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PELVIC INFLAMMATORY DISEASE SUPPLEMENT

Immune Responses to *Neisseria gonorrhoeae*: Challenges and Opportunities With Respect to Pelvic Inflammatory Disease

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Pelvic inflammatory disease and infertility frequently develop after female genital tract infection with *Neisseria gonorrhoeae*, but determining their etiology from among various possibilities presents difficulties. Exploitation of serology to identify the causative agent is complicated by numerous factors, and no immunological test currently exists to determine unequivocally whether an individual currently is, or has been, infected with *N. gonorrhoeae*. The extensive antigenic variability of *N. gonorrhoeae* and its expression of antigens shared with other *Neisseria* species commonly carried in humans render problematic an assay that is specific for all gonococcal strains. However, novel conserved gonococcal antigens identified for potential vaccines may find additional application in diagnostic assays. *N. gonorrhoeae* also interferes with the adaptive immune response, and antibody responses to uncomplicated infection are usually weak. Elucidating the mechanisms whereby *N. gonorrhoeae* manipulates the human immune system may lead to improved understanding of the pathogenesis of pelvic inflammatory disease and infertility.

Keywords. *Neisseria gonorrhoeae*; gonorrhea; pelvic inflammatory disease; diagnosis; immune response; antibodies; T cells; cytokines; antigens.

INTRODUCTION: THE PROBLEM

The concept of immunity to *Neisseria gonorrhoeae*, the causative agent of the human sexually transmitted infection, gonorrhea, has been difficult to grasp, because until recently there has been no clear evidence for a state of immunity to this disease. Repeat infections are fairly common, suggesting that an episode of infection does not usually induce a protective immune response. On the other hand, the presence of antibodies reactive against *N. gonorrhoeae* can be readily demonstrated in most samples of human serum, regardless of any known exposure to the organism. As discussed below, several explanations, with varying degrees of substantive support, can be put forward to account for these apparently conflicting findings. One undoubted contributing factor is that *N. gonorrhoeae* displays a remarkable capacity for antigenic variation involving most of its prominent surface antigens. While many pathogens utilize antigenic variation as an immune escape strategy, few do so to the same extent as *N. gonorrhoeae*. Thus it could be plausibly argued that a gonococcal infection might induce an antibody response, but that a subsequent encounter is not recognized by the host's immune memory because the new strain presents a different set of surface antigens. In addition, *N. gonorrhoeae* possesses several mechanisms to thwart complement-mediated bacteriolysis, which is known to be important in immune defense against the closely related pathogen, *N. meningitidis*. Thus even a low level of cross-reactive antibody against gonococcal surface antigens might be insufficient to trigger this immune defense mechanism, or others that also utilize complement factors such as opsonophagocytosis.

However, several findings over recent decades have cast doubt on this simple interpretation and have shed new light on the subject by revealing ways in which *N. gonorrhoeae* interacts with the immune system and manipulates it for its own benefit. These considerations assume new importance in the present context of evaluating immune responses to genital tract infections for diagnosing pelvic inflammatory disease (PID) or predicting susceptibility to it as a sequela of gonorrhea. The questions to address are:

What serological criteria are there

1. for diagnosing infection with *N. gonorrhoeae*?
2. for predicting immunity to *N. gonorrhoeae*?
3. for predicting the course of gonococcal infection, especially towards PID?
4. for determining the cause of PID?

As none of these questions can be answered positively, it is desirable to consider the state of knowledge concerning immunity to *N. gonorrhoeae*.

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One of the complicating factors in comprehending immunity to *N. gonorrhoeae* is that it is a uniquely human pathogen that has no other known natural hosts. Most nonhuman primates are not naturally susceptible to gonococcal infection, and attempts to infect them experimentally in a manner resembling the human disease have not been successful [1], although efforts continue. Chimpanzees have been infected, and in one instance transmission to another individual was shown [2]. However, it is now impermissible to experiment with these animals and the expense involved would be prohibitive. Currently the only available animal model for gonococcal infection is the estradiol-treated female mouse [3], which has been widely used in several laboratories to investigate aspects of pathogenesis and immunity [4]. Although this model displays evidence of actual infection [5], it does not mimic human disease, and the infection is spontaneously cleared, typically within 1–2 weeks, for reasons that are not fully understood, but may simply be that *N. gonorrhoeae* is uniquely adapted to humans and cannot survive in other animals. The genetic tractability of mice, however, has allowed for the further development of the model by introducing human transgenes that result in the expression of, for example, human carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors for gonococcal opacity (Opa) proteins, or human transferrin as a source of iron (reviewed in [6]).

Performing studies on the immune response to gonorrhea in humans is not a straightforward undertaking. Ethical considerations totally prohibit longitudinal studies on the development of responses in the absence of treatment, and cross-sectional studies to compare responses in infected and uninfected individuals are complicated by numerous factors. These include:

- Difficulties in the recruitment and retention of volunteers willing to follow study protocols
- Identifying matched infected and control subjects to account for confounding factors
- Frequent occurrence of "asymptomatic" infections (subjects may be unaware of their infection), which can make it difficult to ascertain when exposure occurred and hence the duration of infection
- Multiple states of genital infection in women and men, ranging from uncomplicated lower tract infection (cervicitis or urethritis), to complicated upper tract infection (endometritis and salpingitis, or prostatitis, epididymitis, and orchitis), and disseminated systemic infection
- Frequent occurrence of coinfections, especially with *Chlamydia trachomatis*
- Unreliability of self-reporting of previous infection, coupled with lack of adequate records
- Availability of funding for extensive studies with statistical rigor.

There are also technical difficulties relating to quantitative evaluation of immune responses, due especially to the antigenic variability of *N. gonorrhoeae*, which makes defining a standard antigen preparation for specific antibody and cellular assays problematic.

CURRENT STATE OF KNOWLEDGE IN IMMUNITY TO *N. gonorrhoeae*

There have been few systematic, quantitative studies of the immune response to natural human gonococcal infection, probably for reasons related to the difficulties outlined above. Early reports showed that subjects with disseminated gonococcal infection or with salpingitis developed serum complement-dependent bactericidal antibodies against *N. gonorrhoeae* [7, 8]. Evidence indicated that the response to gonococcal salpingitis afforded some level of protection against recurrence of infection [9]. A study among highly exposed sex workers in Kenya revealed statistical evidence of reduced susceptibility to repeat infection with the same porin serovar of *N. gonorrhoeae* [10]. However, this finding was not replicated in another study in North Carolina, albeit that the level of exposure to gonorrhea was likely lower in this population [11]. Studies on cellular aspects of the response to gonococcal infection are also few. Peripheral blood T cells from infected individuals responded to stimulation in vitro with gonococcal porin and secreted inerleukin-4 (IL-4) but not other cytokines, suggesting a mainly T helper 2 (Th2) response [12]. In men experimentally infected with *N. gonorrhoeae* inflammatory cytokines including IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) became elevated in urine [13]. Elevated IL-10 and IL-12 have also been found in cervical mucus of women with gonorrhea [14]. Hedges et al [15, 16] evaluated serum and genital tract antibodies in women with microbiologically confirmed uncomplicated gonococcal cervicitis, using both a stock laboratory strain of *N. gonorrhoeae* and the subjects' own isolates as test antigen. This revealed generally modest immunoglobulin G (IgG) and immunoglobulin A (IgA) antibody responses that were only slightly higher when tested against autologous gonococcal antigen. No evidence was seen for increased responses in subjects documented to have had previous infections, and responses did not increase in subjects who returned for follow-up evaluation approximately 2 and 4 weeks after initial presentation for diagnosis and treatment. When cytokines (limited to IL-1, IL-6, IL-8, IL-10, and transforming growth factor-β [TGF-β]) were evaluated in serum and vaginal secretions, again no evidence was obtained for elevated levels unless coinfections were present [15]. While the study focused mainly on women, similarly unimpressive antibody responses were seen in a few serum and urethral swab samples from men with gonococcal urethritis [16]. These findings led to the speculation that *N. gonorrhoeae*, which typically induces an intense inflammatory reaction revealed by the purulent exudate

in symptomatic infection, somehow interferes with the normal course of an adaptive immune response.

Several mechanisms have now been revealed whereby *N. gonorrhoeae* manipulates host immune responses to favor its own survival (Table 1; [6]). The first evidence was that gonococcal Opa proteins bind to human CEACAM1 on $CD4^+$ Th cells, resulting in their inactivation [17]. Gonococcal porin (PorB) has been shown to inhibit the proliferation of human $CD4^+$ Th cells induced by dendritic cells [21]. Studies in the mouse model have shown that genital gonococcal infection induces innate immune responses driven by Th17 cells, while concomitantly suppressing Th1- and Th2-driven adaptive immune responses by elevating production of the regulatory cytokines TGF-β and IL-10 and generating type 1 regulatory T cells [24, 25]. Reversal of this immunosuppression by means of neutralizing antibodies to TGF-β and/or IL-10 allows the development of Th1-driven adaptive immune responses with the production of antigonococcal antibodies, establishment of immune memory, and generation protective immunity against reinfection [24, 25]. These findings remain to be confirmed in humans, but elevated serum IL-17 and IL-23 (which is involved in Th17 cell maturation [28]) have been reported in patients diagnosed with gonorrhea [29, 30]. A systematic study of cytokine responses, especially including IL-17 and more recently identified cytokines that govern various aspects of immune induction and regulation, in relation to different states of human gonococcal infection in both men and women has not been undertaken and is sorely needed.

Meanwhile, studies in New Zealand have shown that subjects immunized with a meningococcal outer membrane vesicle (OMV) vaccine (MeNZB), which was developed to counter an outbreak of serogroup B *N. meningitidis* infection, were 31% less likely to be diagnosed with gonorrhea than control subjects during the ensuing follow-up years [31]. This represents the first report of a state of immunity, albeit partial, to *N. gonorrhoeae*. It is thought that cross-reactive antibodies against antigens shared between the 2 species might be responsible, but this has not yet been demonstrated. The meningococcal OMV in MeNZB, however, has been incorporated into the recently licensed meningococcal vaccine, Bexsero (GSK), and it has already been found that antibodies induced by immunization with Bexsero recognize gonococcal antigens [32]. Further studies on the impact of immunization with Bexsero on susceptibility to gonococcal infection, and the mechanisms responsible for any effect, are awaited with interest.

Table 1. Neisseria *gonorrhoeae* Manipulates Host Immunity for its Own Advantage

Activity	Reference
Opa proteins interact with CEACAM1 and inactivate $CD4^+$ T cells	[17]
Opa proteins interact with CEACAM3 on neutrophils	[18]
N. gonorrhoeae inhibits intracellular killing mechanisms in neutrophils	[19]
N. gonorrhoeae inhibits killing by antimicrobial peptides using MtrCDE efflux pump	[20]
PorB inhibits DC-induced $CD4^+$ T-cell proliferation	[21]
Gonococcal infection induces Th17-driven innate immune responses	[22, 23]
N. gonorrhoeae suppresses Th1/Th2-driven adaptive immune responses by inducing TGF-β, IL-10, and type 1 regulatory T cells	[24, 25]
N. gonorrhoeae modulates macrophage differentiation into "alternative" M2 pathway	[26]
N. gonorrhoeae induces NLRP3-dependent pyronecrosis of monocytes	[27]

Abbreviations: CEACAM, carcinoembryonic antigen-related cell adhesion molecule; DC, dendritic cell; IL-10, interleukin-10; Opa, opacity; PorB, porin; TGF-β, transforming growth factor-β; Th, T helper.

IDENTIFICATION OF APPROPRIATE GONOCOCCAL ANTIGENS

Numerous gonococcal antigens have been described (reviewed in [4, 33]), several of which have been considered as candidates for vaccine development. However, many of these are subject to phase-variable on-off expression, and show extensive antigenic variation by different mechanisms. Elegant studies over the past few decades have revealed the molecular mechanisms of such variation (Table 2; reviewed in [6]). Inspired by the success of "reverse vaccinology" in identifying conserved antigens in *N. meningitidis* [41], several investigators have applied bioinformatics and immunoproteomics technologies to *N. gonorrhoeae* and have identified numerous candidate antigens for possible use in vaccine development (reviewed in [4, 6, 33, 42]). Several such antigens might also find applicability in diagnostics. However, the 2 objectives have different requirements. For diagnostic purposes such as the development of serological assays for antibodies induced by natural infection, the antigen(s) must not only be conserved and expressed by all (or at least most) strains of *N. gonorrhoeae*, but also they must be sufficiently immunogenic during the natural infection to reliably induce a detectable response. This is not just a property of the antigen itself and its level of expression in vivo, as we have already seen above that *N. gonorrhoeae* has the capacity to actively interfere with antibody generation by inducing regulatory cytokines TGF-β and IL-10. Furthermore, for antigen production it would be desirable to grow *N. gonorrhoeae* under iron-limiting conditions (mimicking the in vivo environment) to promote the expression of Fur-regulated antigens as they occur in vivo [43].

In addition, the antigen(s) need to be unique to *N. gonorrhoeae*. The existence of cross-reactive antigens shared between *N. meningitidis* and *N. gonorrhoeae* has already been demonstrated [44]. Given that *N. meningitidis* is frequently carried asymptomatically as a commensal in the human nasopharynx [45], this likely accounts for the finding of antibodies reactive against *N. gonorrhoeae* in most samples of human serum, despite lack of infection by the latter. Furthermore, commensal species of *Neisseria* are typically

Table 2. Antigenic Variation in *Neisseria gonorrhoeae*

Antigen	Expression	Source of Variation
Lipo-oligosaccharide	Constitutive	Glycan chains synthesized by phase-variably expressed glycosyltransferases [34] Sialylated by sialyltransferase using host-derived cytidine 5′-monophospho-N-acetyl neuraminic acid [35]
Porin (PorB)	Constitutive	Multiple alleles 2 main serovars (Ia, Ib) each with multiple subtypes [36]
Type IV pilus	Phase-variable; can be withdrawn or extended	Pilus fiber gene (*pilE*) recombined at expression locus from multiple *pilS* gene segments in silent loci [37]
Opa proteins	Phase-variable	10–12 *opa* genes scattered throughout genome [38] Pentanucleotide repeats in leader sequence subject to slip-strand replication causing reading frame-shifted on-off expression
Transferrin-binding proteins A and B	Fur dependent, ie, iron regulated	Multiple alleles [39]
All antigens		Horizontal gene exchange within and between *Neisseria* sp. allows for homologous recombination between antigen segments [40]

found in the human oropharynx (Table 3; [46]). While the antigenic profiles of commensal *Neisseria* sp. have not been well studied, it is possible that such organisms can induce in their hosts antibodies that cross-react with *N. gonorrhoeae*. Thus the mere detection of an antibody reactive with *N. gonorrhoeae* is insufficient evidence that it was induced by that organism.

RECENT FINDINGS FROM ANIMAL STUDIES

The finding that suppression of host adaptive immune responses by *N. gonorrhoeae* can be reversed by neutralizing the regulatory cytokines, TGF-β and IL-10, has led to a further finding that the local administration of IL-12, which antagonizes IL-10 and TGF-β [47], during vaginal gonococcal infection in mice restores immune responsiveness against it [48]. In these studies, intravaginal instillation of sustained-release, microencapsulated IL-12 in mice infected with *N. gonorrhoeae* induced IFN-γ–secreting Th1 cells and antigonococcal IgG and IgA antibodies, leading to faster clearance of the infection. Moreover, when these animals were challenged a second time with *N. gonorrhoeae* without further treatment, the repeat infection was cleared more rapidly than in previously untreated but infected mice [48]. Resistance to repeated infection persisted for at least 6 months and was accompanied by recall of Th1 cells and antibody responses, and moreover was revealed equally well against challenge with antigenically different strains of *N. gonorrhoeae* [49]. Resistance was dependent on both the generation of IFN-γ and the presence of B cells, presumably to produce antibodies [49]. These findings implied that microencapsulated IL-12 functioned as an adjuvant that in effect turned the infection into a live vaccine, as administration of IL-12 in the absence of gonococcal infection had no effect [48]. In support of this hypothesis, microencapsulated IL-12 was subsequently found to serve as an adjuvant for a vaginally administered vaccine consisting of gonococcal OMV. Mice immunized intravaginally with gonococcal OMV plus microencapsulated IL-12 generated Th1-driven immune responses with antigonococcal IgG and IgA antibody production, and resisted challenge with the same or different strains of *N. gonorrhoeae* for up to 6 months [50]. These findings show that it may be possible to induce a state of protective immunity against gonococcal infection if the capacity for *N. gonorrhoeae* to suppress adaptive immune responses against it can be overcome. In turn, this suggests novel approaches to vaccine development.

Table 3. *Neisseria gonorrhoeae* Shares Antigens With Other *Neisseria* Species Carried as Commensals

Neisseria Species	Individuals Colonized, %, Male/ Female
N. meningitidis carried asymptomatically in human nasopharynx [45]	
Commensal *Neisseria* species present in human oropharynx [46]	
N. perflava/sicca	97/ 95
N. mucosa	23/ 28
N. flava	28/ 22
N. cinerea	27/ 27

Most individuals have antibodies detectable against *N. gonorrhoeae* regardless of gonococcal infection.

APPLICABILITY TO HUMANS—FUTURE STUDIES

The extent to which these findings apply also to human infection remains to be investigated. In particular, human immune responses including antibodies (in both genital secretions and the circulation), as well as T cells and cytokines, especially including more recently defined cell lineages and cytokines, need to be systematically and quantitatively examined in all states of gonococcal infection. State-of-the-art technologies for assaying multiple cytokines in limited amounts of samples should make it possible to reassess previous findings, which were limited in

the range of cytokines examined as well as the methods available for their assay. The limited knowledge that is already available suggests that responses increase with more advanced inflammatory states of upper tract infection in women, and in disseminated systemic infection, implying that eventually the immune system overcomes any suppression induced by *N. gonorrhoeae* during initial uncomplicated lower tract infection. If this is correct, then it may become possible to relate the generation of particular aspects of host responses to the pathological sequelae such as PID and infertility.

Meanwhile, the quest to develop specific immunological tests for the diagnosis of gonococcal infection, to predict the likelihood of developing PID, or to assign the etiological cause of cases of PID, will not only require an improved understanding of human immune responses to *N. gonorrhoeae* and how this organism manipulates these for its own benefit, but also require the identification of specific antigenic targets against which such tests can be designed. The essential properties required of such gonococcal antigens are shown in Table 4. Reinvigorated efforts to develop a vaccine against gonorrhea have led to the description of numerous novel conserved antigens expressed by *N. gonorrhoeae*, and although the requirements for vaccine antigens differ from those for diagnostic antigens, there is reason to hope that some might be found suitable for development for the latter purpose.

Notes

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Table 4. Issues in Developing a Serological Test for *Neisseria gonorrhoeae*

Identification of target antigen(s):
Expressed by all/most strains of *N. gonorrhoeae*
Preferably constitutive
Relatively abundant
Immunogenic during natural infection
Unique to *N. gonorrhoeae*
Not present in *N. meningitidis* or commensal *Neisseria* sp.
N. gonorrhoeae suppresses adaptive immune responses, ie, antibodies
More information required about responses in complicated upper tract infection

Potential conflicts of interest. The author serves as a paid Consultant and Chief Scientific Officer for Therapyx, Inc., which is developing sustained-release microparticulate adjuvants for use in inflammatory disease therapy and gonococcal vaccine development. The author has submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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