Bypassing Phase Variation of Lipooligosaccharide (LOS): Using Heptose 1 Glycan Mutants To Establish Widespread Efficacy of Gonococcal Anti-LOS Monoclonal Antibody 2C7

Srinjoy Chakraborti,^a Sunita Gulati,^a Bo Zheng,^a Frank J. Beurskens,^a Janine Schuurman,^b Peter A. Rice,^a Sanjay Ram^a

^aDivision of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts, USA

^bGenmab, Utrecht, The Netherlands

ABSTRACT The sialylatable lacto-*N*-neotetraose (LNnT; Gal-GlcNAc-Gal-Glc) moiety from heptose I (HepI) of the lipooligosaccharide (LOS) of *Neisseria gonorrhoeae* undergoes positive selection during human infection. Lactose (Gal-Glc) from HepII, although phase variable, is commonly expressed in humans; loss of HepII lactose compromises gonococcal fitness in mice. Anti-LOS monoclonal antibody (MAb) 2C7, a promising antigonococcal immunotherapeutic that elicits complement-dependent bactericidal activity and attenuates gonococcal colonization in mice, recognizes an epitope comprised of lactoses expressed simultaneously from HepI and HepII. Glycan extensions beyond lactose on HepI modulate binding and function of MAb 2C7 *in vitro*. Here, four gonococcal LOS mutants, each with lactose from HepII but fixed (unable to phase-vary) LOS HepI glycans extended beyond the lactose substitution of HepI (lactose alone, Gal-lactose, LNnT, or GalNAc-LNnT), were used to define how HepI glycan extensions affect (i) mouse vaginal colonization and (ii) efficacy *in vitro* and *in vivo* of a human IgG1 chimeric derivative of MAb 2C7 (2C7-Ximab) with a complement-enhancing E-to-G Fc mutation at position 430 (2C7-Ximab-E430G). About 10-fold lower 2C7-Ximab-E430G concentrations achieved similar complement-dependent killing of three gonococcal mutants with glycan extensions beyond lactose-substituted HepI (lactose alone, LNnT, or GalNAc-LNnT) as 2C7-Ximab (unmodified Fc). The fourth mutant (Gal-lactose) resisted direct complement-dependent killing but was killed approximately 70% by 2C7-Ximab-E430G in the presence of polymorphonuclear leukocytes and complement. Only mutants with (sialylatable) LNnT from HepI colonized mice for >3 days, reiterating the importance of LNnT sialylation for infection. 2C7-Ximab-E430G significantly attenuated colonization caused by the virulent mutants.

KEYWORDS *Neisseria gonorrhoeae*, gonorrhea, lipooligosaccharide, monoclonal antibodies

Gonorrhea is the second most common bacterial sexually transmitted infection, with 86.9 million new cases estimated to occur annually (1, 2). There were 583,405 cases of gonorrhea reported to the Centers for Disease Control and Prevention in 2018, which represents a 63% increase since 2014 (5 years) and an 82.6% increase since a historic low in 2009 (<https://www.cdc.gov/std/stats18/gonorrhea.htm>). *Neisseria gonorrhoeae*, the etiologic agent of gonorrhea, has become resistant to multiple conventional antibiotics, including third-generation cephalosporins, macrolides, quinolones, and tetracyclines (3). Furthermore, there are no safe and effective vaccines licensed against *N. gonorrhoeae*, necessitating the need for developing novel therapeutics.

The gonococcus has acquired resistance to multiple antibiotics without any apparent decrease in fitness. Antibodies that target critical bacterial virulence mechanisms

Citation Chakraborti S, Gulati S, Zheng B, Beurskens FJ, Schuurman J, Rice PA, Ram S. 2020. Bypassing phase variation of lipooligosaccharide (LOS): using heptose 1 glycan mutants to establish widespread efficacy of gonococcal anti-LOS monoclonal antibody 2C7. *Infect Immun* 88:e00862-19. <https://doi.org/10.1128/IAI.00862-19>.

Editor Manuela Raffatellu, University of California San Diego School of Medicine

Copyright © 2020 American Society for Microbiology. All Rights Reserved.

Address correspondence to Sanjay Ram, sanjay.ram@umassmed.edu.

Received 11 November 2019

Accepted 23 November 2019

Accepted manuscript posted online 9 December 2019

Published 22 January 2020

have an advantage over conventional antibiotics because resistance, if it were to develop, would impair bacterial fitness (4–6). As a result, such attenuated escape mutants would be cleared by host immune defenses. We previously identified a murine monoclonal antibody (MAB), 2C7 (2C7-murine), that reacted with 95% or 100% of *N. gonorrhoeae* recovered directly from cervical secretions (7) or minimally passaged clinical isolates (10), respectively, and shortened the duration and reduced the burden of infection in mice (8). The minimal structure required for MAb 2C7 binding (i.e., the 2C7 epitope) requires lactose extensions simultaneously from the heptose I (HepI) and HepII core glycans (see Fig. S1 in the supplemental material) (9). While the addition of lactose from HepI requires two constitutively expressed lipooligosaccharide (LOS) glycosyltransferases, LgtF and LgtE, initiation of HepII lactose expression requires LgtG, which is controlled by the phase-variable gene *lgtG* (Fig. S1). Expression of HepII lactose facilitates colonization of the mouse vagina by gonococci; *lgtG* deletion mutants are attenuated in mice (8, 10).

We recently discovered that the lactose extension from HepII could be replaced with N-acetylneuraminic acid (Neu5Ac), which inhibited complement C3 fragment deposition (10) and engaged Siglec receptors (11), which may downregulate the host inflammatory response to infection. As stated above, resistance to MAb 2C7 because of *lgtG* being phase-varied off would result in considerable loss of bacterial fitness.

LOS phase variation in wild-type (WT) strains of *N. gonorrhoeae* precludes unambiguous assignment of a specific role to hexose-length and -substitution of HepI in pathogenesis and infection. To better understand the role that HepI-substituted glycans play in infection, we first examined the effect of individual HepI structures upon infection in the mouse genital tract. Individual HepI-substituted hexoses were each fixed “on,” using isogenic gonococcal mutants with defined LOS structures that lacked the ability to phase vary. A second step, to evaluate MAb 2C7 as an immunotherapeutic in this context, used chimerized MAb 2C7 and its modified Fc (HexaBody Technology) (12, 13), which enhances complement activation and increases bactericidal activity against *N. gonorrhoeae* (14). We had shown previously that HepI glycan extensions modulate the functional activity of MAb 2C7 *in vitro* (15). In order to better predict treatment/prevention with 2C7 antibody, here we examined how the function of chimeric MAb 2C7 *in vivo* was influenced by changes in individual HepI substitutions on *N. gonorrhoeae*.

RESULTS

Fc mutations in 2C7-Ximab(s) do not alter binding to HepI LOS mutants; HepI LOS glycans modulate 2C7-Ximab binding. Binding of the three human IgG1 chimeric derivatives of MAb 2C7 (2C7-Ximabs) that contained unmodified or wild-type (WT) Fc, the complement-enhancing E430G Fc, or the complement-inactive D270A/K322A (Null) Fc to four *N. gonorrhoeae* HepI LOS mutants (14) (Fig. 1A shows a schematic of their LOS structures) was measured by flow cytometry (FCM). Each HepI mutant bound similar amounts of all three 2C7-Ximabs (Fig. 1B). However, the amount of antibody bound varied across the four HepI mutants. Maximum 2C7-Ximab binding was observed with the 2-Hex/G+ mutant followed by the 4- and 5-Hex/G+ mutants, while 3-Hex/G+ bound the least (Fig. 1B; see also Fig. S2 in the supplemental material), findings consistent with our prior observations with murine MAb 2C7 (15). Only the 2-Hex/G+ mutant strain showed binding above control values when 2C7-Ximab-E430G was tested at a lower concentration (3 μ g/ml; average fluorescence from three experiments was 126 [$\sim$ 2.6-fold over baseline control values]); there was no binding of Ximab to the remaining mutants, above control, at 3 μ g/ml (data not shown).

The E430G Fc mutation enhances serum bactericidal efficacy of 2C7-Ximab. Introducing the E430G mutation increases Fc hexamer formation following binding of Ab to a surface, which in turn enhances C1q engagement and downstream, complement-dependent cytotoxicity (a mechanism similar to bactericidal activity) (13). The 2-Hex/G+ mutant was chosen for these experiments because it bound the largest amount of 2C7-Ximab. Consistent with previous data with wild-type strain MS11

FIG 1 Binding of 2C7-Ximabs to *N. gonorrhoeae* with various HepI glycan lengths as extensions from HepI LOS. (A) Schematic structures of the LOS glycans of the four *N. gonorrhoeae* mutants used in this study, which have been described previously (15). Note that the mutant strains may express LOS molecules with structures of lower molecular mass in addition to expected structures shown, because transport of LOS molecules to the outer membrane from the site of assembly on the cytoplasmic side of the inner membrane may occur prior to the addition of glycans by all Lgts that are fixed on. As an example, the 5-Hex/G+ mutant also expresses the 4-Hex/G+ structure. (B) Each of the three 2C7-Ximabs binds similarly to the indicated MS11 HepI LOS mutant. Binding of 2C7-Ximabs possessing WT Fc, E430G Fc, or Null Fc to each LOS mutant was measured by flow cytometry. Concentrations of Ximabs tested are indicated on the x axis. The median fluorescence intensity (MFI) of binding (mean [range] from 3 experiments) is shown on the y axis (log₁₀ scale). The dashed line in each panel represents the fluorescence reading (MFI) of the conjugate control (bacteria plus anti-human IgG FITC).

(14), serum bactericidal assays with the 2-Hex/G+ mutant using 20% human complement (IgG- and IgM-depleted normal human serum [NHS]) as the complement source confirmed higher bactericidal activity of E430G than WT Fc (Fig. 2). As expected, the Null Fc that was engineered to decrease C1q binding did not cause any bacteriolysis (>100% survival even at 316 µg/ml).

Enhanced bactericidal activity of 2C7-Ximab E430G Fc is associated with increased C1q binding. C1q binding in the presence of 31, 56, or 100 µg/ml of each of the three 2C7-Ximabs incubated with the 2-Hex/G+ mutant was measured by FCM (Fig. 3A) to associate increased bactericidal activity of the E430G Fc molecule with increased C1q recruitment (12, 13). E430G Fc bound significantly more C1q than the WT Fc at all concentrations tested. As expected, the Null Fc MAb bound very low levels of C1q. C1q recruitment in the absence of 2C7-Ximab was minimal and is indicated by the dashed horizontal line labeled control in Fig. 3A. The requirement for C1q in killing

FIG 2 E430G Fc mutation enhances serum bactericidal activity of complement-dependent 2C7-Ximab against MS11 2-Hex/G+. MS11 2-Hex/G+ was incubated with either 2C7-Ximab E430G Fc or WT Fc at the concentrations indicated on the x axis, followed by the addition of 20% human complement. Survival at 30 min is shown on the y axis. Each bar represents the mean (±range) from two separate experiments. Groups were compared using 2-way ANOVA (*, $P = 0.013$; ****, $P < 0.0001$). The Null Fc did not show any bactericidal activity (>100% survival at 316 µg/ml).

FIG 3 E430G Fc mutation enhances C1q engagement, which is required for the bactericidal activity of 2C7-Ximab E430G Fc. (A) Enhanced C1q recruitment by 2C7-Ximab E430G Fc. MS11 2-Hex/G+ was incubated with the concentrations of 2C7-Ximab indicated on the x axis, followed by the addition of purified C1q (30 µg/ml). Bound C1q was detected by FCM. For each experiment, median fluorescence values were expressed as a percentage of the readings seen with E430G Fc used at a concentration of 100 µg/ml. Each bar represents the mean ($\pm$ range) from 2 replicate experiments. Comparisons across groups were made using 2-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B) C1q is required for bactericidal activity of 2C7-Ximab E430G Fc. Serum bactericidal assays with the 2-Hex/G+ mutant were performed with 20% C1q-depleted NHS (Δ C1q NHS) or Δ C1q NHS reconstituted with pure C1q (70 µg/ml). Each bar represents the mean ($\pm$ range) from 2 replicates. Comparisons across the four groups were made using one-way ANOVA (F value, 15.67; $P = 0.011$), and pairwise comparisons were made with Tukey's multiple-comparison test.

of *N. gonorrhoeae* by E430G Fc is shown in Fig. 3B, where C1q-depleted NHS (abbreviated as Δ C1q NHS in Fig. 3B) failed to kill bacteria; the addition of purified C1q to physiological concentrations restored bactericidal activity (100% killing). Collectively, these observations suggest that the enhanced bactericidal activity of E430G Fc is directly linked to its ability to increase C1q recruitment, thereby invoking classical pathway activation.

Bactericidal activity of 2C7-Ximab E430G Fc is modulated by HepI LOS glycan extensions. Bactericidal activity of 2C7-Ximab E430G against the other three HepI LOS mutants (3-Hex/G+, 4-Hex/G+, and 5-Hex/G+) was tested using 20% human complement and 31, 100, or 178 µg/ml Ximab; the 2-Hex/G+ mutant was included as a comparator. Consistent with data shown in Fig. 3A, only the 2-Hex/G+ mutant was killed by E430G Fc at 31 µg/ml, while the 4-Hex/G+ and the 5-Hex/G+ mutants were killed (<50% survival) only at concentrations of 100 µg/ml and 178 µg/ml E430G Fc (Fig. 4). The 3-Hex/G+ mutant was fully serum resistant (>100% survival) even at 178 µg/ml (Fig. 4) and remained resistant even when the Ximab concentration was increased to 500 µg/ml (data not shown). Thus, complement-dependent killing of these LOS mutant strains paralleled 2C7-Ximab binding, similar to observations with murine MAbs 2C7 (15).

FIG 4 Bactericidal efficacy of 2C7-Ximab E430G Fc is modulated by the length of HepI glycan extensions. Serum bactericidal assays were performed with 20% human complement and 2C7-Ximabs at concentrations indicated on the x axis. Each bar represents the mean ($\pm$ range) from 2 replicates. Comparisons across mutants at each 2C7-Ximab concentration were carried out by two-way ANOVA; pairwise comparisons were made with Tukey's multiple-comparison test. ****, $P < 0.0001$; **, $P = 0.027$; *, $P = 0.017$.

FIG 5 2C7-Ximab E430G Fc deposits more C3 on the 3-Hex/G+ mutant than WT Fc and enhances killing by human PMNs. (A) C3 deposition on MS11 3-Hex/G+ in the presence of complement and 2C7-Ximab E430G Fc and WT Fc. A control reaction included bacteria incubated with 20% complement alone and is shown by the dashed line. In each experiment, the geometric mean fluorescence was calculated as a percentage of the fluorescence seen with complement and E430G Fc at a concentration of 200 µg/ml. The mean (range) from two independent experiments is shown. Groups were compared using 2-way ANOVA. (B) 2C7-Ximab E430G Fc facilitates complement-dependent opsonophagocytic killing of 3-Hex/G+ (created in the background of MS11 ΔOpa) by human PMNs. The Ximabs were each used at a concentration of 100 µg/ml. Percent survival (number of CFU at 60 min relative to CFU at 0 min) is shown on the y axis. Each bar represents the mean (±range) percent survival of 3 separate experiments using PMNs from a single donor. A comparison of killing across the six groups that contained PMNs was performed by one-way ANOVA (F value, 9.047; $P = 0.0009$), and pairwise comparisons were made with Tukey's multiple-comparison test.

3-Hex/G+ opsonized with 2C7-Ximab(s) is killed by PMNs in a complement-dependent manner. We had shown previously that despite resistance of 3-Hex/G+ to direct complement-mediated bacterial killing, C3 fragment deposition mediated by murine MAb 2C7 facilitated opsonic killing by human polymorphonuclear leukocytes (PMNs) *in vitro* (15). Here, we examined the ability of 2C7-Ximabs to kill complement-opsonized 3-Hex/G+ despite the mutant strain's resistance to direct antibody killing alone. First, we measured deposition of C3 fragments on 3-Hex/G+. Both 2C7-Ximab-E430G (E430G Fc in Fig. 5A) and 2C7-Ximab (WT Fc) deposited C3 on the 3-Hex/G+ mutant above baseline control levels (indicated by the horizontal dashed line in Fig. 5A). E430G Fc deposited more C3 than WT Fc; differences were statistically significant at a higher concentration of E430G Fc (200 µg/ml) (Fig. 5A). We then asked whether opsonization with 2C7-Ximab, either alone or in conjunction with human complement, could kill the 3-Hex/G+ mutant in the presence of freshly isolated human PMNs, and if so, whether E430G Fc was more efficient in PMN killing than WT Fc. This experiment was performed using a 3-Hex/G+ mutant of *N. gonorrhoeae* strain MS11

FIG 6 Expression of lacto-N-neotetraose (LNnT) from Hepl promotes gonococcal vaginal colonization in mice. Premarin-treated wild-type BALB/c mice ($n = 8/\text{group}$) were each infected with $\sim 10^6$ CFU of 2-Hex/G+, 3-Hex/G+, 4-Hex/G+, 5-Hex/G+, or WT/G+. Vaginas were swabbed daily to enumerate CFU. The data shown include all of the saline-treated animals shown in Fig. 7, which were the negative controls to assess the efficacy of 2C7-Ximab E430G. (A) Kaplan-Meier (KM) curves showing time to clearance of infection. Mutants were compared pairwise using the Mantel-Cox log rank test, and significant differences across groups are indicated in the table beneath the graph. Significance was set at 0.005 (Bonferroni's correction for 5 groups). (B) CFU versus time. The mean $\log_{10}$ concentration of bacteria on each day was plotted versus time. (C) Area under the curve (AUC) analysis. Each data point represents the AUC ($\log_{10}$ CFU) for each mouse. Horizontal lines represent the median (95% confidence interval) for each group. Groups were compared using one-way ANOVA (Kruskal-Wallis nonparametric test), with pairwise comparisons performed with Dunn's multiple-comparison test. The χ^2 with ties (Kruskal-Wallis statistic; four degrees of freedom) was 33.89 ($P < 0.0001$).

where all 11 Opa proteins had been deleted (MS11 ΔOpa) (15, 16). Elimination of Opa(s) avoids interactions with CEACAM3 on human PMNs that would result in nonopsonic killing (17, 18) and thereby confound interpretation of results. PMNs failed to kill bacteria opsonized with either of the 2C7-Ximabs alone (E430G Fc or WT Fc) at 100 $\mu\text{g}/\text{ml}$ (Fig. 5B, 4th and 6th columns, respectively). However, a significant decrease in gonococcal survival was observed when bacteria were incubated with PMNs in the presence of both 2C7-Ximab-E430G (E430G Fc) and complement (Fig. 5B, 5th column). In contrast, WT Fc plus complement did not reduce survival significantly (Fig. 5B, 7th column). Thus, enhanced complement activation mediated by E430G Fc also promotes opsonophagocytic killing of *N. gonorrhoeae* *in vitro*.

Expression of lacto-N-neotetraose extending from Hepl-lactose (4-Hex) promotes vaginal colonization in mice. The following experiment had two objectives: (i) to determine the role of defined (i.e., fixed) Hepl glycan extensions in establishing colonization of the mouse vagina (Fig. 6) and (ii) to assess the ability of 2C7-Ximab-E430G (E430G Fc) to attenuate colonization by each LOS mutant (Fig. 7). Hepl glycan structures affect pathogenesis and virulence in the human male volunteer infection model of gonorrhea (19, 20), where positive selection occurs for bacteria that express the sialylatable lacto-N-neotetraose (LNnT) structure. The importance of LNnT sialylation for colonizing the mouse genital tract has been demonstrated previously (21, 22). Only the *lgtG* "on" (G+) mutants were used here, because genetic deletion of *lgtG* significantly attenuates gonococcal infection in mice (8, 10, 23). Therefore, we included a mutant with only *lgtG* locked on (WT/G+), which, unlike the other four mutants, permitted phase variation only of Hepl glycans. This mutant would address a role for Hepl glycan extensions (if any) independently in their ability to colonize mice.

FIG 7 Efficacy of 2C7-Ximab E430G against the Hepl LOS mutants. Mice were infected with each of the five LOS mutant strains as described in the legend to Fig. 5. The solid black lines/solid black circles in each graph (saline controls) are the same as those shown in Fig. 5. In addition to control mice, two groups of mice, each infected with each mutant gonococcal strain ($n = 6/\text{group}$), were treated with 2C7-Ximab E430G Fc intravaginally at either $1 \mu\text{g}/\text{day}$ or $0.1 \mu\text{g}/\text{day}$. The top row shows Kaplan-Meier curves for time to clearance of infection. Comparisons across groups were made with the Mantel-Cox log-rank test. Significance was set at 0.017 (Bonferroni's correction for 3 groups). The middle row shows $\log_{10}$ CFU versus time, and the bottom row shows consolidated bacterial burdens over time using AUC analysis. AUCs were analyzed by one-way ANOVA, and pairwise comparisons were made with Dunn's multiple-comparison test.

Mice were infected with 10^6 CFU of *N. gonorrhoeae* intravaginally. The control groups ($n = 8/\text{group}$) received saline, and treatment groups received 2C7-Ximab ($n = 6/\text{group}$). Vaginas were swabbed and cultured daily to monitor the burden of infection. Data with the saline control groups, shown separately in Fig. 6, revealed the role of Hepl glycans in vaginal colonization. 4-Hex/G+ possessing the LNnT as a terminal LOS structure on Hepl (fixed on), which is sialylatable, and WT/G+, capable of phase varying LNnT as a sialylatable terminal LOS structure on Hepl, both colonized mice most effectively. The median time to clearance of infection with each of these mutants was 7 days (Fig. 6A). The 5-Hex/G+ mutant also expresses the sialylatable LNnT structure in addition to GalNAc-LNnT (15). This mutant showed intermediate infectivity (median time to clearance, 5.5 days). The 2- and the 3-Hex/G+ mutants, which can be sialylated via $\alpha 2$ -6 linkages to the terminal Gal residues on Hepl (10) and Hepl (24), respectively, colonized mice only transiently (median time to clearance for both mutants was 3 days) (Fig. 6A). Values of $\log_{10}$ CFU versus time and overall burdens of infection (as measured by area under the curve [AUC]) are shown in Fig. 6B and C, respectively, and parallel the clearance data shown in Fig. 6A. 2-Hex/G+ and 3-Hex/G+ showed significantly lower overall burdens of infection, as shown by AUC analysis (Fig. 6C). These results reiterate the importance of expression of the LNnT LOS, which is sialylated through an $\alpha 2$ -3 linkage, and correspond to the findings in the human challenge model (men) (19, 20) and may account for the infrequency with which these variant forms are encountered in natural human infection (10, 25).

E430G 2C7-Ximab shortens duration of colonization in mice infected with virulent mutants. In the same experiment, we also evaluated the effect of intravaginally

administered 2C7-Ximab-E430G Fc (E430G Fc) in promoting clearance of each of the mutants in mice (Fig. 7). Kaplan-Meier curves for times to clearance effected by 10-fold differences in 2 doses of E430G Fc are shown in the top row, numbers of CFU versus time in the middle row, and AUC in the bottom row, with each column representing data with the indicated mutant. For the 2-Hex/G+ and 3-Hex/G+ mutants, the median times to clearance of infection in untreated control saline-treated mice (3 days) were similar to those of mice treated with E430G Fc (2.5 to 3 days). Bacterial burdens comparing treated and untreated groups were also similar. Thus, therapy with E430G Fc did not confer any apparent measurable benefit in these ineffectual mutants. Median times to clearance of WT/G+, 4-Hex/G+, and 5-Hex/G+ mutants were 2 to 2.5 days in mice treated with 1 μ g/day E430G Fc, which increased minimally to 2.5 to 3 days in mice treated with 0.1 μ g/day, and both doses were significantly effective compared to clearance in control saline-treated animals. Bacterial burdens (AUCs) in the treated groups of these three mutants were also significantly lower than those of untreated groups. 2C7-Ximab-E430G Fc performed comparably at both doses tested (1 μ g/day and 0.1 μ g/day).

DISCUSSION

N. gonorrhoeae has become increasingly resistant to antimicrobial agents, including third-generation cephalosporins such as ceftriaxone and cefixime; several highly resistant ceftriaxone strains have resulted in therapeutic failure, reported worldwide (26–29), and cefixime has been removed from public health guidelines as recommended therapy (30). Azithromycin, recently recommended for use in combination with ceftriaxone as first-line treatment of gonorrhea (31), increasingly has become ineffective in many parts of the world (3, 32–34). These developments will seriously limit treatment options (3, 34). Prior work by our group had identified a monoclonal antibody (2C7-murine) that recognized about 94% of gonococcal infections directly in cervical samples and 95% of strains that were isolated (7, 15). A survey of 75 minimally passaged isolates from Nanjing, China, showed that all (100%) isolates bound MAb 2C7, which was bactericidal against all the isolates that were tested (10). These encouraging results led to the development of 2C7-Ximab(s) as a potential antigonococcal immunotherapeutic (14).

Because congenital and acquired complement deficiencies have been associated with a predisposition to recurrent neisserial infections, including disseminated gonococcal infection (35–38), we reasoned that introducing the complement-enhancing E430G mutation in Fc of 2C7-Ximab would enhance its efficacy. Enhancing complement activation is important to overcome several complement evasion mechanisms that *N. gonorrhoeae* possesses, including binding of soluble complement inhibitors FH and C4BP (39, 40). In accordance with a key and perhaps exclusive role of complement in host defenses against *N. gonorrhoeae*, we recently showed that 2C7-Ximab-E430G showed greater activity than 2C7-Ximab (with unmodified Fc) against gonococci in the mouse vaginal colonization model (14). Furthermore, an intact complement system was necessary and sufficient for activity of MAb 2C7 in the mouse model (14).

Phase variation of gonococcal LOS also plays an additional important role in virulence. Here, we have shown that gonococci expressing/displaying different surface glycans evade host immunity differentially, which results in infections that vary in duration. Loss of terminal GalNAc from the 5-Hex structure (phase-variable *lgtD* turned off) decreases binding of natural IgM in human serum and increases resistance to complement (41, 42). Terminal GalNAc on LOS interacts with C-type lectin macrophage galactose-binding lectin (MGL) and skews immunity toward a Th2 lineage, whereas terminal GlcNAc (representing the LNNt structure) is recognized by dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and induces significantly more IL-10 production (43). Both mechanisms allow for selective manipulation of dendritic cell function, thereby shifting subsequent immune responses in favor of bacterial survival (43). Unsialylated LNNt serves as a ligand for the asialoglycoprotein receptor (ASGP-R) and facilitates entry of gonococci into male urethral

epithelial (44) and M-180 (45) cells. 2C7 antibodies bind well to all LOS glycoforms except the 3-Hex/G⁺ mutant (the P^K-like structure or L1 LOS immunotype in clinical isolates), whose natural counterpart is rarely identified as a cause of gonococcal infection (10, 25). While increasing the amount of Fc-enhanced MAb in the bactericidal assay did not result in direct complement-dependent killing of the 3-Hex/G⁺ mutant because of low binding, nonetheless C3 deposition was increased sufficiently to render the 3-Hex/G⁺ mutant susceptible to opsonic killing by PMNs.

Using well-defined *N. gonorrhoeae* LOS mutants that eliminate microheterogeneity of LOS structures that occur among wild-type strains, we confirmed enhanced potency of 2C7-Ximab E430G Fc over 2C7-Ximab WT Fc, similar to that of wild-type *N. gonorrhoeae* strains FA1090, MS11, and 15253 (14). We show here that LOS glycans modulate the binding and functional efficacy of all 2C7-Ximabs, akin to our observations with murine MAb 2C7 (15). A comparison between the E430G Fc and WT Fc revealed that E430G Fc promotes complement activation, measured by either serum bactericidal activity or C3 deposition, on all four LOS mutant strains. Efficacy of 2C7-Ximabs in the mouse vaginal colonization model against wild-type strains requires an intact complement system (14), but it was not possible to test efficacy of 2C7-Ximabs in mice against the 2- or 3-Hex/G⁺ mutants, because these mutants did not colonize mice beyond 3 days. We attempted colonization of mice with a gonococcal strain, called 398078, predominantly expressing the 3-Hex glycoform (24), but were unable to recover any gonococci by vaginal swabbing beyond day 2 (S. Gulati, P. A. Rice, and S. Ram, unpublished data). 3-Hex LOS can also be modified with Neu5Ac, which, like LNNt LOS, enhances gonococcal serum resistance, albeit to a lesser degree (24). In contrast to LNNt LOS sialylation, which occurs through an α 2-3 linkage and enhances binding of human FH, sialylation of 3-Hex occurs through an α 2-6 linkage and does not enhance binding of human FH (24). Sialylation of the 2-, 3-, and 4-Hex/G⁺ mutants each inhibits mouse C3 deposition (10), which suggests that attenuation of the 2- and 3-Hex/G⁺ mutants involves a mechanism(s) distinct from complement inhibition. The low virulence of the 3-Hex mutant in mice is consistent with the relative infrequency of clinical counterparts (P^K or L1 immunotypes) identified in clinical isolates of *N. gonorrhoeae* (10). The 2-Hex/G⁺ mutant likewise does not possess sialylatable LNNt LOS, which may account for its reduced virulence. However, a natural counterpart, *N. gonorrhoeae* strain 15253, possesses lactose substitution of HeplI (*lgtG* intact) but is truncated at HeplI, because *lgtB* to *lgtD* are deleted and *lgtA* is phase-varied "off" (46; see also Fig. S1 in the supplemental material); strain 15253 maintains infection in 50% of mice for 7 days (8).

Challenge with untreated mutants with longer HeplI LOS structures (4- and 5-Hex/G⁺) that possess LNNt and therefore are modifiable by α 2-3-linked Neu5Ac resulted in durable infection in mice (47). Exhibiting LNNt is critical to establish infection both in human male volunteers (19, 20) and in the female BALB/c mouse model (21, 22). The 5-Hex/G⁺ mutant also expresses the LNNt structure as part of its LOS repertoire (15), which may explain its intermediate infectivity. Targeting of GalNAc by antibody (42) or innate immune receptors (43) that would facilitate clearance of infection may be offset by the anti-inflammatory properties of sialylated LNNt that include complement down-regulation and engagement of Siglec receptors (11, 48).

Two studies have addressed the role of phase variation of HeplI glycans *in vivo*. In the first, human male volunteers were experimentally challenged with a wild-type *N. gonorrhoeae* variant whose HeplI glycan was predominantly 2-Hex (MS11 mkA) (20). Sequential sampling of urethral contents demonstrated that recovered bacteria expressed 2-Hex HeplI glycans transiently until the onset of dysuria. However, with further progression of infection, the HeplI LOS phenotypes transitioned to predominantly 4-Hex and 5-Hex (also called the MS11 mkC variants) (20). The second study showed that the required infectious dose of MS11 mkA (mostly 2-Hex) was higher than the dose of MS11 mkC (LNNt LOS) (19). It should be noted that although MS11 mkA predominantly expresses the 2-Hex structure from HeplI, it also expresses a small amount of the 4-Hex (LNNt) structure. Therefore, the contribution of the 2-Hex structure alone in initiation of infection could not be ascertained. Nevertheless, these studies in the human challenge

model provide evidence that *N. gonorrhoeae* expressing predominantly truncated Hepl glycans are less efficient in initiating and sustaining infection. Our study employs LOS mutants with fixed glycan structures that cannot phase-vary and strongly suggests that mutants that express 4-Hex (LNnT) Hepl glycans are necessary for durable infection, thereby affirming the findings of Schneider and colleagues (20) in the human challenge model. While we acknowledge the limitation of the mouse model for studies of this human-specific infection (49), the importance of certain gonococcal virulence factors in humans, in particular lipid A phosphoethanolamine (50) and LNnT LOS sialylation (21, 22), has been mirrored in mice.

2C7-Ximab is effective when administered intravaginally and systemically against wild-type *N. gonorrhoeae* (14). Here, we have confirmed the efficacy of intravaginally delivered 2C7-Ximab-E430G using well-defined LOS mutant strains. Collectively, these studies show that antibodies directed against the 2C7 LOS epitope may constitute a promising immunotherapeutic approach to curb infection that will be effective in the face of LOS phase variation of *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Isogenic mutants of gonococcal strain MS11 4/3/1, a variant of MS11 VD300 with an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *pilE* that controls pilus expression (51), where phase variability of Hepl glycan extensions was eliminated by fixing *lgtA*, *lgtC*, and/or *lgtD* in so-called on or off settings in various combinations to yield 2-Hex (Gal-Glc), 3-Hex (Gal-Gal-Glc), 4-Hex (Gal-GlcNAc-Gal-Glc), or 5-Hex (GalNAc-Gal-GlcNAc-Gal-Glc) Hepl substitutions, have been described previously (15). In each of these mutants, *lgtG* was fixed on (G+), as described previously (15), to yield the following mutants: 2-Hex/G+, 3-Hex/G+, 4-Hex/G+, and 5-Hex/G+ (15). In addition, we created a mutant in the parent (or wild-type [WT]) strain MS11 4/3/1, where only *lgtG* was locked on to create WT/G+; the ability of Hepl glycans to phase vary was unaffected in this mutant. As previously described (15), a 3-Hex/G+ LOS mutant was made in the background of MS11 Δ Opa for opsonic killing studies by PMNs, where all 11 *opa* genes were deleted (16).

N. gonorrhoeae from freezer stocks was streaked on chocolate agar (CA) plates with supplemented IsoVitalEx and incubated overnight (~18 h). To prepare an inoculum for *in vivo* experiments, *N. gonorrhoeae* (streptomycin resistant) was harvested from overnight cultures, subcultured onto fresh chocolate agar plates for 6 h at 37°C in the presence of 5% CO₂, and suspended in phosphate-buffered saline (PBS). *N. gonorrhoeae* was isolated from the mouse vagina by culturing swabs on CA plates supplemented with vancomycin (V), colistin (C), nystatin (N), trimethoprim (T), and streptomycin (S) (VCNTS) to suppress growth of commensal microflora. For *in vitro* assays, overnight cultures on CA plates were suspended in liquid medium comprising Morse A, Morse B, and IsoVitalEx for 4 h at 37°C with shaking at 250 rpm.

Construction, expression, and purification of chimeric MAb 2C7 Ab(s). A chimeric MAb 2C7 (2C7-Ximab) was generated by fusing the V_H and V_L sequences of murine MAb 2C7 to the constant domains of human IgG1 heavy (C_H1, C_H2, and C_H3) and light (C_L) chains, respectively (14). In addition to 2C7-Ximab with the unmodified (wild-type) Fc (WT-Fc), two additional molecules were created. One had an E-to-G mutation at position 430 (2C7-Ximab-E430G) that enhances Fc hexamerization following binding of Ab to a surface, which in turn increases C1q engagement and complement activation (12, 13), and the other had simultaneous D270A and K322A mutations (2C7-Null Fc) that reduced C1q binding and abrogated complement activation. Construction, expression, and purification of all three Ximabs were described previously (14).

Sera and complement reagents. Human complement was prepared as described previously (15, 52). All steps were carried out at 4°C. Briefly, normal human serum to which NaCl and EDTA were added to final concentrations of 1 M and 10 mM, respectively, was depleted of endogenous IgG and IgM by sequential passage over protein A/G (Pierce, USA) and anti-human IgM (Sigma) columns. Subsequently, the flowthrough was spin concentrated and dialyzed/equilibrated against PBS–0.1 mM EDTA, filter sterilized, and stored in single-use aliquots at –80°C. C1q-depleted sera and purified C1q were purchased from Complement Technology, Inc., USA.

2C7-Ximab binding assays. The four Hepl LOS mutants (10⁷ CFU each) were incubated with 10, 31, or 100 μ g/ml of each of the three 2C7-Ximabs for 30 min at room temperature. Bound Ximab was detected with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Sigma) for 15 min. Fluorescence was measured by flow cytometry (FCM) using an LSRII (BD Biosciences, Franklin Lakes, NJ). FCM data were analyzed using FlowJo (v 10.2; Tree Star, Ashland, OR).

Complement C1q binding and C3 deposition assays. Approximately 10⁷ CFU of *N. gonorrhoeae* was incubated (in the presence or absence of 2C7-Ximab[s]) either with 20% complement (for C3 deposition) or with 30 μ g/ml C1q (for C1q binding) for 15 min. C3 deposited on bacteria was stained with FITC-conjugated sheep anti-human C3c (Bio-Rad) for 15 min. Paraformaldehyde (2%) was added to reaction mixtures containing C1q and incubated for an additional 10 min at 37°C. Bacteria were washed and associated bacteria were detected with biotinylated anti-C1q (JL-1 clone; Abcam) for 30 min, followed by streptavidin-DyLight633 (Thermo Fisher) for 15 min. Fluorescence was measured with a BD

LSRII instrument (BD Biosciences, Franklin Lakes, NJ) and analyzed using the FlowJo analysis software program (version 7.2.5; Tree Star, Ashland, OR).

Serum bactericidal assays. Serum bactericidal assays were performed as described previously (15). About 2,000 CFU of bacteria was incubated at 37°C in the presence of complement and/or Ab(s). C1q-depleted serum with or without added purified C1q (to a final concentration of 70 μ g/ml) was used in some experiments. Duplicate aliquots of reaction mixtures were plated at 0 and 30 min, and percent survival was calculated by expressing the number of CFU at 30 min as a percentage of the number of CFU at 0 min.

PMN killing and association assays. PMN killing assays were performed as previously described (15, 53). Briefly, PMNs were isolated from freshly drawn venous blood over a Percoll gradient (density, 1.130 g/ml; GE Lifesciences) after initial separation over Histopaque K119 (Sigma). PMNs then were adhered onto tissue culture-coated plastic coverslips (Sarstedt, Germany) primed with interleukin-8 (IL-8), following which they were infected with either opsonized or unopsonized *N. gonorrhoeae* at a multiplicity of infection of 1. Bacteria were centrifuged onto the adherent PMNs at $400 \times g$ for 4 min at 10°C to achieve synchronous infection (54). Cells were washed and lysed using 1% saponin in PBS at 0 min (taken immediately after the 10°C centrifugation step), and parallel wells were similarly treated at 60 min, serially diluted in GC broth, and plated to determine numbers of viable CFU. Survival was expressed as the percentage of CFU at 60 min relative to CFU at 0 min.

In vivo infection studies. Six- to 8-week-old female BALB/c mice (Jackson Laboratories) were infected with *N. gonorrhoeae* as described previously (8, 14). Briefly, mice were selected in the diestrus phase of the estrous cycle and administered 0.1 mg Premarin (Pfizer) subcutaneously as a single daily dose on days -2 , 0 (day of infection), and $+2$. Premarin is a mixture of sodium estrone sulfate and sodium equilin sulfate and, as concomitant components, sodium sulfate conjugates of 17α -dihydroequilin, 17α -estradiol, and 17β -dihydroequilin. Mice were also given two daily intraperitoneal doses of 0.6 mg of vancomycin (Mylan Pharmaceuticals) and 0.3 mg of streptomycin (X-gen Pharmaceuticals) on days -2 , -1 , and 0 to minimize commensal vaginal flora. Premarin, streptomycin, and vancomycin were all diluted in sterile water. On day 0, mice were infected intravaginally with 10^6 CFU of one of the five *N. gonorrhoeae* mutants: WT/G+, 5-Hex/G+, 4-Hex/G+, 3-Hex/G+, or 2-Hex/G+. Animals in the treated groups ($n = 6$ /group) received 1 or 0.1 μ g of 2C7-Ximab E430G Fc in saline intravaginally once a day beginning on day 0. Control groups ($n = 8$ /group) were administered saline using the same dosing schedule. Vaginal swabs were collected daily for 9 days; bacteria in swabs were eluted in 100 μ l saline. Bacterial burdens were monitored by quantitative cultures of vaginal swab eluates on CA-VCNTS agar. Mice were deemed to have cleared the infection after three successive negative cultures.

Statistical analyses. One-way analysis of variance (ANOVA) was employed to compare multiple groups; pairwise comparisons were made using Tukey's or Dunn's *post hoc* tests for parametric and nonparametric data, respectively. Two-way ANOVA was used to compare groups when concentration was a variable. *In vivo* clearance experiments were tested for time to clearance, longitudinal trends in mean $\log_{10}$ CFU, and the cumulative burden of infection by AUC analysis (8). Kaplan-Meier survival curves yielded the median times to clearance; Mantel-Cox log rank test was used to compare times to clearance between groups. Significance was set using Bonferroni's correction for number of pairwise comparisons when more than two groups were compared. AUCs were compared using ANOVA (Kruskal-Wallis nonparametric testing), and comparisons across groups were made with Dunn's multiple-comparison test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Nancy Nowak for technical assistance.

This work was funded by the National Institutes of Health/National Institute of Allergy and Infectious Diseases grants AI114710 (to P.A.R. and S.G.), AI118161 (to S.R.), and AI114790, AI141181, and AI132296 (to P.A.R. and S.R.).

We thank Ashild Vik and Michael Koomey (University of Oslo, Oslo, Norway) for providing strain MS11 4/3/1 and Daniel C. Stein (University of Maryland, College Park, MD) for the Opa $^-$ MS11 mutant. We also thank the Flow Cytometry Core Facility at the University of Massachusetts Medical School for assistance.

F.J.B. and J.S. have stock and/or warrants in Genmab.

REFERENCES

- Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, Chico RM, Smolak A, Newman L, Gottlieb S, Thwin SS, Broutet N, Taylor MM. 2019. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. Bull World Health Organ 97:548–562. <https://doi.org/10.2471/BLT.18.228486>.
- Anonymous. 2016. Report on global sexually transmitted infection surveillance 2015. World Health Organization, Geneva, Switzerland.
- Unemo M, Shafer WM. 2014. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev 27:587–613. <https://doi.org/10.1128/CMR.00010-14>.

4. Berry JD, Gaudet RG. 2011. Antibodies in infectious diseases: polyclonals, monoclonals and niche biotechnology. *Nat Biotechnol* 28:489–501. <https://doi.org/10.1016/j.nbt.2011.03.018>.
5. Casadevall A, Dadachova E, Pirofski LA. 2004. Passive antibody therapy for infectious diseases. *Nat Rev Microbiol* 2:695–703. <https://doi.org/10.1038/nrmicro974>.
6. Saylor C, Dadachova E, Casadevall A. 2009. Monoclonal antibody-based therapies for microbial diseases. *Vaccine* 27(Suppl 6):G38–G46. <https://doi.org/10.1016/j.vaccine.2009.09.105>.
7. Gulati S, McQuillen DP, Mandrell RE, Jani DB, Rice PA. 1996. Immunogenicity of *Neisseria gonorrhoeae* lipooligosaccharide epitope 2C7, widely expressed in vivo with no immunochemical similarity to human glycosphingolipids. *J Infect Dis* 174:1223–1237. <https://doi.org/10.1093/infdis/174.6.1223>.
8. Gulati S, Zheng B, Reed GW, Su X, Cox AD, St Michael F, Stupak J, Lewis LA, Ram S, Rice PA. 2013. Immunization against a saccharide epitope accelerates clearance of experimental gonococcal infection. *PLoS Pathog* 9:e1003559. <https://doi.org/10.1371/journal.ppat.1003559>.
9. Yamasaki R, Koshino H, Kurono S, Nishinaka Y, McQuillen DP, Kume A, Gulati S, Rice PA. 1999. Structural and immunochemical characterization of a *Neisseria gonorrhoeae* epitope defined by a monoclonal antibody 2C7; the antibody recognizes a conserved epitope on specific lipooligosaccharides in spite of the presence of human carbohydrate epitopes. *J Biol Chem* 274:36550–36558. <https://doi.org/10.1074/jbc.274.51.36550>.
10. Ram S, Gulati S, Lewis LA, Chakraborti S, Zheng B, DeOliveira RB, Reed GW, Cox AD, Li J, St Michael F, Stupak J, Su XH, Saha S, Landig CS, Varki A, Rice PA. 2018. A novel sialylation site on *Neisseria gonorrhoeae* lipooligosaccharide links heptose II lactose expression with pathogenicity. *Infect Immun* 86:e00285–18. <https://doi.org/10.1128/IAI.00285-18>.
11. Landig CS, Hazel A, Kellman BP, Fong JJ, Schwarz F, Agarwal S, Varki N, Massari P, Lewis NE, Ram S, Varki A. 2019. Evolution of the exclusively human pathogen *Neisseria gonorrhoeae*: human-specific engagement of immunoregulatory Siglecs. *Evol Appl* 12:337–349. <https://doi.org/10.1111/eva.12744>.
12. de Jong RN, Beurskens FJ, Verploegen S, Strumane K, van Kampen MD, Voorhorst M, Horstman W, Engelberts PJ, Oostindie SC, Wang G, Heck AJ, Schuurman J, Parren PW. 2016. A novel platform for the potentiation of therapeutic antibodies based on antigen-dependent formation of IgG hexamers at the cell surface. *PLoS Biol* 14:e1002344. <https://doi.org/10.1371/journal.pbio.1002344>.
13. Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, Voorhorst M, Ugurlar D, Rosati S, Heck AJ, van de Winkel JG, Wilson IA, Koster AJ, Taylor RP, Saphire EO, Burton DR, Schuurman J, Gros P, Parren PW. 2014. Complement is activated by IgG hexamers assembled at the cell surface. *Science* 343:1260–1263. <https://doi.org/10.1126/science.1248943>.
14. Gulati S, Beurskens FJ, de Kreuk BJ, Roza M, Zheng B, DeOliveira RB, Shaughnessy J, Nowak NA, Taylor RP, Botto M, He X, Ingalls RR, Woodruff TM, Song WC, Schuurman J, Rice PA, Ram S. 2019. Complement alone drives efficacy of a chimeric antigonococcal monoclonal antibody. *PLoS Biol* 17:e3000323. <https://doi.org/10.1371/journal.pbio.3000323>.
15. Chakraborti S, Lewis LA, Cox AD, St Michael F, Li J, Rice PA, Ram S. 2016. Phase-variable heptose I glycan extensions modulate efficacy of 2C7 vaccine antibody directed against *Neisseria gonorrhoeae* lipooligosaccharide. *J Immunol* 196:4576–4586. <https://doi.org/10.1049/jimmunol.1600374>.
16. Stein DC, LeVan A, Hardy B, Wang LC, Zimmerman L, Song W. 2015. Expression of opacity proteins interferes with the transmigration of *Neisseria gonorrhoeae* across polarized epithelial cells. *PLoS One* 10:e0134342. <https://doi.org/10.1371/journal.pone.0134342>.
17. Roth A, Mattheis C, Muenzner P, Unemo M, Hauck CR. 2013. Innate recognition by neutrophil granulocytes differs between *Neisseria gonorrhoeae* strains causing local or disseminating infections. *Infect Immun* 81:2358–2370. <https://doi.org/10.1128/IAI.00128-13>.
18. Sadarangani M, Pollard AJ, Gray-Owen SD. 2011. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic *Neisseria*. *FEMS Microbiol Rev* 35:498–514. <https://doi.org/10.1111/j.1574-6976.2010.00260.x>.
19. Schneider H, Cross AS, Kushner RA, Taylor DN, Sadoff JC, Boslego JW, Deal CD. 1995. Experimental human gonococcal urethritis: 250 *Neisseria gonorrhoeae* MS11mkC are infective. *J Infect Dis* 172:180–185. <https://doi.org/10.1093/infdis/172.1.180>.
20. Schneider H, Griffiss JM, Boslego JW, Hitchcock PJ, Zahos KM, Apicella MA. 1991. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. *J Exp Med* 174:1601–1605. <https://doi.org/10.1084/jem.174.6.1601>.
21. Wu H, Jerse AE. 2006. Alpha-2,3-sialyltransferase enhances *Neisseria gonorrhoeae* survival during experimental murine genital tract infection. *Infect Immun* 74:4094–4103. <https://doi.org/10.1128/IAI.00433-06>.
22. Lewis LA, Gulati S, Burrows E, Zheng B, Ram S, Rice PA. 2015. Alpha-2,3-sialyltransferase expression level impacts the kinetics of lipooligosaccharide sialylation, complement resistance, and the ability of *Neisseria gonorrhoeae* to colonize the murine genital tract. *mBio* 6:e02465-14. <https://doi.org/10.1128/mBio.02465-14>.
23. Lam J, Gray-Owen SD. 2017. Genetic adaptation contributing to increased gonococcal fitness during vaginal infection of CEACAM-humanized mice. Master's thesis. University of Toronto, Toronto, Canada. <https://space.library.utoronto.ca/handle/1807/77840>.
24. Gulati S, Cox A, Lewis LA, Michael FS, Li J, Boden R, Ram S, Rice PA. 2005. Enhanced factor H binding to sialylated Gonococci is restricted to the sialylated lacto-N-neotetraose lipooligosaccharide species: implications for serum resistance and evidence for a bifunctional lipooligosaccharide sialyltransferase in gonococci. *Infect Immun* 73:7390–7397. <https://doi.org/10.1128/IAI.73.11.7390-7397.2005>.
25. McLaughlin SE, Cheng H, Ghanem KG, Yang Z, Melendez J, Zenilman J, Griffiss JM. 2012. Urethral exudates of men with *Neisseria gonorrhoeae* infections select a restricted lipooligosaccharide phenotype during transmission. *J Infect Dis* 206:1227–1232. <https://doi.org/10.1093/infdis/jis481>.
26. Camara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, Ardanuy C. 2012. Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. *J Antimicrob Chemother* 67:1858–1860. <https://doi.org/10.1093/jac/dks162>.
27. Lahra MM, Ryder N, Whitley DM. 2014. A new multidrug-resistant strain of *Neisseria gonorrhoeae* in Australia. *N Engl J Med* 371:1850–1851. <https://doi.org/10.1056/NEJMc1408109>.
28. Lee K, Nakayama SI, Osawa K, Yoshida H, Arakawa S, Furubayashi KI, Kameoka H, Shimuta K, Kawahata T, Unemo M, Ohnishi M. 2019. Clonal expansion and spread of the ceftriaxone-resistant *Neisseria gonorrhoeae* strain FC428, identified in Japan in 2015, and closely related isolates. *J Antimicrob Chemother* 74:1812–1819. <https://doi.org/10.1093/jac/dkz129>.
29. Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, Nakayama S, Kitawaki J, Unemo M. 2011. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea? Detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 55:3538–3545. <https://doi.org/10.1128/AAC.00325-11>.
30. Centers for Disease Control and Prevention. 2012. Update to CDC's sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer a recommended treatment for gonococcal infections. *MMWR Morb Mortal Wkly Rep* 61:590–594.
31. Weston EJ, Workowski K, Torrone E, Weinstock H, Stenger MR. 2018. Adherence to CDC recommendations for the treatment of uncomplicated gonorrhea—STD Surveillance Network, United States, 2016. *MMWR Morb Mortal Wkly Rep* 67:473–476. <https://doi.org/10.15585/mmwr.mm6716a4>.
32. Fifer H, Natarajan U, Jones L, Alexander S, Hughes G, Golparian D, Unemo M. 2016. Failure of dual antimicrobial therapy in treatment of gonorrhea. *N Engl J Med* 374:2504–2506. <https://doi.org/10.1056/NEJMc1512757>.
33. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M. 2015. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PLoS One* 10:e0143304. <https://doi.org/10.1371/journal.pone.0143304>.
34. Unemo M, Nicholas RA. 2012. Emergence of multidrug-resistant, extensively drug-resistant, and untreatable gonorrhea. *Future Microbiol* 7:1401–1422. <https://doi.org/10.2217/fmb.12.117>.
35. Figueroa JE, Densen P. 1991. Infectious diseases associated with complement deficiencies. *Clin Microbiol Rev* 4:359–395. <https://doi.org/10.1128/cmr.4.3.359>.
36. Boelaert J, Joos R, Criel A, Van Landuyt HW, Daha MR. 1985. C5 deficiency in a white family. *Arch Intern Med* 145:1333. <https://doi.org/10.1001/archinte.1985.00360070215046>.
37. Petersen BH, Lee TJ, Snyderman R, Brooks GF. 1979. *Neisseria meningitidis* and *Neisseria gonorrhoeae* bacteremia associated with C6, C7, or C8 deficiency. *Ann Intern Med* 90:917–920. <https://doi.org/10.7326/0003-4819-90-6-917>.
38. Crew PE, Abara WE, McCulley L, Waldron PE, Kirkcaldy RD, Weston EJ,

- Bernstein KT, Jones SC, Bersoff-Matcha SJ. 12 November 2018. Disseminated gonococcal infections in patients receiving eculizumab: a case series. *Clin Infect Dis* <https://doi.org/10.1093/cid/ciy958>.
39. Blom AM, Ram S. 2008. Contribution of interactions between complement inhibitor C4b-binding protein and pathogens to their ability to establish infection with particular emphasis on *Neisseria gonorrhoeae*. *Vaccine* 26:149–155. <https://doi.org/10.1016/j.vaccine.2008.11.049>.
 40. Welsch JA, Ram S. 2008. Factor H and Neisserial pathogenesis. *Vaccine* 26(Suppl 8):140–145. <https://doi.org/10.1016/j.vaccine.2008.11.060>.
 41. Balthazar JT, Gusa A, Martin LE, Choudhury B, Carlson R, Shafer WM. 2011. Lipooligosaccharide structure is an important determinant in the resistance of *Neisseria gonorrhoeae* to antimicrobial agents of innate host defense. *Front Microbiol* 2:30. <https://doi.org/10.3389/fmicb.2011.00030>.
 42. Griffiss JM, Jarvis GA, O'Brien JP, Eads MM, Schneider H. 1991. Lysis of *Neisseria gonorrhoeae* initiated by binding of normal human IgM to a hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain-specific, properdin-binding-dependent alternative complement pathway activation. *J Immunol* 147:298–305.
 43. van Vliet SJ, Steeghs L, Bruijns SC, Vaezizad MM, Snijders Blok C, Arenas Busto JA, Deken M, van Putten JP, van Kooyk Y. 2009. Variation of *Neisseria gonorrhoeae* lipooligosaccharide directs dendritic cell-induced T helper responses. *PLoS Pathog* 5:e1000625. <https://doi.org/10.1371/journal.ppat.1000625>.
 44. Harvey HA, Jennings MP, Campbell CA, Williams R, Apicella MA. 2001. Receptor-mediated endocytosis of *Neisseria gonorrhoeae* into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor. *Mol Microbiol* 42:659–672. <https://doi.org/10.1046/j.1365-2958.2001.02666.x>.
 45. Song W, Ma L, Chen R, Stein DC. 2000. Role of lipooligosaccharide in Opa-independent invasion of *Neisseria gonorrhoeae* into human epithelial cells. *J Exp Med* 191:949–960. <https://doi.org/10.1084/jem.191.6.949>.
 46. Erwin AL, Haynes PA, Rice PA, Gotschlich EC. 1996. Conservation of the lipooligosaccharide synthesis locus *lgt* among strains of *Neisseria gonorrhoeae*: requirement for *lgtE* in synthesis of the 2C7 epitope and of the beta chain of strain 15253. *J Exp Med* 184:1233–1241. <https://doi.org/10.1084/jem.184.4.1233>.
 47. Apicella MA, Mandrell RE, Shero M, Wilson ME, Griffiss JM, Brooks GF, Lammel C, Breen JF, Rice PA. 1990. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: an immunoelectron microscopic analysis. *J Infect Dis* 162:506–512. <https://doi.org/10.1093/infdis/162.2.506>.
 48. Jones C, Virji M, Crocker PR. 2003. Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol Microbiol* 49:1213–1225. <https://doi.org/10.1046/j.1365-2958.2003.03634.x>.
 49. Edwards JL, Jennings MP, Apicella MA, Seib KL. 2016. Is gonococcal disease preventable? The importance of understanding immunity and pathogenesis in vaccine development. *Crit Rev Microbiol* 42:928–941. <https://doi.org/10.3109/1040841X.2015.1105782>.
 50. Hobbs MM, Anderson JE, Balthazar JT, Kandler JL, Carlson RW, Ganguly J, Begum AA, Duncan JA, Lin JT, Sparling PF, Jerse AE, Shafer WM. 2013. Lipid A's structure mediates *Neisseria gonorrhoeae* fitness during experimental infection of mice and men. *mBio* 4:e00892-13. <https://doi.org/10.1128/mBio.00892-13>.
 51. Wolfgang M, van Putten JP, Hayes SF, Dorward D, Koomey M. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J* 19:6408–6418. <https://doi.org/10.1093/emboj/19.23.6408>.
 52. Ray TD, Lewis LA, Gulati S, Rice PA, Ram S. 2011. Novel blocking human IgG directed against the pentapeptide repeat motifs of *Neisseria meningitidis* Lip/H.8 and Laz lipoproteins. *J Immunol* 186:4881–4894. <https://doi.org/10.4049/jimmunol.1003623>.
 53. Stohl EA, Dale EM, Criss AK, Seifert HS. 2013. *Neisseria gonorrhoeae* metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H2O2- and neutrophil-mediated killing. *mBio* 4:e00399-13. <https://doi.org/10.1128/mBio.00399-13>.
 54. Stohl EA, Criss AK, Seifert HS. 2005. The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Mol Microbiol* 58:520–532. <https://doi.org/10.1111/j.1365-2958.2005.04839.x>.