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Neisseria gonorrhoeae: Drug Resistance, Mouse Models, and Vaccine Development

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Abstract

Gonorrhea, an obligate human infection, is on the rise worldwide and gonococcal strains resistant to many antibiotics are emerging. Appropriate antimicrobial treatment and prevention, including effective vaccines, are urgently needed. To guide investigation, an experimental model of genital tract infection has been developed in female mice to study mechanisms by which *Neisseria gonorrhoeae* evades host-derived antimicrobial factors and to identify protective and immunosuppressive pathways. Refinements of the animal model have also improved its use as a surrogate host of human infection and accelerated the testing of novel therapeutic and prophylactic compounds against gonococcal infection. Reviewed herein are the (*a*) history of antibiotic usage and resistance against gonorrhea and the consequences of resistance mechanisms that may increase gonococcal fitness and therefore the potential for spread, (*b*) use of gonococcal infection in the animal model system to study mechanisms of pathogenesis and host defenses, and (*c*) current status of vaccine development.

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INTRODUCTION

Neisseria gonorrhoeae (gonococcus) is an obligate human pathogen and the etiological agent of gonorrhea, an ancient disease (Leviticus 15:1–3, Old Testament). Gonococcal transmission occurs primarily from an infected individual by direct contact between the mucosal membranes of the urogenital tract, anal canal, oropharynx, and occasionally eye (conjunctivitis), usually during sexual activity, and often results in urethritis in men and cervicitis in women. Most symptomatic men with gonococcal urethritis seek medical attention and cease to be infectious after treatment. Asymptomatic men constitute about two-thirds of all infected men at any point in time and are the principal source of spread of infection. More than half of infected women may be asymptomatic, but often they manifest physical signs of inflammation upon direct (speculum) examination of the cervix. Untreated cervical infection may ascend upward to the uterus and fallopian tubes and can cause pelvic inflammatory disease (endometritis and/or salpingitis), which may result in sterility and/or chronic pelvic pain. Rarely, ectopic pregnancy that occurs in a fallopian tube, scarred from a prior infection, may lead to severe hemorrhage. Occasionally *N. gonorrhoeae* enters the bloodstream, disseminates, and causes skin and/or joint/tendon infection and, rarely, endocarditis or meningitis (102). Infected mothers may transmit gonococcal infection to their newborns during delivery that can result in eye infection (ophthalmia neonatorum) and/or, rarely, disseminated infection. Rectal or pharyngeal gonorrhea is often asymptomatic. Gonococcal infection also facilitates the transmission and acquisition of HIV (17).

Gonorrhea is a major global public health problem and the World Health Organization estimated that in 2008, there were 78.3 million new cases in adults (15–49 years of age) worldwide (79). In the United States, the Centers for Disease Control and Prevention (CDC) report gonorrhea as the second most common notifiable bacterial infection (15). In 2015, approximately 395,000 cases of gonorrhea were reported, an increase of 27% from 2012. The national gonorrhea rate in 2015 was 124 new cases per 100,000 population, representing a one-year increase of 13% and the highest rate among industrialized countries (15). The true number of cases is much higher, particularly from resource-poor settings where the lack of laboratory testing/sensitive diagnostics and incomplete case reporting contribute to underrecognition and -reporting.

Gonorrhea is on the rise worldwide and strains resistant to many antibiotics are emerging. Appropriate treatments and preventive measures, including effective vaccines, are urgently needed. To guide research in this direction, a human urethral challenge model in men (46) and an experimental genital tract infection in female mice (51) have been developed to study mechanisms by which *N. gonorrhoeae* evades host-derived antimicrobial factors and to identify protective and immunosuppressive pathways. Refinements of the animal model have improved the use of mice as surrogate hosts of gonorrhea and accelerated the testing of novel therapeutic and prophylactic compounds against gonorrhea. Herein, we review the (*a*) history of antibiotic usage and resistance and the consequences of resistance mechanisms that may increase gonococcal fitness and therefore the potential for spread, (*b*) use of gonococcal infection in animal model systems to study mechanisms of pathogenesis and host defenses, and (*c*) current status of vaccine development.

ANTIBIOTIC RESISTANCE

History of Gonorrhea Treatments and the Development of Antibiotic Resistance

In the absence of a vaccine, effective antibiotic therapy of gonorrhea is critical to cure infected persons and to reduce spread of infection in the community. The efficacy of antibiotic treatment regimens that cure gonorrhea is now threatened by the international emergence and spread of strains resistant to currently (and previously) used antibiotics (83, 119, 121).

Scientific advances in rational development of chemotherapeutics in the early twentieth century set the stage for safe and effective treatments that over the decades have been largely evidence based. The mode of action of representative antibiotics used to treat gonorrhea since 1938, the length of time they have remained effective, and the mechanisms of resistance that led to their discontinuation are summarized in **Table 1** (121). The antibiotic pipeline began with the use of sulfonamides that were effective for only a few years because of the rapid development of resistance due to *folP* mutations (**Table 1**). Fortunately, gonococci were found to be remarkably sensitive to the new miracle drug penicillin (mean inhibitory concentration [MIC] values of 0.01 μg/mL were common), which was brought into clinical practice in 1943–4. In subsequent years, the dose of penicillin was increased to counteract diminished susceptibility of some gonococcal strains. For some individuals (e.g., those with penicillin allergies), alternative antibiotics to treat gonorrhea were effective. Tetracycline was one of these antibiotics, but emergence of resistant strains possessing *tetM* caused its removal from treatment regimens in 1986 [the TetM protein binds to ribosomes and protects them from tetracycline (**Table 1**)]. One year later, penicillin was also removed, because strains with chromosomal mutations (**Table 1**) emerged and proliferated, collectively increasing MICs to penicillin above the breakpoint (>2 μg/mL). Nevertheless, the availability of highly active quinolones and cephalosporins gave reason for confidence, naive in hindsight, that gonorrhea treatments would remain effective. Unfortunately, within a few years of its introduction, ciprofloxacin-resistant strains emerged due to mutations in *gyrA* and *parC* (**Table 1**) that encoded subunits of DNA gyrase and topoisomerase IV, respectively. Predictably, this class of antibiotics was removed from the CDC-recommended guidelines in 2007 (13). Third-generation cephalosporins (ceftriaxone and cefixime) remained as the last line of antibiotics for empiric monotherapy. Gonococcal strains with decreased susceptibility to these β-lactams and occasionally strains that have failed treatment with these agents have emerged in the past decade, resulting in *N. gonorrhoeae* now being assigned to the infamous superbug list. In response to increasing numbers of strains with decreased susceptibility to oral cefixime,

Table 1 Summary of antibiotics used for treatment of gonorrhea

Antibiotic class mutations/mechanisms	Effective years[a]	Principal resistance
Sulfonamides	1938–1942	SNPs in *folP* that reduce sulfonamide affinity for DHPS enzymes
β-Lactams: penicillin	1944–1987	Plasmid-mediated β-lactamase (TEM-1 and TEM-35); *penA* mutations that decrease acylation of PBP2; *ponA* mutations that decrease acylation of PBP1; *mtrR* promoter mutations that increase levels of the MtrCDE efflux pump; *pilQ* mutations that decrease influx
Tetracycline	~1962–1986	*tetM*; ribosome protection; SNPs in *rpsJ*; reduction of affinity for 30S ribosomal subunit protein S10 (V57M)
Doxycycline	~1967–1986	*mtrR*; *porB1B* SNPs (see above)
Spectinomycin	~1962–1986[b]	16S rRNA SNP (C1192U) reduces spectinomycin binding; *rpsE* mutations (30S ribosomal protein 5) disrupt spectinomycin binding
Quinolones	~1990–2007	QRDR mutations in *gyrA* and *parC* reduce drug binding to DNA gyrase and topoisomerase IV, respectively; overexpression of NorM efflux pump
Macrolides: azithromycin	Early 1980s–present[c]	23S rRNA SNPs result in a 23S rRNA target (peptidyltransferase loop of domain V) with a reduced affinity for the 50S ribosomal macrolide target; *mtrR* promoter mutations (see above); *erm* genes (*ermB*, *ermC*, and *ermF*) encoding rRNA methylases that methylate nucleotides in the 23S rRNA target that block the binding of macrolides; MacAB efflux pump overexpression increases the MICs of macrolides; *mef*-encoded efflux pump exports macrolides out of the bacterial cell and increases the MICs of macrolides
Third-generation cephalosporins	~1980–present	Non-β-lactamase mechanisms described; highly remodeled PBP2 due to mosaic *penA*

[a]The effective years listed are restricted to the United States.
[b]Spectinomycin is no longer available in many countries, including the United States, but is still widely used in some countries, such as China.
[c]Together with ceftriaxone, azithromycin is used in dual antibiotic therapy.
Table modified with permission from The American Society for Microbiology (121). Abbreviations: DHPS, dihydropteroate synthase; MIC, mean inhibitory concentration; PBP, penicillin-binding protein; QRDR, quinolone resistance determining region; SNP, single nucleotide polymorphism.

in 2012, the CDC recommended that cefixime no longer be used, and subsequently, dual antibiotic therapy consisting of ceftriaxone (injectable) and azithromycin (oral) was recommended to treat uncomplicated gonorrhea (7, 14). Now, azithromycin-resistant strains are increasingly prevalent, particularly in China (70), and there are increasing reports of strains that have failed therapy with cefixime/ceftriaxone. Worryingly, a strain resistant to the currently recommended dual antibiotic regimen of ceftriaxone and azithromycin was recently isolated from an infected individual (32).

The antibiotic pipeline for treatment of gonorrhea is now nearly closed and needs reopening with new drug discovery efforts (120). New antimicrobials, some of which are in clinical trials (e.g., the novel bacterial topoisomerase inhibitor spiropyrimidinetrione ETX0914, and the fluoroketolide solithromycin), have been reviewed (121, 122).

Antibiotic Resistance and Fitness in *N. gonorrhoeae*

Resistance mechanisms affect therapeutic options, but an equally important concern is the consequence of resistance to spread of gonococcal infection in the community. Traditionally, antibiotic resistance is thought to be accompanied by a cost in fitness, particularly when it results

from mutations in genes that are important for basic cellular functions (5). However, some resistance mutations in gonococci provide a growth benefit in vitro or increase fitness when tested in mouse models of infection/pathogenesis (62, 126, 127) (see below). This may result from compensatory mutations that offset the detrimental effects of resistance mutations. Increased microbial fitness during infection can also be a direct consequence of the resistance mutation itself, for example, mutations that increase the efflux of antimicrobial substances (126, 127).

Two mechanisms of antibiotic resistance in *N. gonorrhoeae* that also increase in vivo fitness have been identified. The first includes resistance mutations in the repressor gene (*mtrR*) or its promoter region that repress transcription of the gonococcal *mtrCDE* operon; the second is a novel MtrR-independent promoter mutation that increases *mtrCDE* transcription (82, 126). Overexpression of the MtrCDE active efflux pump that results from these mutations confers clinically relevant resistance to macrolide antibiotics (e.g., erythromycin, azithromycin) and also to penicillin (36, 123). The MtrCDE pump also expels hydrophobic antimicrobial substances, for example, cationic antimicrobial peptides (CAMPs), fatty acids, bile salts, and progesterone (126, 127). Therefore, increased fitness of *mtr* mutants in vivo likely occurs as a result of decreased susceptibility to innate antimicrobial substances. In support, clinically relevant *mtr* mutations that confer increasing degrees of erythromycin resistance possess the same hierarchy of resistance to CAMPs and differential fitness advantage in the mouse model (127).

Fluoroquinolones target topoisomerases to inhibit DNA synthesis; interestingly, a commonly isolated $gyrA_{91/95}$ mutation enhances in vivo fitness of *N. gonorrhoeae* in the mouse model (62). A more than tenfold advantage in fitness was also observed for a ciprofloxacin-intermediate $gyrA_{91/95}$ mutant in the model compared to the sensitive wild-type parent strain. Introduction of a second (common) $parC_{86}$ mutation into the $gyrA_{91/95}$ mutant resulted in full resistance to ciprofloxacin, as expected, but carried a significant cost in fitness, measured in vitro. The fitness of the combined $gyrA_{91/95}$, $parC_{86}$ mutant was similar to that in the sensitive wild-type strain in vivo but was outcompeted by the $gyrA_{91/95}$ mutant (62). Prevalence of ciprofloxacin resistance has not declined since the removal of fluoroquinolones from the CDC-recommended list of therapies in 2007. It is possible that $gyrA_{91/95}$ mutations provide a reservoir of partially resistant strains that can then become fully resistant to fluoroquinolones upon acquisition of a single base pair mutation in *parC*.

Selection for compensatory mutations that enhances gonococcal fitness (e.g., diminishes/reverses defects in fitness caused by some resistance mutations) is well documented in the mouse infection model and is also likely to occur in human infections. For example, an *mtrA* mutant, which cannot induce the *mtrCDE* operon (106), is attenuated in the mouse model of infection, as expected. However, compensatory mutations in the *mtrR* structural gene or *mtr* promoter region, which are selected for in vivo, enhance fitness that is similar to or higher than the sensitive wild-type strain (126). High-level fluoroquinolone-resistant mutants that carry *gyr* and *parC* mutations or *gyrA*, *parC*, and *mtr* mutations, whose growth in vitro is attenuated, are outcompeted in the mouse model by the sensitive parent strain or mutants that carry a single resistant allele, *gyrA*, or resistant *gyrA* and *mtr* alleles together. Nevertheless, after a period of decreased recovery, high numbers of ciprofloxacin-resistant isolates sometimes emerge. This suggests that compensatory mutation(s) can occur that relieves a defect in fitness. One of these compensatory mutant strains has been examined and found both to carry a different amino acid substitution in the *gyrA* allele and to possess the ability to repair the *mtr* promoter mutation; in vitro studies show that this compensatory mutant grows better than the sensitive parent strain and also maintains enhanced fitness in the mouse model (62).

ANIMAL MODELS

Animal Models of Gonococcal Genital Tract Infection

Animal modeling of *N. gonorrhoeae* infections is challenged by the strict adaptation of *N. gonorrhoeae* to humans. Experimental genital tract infection has been successful only in chimpanzees (2), which are no longer available, and estradiol-treated female mice (51, 118). Several inbred and outbred mouse strains can be infected with *N. gonorrhoeae* provided they are given 17β-estradiol to extend estrus during the reproductive cycle and antibiotics to suppress the overgrowth of commensal flora that occurs under the influence of estradiol (54). Female mice in the proestrus stage of the reproductive cycle can be colonized transiently with *N. gonorrhoeae* (10). Estradiol suppresses cytokine production and therefore is influential in expression of innate receptors and effectors (116, 131), which reduces the usual robust influx of neutrophils in normal mice that occurs after ovulation. Changes in neutrophil function that are estrogen mediated do not seem to play a role; neutrophils from untreated and estradiol-treated mice show no difference in the capacity to kill *N. gonorrhoeae* (114, 130). Several host restrictions limit the ability of mice to mimic gonococcal infection in humans, including human-specific receptors for adherence and invasion pathways, the use of human transferrin and lactoferrin as an iron source, binding to soluble regulators of the complement cascade [factor H (FH), C4-binding protein (C4BP)], and IgA1, the substrate of gonococcal IgA1 protease. In addition, mice lack the opsonophagocytic receptor for IgA, FcαR (CD89) (reviewed in 54). Some restrictions can be alleviated by transgenic (Tg) mice that express human carcinoembryonic antigen cellular adhesion molecules (CEACAMs; 108), human CD46 (55), human transferrin (133), and human FH and C4BP (29, 67).

Characteristics of Experimental Murine Infection

Several lines of investigation using different mouse strains and gonococcal mutants have helped characterize female mice as surrogate hosts for *N. gonorrhoeae*, beginning with the careful examination of colonization kinetics and host innate responses over the course of infection and the demonstration that the reproductive cycle plays a role in colonization patterns.

Colonization and influence of reproductive cycle. *N. gonorrhoeae* infection of BALB/c or C57/BL6 mice persists for an average of 10–12 days and as long as 40 days. Gonococci localize to the cervicovaginal lumen and within genital tract tissue, including the lamina propria. Endometrial cultures are positive in 17–20% of vaginally inoculated mice, which is similar to culture rates for endometrial infection in women, but upper tract infection is not sustained (54). It is therefore likely that establishment of a productive upper reproductive tract infection is prevented by the absence of one or more host-restricted factors. Hormones also play a role in the susceptibility of the upper tract; recently, transcervical inoculation directly into the uterus of diestrus-stage or progesterone-treated mice with *N. gonorrhoeae* was shown to result in short-term upper tract infection, increased host responses, and bacterial invasion into tissue (49).

Reproductive hormones also influence lower genital tract infection, and the relationship between the stages of the reproductive cycle and susceptibility to *N. gonorrhoeae* in mice is consistent with reports that *N. gonorrhoeae* was more frequently obtained during the proliferative (high-estradiol) stage and not in the luteal stage of the menstrual cycle in infected women (50, 57, 61). In one study, several consecutive negative cultures were obtained from infected women during the luteal phase, followed by isolation of *N. gonorrhoeae* upon transition to the next cycle (61). A similar recovery pattern occurs in female mice in which high numbers of gonococci are isolated

early in infection, followed by a period of significantly reduced recovery or negative cultures and then a rebound in the number of gonococci recovered (51, 112). This fluctuating pattern does not occur in estradiol-treated, ovariectomized mice. Hormonally driven selection for gonococcal opacity (Opa) protein expression occurs during the proliferative phase in both human cervical infection (50) and infection of female mice (18, 112). Collectively, these observations illustrate the sophisticated adaptation of the gonococcus to the female host.

Inflammatory response and the Th17 pathway. *N. gonorrhoeae* colonizes estradiol-treated C57/BL6 and BALB/c mice to similar levels, but in contrast to BALB/c mice, C57/BL6 mice do not have a significant proinflammatory cytokine/chemokine response or neutrophil influx during infection (54, 84). Significant increases of vaginal IL-6, TNF-α, KC, and MIP-2 are detected in BALB/c mice within 3–5 days of infection and a peak in vaginal polymorphonuclear neutrophils (PMNs) occurs at day 5. Genetic factors that may be responsible for this difference include the absence of secretory phospholipase A2 (sPLA2) in C57/BL6 mice, which can accelerate the inflammatory response through the production of lipid mediators and influence PMN migration and the production of proinflammatory cytokines and chemokines from immune cells (54).

TLR4 controls gonococcal colonization during murine infection. Significantly fewer gonococci are isolated from wild-type BALB/c (TLR4/*lps*^{n}) mice versus BALB/c mice with the defective TLR4/*lps*^{d} allele (85). Phosphoethanolamine (PEA) substitution at the 4′ position of gonococcal lipid A increases interaction with murine or human TLR4 to cause higher levels of NFκB expression and results in higher levels of proinflammatory cytokines (56, 71). Lower levels of vaginal TNFα, IL-1β, and MIP-1α are detected in mice infected with *lptA* mutants lacking PEA substitution compared to the wild-type strain (86). *N. gonorrhoeae* may capitalize on the inflammatory response to suppress inhibitory commensal flora and promote transmission. PEA-decorated lipid A also benefits the gonococcus by increasing resistance to CAMPs (e.g., cathelicidins and defensins), other antimicrobial proteins, and complement-mediated killing (66). The demonstration that an *lptA* mutant was attenuated in the human urethral challenge model and the female mouse model (45) supports the importance of this lipid A modification.

Th17 responses can be elevated in human subjects infected with *N. gonorrhoeae* compared to healthy controls (33). In mice, the inflammatory response to gonococcal infection is driven by the Th17 pathway. Differentiation and proliferation of Th17 cells are induced by gonococcal stimulation of antigen-presenting cells to produce IL-6, TGFβ, IL-1β, and IL-23. Binding of IL-17 to IL-17 receptor A, present on endothelial and stromal cells, results in the production of cytokines/chemokines, which recruit PMNs to the site of infection. Stimulation of mouse splenic mononuclear cells with *N. gonorrhoeae* or gonococcal outer membrane vesicles (OMVs) releases Th1 but not Th2 cytokines (31). Th1 cytokines are also produced by cells from draining iliac lymph nodes in infected mice (74) and by vaginal explants incubated with *N. gonorrhoeae*, which also produce downstream chemokines. Th17 responses likely originate from CD4^{+} T cells (Th17 cells) and γδ T cells, which are abundant on mucosal surfaces (30). The Th17 response in mice is also dependent on gonococcal lipid A–mediated signaling through TLR4 (30, 85).

Evasion of Innate Immune Defenses

A critical evolution behind the success of the gonococcus as a pathogen, as with other pyogenic bacteria, has been selection for evasion mechanisms against host innate effectors. The importance of several gonococcal evasion mechanisms has been tested using genetically defined mutants of *N. gonorrhoeae* with reduced or increased susceptibility to host innate effectors in normal mice and mice that are genetically deficient in these defenses.

Cationic antimicrobial proteins. Secretions from the female genital tract contain hydrophobic antimicrobial substances, including bile salts, fatty acids, progesterone, and CAMPs. CAMPs are bactericidal and immunomodulatory; epithelial cells can induce their expression, or they can be delivered to the site of infection within phagocytic granules. Gonococci are well equipped to defend against CAMPs. The MtrC-MtrD-MtrE active efflux pump captures and expels the human and murine cathelicidin LL-37 and cathelicidin-related antimicrobial peptide (CRAMP), respectively (127); surface binding of CAMPs is reduced by chemical modifications of lipooligosaccharide (LOS). As discussed, PEA substitution at the 4′ position of lipid A increases gonococcal resistance to CAMPs (66).

Complement. Complement is present at relatively high basal levels in the female genital tract and in inflammatory exudates, but the gonococcus has evolved several mechanisms to evade complement. Most strains that express the porin (Por) molecule PorB.1A and many PorB.1B strains are inherently resistant to complement-dependent direct killing by normal human serum (NHS) due to the binding of FH or C4b-binding protein (C4BP) to surface-exposed PorB loops. FH and C4BP are host-restricted soluble regulators of the alternative and classical pathways of complement, respectively. PorB-mediated binding of FH and C4BP protects the pathogen by decreasing complement activation at the gonococcal surface (96, 97). This mechanism of complement evasion likely is instrumental for bloodstream infection based on the strong correlation between strains that disseminate and their ability to resist killing by NHS (96). LOS sialylation or PEA substitution of lipid A also protects *N. gonorrhoeae* from complement-dependent killing (66, 68) by binding human FH (98) or C4BP, respectively. LOS sialylation is also important in protecting against serum-mediated opsonophagocytic uptake by PMNs (35, 129).

Neutrophil killing. *N. gonorrhoeae* has a remarkable capacity to resist killing by neutrophils through decreasing gonococcal uptake by PMNs and intracellular killing, once ingested. LOS sialylation reduces C3b-dependent uptake (35, 60, 98, 101). In addition, phase-variable expression of Opa proteins results in gonococcal variants that do not bind to neutrophil CEACAMs and thus evade nonopsonic uptake (3, 113). PMNs ingest gonococci during infection as evidenced by Gram stains of patient exudates. In vitro, approximately 50% of gonococci within PMNs remain viable; the mechanisms for intracellular survival have not been fully elucidated. PMN oxidative defenses do not challenge *N. gonorrhoeae*, based on studies with human chronic granulomatous disease neutrophils (21, 100), pharmacological inhibitors (21), and experimentally infected NADPH oxidase–defective mice (130). Gonococcal antioxidant factors such as catalase, cytochrome *c* peroxidase, or quenching through manganese uptake do not protect against human or murine neutrophil killing in vitro or during experimental murine infection (109, 110, 130). Instead, the gonococcus may actively suppress induction of the phagocytic respiratory burst in phagocytes (22) or delay fusion of the phagosome with primary granules (58). In addition, gonococcal lytic transglycosidases release peptidoglycans extracellularly and protect organisms from neutrophils by contributing to envelope integrity, thereby limiting bacterial exposure to select antimicrobial proteins in neutrophil granules (95).

Suppression of the Adaptive Immune Response

In recent years, emphasis on the lack of a protective response following gonococcal infection has turned to identifying immunosuppressive pathways (9, 27, 81, 87, 136). Induction of apoptosis in antigen-presenting cells through the NLRP3 inflammasome pathway (27) and inhibition of dendritic cell–induced proliferation of T cells (136) are two major pathways. In addition, gonococcal Opa proteins that bind CEACAM1 downregulate proliferation of activated CD4+ T cells and

B cells (9, 87). Th1- and Th2-driven adaptive immune responses are suppressed during murine infection by mechanisms dependent on TGF-β and IL-10 (74, 75) and by induction of type 1 regulatory T (Treg) cells (73). Higher numbers of TGF-β1+ CD4+ T cells and a subset of CD4+ CD25+ Foxp3+ T cells are found in regional lymph nodes of *N. gonorrhoeae*–infected BALB/c mice. Increased numbers of TGF-β1+ CD11b+ macrophages present in the genital tracts of infected mice could favor the differentiation of Treg cells and thereby suppress immune responses (48). Treatment of mice with blocking antibodies against TGFβ reverses immunosuppression that enables several Th1- and Th2-dependent responses to flourish, resulting in circulating/vaginal antigonococcal antibody production, immunological memory, and protective immunity against reinfection (72, 76).

Understanding mechanisms of immunosuppression in gonococcal infection may reveal sites of potential therapeutic and preventive intervention. Suppression of Th1 cell proliferation that occurs during murine infection is mediated via induction of PDL-1 and PDL-2 expression by dendritic cells. These ligands induce apoptosis of Th1 cells expressing PD1 that results in release of IL-10, which is immunoregulatory and stimulates type 1 Treg cells (136). As proof of concept, to enhance a greater Th1 response, IL-12 administration accelerated clearance of infection, elicited a memory response, and resulted in protection against reinfection with the same strain (72). These findings may serve as an example of the use of novel cytokine-based therapies to target infected individuals, who are at highest risk for gonorrhea. Adjuvants that elicit cytokines to enhance prevention may also help to inform the identification and development of vaccine candidates that are best suited to be combined with optimal adjuvants.

VACCINES

Human Gonococcal Vaccine Trials

The development of vaccine candidates effective against gonococcal infection is challenging because the correlates of immune protection in humans are not fully known (107). Gonococcal surface antigenic determinants that may appear suitable are often antigenically variable, modifying their epitopes by antigenic or phase variation (105), which complicates vaccine development by creating an ever-changing surface. The ability to modify surface determinants is beneficial for gonococcal organisms and may result in evasion strategies that increase fitness and facilitate environmental adaptation. A vaccine response that bypasses these adaptations is essential. Unfortunately, adaptive immune responses to highly conserved gonococcal antigens during infection have not been shown to elicit protection against future bouts of infection; in fact, repeat infections are common because robust protective immune responses are not elicited but also because prior infection may enhance susceptibility (91, 104). Levels of genital and serum antibodies elicited, taken as a whole, in men and women with uncomplicated gonococcal urethritis and cervicitis are modest (63), and it remains unclear how responses targeted to specific antigens influence future susceptibility.

In recent times, only two vaccines have entered full-fledged field trials in humans. Both were unsuccessful. The first was a crude, whole-cell vaccine prepared from a single strain of *N. gonorrhoeae* given to an Aboriginal population of Inuit in northern Canada, whose yearly incidence of gonorrhea in sexually active persons was nearly 25%. Vaccinated subjects sustained a cumulative infection rate of 30% (versus 24% in placebo recipients; $p = 0.78$) in the year following vaccination (37). The second was a single-antigen pilus vaccine used in a large-scale field trial conducted in high-risk US military personnel stationed in Korea. Vaccinated male subjects sustained a cumulative infection rate of 6.9% (versus 6.5% in placebo recipients) during the study period (8).

Neither vaccine candidate displayed a sufficient subset of antigenic determinants likely to have been shared across the wild-type strains that exposed the vaccinees.

In the most recent American trial, which took place in 1985 (103), a placebo/control, human challenge trial was performed. Sixty-three male volunteers were either immunized with a vaccine prepared from the outer membranes of a single strain of *N. gonorrhoeae* or given a placebo. Two to four weeks after completing the vaccination course, men were challenged with intraurethral administration of the strain used to prepare the vaccine. No significant difference in infection after challenge was observed in the two groups, but resistance to infection was high: 46% of vaccinees and 36% of placebo recipients resisted infection. The goal for vaccine preparation was enrichment for the Por protein. The proposed mechanism of protection, had it occurred, was to generate complement-fixing antibodies directed against Por that were directly bactericidal (and presumably opsonophagocytic) to gonococci (47) in the urethra. Methods for preparing pure Por were not reliable at the time of the vaccine trial in 1985 (103), and preparations were contaminated with other outer membrane constituents, particularly LOS and reduction modifiable protein (Rmp), which together with Por also stimulated antibody responses in the vaccinees. Not completely appreciated in 1985 were the complex interactions of antibodies directed against these antigens that influenced the net effect upon complement-dependent bactericidal activity.

A graded risk of acquiring gonorrhea in both vaccine and placebo recipients in this trial (103) was not considered prospectively in choosing the cohorts, because at that time, protective immunity against gonorrhea, although in some cases suspected (11) and later studied in more detail (44, 92, 93), was not defined in sufficiently specific terms to permit immunologic stratification of volunteers into different categories. In a look back at the vaccine trial (103), volunteers were retrospectively stratified for immunologic risk, and the question of whether or not susceptibility to infection after intraurethral inoculation was influenced by the vaccine was addressed. The ratio of the concentration of Por and LOS antibodies to that of Rmp antibody (Por-Ab + LOS-Ab/Rmp-Ab) was positively correlated with protection in both vaccine and placebo recipients (more so in placebo recipients because of the disproportionate antibody response elicited by Rmp). Rmp is a highly immunogenic outer membrane component that is conserved (shared) across different strains of *N. gonorrhoeae* (6, 128) and elicits antibody that blocks complement-dependent killing of *N. gonorrhoeae* by anti-Por and anti-LOS antibodies (104). None of the antibody levels alone correlated with protection against challenge. Furthermore, positive and negative changes in bactericidal activity (the latter caused by Rmp blocking antibody elicited by the vaccine) correlated, respectively, with increased protection from and susceptibility to infectious challenge when this variable was considered independently. This study emphasizes that the use of a placebo group and stratification for preexisting immunity will be important considerations in the future design of gonococcal vaccine trials that involve vaccine candidates to which there may already be partial immunity (103).

Proposed Human Vaccine Model

A proposed predictor of protection against gonococcal infection and therefore a surrogate for vaccine efficacy has been an increased level of complement-dependent bactericidal antibody activity directed at *N. gonorrhoeae* (103). Bactericidal antibodies, in addition to killing gonococci directly, promote binding to and ingestion by phagocytes via Fc and complement receptors, which may dispose of infecting organisms (38, 39). However, numerous variables contribute to the efficacy of bactericidal antibodies. In the context of natural infection, for example, the major antigenic targets of bactericidal antibodies are Por and LOS components of the gonococcal outer membrane, which themselves may vary antigenically and in their ability to induce bactericidal antibodies during infection. In addition, effective bactericidal function of antibodies directed against these antigens is downregulated by the presence of blocking antibodies, directed against Rmp (104). Bactericidal

antibody function is also downregulated by the soluble complement regulators C4BP and FH discussed above (96–98). Together, regulatory events appear to make bactericidal antibody activity a variable parameter; however, regulation by any cause can be overcome by sufficiently high levels of bactericidal antibodies, a condition that rarely occurs in natural infection but may be achievable with an appropriate vaccination strategy.

A number of gonococcal surface components that elicit bactericidal antibodies have been identified and are being pursued as vaccine candidates. Some of these targets do not promote bactericidal antibody activity in natural infection and were not predicted as potential candidates from immune responses occurring during infection; they represent examples where nurture trumps nature, because an immune response can be forced under conditions of vaccination that are not seen in natural infection. In addition, bactericidal antibody responses to several of the antigens may target important physiologic functions that if disrupted could compromise *N. gonorrhoeae* further; these include colonization and invasion (1, 12, 19, 23, 26, 28, 34, 43, 59, 78, 111, 124), nutrient acquisition (20, 24, 25, 46, 64, 89, 94, 96, 115), and immune evasion (16, 39, 41, 47, 65, 69, 80, 97, 98) (**Table 2**). Additional vaccine candidates that elicit bactericidal antibodies (**Table 2**) have been identified by proteomic analysis of *N. gonorrhoeae* surface proteins (111, 137) and bioinformatic analysis in *N. gonorrhoeae* of an adhesin complex protein (ACP) homolog that was originally identified in *N. meningitidis* (1). Other vaccine candidates that target function but are not known to elicit bactericidal activity are also discussed in two reviews by one of us (A.E.J.) and colleagues (52, 53).

In an early experiment performed in mice, immunization with gonococcal outer membrane elicited vaginal and serum antibodies against several outer membrane proteins. Taken as a whole, these antibodies were bactericidal and accelerated clearance of gonococcal organisms (90) (**Table 3**). However, a similar experiment that faithfully reproduced the vaccine experiment above, reported eleven years later, failed to protect immunized mice or accelerate clearance of gonococci (134). Using an alternative approach that favored a Th1 response, mice underwent immunization (were primed) with PorB-expressing Venezuelan equine encephalitis virus replicon particles followed by boosting with recombinant PorB (rrPorB) (135). Although the antibodies elicited were not bactericidal, clearance of infection occurred significantly faster in immunized mice compared to controls (134).

A highly conserved structure characterized by lactose substitutions at HepI and HepII in the LOS core is a widely expressed epitope (2C7 epitope) that is highly immunogenic in human gonococcal infection (38). The corresponding monoclonal antibody (2C7 mAb) is bactericidal and promotes opsonophagocytic killing of *N. gonorrhoeae* (38). Although the 2C7 epitope is phase variable, under control of the *lgtG* glycosyltransferase gene that contains a poly-C tract to enable slip strand mispairing (4), the 2C7 epitope is nevertheless expressed in more than 95% of gonococci in situ in human infection (38) and promotes survival of the microbe in the mouse experimental model (41). Antibodies against a peptide mimic of the highly conserved 2C7-LOS epitope are also bactericidal (80). Immunization of mice with a multiantigen form of the 2C7-LOS peptide mimics elicited vaginal and bactericidal serum antibodies and accelerated clearance of gonococcal infection. Although mice responded to immunization with a Th1-biased IgG antibody response, passive administration of the IgG3 (λ) 2C7 mAb (a non-Th1 IgG subclass) directed against the LOS epitope also enhanced clearance (41). In the 1985 vaccine study performed in humans, discussed above (103), anti-Rmp blocking IgG antibody, also elicited by the outer membrane–based vaccine used in that study, appeared to have decreased the protective efficacy of the vaccine. Clearance of infection in mice administered 2C7 vaccine antibody was also delayed by anti-Rmp antibody, but an increased stoichiometric ratio ($\geq$3) of 2C7-Ab to Rmp-Ab overcame the effect of anti-Rmp antibody in prolonging infection (40). This suggests that eliciting a robust response to a protective vaccine in humans can override relevant immune mechanisms that may be subversive.

Table 2 ***Neisseria gonorrhoeae*** **vaccine candidates that elicit bactericidal antibodies**

Antigen	Function	Expression	Variability	Immunogenicity[a]
Colonization and invasion				
PilQ (26)	Outer membrane channel for pilus extrusion	Stable	Conserved at C terminus	Antibodies elicited by *N. meningitidis* homologs are bactericidal (43)
Opa (12, 19, 23, 124)	Adherence, invasion	Phase variable	Variable	Bactericidal antibody (12, 19, 23)
OpcA (59, 78)	Adherence, invasion	Stable	Conserved	Antibodies elicited by *N. meningitidis* homologs are bactericidal (59)
PorB (28, 34)	Adherence, invasion	Stable, essential	Variable	Bactericidal antibody (34)
Nutrient acquisition				
TbpA	Transferrin-binding protein	Induced in iron-limiting conditions	TbpA semiconserved (>90%) with hypervariable segments	Bactericidal antibody (94)
TbpB (46)	Transferrin-binding protein	Induced in iron-limiting conditions	TbpB variable with conserved segments	Bactericidal antibody (94)
LbpA (89)	Lactoferrin-binding protein	Induced in iron-limiting conditions	LbpA semiconserved (including most of the exposed loops)	Antibodies elicited by *N. meningitidis* homologs are bactericidal, but cross-reactivity (in *N. meningitidis*) is limited (89)
LpbB (89)	Lactoferrin-binding protein	Induced in iron-limiting conditions	LbpB variable	Antibodies elicited by *N. meningitidis* homologs are bactericidal, but cross-reactivity (in *N. meningitidis*) is limited (89)
ZnuD (115)	Zinc transporter	Induced by zinc limitation	Conserved	Antibodies elicited by the *N. meningitidis* homolog (ZnuD) are bactericidal (115)
MtrE	Surface-exposed channel of the MtrC-MtrD-MtrE (24) and FarA-FarB-MtrE (64) active efflux pumps	Stable	Highly conserved	Bactericidal antibody (25)

(*Continued*)

Table 2 (*Continued*)

Antigen	Function	Expression	Variability	Immunogenicity[a]
Immune evasion				
PorB (16, 47, 97, 98)	Binds C4BP and FH (also critical for nutrient acquisition)	Stable, essential	Variable	Antibodies elicited by *N. meningitidis* PorB cyclic loop peptides are bactericidal (16)
NspA (65, 69)	Binds FH	Stable	Highly conserved	Antibodies elicited by *N. meningitidis* homolog (NspA) are bactericidal (69)
2C7 epitope; inner glycose core of LOS characterized by lactose substitution of both HepI and HepII (38)	Promotes survival of *N. gonorrhoeae* in humans (38) and experimental mice (41)	Under control of a phase-variable gene (*lgtG*) (4), the 2C7 epitope is nonetheless widely expressed	Highly conserved	Bactericidal (39, 41, 80) and opsonophagocytic (39) antibodies elicited by 2C7 epitope mimics[b] (39, 41, 80)
Proteomic analysis of *N. gonorrhoeae* surface proteins (137)				
BamA[c] (NGO1801)	Outer membrane protein assembly factor	Stable, essential[d]	Highly conserved	Bactericidal antibody (137)
LptD[e] (NGO1715)	LOS assembly	Stable, essential	Highly conserved	Bactericidal antibody (137)
TamA[f] (NGO1956)	Translocation assembly	Stable	Highly conserved	Bactericidal antibody (137)
MetQ (NGO2139[g])	Methionine transport adhesin (111)	Stable	Highly conserved	Bactericidal antibody (111, 137)
NGO2054[h]	Unknown	Stable	Highly conserved	Bactericidal antibody (137)
Bioinformatic analysis				
Ng-ACP (NGO1981) (1)	Adhesin complex protein	Stable	Highly conserved	Bactericidal antibody (1)

[a]Bactericidal antibodies directed against *N. gonorrhoeae* unless stated otherwise (directed against *N. meningitidis*).
[b]Including a 2C7 monoclonal anti-idiotope antibody (39) and a peptide mimic of the 2C7 epitope (41, 80).
[c]Outer membrane protein assembly factor BamA.
[d]Not influenced by normal human serum, iron depletion, or oxygen deprivation.
[e]LOS-assembly protein LptD.
[f]Translocation and assembly module protein TamA.
[g]*N. meningitidis* homolog (GNA1946).
[h]Putative uncharacterized protein.
Table adapted from References 52 and 53, with permission. Abbreviations: FH, factor H; LOS, lipooligosaccharide.

Table 3 Gonococcal vaccine candidates that accelerate clearance of cervicovaginal *Neisseria gonorrhoeae* in the experimental mouse model

Antigen	Expression	Variability	Immunogenicity[a]
OM (90)	Stable, essential	OM antibodies[a] recognize multiple bands by Western blot	Antibodies elicited by OM immunization are bactericidal (90)
rrPorB-VRP (134) prime and boost (rrPorB), both administered via footpad (135)	Stable	Variable	Antibodies elicited by rrPorB-VRP (prime) and rrPorB (boost) are nonbactericidal (134)
2C7 epitope (38, 41); inner glycose core of LOS characterized by lactose substitution of both HepI and HepII	Phase variable but expressed by >95% of isolates in vivo in human infection; essential to maintain infectivity	Common epitope in otherwise variable LOS	2C7 antibodies elicited by a 2C7 vaccine candidate[b] are bactericidal; both active and passive immunization accelerate clearance of infection (41)

[a]Antibodies elicited by immunization of mice.
[b]Vaccine candidate configured as a 2C7 peptide mimic (41).
Abbreviations: LOS, lipooligosaccharide; OM, outer membrane; VRP, virus replicon particles.

SUMMARY

Gonococcal infection is on the rise worldwide and gonococcal strains resistant to many antibiotics are emerging. Appropriate treatments that include effective vaccines are urgently needed. Characterization of antibiotic resistance mechanisms and identification of the geographic distribution of *N. gonorrhoeae* strains that harbor defined resistance patterns is essential. Confronting gonococcal fitness and antibiotic resistance simultaneously is important to eradicating infection in individuals but also in controlling infection in the community. This approach holds the possibility that new antimicrobial strategies can be developed and applied strategically for maximal impact. Development of the experimental mouse model of gonococcal infection has advanced the preclinical testing of antimicrobials and also provided understanding of a number of immunologic mechanisms that may be relevant in human infection. In particular, defining suppression of the adaptive immune response may lead to an understanding of why gonococcal infection in humans does not result in protection on reexposure, thereby providing clues about how to break tolerance. Finally, the systematic examination of different vaccine strategies and mechanisms elicited may better define a potentially protective immune response and/or correlates of infection to provide insight into strategies that will lead to successful clinical trials.

FUTURE ISSUES

1. It is important to develop adjunctive therapies to supplement remaining effective antibiotics, which currently are on the verge of disappearance. Examples include (*a*) histone deacetylase inhibitors that result in increasing production of endogenous antimicrobial peptides (132), (*b*) compounds, including monoclonal antibodies, that combat complement evasion strategies utilized by *N. gonorrhoeae* (99), and (*c*) monoclonal antibodies directed against promising vaccine candidates that could be adapted for human use to improve effector function.

2. Reversal of *N. gonorrhoeae*–mediated immunosuppression is necessary to facilitate a protective memory response elicited by vaccines (136).
3. Identification of promising vaccine candidates by analysis of antigen expression on gonococci directly, in vivo, that will induce protective immune responses when configured as vaccines is particularly important. Transcriptome analysis of genital specimens from infected individuals holds the possibility that upregulated transcriptomes (77) will identify these protein antigens that may encompass virulence factors and antigens that otherwise are robustly expressed in the context of infection.
4. Modification of existing (and licensed) group B *N. meningitidis* vaccines that may already provide partial protection against gonorrhea (88) will play an important role in bolstering vaccine responses effective against *N. gonorrhoeae*. Several candidate antigens may already be present in *N. meningitidis* and others that are specific to *N. gonorrhoeae* could be introduced (**Tables 2**, **3**).
5. Continued investigation of the impact that *Chlamydia* coinfection and, in women, other components of the microbiome have on host response, transmission, and vaccine efficacy (42, 117, 125) is required.

DISCLOSURE STATEMENT

P.A.R. holds patents on a vaccine candidate that is discussed in this review.

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