Expression of Paragloboside-like Lipooligosaccharides May Be a Necessary Component of Gonococcal Pathogenesis in Men

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Summary

To learn how lipooligosaccharide (LOS) phase variations affect pathogenesis, we studied two male volunteers who were challenged intraurethrally with *Neisseria gonorrhoeae* that make a single LOS of 3,600 daltons and sequentially followed LOS expression by gonococci as urethritis developed. LOS variation occurred in vivo. Signs and symptoms of gonorrhea began with the appearance of variants making 4,700-dalton LOS that are immunochemically similar to glycosphingolipids of human hematopoietic cells (Mandrell, R. E., J. M. Griffiss, and B. A. Macher. 1989. *J. Exp. Med.* 168:107) and that have acceptors for sialic acid. A variant that appeared at the onset of leukorrhoea was shed by 34/36 men with naturally acquired gonorrhea at the time they sought medical attention; the other two shed the variant associated with dysuria. None shed the challenge variant. These data show that in vivo phase shifts to higher molecular mass LOS that mimic human cell membrane glycolipids are associated with the development of gonococcal leukorrhea.

Outer membrane glycolipids of Gram-negative bacteria that cause disease along the respiratory or genital mucosae are relatively small (<7,000-dalton) lipooligosaccharides (LOS)[1] whose multiantennary oligosaccharide structures mimic those of human cell membrane glycosphingolipids (GSL) (1–4). During a study of the effect of piliation on infectivity, human volunteers developed gonorrhea after intraurethral challenge with a piliated *Neisseria gonorrhoeae* strain (5) that made a single LOS of 3,600 daltons. We made serial analyses of the LOS made by the organisms infecting two of the volunteers to learn whether LOS phase variations occurred during infection. We then confirmed the results by studying men with naturally acquired gonorrhea.

[1] *Abbreviations used in this paper:* GSL, glycosphingolipids; LOS, lipooligosaccharide.

Materials and Methods

Bacteria. The heavily piliated, nonopaque (5) challenge strain, MS11mk variant A, and its other variants were cultured as previously described (6).

We obtained gonococcal strains cultured from patients attending local sexually transmitted transmitted disease clinics as 24- or 48-h cultures on Martin-Lewis medium and confirmed their species (7). We used the growth on these primary cultures to make proteinase K (PK)-treated whole cell lysates (8).

LOS Analyses. LOS in PK-treated lysates were characterized by SDS-PAGE and immunoblotting (6) with the use of mAbs 2-1-L8 (7), 3F11, 6B4, and 1-1-M (9).

Infection of Volunteers. Male volunteers were challenged with different inocula of variant A (5). Beginning 3 h after challenge, urine samples were collected twice daily until the infections were terminated by antibiotic treatment. Aliquots of centrifuged urine sediments were cultured and then stored frozen at −90°C. We recovered bacteria retrospectively only from frozen sediments from two volunteers who were challenged with 10^8 organisms; bacteria in the other sediments did not survive freezing.

We sought LOS phase variants in nitrocellulose lifts of colonies by reacting them sequentially with mAbs 3F11 or 1-1-M and 2-1-L8 (6). Progeny were studied by SDS-PAGE and immunoblot (6).

Results and Discussion

Gonorrhea in men is characterized by the onset of urethral dysuria and leukorrhoea after a variable incubation (10, 11). Excretion of viable gonococci initially decreased in both volunteers and then steadily increased until dysuria and leukorrhoea began between 48 and 96 h after challenge (Fig. 1).

Gonococcal LOS participate in a complex mimicry of human cell surfaces. mAbs 3F11 and 6B4 recognize different epitopes within the Lacto-*N*-neotetraose moiety shared by the 4,800-dalton and larger gonococcal LOS (Fig. 2) and by human GSLs of the paragloboside series (1, 13–15). Gonococci can leave the lactosamine (LacNAc) moiety unsubstituted or add GalNAc that occludes the 3F11 epitope and forms the 1-1-M epitope (Fig. 2 *A*) (14). LacNAc also has acceptors for sialic acid (15, 20). Sialylation variously occludes the 3F11 and 6B4 epitopes; the 6B4 epitope is unaffected by GalNAc substitution (14, 20).

Three LOS variants were found in urine sediments (Fig. 3). Variant A, the challenge variant, made single 3,600-dalton LOS, and bound only mAb 2-1-L8; after the initial decline in the numbers of organisms excreted (Fig. 1), it was recovered in increasing numbers from both volunteers until the onset of leukorrhoea (Table 1). Variant B appeared coincidental with the onset of inflammation and dysuria. In addition to the 3,600-dalton LOS, B made molecules of 4,300, 4,800, and 5,400 daltons (2, 7) (Fig. 2). Variant C appeared after B and coincidental with the onset of urethritis and discharge (Table 1). It made the 4,300-, 4800-, and 5,400-dalton LOS of variant B, and a 5,900-dalton LOS, but not the 3,600-dalton LOS. A fourth variant, D, occurred only during in vitro passage of variant C, and it made all five LOS (Fig. 2).

Table 1. *Gonococcal LOS Variants Grown from Urine Sediments of Volunteers Infected with N. gonorrhoeae*

Study no.	Days post-infection	Symptoms and signs	Retrospective culture
016	0	Dysuria	5/0/0*
	1	No dysuria	10/0/0
	2	Dysuria	172/3/0
	3	Dysuria	612/287/0
	4	Dysuria and discharge	88/678/15
018	0	Dysuria	3/0/0
	1	No dysuria	3/0/0
	2	No dysuria	18/0/0
	3	No dysuria	0/0/0
	4	No dysuria	103/0/0
	5	Dysuria	385/14/0
	6	Dysuria and discharge	2/12/24

Characterization of variants is described in the legend for Fig. 3.
* Variant A colonies/variant B colonies/variant C colonies.

Organisms from volunteer 016 at the onset of discharge show the LOS lineage A to B to C (Fig. 3). Variation from A to B represents the addition of LacNAc to the 3,600-dalton LOS (Fig. 2); the shift from B to C represents the loss of the 3,600-dalton precursor LOS and the acquisition of the 5,900-dalton LOS. The emergence of variant C from within a variant B colony shows that LOS variation can occur in vivo.

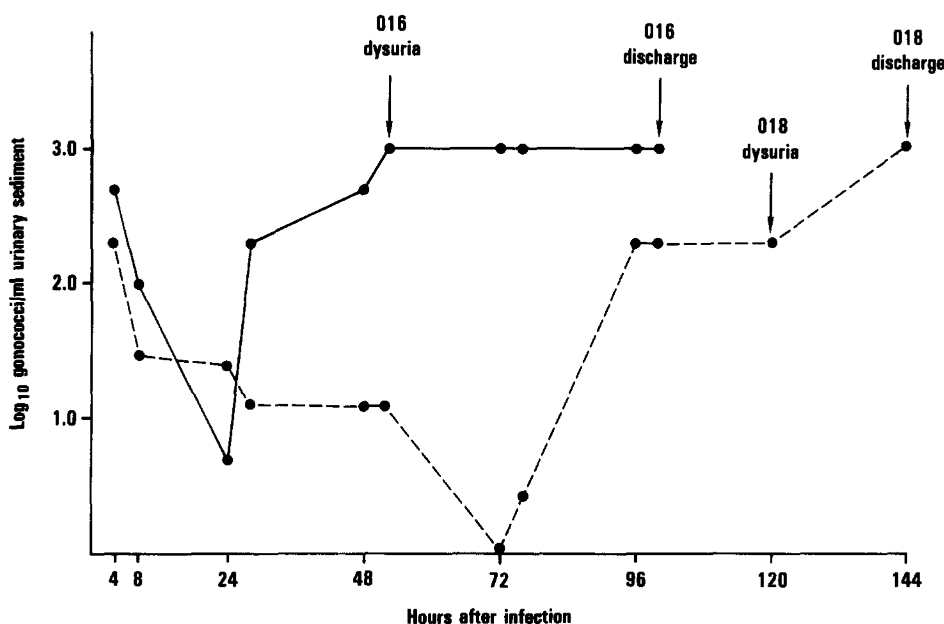

Figure 1. Quantitative cultures of sediments of urine from volunteers 016 and 018 challenged with 10^8 *N. gonorrhoeae* organisms. Aliquots of urine sediment suspensions from 50 ml urine were cultured and colonies enumerated after 24 and 48 h of incubation.

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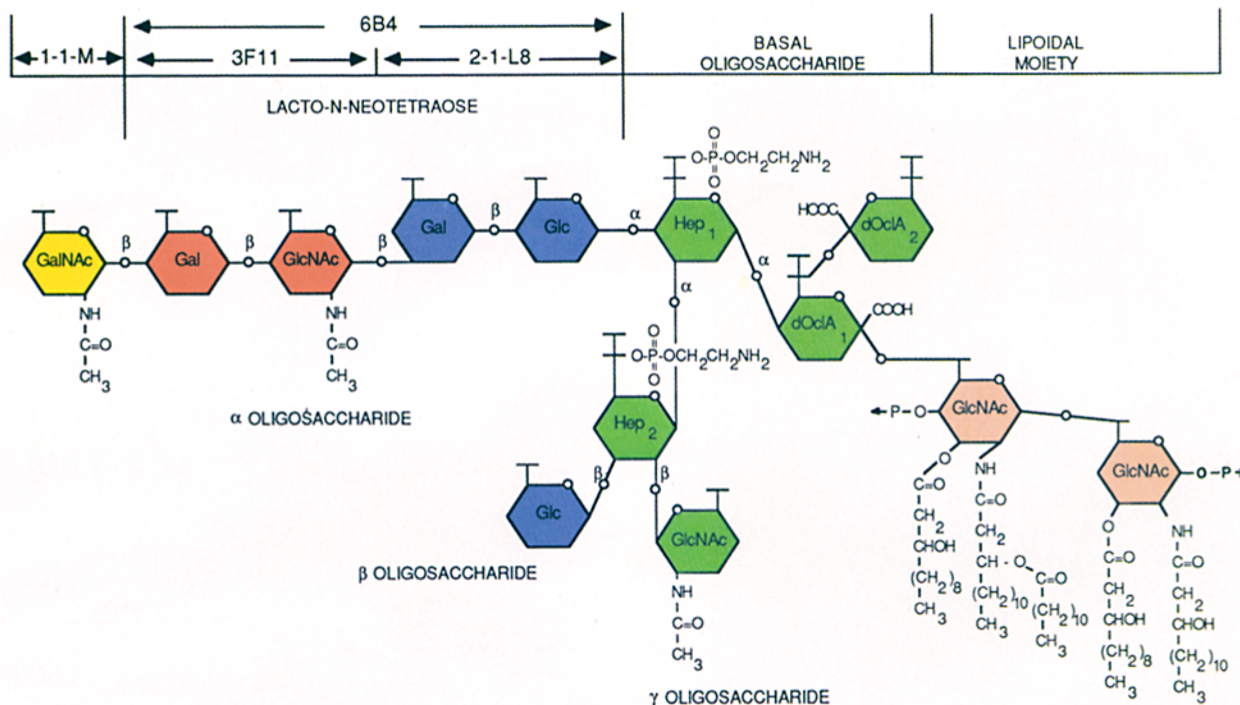

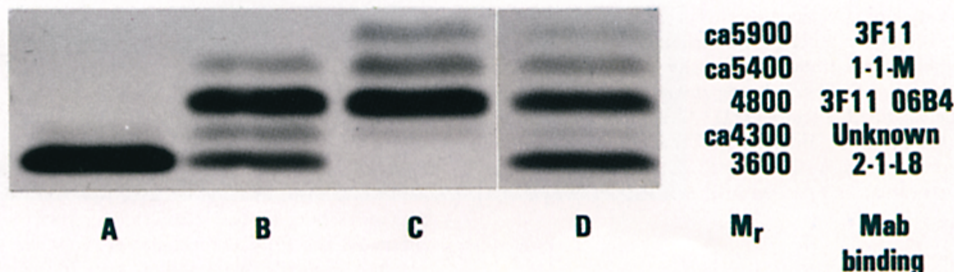

Figure 2. (*A*) Structure of the 5,400-dalton gonococcal LOS drawn from published structures of meningococcal (12) and gonococcal (1, 2, 13–17) LOS. Colors indicate the important antigenic and biosynthetic domains that comprise the epitopes that bind mAbs 2-1-L8 (13, 18, 19), 3F11 and 6B4 (1, 2, 9, 13, 14, 20), and 1-1-M (9, 14). (*B*) SDS-PAGE separation of *N. gonorrhoeae* MS11mk variants A, B, C, and D LOS, and the mAbs that they bind. PK lysates were separated on duplicate SDS-PAGE gels. Lysates of PID2 and ML5 (variant D) were used as marker strains to estimate LOS molecular mass and to reference mAb binding to LOSs. Molecular mass was assigned as previously described (7); this method progressively overestimates real molecular size as determined by mass spectrometry (13). One gel was stained with silver (21) to reveal LOS. The second gel, used to assign mAb binding, was electroblotted to nitrocellulose paper and treated sequentially with mAbs 3F11, 6B4, 1-1-M, and 2-1-L8. The blot was treated with alkaline phosphatase-conjugated goat anti-mouse IgM (A-GAMM) after 3F11 and again after 1-1-M. Binding was detected as a red color produced by the alkaline phosphatase substrate, naphthol-AS-MX-phosphate, and Fast Red TR salt. Binding of mAb 6B4 was detected as a blue color by using horseradish peroxidase-conjugated goat anti-mouse IgM (P-GAMM), and the substrate hydrogen peroxide and 4-chloro-1-naphthol. mAb 2-1-L8 binding was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (P-GAMG) and the same peroxidase substrate.

As expected from the known frequency of LOS phase variation (10^{-3}) (6), we found eight variant C colonies among the 8,000 variant A colonies grown from the inoculum suspension. This means that 10^5 variant C organisms were among the 10^8 variant A organisms with which the volunteers were challenged.

If variant C organisms in the challenge inoculum had had a survival advantage, we should have recovered some as the total numbers of shed organisms increased, but we did not. Instead, we recovered variant C organisms in proportions much greater than in the inocula from both volunteers after the onset of urethral discharge, and they predominated in volunteer 018 (Table 1). Mere persistence of variant C cells present in the challenge would have resulted in no more than one C organism on days 3 and 4 (1/1,000 organisms) for volunteer 016 and no C organisms at any time for the other volunteer, who shed <400 organisms.

Variant B arose only in vivo; we did not find it among

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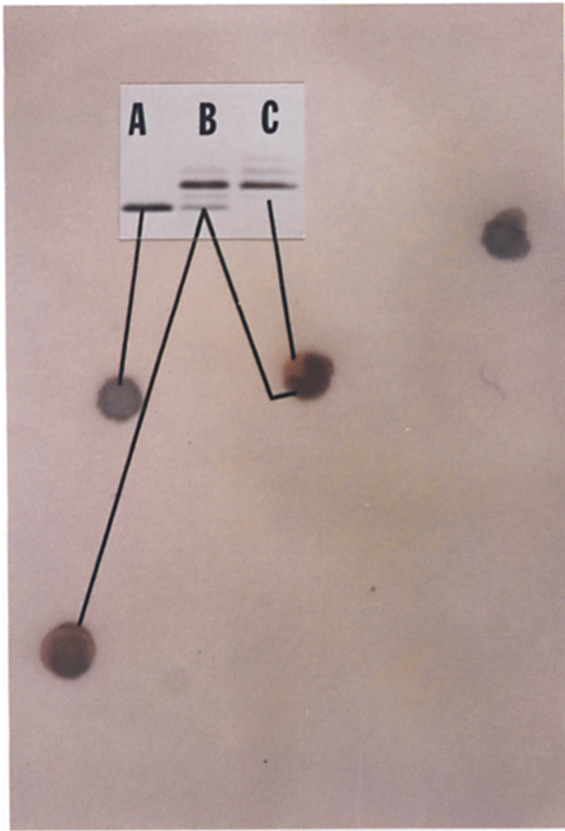

Figure 3. Identification of LOS phase variants in the sediment of a urine passed by volunteer 016 at the onset of leukorrhoea 4 d after infection. Nitrocellulose colony lifts were treated sequentially with mAbs 3F11 and 2-1-L8 as described for Fig. 2 *B*. The challenge variant A colonies (3F11/2-1-L8$^+$) are blue; the variant B colonies (3F11$^+$/2-1-L8$^+$) are violet (red plus blue). Variant C organisms (3F11$^+$/2-1-L8$^-$) appear as a red sector within a violet variant B colony. A silver-stained SDS-PAGE-separated gel of PK lysates of each variant is provided in the inset.

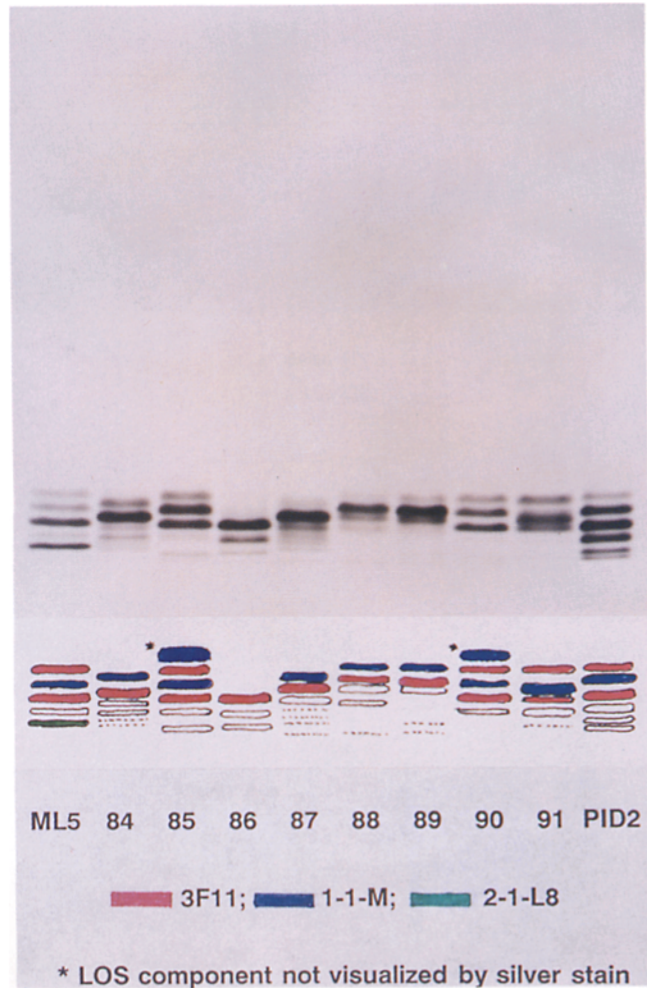

Figure 4. Representative SDS-PAGE (*top*) and immunoblot (*bottom*) analysis of LOS made by *N. gonorrhoeae* strains recovered on primary culture from men seeking medical attention for urethral gonorrhoea. ML5 (MS11mk variant D) and PID2 are included as molecular mass and mAb binding reference standards. Immunoblots were treated sequentially with mAbs 3F11, 1-1-M, and 2-1-L8, as described in Figs. 2 and 3. Outlines of the silver-stained LOS in the top were colored to indicate mAb binding.

the challenge or in vitro passed organisms. Its abundance during development of symptomatic urethritis suggests that it is an intermediate step necessary for the in vivo transition from variant A to C as inflammation (dysuria) progresses to leukorrhoea (discharge). The presence of a variant C sector within a variant B colony (Fig. 3) is a clear demonstration that variant C cells did not have to be present de novo.

In contrast with the in vivo transition from variant A to B to C, a one-step change from variant A to C occurred in vitro. This would suggest that induction of variant B cells requires either an in vivo signal, or that they have an advantage that leads to their selection and persistence. Variant B expresses the Lacto-*N*-neotetraose acceptor for CMP-NANA (15). The acceptor site is occluded by the terminal GalNAc on 1-1-M-binding LOS (14). Sequential competition between sialylation and galactosylation with the terminal GalNAc could explain the association between LOS phase variance and symptoms of infection. This model is consistent with our finding that gonococci within PMNs of naturally acquired urethral gonorrhea have sialylated surfaces that occlude binding by mAbs that recognize the Lacto-*N*-neotetraose LOS substituent (20). It implies that the nonsialylated A variants established the infection and the potentially sialylated B and C variants caused disease.

To confirm this model we assessed whether gonococci recovered from men with naturally acquired gonorrhoea expressed LOS that bound mAbs 2-1-L8 and 3F11 (Fig. 4). Strains from 36 men bound mAb 3F11 to one or more LOS of >4,700 daltons, and of these only two bound mAb 2-1-L8. Thus, 34 (94.4%) were shedding organisms with characteristics of variant C, and two (5.6%) were shedding organisms with characteristics of variant B, when they sought medical attention. None were shedding variant A. Half of the men's strains also bound mAb 1-1-M.

These observations support the conclusion that persistent colonization and development of symptomatic gonorrhea re-

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quires the expression by the infecting strain of variants making higher molecular mass LOS, at least one of which shares an oligosaccharide with paragloboside, a GSL of human cells. Expression of this shared oligosaccharide and its potential subsequent sialylation is likely to be a necessary component of virulence for the male urethra.

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