl transferases contained tracts of poly-G within the coding frame coding for runs of glycines in the predicted proteins. This suggested that the high frequency antigenic variation of GC LOS could be due to slipped strand mispairing during replication, causing premature termination of translation. This form of variation has been seen in the case of the pilC protein (10), a GC pilus accessory protein imparting adhesiveness to epithelial cells (11). The glycosyl transferases that might be subject to this form of variation are *lgtA*, the GlcNAc transferase; *lgtD*, the GalNAc transferase; and *lgtC*, the Gal transferase responsible for the addition of αGal1-4βGal.

This report demonstrates that high frequency genetic variation of LOS structure is attributable to changes in the number of G residues in the poly-G tracts in genomic DNA.

Materials and Methods

Colony Immunoblots. GC on GC agar (12) were grown overnight, suspended and diluted in proteose peptone liquid medium

(9), spread on GC agar, and allowed to grow for ~18 h to yield ~1,000 small colonies. The agar cultures were overlaid with nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH), lifted off, and air-dried for 60 min. The plates were reincubated at 30°C in a candle jar. The filters were wetted, immersed individually in petri dishes in 3% gelatin in 150 mM NaCl in 10 mM Tris-HCl, pH 7.5, with 0.02% NaN_3, and rocked for 60 min. Subsequently all reactions were carried out in 1% gelatin in the same buffer with rocking. The filters were washed once for 10 min and reacted for 60 min with a 1:2,000 dilution of mAb 17-1-L1 (L1). They were washed four times and placed in a 1:2,000 dilution of alkaline phosphatase–conjugated anti–mouse immunoglobulin antiserum (Cappel, Organon Teknika Co., West Chester, PA). The filters were washed four times for 10 min each, washed for 1 min with 50 mM Tris base containing 3 mM $MgCl_2$, and then stained as previously described (13).

Purification and Electrophoresis of LOS. LOS was prepared by a modification of the hot phenol extraction method and analyzed electrophoretically (9).

PCR Reactions. GC genomic DNA (usually 100 ng) was used as a template. The PCR conditions consisted of initial denaturation of DNA for 2 min at 94°C, followed by 25 cycles of 60°C for 60 s, 72°C for 30 s, and 94°C for 30 s. One additional cycle with a 4-min extension time at 72°C was performed. The reagents used were part of a Gene Amp kit (Perkin Elmer Corp., Branchburg, NJ) with 0.5 μl of *Taq* polymerase per 100-μl reaction and primers at 0.1 μM. The primers are described by their position in the sequence deposited in Genbank under accession number U14554 (9). To amplify an 893-bp fragment containing the poly-G region of *lgtA*, a primer pair matching 309–328 and the reverse complement of 1182–1202 was used; the first primer was biotinylated. To amplify a 467-bp fragment containing the poly-G region of *lgtC*, primers matching 2249–2275 and the reverse complement of 2693–2716 were used; the first primer was biotinylated.

DNA Sequencing. DNA sequencing was performed using the Sequenase II kit (United States Biochemical Corp., Cleveland. OH). The PCR products were purified by absorption to streptavidin-coated magnetic beads, and single-stranded template was eluted with NaOH and sequenced (14). Plasmid was purified by the spin-prep method (Qiagen Inc., Chatsworth, CA). The poly-G region in *lgtA* was sequenced in both directions using primers matching 607–623 and the reverse complement of 797–813. Similarly, the region in *lgtC* was sequenced on both strands using primers matching 2410–2426 and the reverse complement of 2573–2589.

Cloning of lgtA and lgtC Region. Genomic DNA was prepared from F62 Δ1 and variants 1-1, 2-1, and 1-6. 12-μg aliquots of DNA were digested with ClaI, and the digests were separated by gel electrophoresis on 0.8% agarose (SeaPlaque; FMC Corp., Rockland, ME), the region containing DNA around 2.3–3.0 kb excised, and the DNA purified using Geneclean II (BIO 101, Inc., La Jolla, CA). The fragments were ligated into ClaI-cut pBluescript KS$^+$, transformed into XL1-Blue MRF′-competent cells, and the desired clone isolated by plating on LB agar containing 50 μg/ml carbenicillin and 200 μg/ml erythromycin and incubating at 30°C. For variants A and C of strain MS11, the genomic DNA was digested with ClaI and PstI and, after size fractionation and purification as above, ligated into pBluescript II SK$^-$ cut with the same restriction enzymes. The desired clones were identified by colony hybridization (15) using ^{32}P-labeled oligonucleotide matching the reverse complement of 2573–2589.

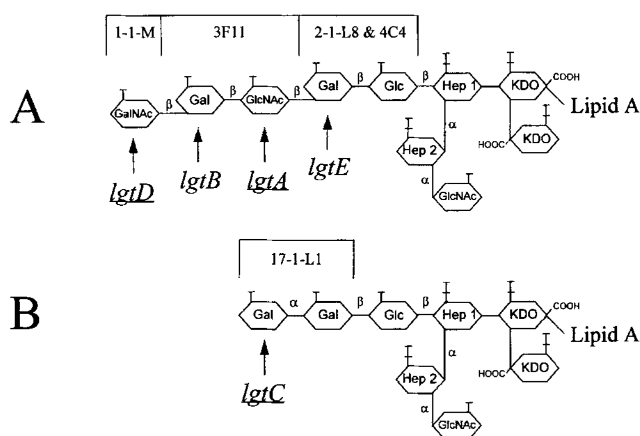

Figure 1. Structures of gonococcal LOS. (*A*) The complete structure that has been reported for strain F62 (1) and MS11 variant C (2). (*B*) The alternative LOS structure, which has been observed in strain 1291b (3) and in the mutant strain F62 Δ1 (9). The second structure differs from the first in that it bears a terminal α1-4–linked galactose instead of a β1-3–linked *N*-acetyl glucosamine. As indicated in the figure, this alternative LOS structure can be recognized by its reactivity with mAb 17-1-L1. The reactivities of a number of well-characterized mAbs are shown. The LOS glycosyl transferase gene (*lgtA, B, C, D, E*) responsible for the addition of each of the residues are indicated. The underlined *lgt* genes contain poly-G tracts in their coding frames. *KDO*, keto deoxyoctulosonic acid; *HEP*, heptose.

Results

Changes in the poly-G tract of *lgtC* should affect the addition of the terminal Gal in the LOS structure αGal1-4βGal1-4βGlc1-R. The α-Gal transferase encoded by *lgtC* appears to compete poorly with the GlcNAc transferase for substrate; its activity is evident only if *lgtA* is silent (9). We therefore used mutant F62 Δ1 with a deletion in *lgtA*, which produces an LOS with the structure shown in Fig. 1 *B* (9). Colony immunoblots were performed with mAb 17-1-L1 and briefly stained with safranin to visualize the colonies that were not immunoreactive. We isolated three independent colonies that did not react with the antibody. LOS from these clones, designated as variants 1-1, 2-1, and 1-6, was prepared and compared by SDS-PAGE (Fig. 2) with the F62 Δ1 parent and F62 Δ5 mutant known to produce a LOS with an α chain limited to a lactosyl group (9).

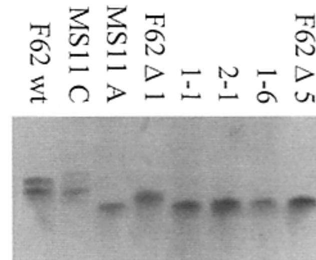

Figure 2. Silver-stained SDS-PAGE of LOS preparations. F62 wild type (wt) gives rise to two principal LOS bands, the lacto-*N*-neotetraose structure with and without the terminal GalNAc substitution. F62 Δ1 expresses mainly Galα1-4Galβ1-4Glc as α chain, whereas F62 Δ5 is limited to a lactosyl group (9). Note that F62 wt and the two MS11 variants give patterns identical to those published by Kerwood et al. (2). The LOS of variants 1-1, 2-1, and 1-6 are clearly smaller than the F62 Δ1 parent, but similar to F62 Δ5.

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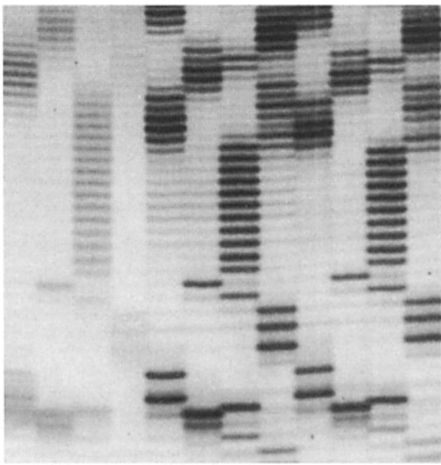

Figure 3. Sequence of PCR products from F62 variants. Genomic DNA of F62 Δ1 (*lgtA*Δ) and of variants 1-1 and 2-1 derived from this strain that no longer reacted with mAb 1-17-L1 was isolated. The genomic area containing the poly-G region of *lgtC* was amplified by PCR and the products sequenced as described in Materials and Methods. F62 Δ1 has 10 G residues, which is identical to the F62 wt (9). Variant 2-1 clearly has 11 Gs. Variant 1-1 has a larger number of Gs that cannot be accurately estimated by the PCR technique.

Each variant produced LOS that was smaller than that of the parent F62 Δ1 and was similar to the LOS of F62 Δ5.

We sequenced the poly-G region of the *lgtC* gene of the parent (F62 Δ1) and two of the variants by a PCR method; the results are shown in Fig. 3. The sequence of F62 Δ1 appears to have 10 Gs (identical to F62 wild type), which puts *lgtC* in the "ON" configuration, that is, the remainder of the coding sequence is in frame (9). Variant 2-1 appears to have one more G, which puts the gene in the "OFF" configuration. Variant 1-1 has a larger number of Gs, but the exact number can not be determined. This is a shortcoming of the PCR technique, which during amplification is also subject to slipped-strand mispairing and accumulates a population of products with different lengths of the poly-G tract. We were unable to improve the fidelity of the PCR reaction by modifying conditions or by the use of other polymerases, including those with intact proofreading function.

Since the F62 Δ1 mutant contained an erythromycin resistance gene in proximity to *lgtC*, it was simple to clone this area of the genomic DNA and to sequence *lgtC* in the resulting plasmids. The results are listed in Table 1 and confirmed that F62 Δ1 had 10 Gs, variant 2-1 had 11 Gs, and showed that variant 1-1 had 17 Gs. The independently isolated variant 1-6 also had 17 Gs. Thus, all three variants selected for lack of reactivity with mAb 1-17-L1 showed shifts in the *lgtC* poly-G region that caused premature termination of translation. We used variant 1-6 to perform colony blots and found that the reversion rate to reactivity with mAb 1-17-L1 was 3×10^{-3}.

Table 1. *Number of G Residues Found in Poly-Tracts in Plasmid Clones*

	G residues	
Plasmid clone	*lgtA*	*lgtC*
F62 Δ1		10*
Var 1-1		17
Var 2-1		11
Var 1-6		17
MS11 Var A	12	8
MS11 Var C	11‡	8

*10 G is in frame with the remainder of the coding frame of *lgtC*.
‡11 G is in frame with the remainder of the coding frame of *lgtA*.

We next wished to study the role of shifts in the poly-G region of *lgtA*. We took advantage of a closely studied example of antigenic variation observed in a study in which volunteers were infected with strain $MS11_{mk}$ expressing a 3,600 mol wt LOS phenotype (2). Over the course of the infection, an increasing proportion of GC isolated from the volunteers were variants that produced a higher molecular weight LOS. Two variants, B and C, predominated (16). Nuclear magnetic resonance and immunochemical analysis showed that the α chain of variant A was a lactosyl group, whereas variant C produced a complete pentasaccharide α chain. These variants were kindly provided by Dr. Herman Schneider, Walter Reed Army Institute of Research, and Fig. 2 shows that the pattern of the LOS prepared in our laboratory is like that previously published (2). We sequenced the *lgtA* and the *lgtC* regions and found that *lgtC* contained 8 Gs in both strains ("OFF"). The *lgtA* region changed from 12 in variant A ("OFF") to 11 Gs in variant C ("ON") (see Fig. 4). Thus, the loss of a single G residue accounted for the regained ability to synthesize the full LOS. To be certain of the number of G residues in the *lgtA* gene in these two variants, we also cloned the relevant re-

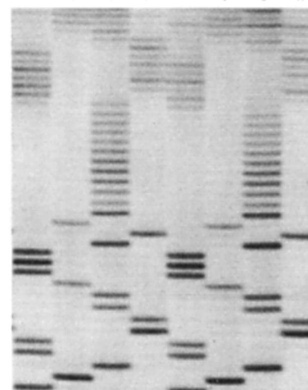

Figure 4. Sequence of PCR products from MS11 variants. Genomic DNA of MS11 variant A and variant C were isolated, the area containing the poly-G region of the *lgtA* gene was amplified by PCR, and the products were sequenced as described in Materials and Methods. MS11 variant A had 12 Gs, which places *lgtA* out of frame and limits the α chain to a lactosyl group. Variant C, an isolate from an infected volunteer, had 11 Gs and produced a complete LOS.

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gions and performed direct sequencing and obtained the same answer (see Table 1).

We were unable despite repeated colony blots to isolate from variant A variants producing the 1-17-L1 epitope. Such variants should have been easy to detect because we were looking for gain in reactivity with the mAb. The failure to isolate antibody-reactive variants suggests that the run of 8 Gs in *lgtC* of that strain is not sufficiently long to expand or contract at a perceptible frequency.

Discussion

We have described the molecular mechanism for the LOS phase variations that have been observed by previous investigators. However, the selective advantage to the organism of this genetic mechanism is unclear. The correlation of LOS structure with function is in its early stages. A large proportion of human bactericidal antibodies are to LOS epitopes, and its structure influences the bacteriolytic effects of human serum on GC. Some GC strains when grown in vitro are not killed by human serum (Ser^R) and are common among isolates from patients with disseminated GC infection (17). However, most GC strains are sensitive (Ser^S). Many Ser^S strains become phenotypically Ser^R when incubated in the presence of cytidine monophosphate *N*-acetyl neuraminic acid (CMP-NANA). GC possess a sialyl transferase that is capable of using exogenous CMP-NANA to sialylate its LOS (18). Concentrations of CMP-NANA in vivo are sufficient to support this reaction (19). The reaction depends on the LOS being a competent substrate. It is well established that the lacto-*N*-neotetraose chain is the substrate for the sialylation leading to the Ser^R phenotype, and that the lactosyl α chain is not modified (20). The resistance may be due to inability of antibodies to bind to the modified LOS, but there also appears to be a general defect of effective complement deposition (21, 22).

The ability of GC to adhere to epithelial cells is a fundamental attribute of their virulence. Preeminent among GC adhesins are pili. A 110-kD protein, pilC, present in small amounts in pili, is responsible for adherence to epithelial cells. The expression of this protein is subject to high frequency variation by a frame-shift mechanism due to a stretch of G residues early in the coding frame (10, 11). The opacity proteins (opa) are also important adhesins. This is a family of 11 outer membrane proteins that are subject to high frequency variation by a frame-shift mechanism that involves a variable number of repeats of the pentameric sequence CTCTT (23, 24). Most of the opa proteins promote adherence to polymorphonuclear phagocytes, and sialylation of the LOS strongly inhibits this opa protein–mediated adherence to polymorphonuclear phagocytes (25). One particular opa protein in strain MS11 not only promotes adherence to epithelial cells, but is essential for invasion of the cells (26). van Putten (6) has shown that sialylation of LOS markedly inhibits epithelial cell invasion without greatly altering adhesion. His studies suggest that in the mucosal infection, LOS that cannot be sialylated may be important for cell invasion. On the other hand, Griffiss et al. (27) reported that sialylation enhanced invasiveness in HEC-1-B cells.

GC has clearly evolved in this locus alone a very elegant system to shift readily between four different LOS structures, and at least one of these is subject to further modification by sialylation. That each of the various α chain structures that the GC can produce is a mimic of a host carbohydrate structure has raised the question of what the role of LOS may be in the mucosal infection. The host uses a large number of ligand-binding proteins recognizing the rich array of carbohydrate structures on glycolipids and glycoproteins for its own homeostatic purposes. The C-lectins, the S-lectins, and the sialoadhesins have binding specificities for structures mimicked by LOS. Thus, it is likely that these structures on the GC would also be recognized and may contribute in important ways to the mucosal infection.

This ability to shift the expression among a number of different LOS structures is not peculiar to the GC, but exists also in the meningococcus, where the genetics is likely to be very similar. The laboratories of Moxon and Hansen (28–30) have shown that this also occurs in another mucosal pathogen, *Haemophilus influenzae*, where at least four genes are subject to phase variation. In this organism, the mechanism is also by slipped-strand mispairing due to repeated tetrameric sequences, which can be either CAAT or GCAA. *H. influenzae* produces chemically similar LOS, and also sialylates its LOS (31). The existence of this genetic capability in *H. influenzae* and the pathogenic *Neisseria* ssp. argues that organisms with phase-variable LOS have a selective advantage. It strongly suggests that specific LOS structures afford an advantage in one biological niche, but a disadvantage in another host environment, and that these mucosal pathogens negotiate this dilemma by phase variation.

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