Urethral Exudates of Men with Neisseria gonorrhoeae Infections Select a Restricted Lipooligosaccharide Phenotype During Transmission

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Neisseria gonorrhoeae lipooligosaccharides (LOSs) induce immunoglobulin G that protects men from experimental infection. This raises the possibility that an LOS vaccine might prevent gonorrhea. Gonococci make different LOS molecules, depending on whether 3 genes, lgtA, lgtC, and lgtD, are in frame (IF) or out of frame (OOF). Mispairing of polymeric guanine (polyG) tracts within each gene determines its frame during replication. We amplified lgtA, lgtC, and lgtD from diagnostic slides of urethral exudates and sequenced their polyG tracts. We found that lgtA in exudative bacteria is IF and that lgtC is OOF. The frame of lgtD varied widely: it was OOF in most but not all cases. This genotype would result in synthesis of polylactosamine α chains that could be sialylated. Polylactosamine α chains would enhance virulence, and their sialylation would enable gonococci to survive within polymorphonuclear cells; however, an active LgtD in a few bacteria could provide a survival advantage in other sites of infection.

Some men who became infected after the Neisseria gonorrhoeae strain MS11mkC was introduced into their urethra [1] made immunoglobulin G (IgG) antibodies against the organisms’ outer membrane lipooligosaccharides (LOSs) [2]. These men were significantly less likely to become infected when challenged later with a lower inoculum than were men who did not have an IgG response to LOS [2]. This raises the possibility of preventing gonorrhea with a vaccine. However, gonococci can make many different LOS molecules [1, 3–6] that each can form several different antigens [4]. If many LOS molecules are made by gonococci during transmission, a successful LOS vaccine might need to encompass several antigens. Alternatively, the molecular environment of urethral exudates might select for stable expression of a limited number of LOS molecules [1, 7], which would simplify determination of the protective LOS antigens.

MS11mkC was isolated from the urethral exudate of men infected with MS11mkA, an LOS variant that has a truncated Lc2 α chain (designation of LOS glycoses follows nomenclature available at: http://www.chem.qmul.ac.uk/iupac/misc/glylp.html) [1]. MS11mkC makes paraglobosyl LOSs that have lacto-N-neotetraose (nLc4) α chains (Figure 1 and Supplementary Materials), some of which have polylactosamine (nLc6–8) extensions and approximately 10%–12% of which are capped by a terminal N-acetylgalactosamine (GalNAc) substitution that creates gangliosyl GalNAc-nLc6–8 α chains [1, 5]. This structural motif differs from that of other well-studied strains [8–11], and MS11mkC is more virulent than FA1090, a strain that also has been used in human challenge studies [12, 13]. This raises the possibility that MS11mkC may not be typical of gonococci that circulate in core mixing populations.
and that data from studies that have used it will not be useful for the development of an LOS vaccine.

LOS α chain structures are determined by the activity of glycosyl transferases (GTx) encoded by \textit{lgtA}, \textit{lgtC}, and \textit{lgtD}, each of which has an internal homopolymeric series of guanines, termed the polyG tract, situated between Pribnow (TATAAT) boxes and −35 consensus elements [4, 15, 16]. The number of guanines in the tracts determines whether the gene is in frame (IF) and fully transcribed or out of frame (OOF) and not fully transcribed [4, 15, 16]. Independent of frame, the number of guanines affects promoter strength and downstream transcription [8, 17, 18].

To determine whether single or multiple LOS glycoforms are present in the urethral exudates of men with acute gonorrhea and whether MS11mkC is typical of gonococci in naturally acquired infections, we scraped material from Gram-stained slides of urethral exudates, amplified gonococcal genes in the recovered material by polymerase chain reaction (PCR), prepared multiple clones from each slide, and sequenced the \textit{lgtA}, \textit{lgtC}, and \textit{lgtD} polyG tracts of all the clones. We then compared the bacteria in the exudates with MS11mkC.

**METHODS**

**Gonococcal DNA**

Gram-stained slides, without personal identifiers, that were used to diagnose gonorrhea in men seen at a Baltimore City Health Department (BCHD) clinic during 2010 were stored at room temperature and transported by hand to San Francisco by one of the authors (J. M. G.). The study was approved by the institutional review boards of the Johns Hopkins University School of Medicine, the BCHD, and the University of California San Francisco; written informed consent was obtained prior to participation. Slides were viewed under oil, and those with >2 polymorphonuclear cells (PMNs) per high-power field with internalized gonococci were selected.

After wiping off oil, material was scraped from the slide with a razor and suspended in 100 μL of sterile water. Ten microliters of this suspension was used in 20-μL PCR reactions for amplification of \textit{lgtA}, \textit{lgtC}, and \textit{lgtD}. Primers [4] were from Integrated DNA Technologies (Coralville, IA). In addition to genomic DNA template from slides, PCR mixtures contained 2 μM primer pairs (Integrated DNA Technologies, Coralville, IA), 1 mM dNTPs (Invitrogen, Carlsbad, CA), 1% dimethyl sulfoxide (Finzymes, Espoo, SF), 1 × GC buffer (Finzymes, Espoo, SF), and 0.5 mL Phusion High Fidelity Polymerase (Finzymes, Espoo, SF). Reaction conditions and thermocycler programs [4] were modified because of high guanine and cytosine contents and the low and varying yields of bacterial DNA from the slides. Two rounds of PCR were done for each gene to increase yield. Two microliters of PCR product from each initial PCR was then used as template in nested PCRs. Different conditions were used to amplify each gene, and amplicons from the set of conditions that yielded the most amplicons were used for cloning. Detailed methods, including the primers used and modified PCR programs, can be found in Supplementary Tables 1–3.

**Cloning and Transformation**

Nested PCR products were purified from a preparative 1.4% agarose gel, using the QIAquick Gel Extraction Kit (QiaGen,
Valencia, CA) according to the manufacturer’s instructions, and these amplicons were cloned into the pCR4-TOPO vector by use of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). One Shot Competent Cells provided in the TOPO TA kit were transformed with the vectors and grown on Luria-Bertani plates that contained 50 μg/mL kanamycin.

**Plasmid Preparation and Analysis**

Plasmids from 15–30 transformants were purified using the QIAprep Spin Miniprep Kit (Quiagen, Valencia, CA) and analyzed by restriction digest with EcoRI (New England Biolabs, Ipswich, MA). Digestion products were visualized by 1.4% agarose gel and staining with SBRY Safe stain (Invitrogen, Carlsbad, CA).

**Sequencing and Analysis**

Sequetech (Mountain View, CA) performed the sequencing. The DNA sequence for the lgtABCDE region of gonococcal strain F62 (National Center for Biotechnology Information accession number U14554) was used to orient the experimentally generated sequences to those reported in the literature and to confirm correct template amplification. Each transformant that contained the correct size insert was sequenced 2 times (2 forward and 2 reverse). A consensus count of guanine/cytosine in the polymeric tract was based on agreement among ≥3 sequences for each transformant. Slides were analyzed for a specific gene until a mean was reached for each gene that gave an overall SD of less than one whole integer.

**MS11mkC**

The MS11mkC lgt operon was sequenced as for strain 1291 [4].

**RESULTS**

We first combined the guanine count (ie, the number of guanines in the polyG tracts) for all of the clones of each gene from all of the slides (pooled data). If the exudative environment selected a dominant gene expression, the guanine count would be normally distributed around the number of guanines selected by the PMN intracellular environment, and the breadth of the distribution would indicate the strength of the selective pressure. If the pooled data were not normally distributed, we could conclude that the exudative environment exerted no selective pressure on expression of that gene. The distributions for each gene are in Figure 2.

The pooled guanine counts for lgtA and lgtC were normally distributed (Figure 2). LgtA had a mode of 11 guanines and a span of 9–13 guanines, which is consistent with moderately strong selection of a gene with 11 guanines (Figure 2A). An lgtA with 11 guanines would be IF, as frame for lgtA is 11 ± 3nGs, and the organisms in the exudates would have functioning LgtA β1 → 3 glucosaminyl transferases.

The guanine count mode for lgtC was 9, with a narrow span of 8–10 (Figure 2B). Frame for lgtC is 10 ± 3nGs, so the guanine count showed strong selection for an OOF lgtC and bacteria that do not have functioning α1 → 4 galactosyl transferases at the time of potential transmission.

The lgtD guanine count spanned 4 frames, as determined from the upstream start codon, and were designated frames 1 (8–10 guanines), 2 (11–13 guanines), 3 (14–16 guanines), and 4 (17–19 guanines), with the first guanine count for each frame being the IF count (Figure 2C). This distribution is consistent with only modest selective pressure for any one guanine count; however, the counts were not distributed equally within each frame. The guanine counts were concentrated in frames 2 and 3, and the modes of those frames were 13 and 15 guanines, respectively, which both yield OOF genes and nonfunctional LgtDs. If lgtD guanine counts were the least common.

To ensure that the pooled data were not biased by any one patient’s bacteria, we analyzed the clones from each patient’s slide individually (Table 1). The guanine counts were averaged to produce means for that patient for each gene, and the individual means then were averaged to generate an overall guanine count mean and 95% confidence interval (CI). To evaluate the consistency of the results and ensure that it was not slippage during PCR that accounted for the variation, we amplified genes from the same patient’s slide 2 or 3 times and averaged those results (Table 1).

The mean lgtA guanine count (±SD) for 6 patients was 10.9 ± 0.42 (95% CI, 10.51–11.29). Because there is a single whole integer, 11, within the CI, we can conclude that this is the number of lgtA guanines in almost all gonococci during transmission and that we sequenced lgtA polyG tracts from enough patients to determine that this gene is IF in almost all of the bacteria. Consequently, we are confident that the gonococci in urethral exudates express paraglobosyl nLc4 LOS α chains.

The lgtC overall mean (±SD) from 7 men was 9 ± 0.11 guanines (95% CI, 8.92–9.10). Not only is there a single whole integer, 9, within the CI, but the interval was very narrow. Thus we can conclude that essentially all of the gonococci in the urethral exudates of infected men have an OOF lgtC with 9 guanines.

Given the distribution of lgtD guanine counts in the pooled data, we generated means within frames 2 and 3 of lgtD for individual men. The overall mean (±SD) was 11.99 ± 0.88 guanines (95% CI, 11.31–12.67) for frame 2 of lgtD and 14.98 ± 0.54 guanines (95% CI, 14.56–15.40) for frame 3 of lgtD (Table 1). Frame for lgtD is 11 ± 3nGs, so the single whole integers within these CIs, 12 and 15, respectively, would yield OOF lgtDs. We conclude that there is strong selection for an OOF lgtD gene and that most, but not all, gonococci in urethral exudates have a nonfunctioning LgtD β1 → 3 galactosaminyl transferase.

The MS11mkC lgtA, lgtC, and lgtD polyG tracts were consistent with those of the exudative bacteria (Table 1) and...
predictive of the structures reported for this strain [1, 7]. \textit{lgtA} was IF, with 11 guanines, as it was in the exudative bacteria, and its functional LgtA would catalyze production of its poly-lactosaminyl nLc4–8 α chains. \textit{lgtC} is OOF in MS11mkC with 9 guanines, as it is in the exudative bacteria [7]. \textit{lgtD} was OOF with 13 guanines.

**DISCUSSION**

These results document that the exudative environment within which gonococci exit the male urethra during gonorrhea selects for the same restricted LOS phenotype as that of MS11mkC. Thus MS11mkC can be used as the type strain for the development of an LOS vaccine.

They also raise intriguing questions about the pathogenetic advantage this phenotype provides the organism and why the 3 genes are regulated differently.

The upstream \textit{glyS} promoter also promotes \textit{lgtABCDE} [17], but downstream promotion is weak, and the operon contains multiple internal promoter sequences [16]. The polyG tracts of the 3 genes are between Pribnow boxes (TATAAT) and –35 consensus elements, and the number of guanines in the tracts affects promoter strength. The \textit{lgtA} polyG tract in F62 has 17 guanines, which places the 2 elements too far apart for downstream promotion, and although \textit{lgtC} is IF [17], it is not transcribed [8]. When \textit{lgtC} is transformed from F62 into MS11mkC, it is transcribed [18], which suggests that \textit{lgtC} has been repromoted by the MS11mkC \textit{lgtA} Pribnow box. \textit{lgtC}
polyG tracts of 9 would provide strong downstream repromotion of lgtE, which encodes the transferase that initiates α chain extension. Thus, the polyG tracts control LOS synthesis in 2 ways. Although this arrangement may be advantageous to the organisms, it does not explain how nontranscription of lgtC serves them.

The advantage of not transcribing lgtD is clearer. The organisms’ LOS α chains are mostly sialylated within PMNs [7], and capping lactosamine moieties with GalNAc would prevent this. LOS sialylation enables gonococcal survival within PMNs [19] but also prevents infection of the male urethra [20], so a hypervariable lgtD would leave some of the organisms with unsialylated LOS.

Capping of nLc4 α chains with GalNAc also prevents LgtA-catalyzed polylactosamine formation [5, 8]. Strain F62 has an IF lgtD (17 guanines [17]); approximately 45% of its nLc4 α chains are capped with GalNAc [10], and it does not make polylactosaminyl α chains [8]. How polylactosamine α chains affects pathogenesis is unknown, but extension of the lactosamine terminal Gal residue further from the cell membrane would be expected to enhance whatever role it or its sialylated derivative might play. Polylactosamine α chains could explain the enhanced virulence of MS11mkC, compared with that of FA1090 [12, 13].

It seems reasonable to assume that the greater the number of guanines in the polyG tracts, the greater the chance that slipped-strand mispairing will occur during both DNA replication and RNA transcription. Consistent with this notion is the finding that lgtD had the highest guanine counts of the 3 genes in the exudative bacteria and the most variation in counts, whereas lgtC had the lowest guanine count and the least variation. Frequent lgtD slippage would facilitate selection of bacteria with and bacteria without a functional LgtD transferase at different times during pathogenesis and in different sites of infection.

Although lgtD was OOF in MS11mkC with 13 guanines, the production of relatively small amounts of LgtD-catalyzed GalNAc-nLc4-8 α chains [5] can be explained by slip strand mispairing of RNA during transcription.

About one-third of the slides yielded no amplicons. When present in large amounts relative to prokaryotic DNA, human DNA is known to interfere with the amplification of bacterial genes by PCR, and this is the most likely explanation. Other possibilities are that wiping off the immersion oil removed material or that the oil prevented complete solubilization of...
DNA. It is possible that gonococci that are present in small numbers or are primarily outside of PMNs would be expressing different LOS molecules than those that yielded amplicons, but this is conjectural. In such cases the ratio of human to bacterial DNA would not favor successful amplification.

Since LOS expressed by gonococci within urethral exudates is homogeneous and identical to that of MS11mkC, an LOS vaccine for the prevention of transmission from men to their partners need target only the antigens conforming by the nLc₄₋₈ α chain [4]. This will, however, require a precise determination of the nLc₄₋₈ α chain antigens that induce protective IgG [4]. A study quantifying IgG antibodies that bind 4 nLc₄₋₈ α chain antigens in the sera of men who have had documented heterosexual exposures to gonococcal infections is ongoing at the BCHD clinics.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

_Acknowledgments._ Mingfeng Liu and Gary Jarvis provided many helpful suggestions during the study and preparation of the manuscript, Sasha Patadia provided technical support, and Connie John helped prepare Figure 1. This is publication 110 from the Centre for Immunoc hemistry.

_Financial support._ This work was supported by the National Institutes of Health (grant number AI065605 to J. M. G.), administered by the North-Immunochemistry.

_Potential conflicts of interest._ All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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