Sialylation Lessens the Infectivity of *Neisseria gonorrhoeae* MS11mkC

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Materials and Methods

Male volunteers. The study was done at Walter Reed Army Institute of Research. Volunteers were 18- to 50-year-old men who were in good health, as determined by medical histories, physical examinations, urinalyses, complete blood cell counts, electrocardiograms, serum creatinine levels, complement activity (C'H), liver function tests, and serologic tests for syphilis, human immunodeficiency virus, and hepatitis B virus. Five days before challenge, urethral swab specimens from each volunteer were cultured for *N. gonorrhoeae* and *Chlamydia trachomatis* and examined microscopically for *Trichomonas vaginalis*. Volunteers with a history of sexually transmitted diseases in the past year or of allergy to ciprofloxacin were excluded.

Sialylation of gonococci. The challenge gonococcal strain, mkC, had been isolated from a human volunteer infected in a previous challenge study [18] and maintained in the lyophilized state. The strain was revived and grown on GCDC agar medium (GC agar; Difco, Detroit) supplemented with 3 g of glucose, 8.3 mg of Fe(NO₃)₃·9H₂O, 1 g of L-glutamine, 1 mL of 0.2% cocarboxylase, and 40 mg of L-cysteine per liter [19]. Piliated (P⁺), transparent (Opa⁻) colonies were selected to maintain the P⁺Dpa⁻ phenotype. In its natural state, the 4.8-kDa LOS of mkC binds the monoclonal antibody (MAb) 1B2 [20]. In the presence of CMP-NANA, this LOS becomes sialylated at the lactosamine terminus of its lacto-N-neotetraose moiety, increasing the size of this LOS and ablating its ability to bind MAb 1B2 [13].

On the day of challenge, gonococci were grown in 10 mL of GCDC broth cultures with or without CMP-NANA (50 μg/mL; Sigma, St. Louis) [13]. Cultures were monitored spectrophotometrically, sampled periodically on GCDC agar to assess viability, and spotted on nitrocellulose filters. Colonies arising from cultures were examined with a dissecting microscope to detect P⁻ and P⁺Opa⁻ variants. Lysates prepared from both cultures and treated with proteinase K (PK) (Boehringer Mannheim, San Francisco) [21] were analyzed on replicate gels by SDS-PAGE [22], one of which was stained with silver [20] and the other electroblotted to nitrocellulose. The culture spots and the electroblot were reacted with MAb IB2 [23] to detect changes in MAb binding resulting from LOS sialylation.

Infection of volunteers. It was our intent to administer an inoculum of sialylated or unsialylated mkC gonococci to 7 or 8 subjects...
in each group. Challenge suspensions were made by diluting cultures containing ~5 to 6 × 10⁷/mL late exponential-phase gonococci in trypticase soy broth (TSB; BBL Microbiological Systems, Cockeysville, MD) so that 0.20 mL contained ~5000 gonococci. The 2 inocula were distributed in 4-mL aliquots into screw-cap test tubes and arranged in a double-blind fashion so that neither those preparing the inocula nor those responsible for their administration could identify which inoculum volunteers received. The volunteers were infected by intraurethral instillation of 0.20 mL of inoculum to a depth of 5 cm using a number 8 French pediatric catheter (Minton, Minneapolis) attached to a 1-mL syringe. The subjects did not urinate for 2 h after inoculation. Volunteers who developed a spontaneous urethral discharge or expressed a discharge that contained gram-negative intracellular diplococci were treated with a 500-mg oral dose of ciprofloxacin (Miles Pharmaceuticals, West Haven, CT). All uninfected volunteers were treated with 500 mg of ciprofloxacin at the end of each study. Follow-up cultures were done 1 and 7 days after treatment.

Analysis of urine specimens. Urine specimens were collected 2 h after challenge and at 12-h intervals thereafter. The specimens were centrifuged, and the supernatant urine was decanted. Sediments were smeared on slides for Gram’s staining and serially diluted 10-fold in TSB, and 0.1-mL volumes were plated in triplicate on GCDC agar containing vancomycin, colistin, and nystatin (BBL Microbiologials). The remaining urinary sediment was suspended in Greaves’ solution [24] and stored frozen at −70°C. Colonies were enumerated and opacity types assessed by use of a dissecting microscope. Cultures from infected volunteers were preserved in Greaves’ solution and used to prepare PK lysates.

Results

Sialylation of mkC gonococci. mkC gonococci were seeded in GCDC broth with and without CMP-NANA and grown to late exponential phase. Figure 1 shows that mkC gonococci grown in the absence of CMP-NANA bound increasing amounts of MAb 1B2 as incubation proceeded, reflecting the increasing numbers of 4.8-kDa LOS-expressing gonococci per spot. On the other hand, gonococci grown in the presence of CMP-NANA showed a lower binding of MAb 1B2, indicating the inhibition of sialylation of the mkC gonococci.

Figure 1. Analysis, using monoclonal antibody (MAb) 1B2, of sialylation of mkC gonococci grown in presence (W) or absence (W/O) of CMP-NANA (cytidine monophosphate-N-acetylneuraminic acid). Samples of each culture were spotted at hourly intervals onto nitrocellulose filters and tested for binding of MAb 1B2 to sialic acid recipient epitope of gonococcal 4.8-kDa lipooligosaccharide. Heavy immunostaining indicates binding of MAb 1B2 in culture grown without CMP-NANA; absence of staining indicates inhibition of MAb 1B2 binding by sialylation of 4.8-kDa epitope in CMP-NANA–grown culture.

Figure 2. SDS-PAGE and immunoblot analysis of mkC gonococci grown in presence (W) or absence (W/O) of CMP-NANA (cytidine monophosphate-N-acetylneuraminic acid). A, Silver-stained SDS-PAGE gel. B, Western blot of replica gel in A reacted with monoclonal antibody (MAb) 1B2. Band comparable to 4.8-kDa band in unsialylated mkC lipooligosaccharide (LOS) and proteinase K (PK) lysate is not present in sialylated mkC PK lysate nor is there significant LOS binding of MAb 1B2. Note shift to higher–molecular mass LOS and loss of MAb 1B2 binding in CMP-NANA–grown gonococcal lysates in lanes 4 and 5.
of CMP-NANA showed only minimal binding throughout the incubation period.

In figure 2, LOS from PK lysates of the 2 cultures are compared with phenol-water–extracted mkC LOS [25] for changes in electrophoretic mobility on SDS-PAGE and for changes in MAb IB2 binding on Western blots. Organisms grown without CMP-NANA expressed an LOS with the same mobility (4.8 kDa) and IB2 binding as the control LOS extract. Organisms grown in the presence of CMP-NANA made an LOS larger than 4.8 kDa that did not bind MAb IB2. The increased size of the LOS and its inability to bind MAb IB2 are indications that this LOS was sialylated. The minimal binding of IB2 by the sialylated cultures, similar to that in the culture spots shown in figure 1, suggest the presence of dividing gonococci that have not become fully sialylated.

Infection of volunteers. In a double-blind study, we inoculated 5 volunteers with sialylated mkC gonococci and 6 volunteers with unsialylated gonococci. The infection rate for unsialylated gonococci was 83% (5/6), and for sialylated gonococci, it was 20% (1/5) (P = .08, Fisher’s exact test). Figure 3 shows the relationship of in vivo gonococcal multiplication to the development of gonococcal urethritis. In volunteers 35, 45, and 47, disease onset occurred on day 2 or 3 after inoculation (figure 3A). In these 3 subjects, transition of the infections from the challenge Opa− phenotype to Opa+ preceded disease by 12 h. In 2 other volunteers (42 and 51), disease occurred on day 4 or 5 after inoculation, even though transition from Opa− to Opa+ occurred 2–2.5 days earlier (figure 3B).

The only infection resulting from inoculation with sialylated gonococci (volunteer 39) differed considerably from the unsialylated gonoococcal infections (figure 3C). This volunteer shed 10^2–10^3/mL of predominately (>90%) Opa− gonococcal urinary sediment during the first 62 h after inoculation, equal numbers of Opa− and Opa+ gonococci after 74 h, and only Opa+ gonococci after 86 h. The number of Opa+ gonococci increased to >10^6/mL urinary sediment over the next 3 days, but a urethral discharge did not develop until day 6. The subject received treatment on day 7 after inoculation.

Discussion

The study design allowed for testing of lessened or no differences in infectivity but not for increased infectivity between the sialylated and unsialylated gonococci. As it turned out, there was not sufficient power in the design (5/6 in the unsialylated and 1/5 in the sialylated mkC groups, P = .08) to demonstrate reduced infectivity by the number of volunteers infected. We can only speculate on the effect that larger numbers of volunteers would have had on the statistics. This study confirms our previous observations that Opa− gonococci are infective and persist for various periods after inoculation but are completely supplanted by Opa+ organisms 12–60 h before the onset of symptoms [18].

The structures of 2 of the 4 LOS molecules made by mkC organisms are known [26]. The smaller, 4.8-kDa, LOS structure has lacto-N-neotetraose as its terminal oligosaccharide and can
be sialylated. LOS sialylation has been shown to interfere with killing of gonococci by serum complement and polymorphonuclear leukocytes (PMNL) [1, 4, 6-9, 11]. The larger, 5.4-kDa, LOS has galactosamine β1-3 added to the terminal galactose (Gal) of the lacto-N-neotetraose structure [27] and cannot be sialylated. Its terminal saccharides conform to an epitope shared by a ubiquitous population of human IgM molecules that effectively initiate killing by complement and prevent gonococcal dissemination [15, 28]. In vitro experiments on the infectivity of gonococci for cultured tissue cells are at variance.
We found sialylated gonococci to be more infective for HEC-1-B cervical cells [16], but van Putten [17] found them less infective for Chang conjunctival epithelial cells. We expected that an inoculum of exogenously sialylated mkC gonococci would be at least as infective as unsialylated mkC, however, they not only were not as infective, but the 6-day incubation of the only sialylated mkC infection was 2 days longer than the longest incubation of the unsialylated mkC infections. While the present data are in accord with the observation that sialylation interferes with gonococcal infectivity [17], the role of LOS sialylation in the pathogenesis of gonorrhea is far from clear.

During natural infections, gonococcal LOS are sialylated within PMNL, which comprise much of the discharge of gonorrhea [2]. Presumably, LOS sialylation protects the ingested organism from PMNL killing in vivo, as it does in vitro [2]. Since colonization or stimulation of the influx of PMNL prevents induction of infection in the male urethra, this observed sialylation must have occurred after the initiation of colonization or during uptake by PMNL. Sialylation of human glycoconjugates is known to be anti-inflammatory [5], and sialylation of neisserial LOS could interfere with immune clearance of the organisms [1, 4, 6–9]. Indeed, lacto-N-neotetraose structures on human cells, particularly fetal cells, are usually sialylated, and their sialylation prevents immune recognition or clearance during fetal life [29, 30].

Infection occurs in only 22% of men who are exposed to gonococci through heterosexual intercourse [31]. The relatively modest risk of infection has been attributed to low and inconsistent shedding of organisms from the female cervix, to innate acquired immunity in the male urethra, and possibly to variable infectivity of different gonococcal LOS phenotypes. Variable LOS sialylation is an additional possibility. Sialylation of cervical mucus varies during the menstrual cycle [30]; if LOS sialylation of gonococci also varies, either through changes in availability of CMP-NANA or through actions of human sialyltransferases and sialidases, whose activities are known to change around the time of ovulation, then males often may be exposed to highly sialylated gonococci that are less able to cause infection.

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References


