



# Gonococcal Infection, Immunity, and Resistance



## **Is there protective immunity to gonococcal disease?**

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Few data exist that clearly implicate acquisition of protective immunity from a previous gonococcal infection(s). Although a quantitative failure of the local immune response may explain this, a more likely explanation may lie in the qualitative response to gonococcal infection that fails to result in protective immunity against future incursions. Three reports suggest the possibility that a partially protective immune response may result from infection. A fourth suggests that, in some cases, the immune status of the host may actually be permissive for infection.

In 1946 Mahoney et al. (1) showed that only 26 of 108 male volunteers with a prior history of urethritis developed disease in response to intraurethral inoculation of gonococci, in contrast to 56 of 131 men without an antecedent history of urethritis. Buchanan et al. (2) showed that gonococcal pelvic inflammatory disease (PID) may produce some protective immunity to repeated episodes of salpingitis with gonococci that possess the same protein 1 serotype, but not to localized mucosal reinfections. In a study reported in 1989 (3), 227 prostitutes in Nairobi were followed at 2 week intervals for a 15-month period beginning in March 1985. Women previously infected with serovar-defined gonococci (except serovar 1B-1) were at 2- to 10-fold reduced risk of re-infection with the same serovar. Although protection was serovar specific, it was incomplete. Using this same population, Plummer et al (4) have also shown that serum antibody directed against reduction modifiable protein (Rmp) is associated with enhanced likelihood of re-infection with *N. gonorrhoeae*. Rmp antibody, previously has been shown to block the effect of bactericidal antibodies that are directed against porin (Por) protein and lipooligosaccharide (LOS) antigens (5).

To clarify the role of Rmp antibody in facilitating acquisition of gonorrhea, antibody concentrations to gonococcal Rmp were measured in serum samples of fifty-seven women who were the only sexual contacts of men with gonococcal urethritis. Antibody levels in infected patients were normalized for the influence of acute infection. In the group of women who became infected, the mean level of antibody (IgG) to Rmp protein at the time of exposure was approximately three-fold higher than that of exposed patients who did not acquire infection. A significant association between Rmp antibody level and transmission ( $p < 0.01$ ) was shown to be independent of the number of sexual exposures to the infected male partners (6).

Effective vaccination against gonococcal infection has been an elusive goal. Until host mechanisms that might protect against infection are better characterized and understood, an effective vaccine that targets the genital tract for protection may continue to be out of reach. Recent evidence suggests that mucosal immune responses particularly local (eg vaginal) IgG antibody levels may mirror those measured in serum (7). Therefore, a consideration of data

available on serum based immune mechanisms may shed light on protection against mucosal pathogens such as *N. gonorrhoeae*. In particular, efficacy of most vaccines, targeted to prevent infections acquired by the mucosal route, have shown a correlation between efficacy and levels of IgG antibody in serum that result from vaccination (8).

A successful vaccine candidate against gonorrhea may require one or more of the following elements: (a) generation of serum or mucosal antibodies that either facilitate complement-mediated killing of the organism, and/or enhance phagocytosis and microbial killing by polymorphonuclear leukocytes; (b) ability to stimulate an immune response that blocks the attachment of *N. gonorrhoeae* to host tissues; and (c) evocation of cell-mediated defenses that prevent infection or modify fallopian tube damage (9). With the intent of satisfying one or more of these criteria, at least two gonococcal vaccine candidates have been tested in humans over the past 20 years (10,11). *N. gonorrhoeae* are obligate human pathogens, and therefore the most relevant testing of vaccine candidates can only be performed in humans. Nevertheless, certain useful predictions have been gleaned using animal models of infection. The chimpanzee model first developed by Lucas et al. (12) mimics uncomplicated gonococcal infection in man with respect to incubation period, certain clinical manifestations, sexual transmission, local cellular response in the exudate, *in vitro* culture characteristics of the infected material, and systemic immune responses. Arko et al. (13) inoculated male chimpanzees parenterally with a formalinized whole cell vaccine, and showed strain-related resistance to gonococcal challenge. Buchanan and Arko (14) used purified outer membranes, (prepared from the same strains used in the chimpanzees noted above) to immunize guinea pigs, and again showed strain-specific immunity in a chamber model. The use of animals to assess immunologic responses to vaccines may be particularly useful to elicit immune responses that involve antibody mediated complement dependent killing and opsonophagocytosis, because these may be shared with their human counterpart. One such approach has been taken in the assessment of LOS as a recent vaccine candidate.

Antibody against LOS has been shown to have several important functions: complement activation and bactericidal activity (15,16) and opsonic activity (17,18). Although these properties make LOS an excellent candidate vaccine antigen, considerable LOS heterogeneity is displayed by gonococci *in vivo* (19,20). Certain other limitations preclude the use of LOS as a vaccine antigen. First, the toxicity of the lipid A moiety of LOS limits its potential use as a vaccine immunogen. Second, purification of oligosaccharide (OS) from LOS may modify its antigenicity (21) and may result in a T-cell independent saccharide antigen that may be poorly immunogenic (22, 23).

Alternative strategies to the use of pure saccharide vaccines may include conjugation to a protein carrier and production of anti-idiotope monoclonal antibodies (Mabs) that may act as functional "molecular mimics". An anti-idiotope monoclonal antibody (Mab), called CA1 (Ab2), has been produced in mice against Mab 2C7, which recognizes a widely *in vivo* expressed gonococcal lipooligosaccharide (LOS) epitope (24). Mice immunized with MAb CA1 initially had a 2.5-fold increase in IgG (12-fold after a booster), but no increase in IgM, anti-LOS (Ab1') antibody. Control mice immunized with LOS had a 4.5-fold rise in IgG and a 4-fold rise in IgM anti-LOS antibody. In rabbits, Mab CA1 elicited a 9-fold rise in IgG and a 3.3-fold rise in IgM anti-LOS (Ab1') antibody. Bactericidal activity of Ab1' antibody was one to two orders of magnitude

greater than that resulting from immunization with LOS. Ab1' mediated complete human polymorphonuclear leukocyte (PMNL) phagocytosis of 2C7 epitope positive (but not 2C7 epitope negative) gonococci. Mab CA1 acts as a molecular surrogate (Ab2 $\beta$ ) for the nominal LOS antigen and suggests a promising vaccine candidate for human immunization.

Soon after gonococcal pili were first described in 1971 (25), Buchanan et al. (26) showed that infected patients made antibodies against purified pili and Tramont et al. (27) showed that antibody generated from vaccination of volunteers with a gonococcal pilus could block the attachment of gonococci to human epithelial cells *in vitro*. In a small trial, Brinton et al (28) reported that a parenteral gonococcal pilus vaccine was protective in a human challenge model when the homologous strain was used as the challenge organism. A large randomized, placebo-controlled, double-blind efficacy trial of a purified gonococcal pilus vaccine composed of a single pilus type was tested in 3,250 volunteers (US military personnel stationed in the Republic of South Korea) (10). Vaccinees developed a sustained ELISA antibody response to homologous and heterologous pili, but the titers against heterologous antigen were only 40% as high as the homologous titers. Local antibodies measured from semen were also seen against homologous and heterologous pili. However, there were no increases in antibody titers that inhibited gonococcal attachment, *in vitro*, and this vaccine failed to protect men against gonococcal urethritis in an open field trial. In designing this or a subsequent vaccine trial (see below), a graded risk of acquiring gonorrhea was not considered in study participants, and all enrollees were considered equal with respect to their *a priori* susceptibility to gonorrhea. Although pre-vaccination serum antibody (IgG and IgA) levels against gonococcal pili initially were low in the military personnel, failure to consider antibody status against other common cross-reacting antigens may have undermined the effect of the vaccine. A placebo/control trial using human challenge might have facilitated this analysis.

In the most recent American trial that took place in 1985 (11), a placebo/control, human challenge trial was performed. Sixty-three male volunteers either were immunized with a vaccine prepared from the outer membranes of a single strain of *N. gonorrhoeae*, or were given a placebo. These men were challenged intraurethrally with viable organisms 2-4 weeks after completing the vaccination course. No significant difference in infection after challenge was observed in the two groups, but resistance to infection was high: 49% in vaccinees and 32% in placebo recipients. The goal for the outer membrane derived 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 perhaps opsonophagocytic) to gonococci (29) in the urethra. Methods for preparing pure Por were not totally reliable at that time, and preparations were contaminated with other outer membrane constituents, particularly LOS and 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 resulted in a net effect upon complement dependent bactericidal activity.

A graded risk of acquiring gonorrhea in both vaccine and placebo recipients was not considered prospectively in choosing the cohorts, because at that time, natural protective immunity against gonorrhea, while in some cases recognized (reviewed above), was not defined in specific enough terms that would have permitted immunologic stratification of volunteers into different risk

categories. In a “look back” at that vaccine trial, volunteers were retrospectively stratified for immunologic risk, and the question asked whether susceptibility to infection after intraurethral inoculation was influenced by the vaccine. The ratio of the concentration of Por and LOS antibodies, summed, to Rmp antibody concentration was positively correlated with protection both in vaccine and placebo recipients, but none of these alone correlated with protection against challenge. Furthermore, changes in bactericidal activity, both positive and negative (blocking), elicited by the vaccine, correlated with enhanced protection or increased susceptibility respectively, to infectious challenge, when this variable was considered independently. This study emphasizes that the use of a placebo group and stratification for pre-existing 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.

Experimental challenge of humans to evaluate vaccine candidates offers several advantages. These are: (a) the ability, in some cases, to use genetically constructed deletional mutants, deficient in the vaccine targeted epitope(s), to establish the connection between virulence and the epitope; (b) the ability to assess naturally acquired immunity (placebo recipients), defined in terms of the vaccine candidate in protecting against disease; and (c) the ability to stratify participants on the basis of pre-existing immunity (placebo recipients) and post-immunization responses (vaccine recipients), while challenging participants with different sized inocula to assess the quantitative role of immunity.

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## **Absence of protective immunity from repeat infections by gonococci expressing the same Por protein**

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*Neisseria gonorrhoeae* (GC) is a human pathogen which causes recurrent mucosal infections. Although several of the components of the outer-membrane have been shown to be immunogenic, significant inter- and intra-strain variation limits their capacity to serve as vaccines. Several vaccine development projects have focused on Porin protein I (Por) which is required by the bacteria and is antigenically stable.

Several characteristics of Por make it an attractive vaccine target; i) anti-Por antibodies are produced by complicated and uncomplicated infections, ii) some anti-Por antibodies are opsonic and bactericidal, iii) anti-Por antibodies have been produced in rabbits immunized with either whole gonococci or peptides corresponding to portions of the Por protein, and iv) Plummer *et al.* reported serovar-specific immunity in a study of commercial sex workers in Africa (1). In that study, the serovar distribution of gonococcal isolates from commercial sex workers repeatedly exposed to gonorrhea was analyzed. The authors reported that the study subjects were less likely to be re-infected by gonococcal strains with the same serovar suggesting that there was a serovar-specific -(i.e. Por-based) protective immunity.

In the current study we have re-examined the role of Por in a protective immune response. Subjects who presented to an STD clinic during a 17-month period were enrolled (n=2896). Each subject was cultured for GC, and isolates were serotyped as previously described (2,3). A total of 618 subjects had positive GC cultures and 91 had one or more repeat infections. 30.2% of subjects with repeat infections were re-infected with the same serovar and 69.9% with a different serovar. There was a shift in the predominant serovars in the community over time. Initially PIB-2 was the predominant serovar. By the 11th month of the study, PIB-3 and PIA-6 were the predominant serovars. The result of this serovar shift was that the risk of any one subject's exposure to a particular serovar changed over time. Taking the serovar shift into account, we found no serovar-specific protective immunity. The odds ratio of repeat infections with the same serovar versus a different serovar was 1.54 (i.e. subjects were more likely to be re-infected by the same serovar of GC than expected based on the serovar distribution in the community at the time of infection).

It is known that multiple GC strains can have the same serovar, and there is significant protein sequence variability among Pors of the same serovar. Having identified a group of subjects with multiple infections by GC with the same serovar, we examined the possibility that these infections were due to different GC strains with the same serovar or that the Por of isolates from these subjects differed in primary sequence. 37 isolates from 17 subjects with multiple infections by GC of the same serovar (15 from the current study and 2 from a previous study) were examined by arbitrarily-primed PCR (AP-PCR) and *por* sequence analysis. AP-PCR uses a

single PCR primer and a low-annealing temperature thermocycle. The primer anneals to sites on the PCR template where it matches or almost matches. When the primer anneals to two sites on opposite strands of the template close enough for PCR amplification a product is generated. Typically AP-PCR generates a series of amplified products (the AP-PCR pattern) which are specific to the given template and is therefore indicative of the genotype of the bacteria. One primer (1290) previously used to genotype other pathogenic bacteria was used for AP-PCR. 10 of 17 subjects were found to be re-infected by bacteria with the same 1290 AP-PCR pattern.

The Por sequence of the isolates was determined by the direct sequencing of a PCR amplified product corresponding to the *por* gene. Complete sequence was obtained from sequence corresponding to the start of the mature protein to the start of the 8th proposed surface exposed loop of the protein. Isolates with the same AP-PCR pattern were found to have Por sequences that were 98.9-100% identical (0-4 amino acid differences). In contrast, isolates with different AP-PCR patterns had Por sequences which were < 96% identical. These data demonstrate that patients can have multiple infections by gonococci expressing the identical Por protein and which are likely the same gonococcal strain. These results are not consistent with Por-based immunity following natural infection in this population.

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## **Infectivity of gonococcal mutants in the human challenge model**

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Previous studies with the human challenge model have used only naturally occurring variants of wild type gonococcal strains. We have constructed mutants of strain FA1090 differing from the wild type parent in expression of individual components believed to be important in virulence. In our previous studies, an inoculum of 10<sup>6</sup> cfu of strain FA1090 variant A21 or A22 (Opa<sup>-</sup>, P<sup>-</sup>) resulted in infection in over 90% of volunteers, defined as the presence of a urethral discharge containing gram-negative diplococci, accompanied by positive urine or urethral swab cultures (1).

Pili promote attachment of gonococci to human cells or cell lines *in vitro*, and previous studies involving human challenge with wild type gonococci have suggested that pilus production may be a critical virulence factor (2-4). We constructed a non-reverting pilin-negative mutant of strain FA1090 with a deletion of 260 bp encompassing the promoter and the 5' end of the *pilE* gene. No new antibiotic resistance markers were introduced into the chromosome of the final strain. The FA1090 Pil<sup>-</sup> variant used in human challenge was matched to wild type variant A22 in Opa and LOS expression. 6 subjects were inoculated with 10<sup>6</sup> cfu of the Pil<sup>-</sup> mutant. 2 subjects developed a watery discharge; the remaining 4 subjects showed little or no sign of infection and were asymptomatic. Abundant nonpiliated gonococci were cultured from urine specimens from all 6 subjects throughout the five day trial. In one asymptomatic subject, colony counts from urine specimens rose rapidly for 2 days after inoculation, but then decreased equally rapidly to few or no colonies on days 4 and 5. These results suggest that pilin expression is not absolutely essential for colonization of the male urethra; alternative adhesins may facilitate pilus-independent colonization. However, the clinical manifestations of infection with the Pil<sup>-</sup> mutant differed from those occurring with wild type FA1090, with little or no inflammatory response to the presence of the organisms.

Gonococcal lipooligosaccharide (LOS) is also believed to be important in pathogenesis of gonorrhea. The enzyme phosphoglucomutase (Pgm), which catalyzes interconversion of glucose-1-phosphate and glucose-6-phosphate, is necessary for synthesis of the sugar precursors that are assembled into LOS (5,6). We cloned the *pgm* gene from strain FA1090 using PCR amplimers based on the sequence of *pgm* from strain 1291 (5), and then constructed a deep rough *pgm* mutant of FA1090, using a two-step strategy involving an insertional inactivation cassette containing both a selectable and counterselectable marker (described in more detail in the abstract by Johnston et al). The final mutant contained a linker insertion mutation in *pgm*, and expressed no new antibiotic resistance markers relative to the parent strain. Strain FA1090 *pgm* produced a single truncated LOS species that did not bind Mab 3F11, and had the same growth

rate, serum resistance, and outer membrane protein profile as the parent strain. The mutant was matched to the wild type parent in Opa and pilin type (assessed by determining DNA sequence of *pilE*). None of 9 subjects inoculated with  $10^6$  cfu or 3 subjects inoculated with  $10^7$  cfu of FA1090 *pgm* developed urethritis within a 5 day trial. All but two daily urine specimens from the subjects were culture-negative; the two positive specimens yielded only a few colonies. In contrast, FA1090 A22 infected 8 of 8 subjects receiving  $10^6$  cfu. These results emphasize the importance of intact LOS, which may influence key properties of the organisms and/or affect their interaction with host cells. The results with both mutants also demonstrate the ability of the human challenge model to discriminate among gonococci that are compromised to different extents in ability to cause urethral infection.

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## **Does an experimental gonococcal infection protect human volunteers from subsequent reinfection?**

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We have previously reported that as few as 250 to 1600 MS11mkC gonococci can infect from 30% to 40% of inoculated volunteers, while 36,000 infects 100% (1). These experiments showed that expression of a terminal paragloboside, lacto-N- neotetraose, on its lipooligosaccharide (LOS) was a gonococcal virulence factor. We further showed that in vitro sialylation of this LOS lessened the infectivity of mkC gonococci (2). We sought to test the hypothesis that an experimental mkC gonococcal infection would prevent reinfection with homologous mkC gonococci two weeks after treatment of the initial infection. In the first experiment we inoculated 15 volunteers with a large number, 57,000 mkC gonococci; 14 (93%) became infected (median incubation time=54 hr; range-43 hr to 91 hr). These 14 and a control group of 10 "naive" volunteers were challenged with 8-fold fewer, 7,000 mkC gonococci 2 weeks after the initial infection were treated. Six (43%) previously infected volunteers became infected (median incubation time = 52 hr; range = 45 hr to 91 hr) and 5 of 10 (50%) of the naive volunteers became infected (median incubation time = 93 hr; range-69 hr to 139 hr), indicating that the first infection did not protect against homologous reinfection. We used recovery of gonococci from urinary sediments to monitor the course of each infection. There appeared to be no relationship between dose and infectious course. We found considerable variation among the subjects infected by either the high or low dose in the patterns of increase in the numbers of gonococci shed during the incubation period and the relationship of these numbers to the onset of dysuria and clinical urethritis. The course of our experimental infections is unpredictable, and does not appear to be dose related.. The unpredictability in the course of these experimental infections, both in incubation time and presentation of symptoms, mirrors the variation seen in naturally acquired infections, and appears more subject to individual variation than to dose of gonococci.

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## **Neisserial porins activate naive resting B lymphocytes, inducing proliferation and immunoglobulin secretion**

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The Neisserial porins are immunogenic without the addition of adjuvants and have immunopotentiating activity. They have been demonstrated to augment the immune response to poorly immunogenic substances, like capsular polysaccharides and peptides (1). The mechanism of this action is unclear, but we postulate that their adjuvant ability is related to the porins' effect on lymphocytes and T-B cell interactions. We have previously reported that the porins can induce expression of the costimulatory ligand B7-2 on B lymphocytes and that these porin treated B cells can stimulate T cells (2). Porins, alone, have not been shown to directly stimulate T cells (2). Moreover, the porins induced B cell proliferation and increased expression of class II MHC. B7-2 expression is essential for B lymphocyte dependent T lymphocyte stimulation (3). The induction of B7-2 expression is one possible mechanism of the porins' adjuvant activity. However, other effects of the porins on B lymphocytes, as evidenced by proliferation and expression of activation markers, might also be involved in this mechanism.

To further characterize the porins effect on B lymphocytes, the following experiments were performed. Naive splenic B cells were obtained from murine strain C3H/HeJ (LPS non-responsive) by centrifugation of single cell suspensions of purified splenic B cells over a discontinuous percoll gradient, to insure that the cells obtained were small resting B cells (2,4). B cells (10<sup>6</sup>/ml) were incubated various neisserial porins at increasing concentrations (0.1-10 µg/ml [0.003-0.3 µM]). The porins used were protein IA from gonococcal strain UU1, protein IB from gonococcal strain Pgh 3-2, and class 1 and 3 proteins from meningococcal strain 44/76 (1,2). After two days of incubation, proliferation was measured by <sup>3</sup>H-thymidine incorporation (2). All species of porins tested induced significantly greater B cell proliferation at concentrations as low as 0.1 µg/ml, when compared to control incubations of B cells with media alone.

When the B cell receptor (BCR), containing the surface immunoglobulin, is crosslinked, small resting B cells do not proliferate unless another stimulus is added, e.g. IL4, bacterial lipopeptides, etc. (4). In this experimental model, BCR crosslinking was induced by incubation of B cells with dextran conjugated with anti-murine IgD (anti-IgD-dex). When porin treated B cells were co-incubated with anti-IgD-dex at concentrations of 0.03-3 ng/ml, a synergistic increase in B cell proliferation was found when 3 or 0.3 ng/ml of anti-IgD-dex was used. No significant proliferation was observed when B cells were incubated with anti-IgD-dex at 0.03 ng/ml or if anti-IgD-dex was used alone. These results demonstrate that neisserial porins, unlike BCR crosslinking, cytokines or lipopeptides, can induce B cell proliferation without the addition of other stimuli. In addition, BCR crosslinking will synergistically increase the porin induced proliferation of B cells.

Another measurable read-out of the porins effect on B cells is the induction of secretion of immunoglobulin. Resting B cells were incubated with the neisserial porins as described. After two days, the supernatant of the cell cultures were obtained and the level of IgM was determined by ELISA (4). When incubated with porins at concentrations between 0.1-10 µg/ml (0.3-0.003 µM), B cells were induced to secrete IgM. When porin treated B cells were co-incubated with anti-IgD-dex, synergistic increases in IgM secretion were noted. Interestingly, at the highest concentration of anti-IgD-dex used in these experiments, 3 ng/ml, IgM secretion either was similar to or below the levels measured in wells containing B cells and porins alone. This finding is in contrast to the induction of proliferation when B cells were incubated with porins and anti-IgD-dex at 3 ng/ml. Similar to the proliferation experiments, the lowest concentration of anti-IgD-dex, 0.03 ng/ml, did not increase IgM secretion by porin treated B cells. This study implies that high BCR occupancy and crosslinking, as seen with high antigen concentrations, could have a negative influence on B cell function and immunoglobulin production. Bacterial capsular polysaccharides, with repeating epitopes, may act in a similar manner and crosslink BCR. There is, likely, an ideal concentration of polysaccharide or other antigens that are to be used in vaccines utilizing porins as adjuvants, as too high a concentration of the antigen could "over" crosslink the BCR and inhibit, rather than increase, antibody production.

Finally, CD40 engagement by soluble CD40-L synergistically increased proliferation and IgM secretion of porin treated B cells. CD40L is present on T cells and therefore, the porins might be able to increase B cell activity induced by T-B cell interaction directly as described and also increase T cell activation indirectly by inducing increased B cell expression of the costimulatory molecule B7-2 (5). Purified meningococcal or enterobacterial LPS had no effect on these B cells, demonstrating that this phenomena is not related to possible LPS contamination of the porin preparations

Crosslinking of the specific BCRs is expected to occur when antigens with repeating epitopes encounter B cells, i.e. polysaccharide. The effect of porins on B cell proliferation and antibody secretion, which are synergistically increased by BCR crosslinking, could be another possible mechanism of the porins' adjuvant activity which could improve the immune response to these classic T cell independent antigens.

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## **Selection for Opa<sup>-</sup> phenotypes of *Neisseria gonorrhoeae* in normal human serum**

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The specificity of *Neisseria gonorrhoeae* for its human host and the lack of a suitable animal model has led to the use of a human male intraurethral challenge model to study gonococcal pathogenesis. One consistent observation made using this model, was that the organisms shed by infected volunteers showed a transition from an Opa<sup>-</sup> (transparent) phenotype of the inoculum to an Opa<sup>+</sup> (opaque) phenotype of the recovered organisms (1,2). Multiple members of the Opa protein family are expressed on gonococci (GC) shed during this early phase of infection. Another consistent result from the male volunteer studies was that not many viable input Opa<sup>-</sup> GC could be recovered from the first urine samples, suggesting that most of the input organisms were killed. The reappearance in urine of Opa<sup>+</sup> GC indicates there is a survival advantage for GC expressing an Opa protein.

A powerful bacterial killing mechanism exerted by the human host is antibody and complement mediated killing in normal human serum (NHS). Although the presence of NHS components on the male urethral mucosal surface is still a matter of debate, we believe NHS mediated killing may play a role in the urethra, since the presence of complement has been shown on other genital mucosal surfaces. Moreover, it was recently shown in the human volunteer model, that a rise in cytokine levels in urine, and therewith attraction of neutrophils and possibly serum factors to the site of infection, occurs within hours after instillation (3).

The goal of this study was to see if serum killing of GC could be a mechanism leading to selection of Opa<sup>+</sup> phenotypes, as a possible explanation for the in vivo observations. We therefore studied serum killing of Opa<sup>-</sup>/Opa<sup>+</sup> mixtures of strain MS11, by measuring dose-response relationships, since minor differences in serum sensitivity could be important when serum availability is limited.

Expression of an Opa protein conferred a survival advantage upon the organism; i.e. the Opa<sup>+</sup> GC were always more serum resistant than their isogenic Opa<sup>-</sup> counter-parts, measured both in mixtures and in separate populations. This resulted in selection for the Opa<sup>+</sup> phenotype during serum killing of a mixture of Opa<sup>-</sup> and Opa<sup>+</sup> GC.

Since the type of lipooligosaccharide (LOS) expressed is a major determinant of serum sensitivity, we studied the selection phenomenon in different LOS backgrounds. Selection for Opa<sup>+</sup> occurred in all three LOS phenotypes examined, even in a LOS background that confers total serum resistance to the GC. In this case a monoclonal anti-LOS antibody was added to NHS. These data indicate that the Opa related survival advantage is not due to a difference in LOS expression between the Opa<sup>-</sup> and Opa<sup>+</sup> phenotype.

The micro-environment in the urethra may have a profound effect on the killing of GC. We therefore studied the effect of normal human urine (NHU) on the selection phenomenon. The bactericidal action of NHS was drastically reduced by the presence of NHU in the killing assay. This was probably due to complement inhibitors present in urine, since urine inhibited complement in an antibody-coated sheep erythrocyte lysis assay. However, also in the presence of urine, we observed a distinct selection for Opa<sup>-</sup> organisms during serum killing of an Opa<sup>-</sup>/Opa<sup>+</sup> GC mixture.

Since in vivo most GC appear to be sialylated, we also studied selection of sialylated GC mixtures. We used a phenotype that expresses two major LOS bands on a silver-stained SDS-PAGE gel, with one being the main acceptor for sialic acid. Sialylation of these organisms led to an enhanced serum resistance, but with enough serum present, they were still killed. In Opa<sup>+</sup>/Opa<sup>-</sup> mixtures of these sialylated GC, we also found a survival advantage for Opa<sup>+</sup> GC.

Thus, the expression of an Opa protein confers an enhanced serum resistance upon GC; this leads to selective survival of Opa<sup>-</sup> GC when limited amounts of NHS are present; this phenomenon may contribute to the enhanced appearance of Opa<sup>-</sup> GC, as observed in the human volunteer studies.

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## **In-vitro effects of contraceptive microbicides on *Neisseria gonorrhoeae* infection**

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The objective of this study was to test a number of new agents with contraceptive activity for their potential as microbicides for STD pathogens. Our focus was on their antigonococcal activity (1-3). These compounds were originally formulated for use as vaginal topicals which has the advantage of the minimization of systemic exposure to pharmacologically active agents as well as increased availability of active agents at the target site where protection is most needed. Contraceptive compounds which include anionic quinonyl aldehyde copolymer (TC5-08PS500), anionic bioflavonoids (TC4-01PH000 and TC5-08SH00), anionic b-1,4-polysaccharide (TC5-12CS000) and arylalkenyl homopolymer M<sub>r</sub> 500 Kd (TC6-02-PSS01), anionic aryl alpha-hydroxyl carboxylic acid (TC6-02MAS00) , macrocyclic r-substituted phenol:formaldehyde condensation products (TC6-02C4A00 and TC6-02C6A00) , sulfated hydroxyalkyl homopolymer (TC6-02VAS01) were tested in varying concentrations (0.1-1000 mg/ml) against *N. gonorrhoeae*. The effect on infectivity was measured quantitatively by incubation of the gonococci on microbicide containing gc agar plates and by infection of human fallopian tube organ culture explants (4,5). Determinations were made for tissue cytotoxicity as well as quantification of the compound's ability to reduce or eliminate infectivity by *N. gonorrhoeae*.

The types of contraceptive compounds showing antigonococcal activity were anionic b-1,4-polysaccharide, arylalkenyl homopolymer M<sub>r</sub> 500 Kd, anionic quinonyl aldehyde copolymer, anionic bioflavonoids, macrocyclic r-substituted phenol:formaldehyde condensation products and sulfated hydroxyalkyl homopolymer when assayed using agar plates containing the microbicide. When the anionic b-1,4-polysaccharide was used in concentrations of 1000, 500 and 100 mg/ml kills of 100, 96 and 94 % respectively were obtained. Arylalkenyl homopolymer in concentrations of 1000, 500, 100 mg/ml all achieved 100% kill while 10 mg/ml resulted in 97% kill. The anionic quinonyl aldehyde copolymer was only effective at 1000 mg/ml with a 97% kill. The anionic bioflavonoids were less effective microbicides with TC4-01PH000 being an ineffective compound (<15% kill) while TC5-08SH00 exhibited 76% killing. At concentrations of 100 and 1000 mg/ml the anionic aryl alpha-hydroxy carboxylic acid was effective with 100% killing of the gonococci. However, at concentrations below 100 mg/ml the compound was less effective (~30% kill). Both macrocyclic r-substituted phenol:formaldehyde condensation products (TC6-02C4A00 and TC6-02C6A00) were effective at 1000 mg/ml but only partially effective at concentration of 100 mg/ml or less (<30% kill). The sulfated hydroxyalkyl homopolymer was highly effective at concentrations of 1000 and 100 mg/ml with kills of 100 and 93% respectively.

The arylalkenyl homopolymer, anionic b-1,4-polysaccharide and anionic quinonyl aldehyde copolymer were also effective as antigonococcal microbicides in fallopian tube explants. The arylalkenyl homopolymer was effective with 100% kills at 100 and 1000 mg/ml. The anionic b-1,4-polysaccharide exhibited kills of 100 and 97% at 1000 and 100 mg/ml respectively. The anionic quinonyl aldehyde copolymer produced 100% kill at 1000 mg/ml.

In addition to a compound's ability to function as an effective topical antimicrobial agent it is important that they are not cytotoxic to local mucosal surfaces. These compounds were tested in both a cell and organ culture system for their ability to disrupt cell membranes and mucosal surfaces. None of the compounds, when tested at concentrations which were microbicidal, demonstrated cytotoxicity in either cell or organ culture assays.

A number of the compounds tested demonstrated promise as potential candidates for inclusion as a topically administered microbicide because of their effectiveness as antigonococcal agents. The compounds were effective in vitro at concentrations which are clinically achievable and do not demonstrate in vitro cytotoxicity, which are essential to be a useful candidate. Of the compounds tested the anionic b-1,4-polysaccharide and the arylalkenyl homopolymer were the most promising antigonococcal compounds because of their high degree of bactericidal activity over a wide range of concentrations. These data indicate that there are several compounds which have both contraceptive and microbicide effects against *N. gonorrhoeae* and have potential for further studies to determine their efficacy as topical microbicides.

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## **Epidemiological survey of plasmid mediated tetracycline resistance gonococci in Argentina (April 1993 - April 1996).**

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*Neisseria gonorrhoeae* (NG) with plasmid mediated tetracycline resistance was unknown in our country before 1993. The first strain was detected in April 1993 (1, 2). Three years after, the number of tetM-containing strains increased to 18 isolates, submitted from different areas of the country: Buenos Aires (DF), Jujuy, Tandil and Mendoza.

The strains were sent to the National Reference Center of STD (NRC) from periferic laboratories belonging to the National Network for Gonococcal Surveillance for characterization by sensitivity test (MIC), auxotyping, serogrouping and molecular studies.

The isolates could be divided into two groups by antimicrobial susceptibility test (3): 15 strains with plasmidic resistance to tetracycline (MIC 16 mg/ml) and penicillin (MIC 32 mg/ml) (PP-TRNG) and 3 strains with only plasmidic tetracycline resistance (MIC 32 mg/ml) (TRNG). All the strains were susceptible to cefuroxime, ceftriaxone, ciprofloxacin and spectinomycin.

The plasmid profiles by agarose gel electrophoresis (4) demonstrated the same pattern in all PP-TRNG: 2.6, 3.2 and 25.2 MDa plasmids. The TRNG strains bore the 2.6 and 25.2 MDa plasmids. The presence of tetM-determinant responsible for the high-level of tetracycline resistance was examined by hybridization using Probe 3 (5). The restriction endonuclease analysis with BglII, SmaI and HincII of 25.2 MDa plasmid, demonstrated a Dutch type plasmid (6).

The studies using the relation auxotype/serogroup (7), identified four phenotypic classes of NG isolates. Fourteen strains were arginine requiring, serogroup WI (A-/WI), and was the prevalent class. Two strains were methionine and arginine requiring, serogroup WI (MA-/WI). These strains were the first isolates in Argentina. Only one strain was proline and arginine requiring, serogroup WI (PA-/WI). The strain recently isolated was non-requiring, serogroup WII/III (NR/WII/III); and it strongly differed from the previous three classes.

The class A-/WI involved nine strains of a microepidemic that took place in Tandil city between February and April, 1995 (8). The comparison of the strains of this outbreak with the pattern of one strain previously isolated in Buenos Aires city in 1994, showed similar phenotype and tetM-containing plasmid type (Dutch).

**Conclusions:** From April 1993 to April 1995 all strains were PP-TRNG MA-/WI or A-/WI. These two phenotypes had never been identified among NG strains of our country before 1993, suggesting they were of foreign origin. Since May 1995 we have identified a new class of PP-TRNG, PA-/WI, besides the prevalent A-/WI. This auxotype is less common between our NG.

During the same year, we found for the first time TRNG strains; two of them belonged to the prevalent A-/WI but the latest isolate was classified as NR/WII/III, the most common phenotype among NG in Argentina. It seems that the 25.2 MDa plasmid which has the ability to move itself, has been introduced in our indigenous NG population strains. The treatment of genital infections with tetracycline enhances the danger of dissemination. These strains may then spread through the population but may not necessarily be recognized. In fact, all the PP-TRNG strains confirmed in our laboratory were not initially submitted as TRNG

Taking in account these results, we suspect that the true frequency and geographic distribution of tetM-containing NG in Argentina are underestimated.

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## **Oral inoculation with live attenuated *Neisseria gonorrhoeae* induces a vaginal IgA response.**

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The gonococcus evades the immune system of the host during infection in a number of different ways. These include both a high level of antigenic variation of outer membrane components such as pili, the Opa protein (1,2) and LPS (3,4) and host mimicry, particularly through the sialylation of LPS (5). Such characteristics have rendered the use of both dead whole gonococcus and individual bacterial components, as vaccines, ineffective (6,7). The development of an attenuated strain of *Neisseria gonorrhoeae*, MS11 JKD298 which harbors a mutation in the *aroA* gene (8), allows the potential for vaccination with live gonococcus, thus avoiding these limitations. This *aroA*<sup>-</sup> gonococcus has been shown to display decreased virulence *in vivo* whilst retaining its immunogenicity (8).

In vaccinating against mucosal pathogens, such as *N. gonorrhoeae*, it is essential to elicit a mucosal immune response. In order to do this it is therefore highly desirable to deliver such vaccines to a mucosal surface. In previous studies with MS11 *aroA*<sup>-</sup>, despite the expected reduced virulence *in vivo*, a strong inflammatory response was observed during infection with this strain. The induction of such a response precludes the possibility of delivery to the genitourinary tract, in common with natural gonococcal infection. However, it is now well established that immunization at one mucosal site leads to the induction of immunity not only at that site but also at other, distant mucosal sites (9). Therefore, as an alternative to vaginal vaccine delivery, we have investigated the outcome of oral inoculation with live attenuated gonococcus.

Female C<sub>3</sub>H/He mice (OLAC, UK) were given three oral doses of MS11 JKD298, of between 6x10<sup>6</sup> and 1x10<sup>10</sup> gonococci, by gavage on days 1, 6 and 9. This was followed by a similar boosting dose on day 27. To investigate the systemic antibody (Ab) response, serum samples were prepared from tail vein bleeds on day 21, after the initial inoculation and day 34, following boosting. Most importantly, in order to assess the local mucosal Ab response in the vagina, vaginal washes were carried out with 200 µl of phosphate buffered saline to which a cocktail of protease inhibitors was added immediately after washing. Washes were carried out on day 21, following the first inoculating doses and then on days 34, 36 and 38 after boosting. Levels of specific IgG and IgA were measured by ELISA. To assess the Ab response elicited against a potential infecting gonococcus, ELISA were carried out against a whole cell lysate of the parental wild type strain MS11 JKD288.

Specific vaginal IgA was present in all mice on day 21 after the initial immunizing doses. However, the levels of Ab detected in vaginal washes performed varied greatly between animals, ranging from 1.2 to 24.7 times that detectable in similar washes from unimmunized, control animals. This observed variation was possibly due to differences in the overall response of the different mice or, alternatively, was related to the position of each individual mouse within the

estrus cycle. To assess which of these was the case, following boosting, vaginal washes were taken over a six day period to cover the complete estrus cycle, on days 34, 36 and 38. Once again specific IgA was detected in vaginal washes from all mice. However the levels in consecutive washes from individual mice were highly variable. For one representative animal, IgA levels ranged from 0.6 times to 24.7 times greater than the levels present in washes from control mice. This observed variation in Ab levels between the washes of one individual mouse would suggest that levels of IgA secreted are indeed dependent on the position of each mouse within the estrus cycle. We are currently conducting experiments to assess the position within the cycle of each animal at the time of each vaginal wash. It is hoped that this will reveal whether the production of particularly high levels of IgA correlates with a particular stage of estrus. In addition to IgA, the presence of IgG and IgM within vaginal secretions was also determined. No Ab of either isotype could be detected within the vaginal washes of any of the mice.

A comparison of the systemic Ab response with the mucosal Ab response was carried out by assessing the levels of specific serum IgG and IgA. In common with the mucosal response, IgA was detected within the serum of all mice both after the initial inoculation, on day 21 and following boosting, on day 34. The mean IgA Ab level of all mice was  $12.3 \pm 6.6$  times (day 21) and  $10.8 \pm 4.5$  times (day 34) that detected in sera collected from control animals. In further contrast to the mucosal Ab response, in addition to IgA, IgG was also present within the sera from all animals at both times (means levels of  $14.9 \pm 3.3$  and  $10.6 \pm 2.4$  times that of control sera on days 21 and 34 respectively).

In summary, the above results have shown that oral delivery of attenuated gonococcus does indeed stimulate an immune response whilst avoiding the induction of an inflammatory response in the genitourinary tract. This resulting Ab response is reactive against the parental wild type strain, and thus potentially infective gonococcus. We are currently extending these observations to determine the specificity of both systemic IgG and IgA and mucosal IgA Ab using SDS-PAGE and western blotting. In addition we are also comparing the responses obtained following inoculation at other mucosal surfaces.

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**Production of inflammatory cytokines by human macrophages and polymorphonuclear cells in response to *in vitro* stimulation with *Neisseria gonorrhoeae*.**

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Uncomplicated gonorrhoea is characterized by an intense inflammatory infiltrate consisting predominantly of polymorphonuclear cells (PMN). In our hands, in the guinea-pig subcutaneous chamber model, even in prolonged infections lasting several weeks, the inflammatory response remains acute with a majority of PMN and much fewer numbers of either macrophages or lymphocytes (1,2). The signals elicited by *Neisseria gonorrhoeae* early during infection which stimulate this acute inflammatory response are still not fully characterized. Additionally, host processes which may prevent dissemination and are thus responsible for localization of infection are not known.

Previous work carried out in this laboratory has shown the presence of the inflammatory cytokine TNF- $\alpha$  in chamber fluid removed during infection with *N. gonorrhoeae*, strain MS11 JKD288 or LPS variants of strain GC40, in the guinea-pig (R Demarco de Hormaeche, elsewhere in this book). In addition, experimental infection in male human volunteers has been shown to lead to an increase in the levels of TNF- $\alpha$ , IL-6 and IL-8 detected in urine (3). Whilst these studies suggest that such inflammatory cytokines may be of central importance during gonococcal infection, they do not address what specific cells are responsible for their production or which bacterial components mediate the stimulation. In order to investigate these points we have assessed the production of a range of inflammatory cytokines by human cells following stimulation with a number of bacterial components *in vitro*.

Monocytes and PMN were isolated from the blood of normal volunteers using commercially available centrifugation gradients. Macrophages were further purified from the monocyte fraction by adherence. In addition, the human myeloid cell line, U937, was utilized following differentiation in the presence of phorbol 12-myristate 13-acetate. Cells were stimulated at concentrations between  $0.5 \times 10^6$  and  $1.0 \times 10^6$  cells/ml, with either LPS purified from gonococcal strains MS11 JKD288 and GC40 variant D1 or from *Salmonella typhimurium* (all used at 5  $\mu\text{g/ml}$ ) or whole cell lysates of MS11 JKD288. Levels of TNF- $\alpha$ , GM-CSF, IL-1 $\beta$  and IL-8, present in the culture supernatants, at various time points after stimulation, were assayed by sandwich ELISA

Stimulation of differentiated U937 cells with GC40 D1 LPS and *S. typhimurium* LPS resulted in the production of all cytokines analyzed. IL-8 and TNF- $\alpha$  were detected first, 3 hours after stimulation. Production of TNF- $\alpha$  in response to the two different LPS was comparable and could be detected throughout the whole cell culture period. However, whilst production of IL-8 in response to gonococcal LPS was maintained for over 40 hours, levels decreased after 6 hours in cultures containing *Salmonella* LPS. IL-1 $\beta$  and GM-CSF were detected later, from 6 hours

post stimulation. In common with the production of TNF- $\alpha$ , levels of GM-CSF were similar in the presence of either LPS.

To confirm and extend the above observations macrophages and PMN isolated from normal volunteers were stimulated in a similar fashion. In common with the cell line, stimulation of fresh macrophages resulted in the production of IL-8, TNF- $\alpha$  and IL-1 $\beta$ . However, in contrast GM-CSF could not be detected at any time. IL-8 and TNF- $\alpha$  were again detected first, although some differences between the two systems were observed. An early peak of IL-8 was apparent 1 hour after stimulation of fresh macrophages. Interestingly, this was greater in the presence of gonococcal LPS than *Salmonella* LPS. Levels of IL-8 increased from 6 hours post stimulation. TNF- $\alpha$  was first detected at 6 hours, at which point production was maximal. No differences were observed with the responses to the two LPS types. In contrast to the results obtained with the cell line, IL-1 $\beta$  was detected in greater quantities after stimulation with gonococcal LPS, levels being lower or undetectable following stimulation with *Salmonella* LPS. In addition, presence of IL-1 $\beta$  was not detected until later in the culture, at 22 hours.

Stimulation of PMN with either gonococcal or *Salmonella* LPS gave rise to the production of IL-8. This was detected early in the culture, 3 hours post stimulation and continued throughout.

To investigate the potential contribution of gonococcal components other than LPS in the stimulation of cytokine production by the different cell types, freshly isolated macrophages and PMN were cultured in the presence of either LPS or whole cell lysate *N. gonorrhoea*, strain MS11 JKD288. Macrophage production of both IL-8 and TNF- $\alpha$  and PMN production of IL-8 was increased at least two fold following stimulation with whole cell lysate as compared to LPS. Concentrations of LPS in the cell lysate are estimated to be considerably smaller than the 5  $\mu\text{g/ml}$  of purified LPS utilized in these experiments. The observed increase in cytokine production cannot therefore be accounted to an increase in LPS concentration. Such an increase suggests, therefore, that other gonococcal factors, in addition to LPS, contribute to the stimulation of both of these cell types. We are currently analyzing the production of TNF- $\alpha$  and IL-8 by macrophages, following stimulation with whole cell lysate, in the presence of either polymyxin B or mAb against CD14 to block the effects of the LPS. The results so far also indicate that other factors contribute to cell stimulation. Our recent work has revealed that stimulation of PMN with whole cell lysate, pre treated with proteinase K, results in a two fold reduction in the production of IL-8 indicating that gonococcal proteins contribute strongly to induction of cytokine production.

In summary, therefore, we have developed a system which enables us to analyze the activation of the individual cell types that predominate at sites of infection during gonorrhoea infections, following stimulation of such cells with *N. gonorrhoeae*. In addition we are able to further characterize the individual bacterial components responsible for the different effects observed. The results described here indicate that both gonococcal and *Salmonella* LPS are similarly capable of stimulating the production of inflammatory cytokines TNF- $\alpha$ , IL-8, IL-1 $\beta$  and GM-CSF by human macrophages and IL-8 by human PMN. Some differences in the stimulation of IL-8 production by macrophages by these two different LPS were, however, observed. Further, preliminary results suggest that both gonococcal proteins and LPS may be important in stimulating cytokine production by both macrophages and PMN.

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**Infection with *Neisseria gonorrhoeae* induces acute systemic, but not local, immune and inflammatory responses: effects of concomitant infection with *Trichomonas vaginalis* and/or *Chlamydia trachomatis*.**

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Antibodies to *Neisseria gonorrhoeae* have been detected in both serum and mucosal secretions (1, 2). Despite the apparent presence of such antibodies, re-infection with *N. gonorrhoeae* is common. How *N. gonorrhoeae* is capable of re-infecting a host in the presence of such antibodies is not well understood. It is believed, however, that this organism evades the host immune response by a variety of mechanisms including antigenic variation. In addition, *N. gonorrhoeae* produces IgA1 proteases that may cleave IgA1 *in vivo*, protecting the organism from the immune system in some (as yet) unknown fashion. In this report we have examined how the mucosal and systemic immune and inflammatory responses to *N. gonorrhoeae* are effected by concomitant infection with *Trichomonas vaginalis* and/or *Chlamydia trachomatis*.

Female patients attending the Jefferson County STD clinic (Birmingham, AL) were recruited into a study examining the role of IgA1 proteases in the pathogenesis of *N. gonorrhoeae* induced disease. Informed consent was obtained from each patient prior to enrollment in the study. Where possible, samples of saliva, cervical mucus, vaginal wash, and serum were obtained from the patients during three visits to the STD clinic spaced at two week intervals following their initial visit. In patients infected with *N. gonorrhoeae*, 3/20 were also infected with *T. vaginalis*, 2/20 with *C. trachomatis*, and 3/20 were infected with both of these organisms. In the control patients, (not infected with *N. gonorrhoeae*) 3/31 were infected with *T. vaginalis*, 1/31 with *C. trachomatis*, and 3/31 were infected with both organisms. All infected patients were appropriately treated during their first visit and were tested for infection at subsequent visits. No patient remained infected with *N. gonorrhoeae* after treatment. All samples were treated with an antiprotease containing buffer, and stored at -70° until analyzed.

The concentrations of total IgA1, IgA2, IgG, and IgM immunoglobulins were determined for each sample. Infection with *N. gonorrhoeae* did not effect the concentrations of total IgA1, IgA2, or IgM in any samples. Total IgG concentrations in the vaginal wash, but not other samples, from patients infected with *N. gonorrhoeae* were significantly lower compared to the controls. Whole, formaldehyde treated, *N. gonorrhoeae* were used as the antigen for the estimation of antibody levels reacting to *N. gonorrhoeae* MS11. In addition, the antibody levels reacting to the patients infecting strain were tested (where possible). Antibodies to MS11 were present in all secretions and serum. There were no differences in the local antibody levels comparing *N. gonorrhoeae* infected versus non-infected women. The levels of serum IgA1, but not other immunoglobulin types, reacting to the MS11 strain were significantly higher in women infected with *N. gonorrhoeae* compared to the controls. Infection with either *T. vaginalis* and/or

*C. trachomatis* did not effect the immunoglobulin concentrations or antibody levels at any site, nor did they alter the response (or lack of response) to *N. gonorrhoeae*.

Experimental infection of males with *N. gonorrhoeae* induced a rapid local and systemic cytokine response including IL-1, IL-6, and IL-8 (3). We examined whether these cytokines would be produced during a natural infection in women. The levels of IL-6, IL-8, and IL-1 $\beta$  were quantitated by ELISA. Monoinfection either *N. gonorrhoeae*, *T. vaginalis*, or *C. trachomatis* did not induce a significant local cytokine response compared to the controls. Serum IL-6, but not other serum cytokines, was significantly elevated in patients infected with *N. gonorrhoeae*. This response was not observed in patients infected with *T. vaginalis* or *C. trachomatis* alone. Concomitant infection with *T. vaginalis* and/or *C. trachomatis* did not modify the cytokine responses to *N. gonorrhoeae* (examined in total), however, there was a significant subgroup (3/8) of these patients with highly elevated levels of these cytokines in serum. This effect was not seen in any other group.

The results summarized in this report suggest that there appears to be little or no acute mucosal immune or inflammatory responses to *N. gonorrhoeae*. Instead, IgA1 and IL-6 responses to this organism occur in serum. Concomitant infection with *T. vaginalis* and/or *C. trachomatis* does not affect those responses. There appears to be a subgroup of patients infected with *N. gonorrhoeae* with concomitant *T. vaginalis* and/or *C. trachomatis* infections that have very high levels of circulating inflammatory cytokines. These infections induce periodic cycles of cell mediated immunity. It is possible, therefore, that the high levels of serum cytokines in these patients may reflect an interaction between *N. gonorrhoeae* and PMN or monocytes previously recruited to the site of infection.

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## Effects of *Neisseria gonorrhoeae* urethritis on the concentration of HIV-1 in seminal plasma

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**Objectives.** To determine 1) if *N. gonorrhoeae* increases the concentration of HIV-1 in semen, and 2) if therapy for *N. gonorrhoeae* decreases the concentration of HIV in the semen.

**Rationale.** Gonorrhea has been linked to increased acquisition of HIV in women, with the assumption that mucosal inflammation provides greater access for HIV than normal tissue (1). However, an additional role for gonorrhea, which could biologically explain these findings includes increased infectiousness of male partners with untreated gonorrhea.

**Methods.** In 1996, men presenting with a urethral discharge to the STD Clinic in Lilongwe, Malawi were studied. Urethral swabs were evaluated with a gram stain and culture for *N. gonorrhoeae*. Semen was collected before treatment to measure the concentration of HIV-1 RNA with a modified nucleic acid sequence based analysis (NASBA) assay. This assay had a detection limit of 1,000 copies/ml. All patients were treated for gonorrhea with Gentamicin 240 mg IM (95% cure rate). If gonorrhea was not found on gram stain, either doxycycline or azithromycin were added to the gentamicin. HIV serostatus was determined by repeat ELISA. HIV+ subjects returned at 1 and 2 weeks post-treatment for semen collection and re-evaluation. Subjects who continued to have *N. gonorrhoeae* were treated with ciprofloxacin. An HIV+ control group of dermatology clinic men without urethritis on gram stain was selected and evaluated with semen collection at 2 visits, 2 weeks apart.

**Results.** 206 subjects with urethritis and 125 control subjects were enrolled. 113/205 (55%) of urethritis subjects and 60/125 (48%) of control subjects were HIV+. 55 of the 83 urethritis men who successfully gave semen were infected with *N. gonorrhoeae* by gram stain or culture. HIV-1 RNA concentrations of seminal plasma from 83 HIV+ subjects with urethritis and 43 HIV+ controls were evaluated. Before treatment, the median seminal plasma concentration in the urethritis group was 129,000 copies/ml, and the sub-set of 55 gonorrhea subjects was 178,000 HIV copies /ml. These values were significantly different than the control value of 17,000 copies /ml found at baseline ( $p = .003$  and  $p = .003$ , respectively). At 2 weeks post-treatment, the median concentration in the gonorrhea group was 44,000 HIV copies/ml compared to, 23,000 copies/ml 2 weeks post-baseline in the controls (NS). In the gonorrhea group, the median individual HIV log concentration change comparing pre- and post-treatment values decreased .53 log (3.4 fold) ( $p < .0001$ ). The individual log change in the control group comparing values at baseline and 2 weeks follow-up was -.02 log. Median HIV RNA blood plasma concentrations

were not significantly different comparing the pre and post-gonorrhoea treatment groups and the controls (95,000, 105,000 and 115,000 HIV copies/ml, respectively).

**Conclusion.** HIV infected men with urethritis have a significantly higher concentration of HIV RNA in seminal plasma compared to controls without urethritis. Men with *N. gonorrhoeae* urethritis had the highest level of HIV in seminal plasma. Effective antibiotic treatment for gonorrhoea results in a reduction of HIV in seminal plasma. Blood plasma HIV concentrations do not appear to be affected by gonorrhoea. Therapy for gonorrhoea did not change blood plasma HIV RNA concentrations. Heretofore there have been nine studies related to the concentration of HIV in semen (2) and none have examined the effects of coinfection with STDs. The significant amplification of HIV in semen caused by gonorrhoea suggests at least part of the way treatment of STDs reduces the incidence of HIV (3).

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***In vivo* induction of TNF $\alpha$  by *Neisseria gonorrhoeae*.**

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The main clinical manifestation of gonorrhoea is the appearance of an acute inflammatory exudate containing mainly polymorphonuclear cells (PMN) some of which harbor large numbers of gonococci. This exudate is so typical of gonorrhoea that its examination has been an important aid for diagnosis for many decades. Apart from its diagnostic value, the inflammatory infiltrate may play an important role in the pathogenesis of gonorrhoea, particularly in precluding dissemination. The interaction of gonococci and phagocytic cells is complex and is not clear yet whether the restricted capacity of gonococci for invasion is due to the lack of capacity to invade on the part of the bacteria or to the efficiency of local non specific defense mechanisms like inflammation. The acute inflammatory cell infiltration may play an important part in the localization of gonococcal infection.

During our work on experimental infection in guinea pigs using open ended subcutaneous chambers coated only by connective tissue at each end, we have observed that the inflammatory infiltrate collected in the chambers is similar to that seen in humans. Untreated infections in guinea pigs may last several weeks, but the infection remains localized and the cellular infiltrate retains its acute characteristic with high proportion of PMN (1,2). This is in contrast with other bacterial infections which will progressively attract mononuclear cells, and suggests that the gonococcus may direct the host towards the maintenance of an acute inflammatory response. This may include induction of the production of particular cytokines that would promote the formation of an acute exudate during prolonged infection.

We studied the production of TNF $\alpha$  in guinea pig subcutaneous chambers during infection with LPS variants D1 and D2 of strain Gc40 and strain MS11JKD288. These strains differ in virulence as measured in this model, D1 and D2 are the most and the least virulent respectively, while JKD288 shows intermediate virulence. D2 produces only mild short infections which resolve by self cure. Each animal had two chambers, only one of which was inoculated with  $5 \times 10^4$  gonococci. Samples of chamber fluid were taken from the two chambers in each animal before inoculation, at the time of inoculation and at times during infection. The samples were used to determine TNF $\alpha$  production, kinetics of infection, and the type of cells present in the chambers. TNF $\alpha$  was measured by the biological L929 cells assay using recombinant TNF $\alpha$  as control.

TNF $\alpha$  was first detected at one hour after inoculation. The amounts detected at one hour were small, about 20 U/ml but were consistently found in all infected chambers. At 6 hours after inoculation all infected chambers had high levels of TNF varying between 200 and 800 U/ml. TNF levels peaked in most chambers between 6 and 24 hours. The highest amount detected was 820 U/ml and the lowest was 78. The differences seen in the size of the response in different chambers may represent individual variations between the animals but may clearly be due to a

lack of correspondence between sampling and TNF peak times. Surprisingly, TNF was detected later during infection. Several samples obtained at 4, 7, and 15 days of infection had high levels of TNF and in some cases the highest levels were seen at 7 and 15 days. TNF was not found in samples obtained before inoculation nor at any time point in any of the samples from the non inoculated chambers carried by the same animals.

Viable counts were made on samples obtained at 24 hours and from then on at every subsequent time point. A correspondence between the numbers of cfu and TNF<sub>a</sub> U/ml was found only at 24 hours of infection. On later samples the amount of viable bacteria and detectable TNF appeared to vary independently. The strain used for infection did not have any effect on the size or kinetics of the TNF response.

During prolonged infections with JKD288 a peculiar pattern of TNF<sub>a</sub> production was observed. Several days after the initial peak seen early during infection, TNF production increased again at various times during infection. Some chambers had further TNF peaks at 14 or even 20 days of infection. Again, the size and time of the TNF response did not match variations on the number of viable gonococci in the chambers. No direct correlation was found between variations of TNF values during prolonged infections and variations in numbers of viable gonococci or cells in the chambers. This was expected, as the amount of a given cytokine detected during infection is most likely to be the result of a multifactorial system of stimuli, responses, and feed back mechanisms, with more than one gonococcal factor and host cells operating simultaneously.

In an attempt to find out whether gonococcal components other than LPS could be involved in induction of TNF production, C3H/He (LPS responsive) and C3H/HeJ (LPS non responsive) mice were inoculated intravenously with gonococcal LPS (20 µg), live gonococci (log<sub>10</sub> 8), or dead gonococci (1 mg). TNF was measured in serum from tail vein blood obtained at 1 hour after inoculation. Sera from a group of uninoculated mice were used as controls. C3H/He mice produced high levels of TNF in response to all stimuli. As expected C3H/HeJ mice did not produce TNF after stimulation with purified LPS. They produced only non significant trace amounts after stimulation with live gonococci. After stimulation with 1 mg of dead gonococci C3H/HeJ mice responded with low levels of TNF. This suggests that although gonococcal products other than LPS might induce TNF<sub>a</sub> production, LPS or their combination with LPS may be needed to initiate stimulation.

Local production of TNF<sub>a</sub>, IL-6, and IL-8 has been shown in experimental urethral infections by *N. gonorrhoeae* in human male volunteers (3). Our current work is focused on the analysis of the role these and other cytokines in the subcutaneous chamber model of gonococcal infection which allows the study of prolonged infections and the effect of modulation of cytokine production (4,5).

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**A strategy for constructing mutant strains of *Neisseria gonorrhoeae* containing no new antibiotic resistance markers using a two gene cassette with selectable and counterselectable markers, and its use in constructing a *pgm* mutant for use in human challenge trials**

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*Neisseria gonorrhoeae* is the etiological agent of the sexually transmitted disease gonorrhea and an obligate human pathogen. Our lab, in collaboration with the laboratory of Myron Cohen, is using an experimental infection model with human male volunteers to study the early events in gonococcal urethritis and the requirement for putative virulence factors *in vivo* (1). Constructing genetically defined mutants for use in human challenge experiments is complicated by several restrictions, first of which is our desire to avoid the introduction of new antibiotic resistance markers into the strains to be tested. To create isogenic gonococcal mutants defective in the expression of putative virulence factors, we have adapted a two step mutagenesis strategy that allows for gene replacement without the introduction of new selectable markers in the final strain. The strategy uses a two-gene cassette containing both a selectable marker (*ermC'*) and a counterselectable marker (*rpsL*) (2). The cassette is cloned into the gene of interest, and the inactivated gene is used to replace the wild type gene on the chromosome of a Str<sup>R</sup> strain by transformation and allelic exchange, selecting for erythromycin resistance. The resulting transformant is a Str<sup>R</sup>/Str<sup>S</sup> merodiploid and is relatively Str<sup>S</sup>, because of the dominance of Str sensitivity over resistance. To replace the cassette-containing gene, a second transformation is done using a cloned gene copy with a deletion or linker insertion mutation as the donor, and the Erm<sup>R</sup>/Str<sup>S</sup> transformant as the recipient. Selection for the regain of high level Str<sup>R</sup> results in a final strain with the same resistance phenotype as the original parent strain, but with the gene of interest inactivated. Using this approach, we have successfully engineered several mutants and tested their infectivity in the human challenge model. This technique has proven successful in creating genetically defined mutants for human challenge studies, and is broadly applicable to neisserial mutagenesis.

We demonstrated the feasibility of this approach by constructing a strain producing deep rough lipooligosaccharide (LOS) as a consequence of mutational inactivation of the gene encoding phosphoglucomutase (*pgm*) (3). Gonococcal LOS is believed to play an important role in pathogenesis. LOS undergoes high frequency antigenic variation, demonstrates molecular mimicry of human glycosphingolipids, and contains binding sites for bactericidal antibodies. Sialylation of gonococcal LOS affects several properties of gonococci, including serum resistance and invasion of epithelial cells *in vitro*. To determine if a complete LOS structure is required in pathogenesis, we used the two step counterselection strategy to engineer an isogenic *pgm* mutant of strain FA1090 (FA1090*pgm*). We cloned the *pgm* gene from strain FA1090 using PCR amplimers based on the sequence of the gene from strain 1291 (4), and constructed a mutant of FA1090 with a linker insertion in *pgm*. The mutant strain expressed a single truncated

LOS species that did not bind mAB 3F11, lacked the LNnT structure, and had identical growth rates and outer membrane profiles as the parent strain. Strain FA1090 $pgm$  was non-infectious in the human challenge model. The results of the human challenge trials are described in more detail by Cannon et al in an accompanying abstract.

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## Isolation of the outer membrane of *Neisseria gonorrhoeae*

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The outer surface of *Neisseria gonorrhoeae* interacts with the host to establish and maintain infection. Conversely, host defenses focus on exposed components of the outer membrane. Our studies center on the identification and characterization of outer membrane components. The goal of these studies is to find unique, constitutively expressed, invariant exposed outer membrane proteins which could be used in improved diagnostic assays or immunoprophylaxis. The physical isolation of gonococcal outer membranes is instrumental in identifying and confirming the location of outer membrane proteins.

A variety of methods have been used to isolate gonococcal "outer membrane" proteins for use in structural, functional and Immunological studies. These include solubility of membrane components in N-laurylsarkosine (sarkosyl), where putative outer membrane proteins remain in the sarkosyl insoluble fraction (1,2), isolation of "serotype-specific" outer membrane vesicles (3) using high-salt and shearing followed by size exclusion chromatography and differential centrifugation to recover "naturally-elaborated membrane vesicles" (membrane vesicles released by gonococci during log-phase growth)(4). Isopycnic sucrose gradient isolation, which separates membranes from EDTA-lysozyme spheroplasts based on membrane density (outer membrane  $\sigma = \sim 1.22$ ) has been used to define and characterize the components of many Gram negative bacteria. Definitive, density gradient purification of gonococcal outer membranes has not been reported, possibly due to the unusual o-acetylated peptidoglycan which is refractory to hydrolysis by most lysozyme preparations and the presence of molecules which cause isolated outer membranes to clump such as Opa and Pil.

This paper reports the density isolation of the outer membrane of *N. gonorrhoeae*. The gonococcal outer membrane appeared to be typical of Gram negative bacteria. The gonococcal outer membrane appeared to be typical of Gram negative bacteria. Known outer membrane components such as Por, Rmp, Lip and LOS were restricted to fractions having a density of  $\sim 1.22$  while dehydrogenases, cytochrome oxidase and heme-proteins were restricted to "inner membrane" fractions in the density range of  $\sim 1.1$ . However, comparison of gradient-isolated outer membrane components with those obtained by several common techniques revealed both qualitative and quantitative differences. None of the techniques yielded results that were completely comparable with density-separated outer membranes.

Antiserum raised against the outer membrane fractions has provided a valuable tool to identify such proteins. This antiserum, used to screen genomic expression libraries, identified thirteen clones expressing unique proteins. One of these, the gonococcal homolog of *Hemophilus influenzae* D15 protective surface antigen, has been characterized (see abstract by Reschke, *et al.*) and several others are being evaluated. Future studies are planned to evaluate outer

membranes isolated from gonococci grown under conditions suggested to mimic *in vivo* growth including anaerobic growth, heat-stress, iron limitation and pH-stress.

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## **Generation of polyclonal and monoclonal antiserum to a specific epitope of the MtrC protein of *Neisseria gonorrhoeae***

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Generation of antibody reagents to defined protein epitopes for research and diagnostic purposes requires significant time and effort. Peptides must be chemically linked to carrier molecules such as KLH or BSA which are, themselves, immunogenic. Extensive purification or clonal screening is needed to obtain the desired antibody reagent.

We used recombinant techniques and a multiple antigenic peptide system (MAPS) to generate polyclonal and monoclonal antibodies to a putative surface epitope of the MtrC lipoprotein. MtrC is a central component of an efflux pump (1) that contributes to gonococcal resistance to hydrophobic reagents and antibiotics. Computer analysis identified the sequence <sup>119</sup>-ISKQEYDAAVTAK<sup>-131</sup> (MtrC Antigenic Peptide 1 - MtrC AP1) as a highly antigenic, possibly exposed epitope of this important protein.

DNA encoding the MtrC AP1 sequence was genetically fused to maltose binding protein. The resultant fusion protein was used as the primary immunogen to stimulate MtrC AP1-specific antiserum in rabbits. Boosting immunizations were performed with a synthetic octomeric MAPS immunogen. The resultant antiserum recognized the MtrC protein on Western blots and appeared to bind native MtrC protein *in situ*, showing bactericidal activity. Multiple protocols were used to immunize mice with MtrC AP1. Several clones producing MtrC AP1-specific monoclonal IgG were isolated and expanded. The resultant monoclonal antibodies were characterized by ELISA and Western blotting. These techniques make it possible to readily generate epitope-specific polyclonal and monoclonal antibody to evaluate surface availability, antibacterial activity and to produce antigen capture and signal monoclonal antibodies for diagnostic studies.

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## Replication origins of b-lactamase-producing plasmids of *Neisseria gonorrhoeae*.

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Plasmid replication in Gram-negative bacteria may be divided into two classes, depending on the absence or presence of a plasmid-encoded protein (Rep protein) for replication initiation (1). ColE1-type plasmids exhibit an inhibition-target mechanism, using a counterscript RNA molecule as the main inhibitor in the control of initiation of replication. The other group of replicons uses an essential Rep protein, a cluster of direct repeats (iterons), binding sites for the DnaA protein, and A-T-rich sequences. Unlike ColE1-type plasmids, this latter group does not require Poll for replication initiation.

In isolates of *Neisseria gonorrhoeae*, penicillinase production is plasmid mediated. These plasmids have been separated into a related family of six types, based on their geographical source of isolation and size. They include the prototype Asia-type plasmid (e.g. pJD4, 7426 bp), the Africa-type (e.g. pJD5, 5599 bp), the Toronto-type (e.g. pJD7, 5154 bp), the Rio-type (e.g. pGO4717, 5154 bp, possibly identical to the Toronto-type), the Nnmes-type (e.g. pGF1, 6798 bp), and New Zealand-type (e.g. pAS84/417, 9309 bp) (2) (3).

The origins of replication of pJD4 were localized using branch-point analysis in an *E. coli* background. The plasmid carried three origins of replication, designated ori1 ori2, and ori3. Plasmid pJD5 contains only one of the three origins found in pJD4 (ori1), and EM analysis demonstrated that this origin was the least one preferred in pJD4. The remaining two origins (ori2 and ori3) of pJD4 were confirmed by the construction of pJD9, a deletion derivative of pJD4. DNA sequencing and EM analysis confirmed that the origin of replication found in pJD5 was not present in pJD9. Plasmid replication in pJD4, pJD5, and pJD9 was unidirectional.

Some naturally occurring -lactamase-producing type plasmids of *N. gonorrhoeae* representing each type were tested in an *E. coli* background for their dependence for DNA polymerase I. The Asia-type plasmid, pJD4, is able to maintain itself in both wild-type and polA- hosts, as can its insertion (duplication) derivative pAS84/417 (New Zealand-type), and its deletion derivatives pJD7 (Toronto-type), and pG04717 (Rio-type). DNA sequencing studies supported the concept of a Rep protein dependent mechanism of initiation. These plasmids contained features similar to the *oriC* of *E. coli* (1,4), such as DnaA boxes, integration host factor (IHF) sites, multiple repeats, and A-T rich sites. These features indicate the capacity for an iteron-based mechanism of plasmid initiation (1).

We used classical incompatibility experiments (5) involving resident and incoming plasmids to test for establishment and maintenance of more than one plasmid in a cell to study the incompatibility of pJD9. The origins of replication in pJD9 were found to belong to the incompatibility group W(6). At the present time, we are unable to differentiate whether ori2 or ori3 was the dominating incompatibility determinant. The two origins of pJD9 are further being

investigated for presence of another incompatibility determinant, consistent with the presence of a second origin. pJD9 was found to be incompatible with pJD4, as expected.

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## **Characterization of an 85 kDa outer membrane protein of *Neisseria gonorrhoeae* having homology with the D15 surface protective antigen of *Haemophilus influenzae***

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A gene expressing an 85 kDa outer membrane protein from *Neisseria gonorrhoeae* FA19 was cloned and sequenced. Western blot analysis showed that polyclonal rabbit antiserum directed against a purified gonococcal outer membrane fraction (1 and see Judd, *et al.*) bound to a similar molecular mass protein in gonococcal strains FA635, FA1090, JS1, and F62 and in *N. meningitidis* strains, MP3, MP78, MP81 and HH. Southern blot analysis demonstrated that the protein was encoded by a single copy gene in all of the above strains. Computer analysis of the sequence showed a typical signal peptide (2) indicating the protein was located in the outer membrane. A BLAST search (3) revealed significant homology with a surface antigen of *Haemophilus influenzae* (4), antibodies against which provide protection against challenge in mice (5). Homology was also observed with OMP1 in *Brucella abortis* (6). Both of these organisms are human pathogens which suggests that the 85kDa outer membrane protein described here is possibly virulence associated.

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## **Recombinant CTB-linked mucosal immunogens for inducing antibodies in secretions**

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The generation of antibody responses at the mucosal surfaces, including those of the genital and upper respiratory tracts, is most effectively accomplished by administration of vaccine antigens at one of the inductive sites of the mucosal immune system. While the best known of these is represented by the gut-associated lymphoid tissue, recent attention has turned also to the lymphoepithelial structures of the upper respiratory tract, such as Waldeyer's ring in humans, or its functional equivalent, the nasal lymphoid tissue of rodents. Intranasal (i.n.) immunization of mice or rhesus monkeys with a bacterial protein antigen chemically conjugated to cholera toxin (CT) B subunit induces strong IgA antibody responses in saliva and secretions of the intestinal, respiratory, and genital tracts, as well as serum IgG antibodies (1,2). Furthermore, mice immunized i.n. with pneumococcal surface protein A (PspA) and CTB develop protective immunity against nasopharyngeal carriage of pneumococci as well as against lethal infection (3). Although the enteric adjuvant effect of CTB may require the synergistic action of intact CT, recombinant CTB completely lacking the toxic A subunit functions effectively as an adjuvant by the i.n. route (4).

A more convenient approach for the development of hybrid immunogens, however, is to construct genetically engineered chimeric proteins, but the direct fusion of large segments of protein to CTB usually disrupts the ability of CTB to assemble into G<sub>M1</sub> ganglioside-binding pentamers which are important for immunogenicity. To overcome this problem, we have modified the pET20b(+) vector (Novagen) by ligating DNA specifying the A2 and B subunits of CT into the multiple cloning site to form pCT<sup>deltaA1</sup> (5). DNA encoding a large (42 kDa) segment of a model bacterial protein antigen, AgI/II from *Streptococcus mutans*, was then inserted upstream of and in frame with the CTA2 gene segment, and behind the *peI/B* leader. When this plasmid was expressed in *E. coli* BL21 (DE3), an AgI/II-CTA2 fusion protein and CTB subunit were synthesized separately and assembled into a chimeric protein in which the toxic A1 subunit of CT was totally replaced by the AgI/II segment (5). G<sub>M1</sub> ganglioside-binding activity of the CTB component and antigenicity of the AgI/II segment were demonstrated by G<sub>M1</sub>-ELISA, and the chimeric protein was purified chromatographically and characterized by SDS-PAGE and western blotting. Intragastric or i.n. administration of small doses of this protein to mice generated high levels of salivary and other mucosal IgA and serum IgG antibodies to AgI/II that persisted for a prolonged period, at least 11 months (6). The responses to AgI/II compared favorably with those to CT itself, a well-known potent mucosal immunogen. Similar CTA2/B chimeric proteins have been constructed from group A streptococcal M protein and PspA, and DNA specifying other protein antigens identified as potential protective immunogens can be readily inserted into the plasmid construct. Because CTB-coupled immunogens induce strong mucosal and circulating antibody responses, we believe they may have application in the development of vaccines against numerous mucosally acquired infections, including those of the respiratory and genital tracts.

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## Opacity protein expression by organisms recovered from volunteers infected with transparent *Neisseria gonorrhoeae* MS11mkC

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MS11mkC harbors 12 *opa* loci; 11 loci appear to be fully functional (1-3). In experimental infections where 34 human male volunteers were inoculated intraurethally with piliated (P<sup>+</sup>) transparent (Opa<sup>-</sup>) strains of *Neisseria gonorrhoeae* MS11mkC (4-6), onset of clinical urethritis was preceded or accompanied by a phase change to the opaque (Opa<sup>+</sup>) phenotype (4). Fourteen volunteers were reinfected with the homologous strain three weeks after the initial infection. Outer membrane proteins were extracted from amplified single colonies, or whole plates of primary isolates from infected volunteers. Opa specific MAb 4B12 (provided by Milan Blake) was used to identify opa proteins on western immunoblots and size was estimated by comparison with standard molecular weight size markers (BioRad) using a 420oe scanning densitometer and QS30 software (pdi, New York). Opa protein nomenclature was based on previously described determinations (1, 7-9). Among the ten opa proteins identified, some patterns occurred repeatedly. OpaK, the most frequently detected protein, was identified in gonococci isolated from all but one volunteer, and persisted over days. OpaK adheres to and mediates invasion of epithelial cells, while other opa proteins interact with human leukocytes (8). OpaI and OpaH predominated in isolates from people with blood type O+, and OpaF in type B+ and AB+ individuals. The predominance of specific opa proteins in a given host suggests clonal selection by host-pathogen interactions, in which the organisms expressing proteins that are most efficient in binding to and invading neutrophils (8,9) will find conditions for multiplication more favorable. At the same time, opa phase variation allows constant low-level cycling of the entire opa repertoire during an infection. Thus, gonococci expressing opa proteins with optimal affinity for particular host receptors would become predominant, and persist against a varying background of the other opa proteins during the course of infections.

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## Characterization of *Neisseria gonorrhoeae* strains isolated from a conjunctivitis outbreak.

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Penicillin was introduced for the first time in 1940 in the treatment of gonococcal infection, and in the following decade the use of a single dose was the effective standard treatment. A few years later some strains having a decreased sensitivity appeared, and to control the disease the use of 4.8 million units as therapeutic dose were effective for a while (1). In 1976 the first penicillinase-producing *Neisseria gonorrhoeae* (PPNG) strains brought in simultaneously in England and USA, it was demonstrated that the strains showing high penicillin-resistance level had two types of resistance plasmids (plasmids R) with a molecular size of 3.2 Mda (African type), and 4.4 - 4.7 MDa (Asian type) respectively. Four new types of plasmids R have been described from that time on, and this way of resistance is wide distributed globally (2).

*Neisseria gonorrhoeae* has shown a remarkable adaptation to the evolutionary pressure made by the treatments used in the last 50 years, that is why the epidemiological studies and the disease control are based on the analysis of the distribution and behavior of the antimicrobial-resistant strains (3). Few antimicrobial susceptibility studies by Minimal Inhibitory Concentration Method have been carried out in our country with *N. gonorrhoeae*, and remains unknown which types of plasmids are responsible for the high resistance levels to penicillin and tetracycline.

We aim at knowing the susceptibility to penicillin and to tetracycline, as well as the plasmid profile in a group of *Neisseria gonorrhoeae* strains isolated from patients with conjunctivitis, and in this patients the use of penicillin brought about complications and sequelae.

**Methods.** A total of six strains were sent to the National Reference Laboratory of Pathogen Neisseria at IPK from the Provincial Center of Hygiene and Epidemiology at Camaguey Province. Those strains were confirmed *Neisseria gonorrhoeae* because of their growth in a selective medium of modified Thayer and Martin, Gram staining, positive oxidase and superoxol, and utilization of carbohydrates (4).

The agar dilution base GC plus Isovilatex supplement at 1% was used to determine the minimal inhibitory concentration, following the suggestions of the National Committee for Clinical Laboratory Standards, and of CDC, Atlanta (5). The detection of betalactamase was carried out using chromogenic cephalosporine using disks impregnated with nitrocephin (Unipath).

Extraction of the plasmid was under taken accordingly to the method described in the Protocol Guide and Applications, Promega (6).

**Results.** The conjunctivitis produced by *Neisseria gonorrhoeae* is one of the less frequently clinical manifestations of gonococcal infection. Some authors from all over the world have

described gonococcal conjunctivitis outbreaks affecting different age groups, but mainly < 5 years (7).

A total of 6 cases of this disease in young adults were notified in one province from May to July 1995. It was found neither relation between them nor the source of infection though the clinical and epidemiological analysis was carried out. Each case presented severity of the ophthalmic invasive clinical manifestations and slow response to the antimicrobial therapy.

A complete study of this phenomenon, will necessarily depend on the services of a National Reference Laboratory, and a characterization of each patient isolation would be possible.

When analyzing our results, it was found that all strains were penicillinase-producing, showed high resistance levels to penicillin (MIC > 16 µg/mL), were resistant to tetracycline (MIC > 2-8 µg/mL) and had identical plasmid profiles (2.6 MDa, 3.2 MDa and 24.5 MDa.)

In a study carried out by Ebong et al, 68% of the *Neisseria gonorrhoeae* isolations from a gonococcal conjunctivitis were penicillinase-producing, showed high level of resistance to penicillin, and had any kind of plasmid R (8).

It should be highlighted that plasmid 3.2 MDa together with the conjugative plasmid 24.5 MDa are found in our isolations. Most of the outbreaks caused by penicillinase-producing strains are associated with few phenotypes accordingly to the MIC values and to the plasmid profiles, and many of them have no conjugative plasmids. In 1981, Dillon and Pauzé reported 5 PPNG strains that showed 3.2 and 24.5 MDa plasmid combination (9).

Though this was an unusual fact in literature, now there are authors from Spain, Uruguay, Argentina and Hawaii that have reported high percentages of strains having both plasmid types. Of the total strains that had 3.2 MDa plasmid they reported 50%, 95%, 87% and 58,3% respectively (10).

The homogeneity of the results in the virulence and epidemiological markers used is suggestive, in spite of the fact that this study was carried with few strains. The results contribute to the knowledge of the invasive behavior of the disease, and at the same time, it is made clear how antimicrobial susceptibility and plasmid profile studies help to understand the dynamic of an outbreak, and to determine the transmission patterns of a gonococcal infection, if they are applied early.

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## Antimicrobial susceptibility of 42 *Neisseria gonorrhoeae* strains isolated in 1995 in Cuba.

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Antimicrobial resistance in *Neisseria gonorrhoeae* is an increasing and costly public health problem in the world, which can contribute to increased morbidity and complications associated with gonorrhea, making its management more difficult (1). In 1995, there were 41,185 confirmed cases of this disease reported to the Public Ministry of Health in Cuba (2). In this country, there exists few studies about the susceptibility of gonococcal isolates to antibiotics, which generally deal only with the detection of the  $\beta$  lactamase enzyme (3). Penicillin is the drug recommended for the treatment of uncomplicated gonorrhea in Cuba (4). This study was conducted to determine the susceptibility of 42 gonococcal strains isolated in 1995 in Cuba to the antibiotics recommended by the WHO for the treatment of uncomplicated gonorrhea.

**Methods.** Forty two gonococcal strains isolated from patients with genital infections from some Cuban provinces occurring during the first six months of 1995 were sent to the National Reference Laboratory for Pathogenic *Neisseria* at the Tropical Medicine Institute "Pedro Kouri", Havana. The strains were identified as gonococci by standard procedures (5) and were stored frozen at - 70 °C. in trypticase soy broth (Unipath) containing 20 % glycerol until tested. Antimicrobial susceptibilities to penicillin G, tetracycline, spectinomycin, ceftriaxone, cefotaxime, cefuroxime, and ciprofloxacin were determined on GC agar base medium (Unipath) supplemented with 1% Isovitalex (Becton Dickinson) as described previously (6).

Antibiotics were supplied by the manufacturer as pure powders and used according to their instructions. *N. gonorrhoeae* ATCC 49226 was used as a control strain. A control plate without antibiotics was also included. The susceptibility of a strain to an agent was defined as the MIC, i.e., the lowest concentration inhibiting growth to  $\leq 1$  CFU. The results were interpreted according to the recommendations of the NCCLS and the CDC (1,6).  $\beta$  lactamase production was detected using the chromogenic cephalosporin, Nitrocefin (Unipath). As positive and negative control strains were used *N. gonorrhoeae* WHO E and WHO A, respectively.

**Results:** Susceptibility to penicillin: Of the strains evaluated, 57,1% and 28,6 % resulted resistant and less susceptible, respectively, and only 14,3 % were entirely susceptible. The proportion of penicillinase ( $\beta$  lactamase) producing *N. gonorrhoeae* (PPNG) MIC  $\geq 16,0$   $\mu\text{g/mL}$  to penicillin and  $\leq 16,0$   $\mu\text{g/mL}$  to tetracycline was 45,2 % (20/42).

Susceptibility to tetracycline: The proportion of strains that resulted resistant, less susceptible and susceptible were 43%, 19 % and 38 %, respectively. A total of 4 strains were presumably identified as a plasmid-mediated tetracycline-resistant *N. gonorrhoeae* (TRNG) MIC  $\geq 16,0$   $\mu\text{g/mL}$  to tetracycline/ $\beta$  lactamase negative. A total of 35.7 % of the strains was multi-resistant to both penicillin and tetracycline.

Susceptibility to other antibiotics: All the strains were susceptible to spectinomycin, cefuroxime, cefotaxime, ceftriaxone and ciprofloxacin, and the MIC 90 for such drugs were 10 ug/mL, 0.125 ug/mL, 0.012 ug/mL, 0,008 ug/mL and 0.004 ug/mL, respectively.

**Comments and Recommendations.** In this study we found that the occurrence of less sensitive and resistant strains of *N. gonorrhoeae* to penicillin and tetracycline and also the resistance level encountered for both drugs were very high.

Delgado et al. in some gonococcal strains isolated in 1983-1985 in Cuba, found no resistance to penicillin, but 63 % of the strains presented a diminished susceptibility to this antibiotic (7). However, some studies sporadically carried out in the Havana population have shown a high proportion of  $\beta$  lactamase-producing *N. gonorrhoeae* isolates (3). We can construct the hypothesis that in Cuba there is an endemic-high gonococcal resistance to both penicillin and tetracycline.

Similar results have been reported almost from some countries in the Far East and Africa (8,9). However a recent study developed in Kingston, Jamaica also revealed an extremely high gonococcal resistance to penicillin and/ or tetracycline (10).

Could the same phenomenon be occurring nowadays in other Caribbean islands? We consider it obvious that some investigation on this matter should be done in the future in that geographical area.

As in previous reports from other countries, the rest of the drugs tested were shown to be very effective against *N. gonorrhoeae* (1,8).

Although the data presented here may not be representative of the Cuban population, they represent an attempt to analyze antimicrobial susceptibility of gonococcal isolates in this country. According to our results, neither penicillin nor tetracycline can any longer be regarded for the treatment of uncomplicated gonococcal infections in Cuba. Other regimens which have been proven to be effective against *N. gonorrhoeae*, i.e., wide-spectrum cephalosporins, quinolones or spectinomycin which are recommended by the WHO and the CDC to combat those infections should be used in Cuba.

We recommend that this study be extended to the rest of the Cuban provinces as soon as possible. Thus, it is mandatory to continue monitoring antimicrobial susceptibility in gonococcal isolates in this country, which can provide the necessary information to support local gonorrhea program efforts and guide the selection of the most effective therapeutic agents for the treatment and control of that disease in Cuba.

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## **In vitro induction of inflammatory cytokines by *Neisseria gonorrhoeae* and by gonococcal antigens**

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*Neisseria gonorrhoeae* infections cause a localized inflammatory response which is accompanied by elevated levels of inflammatory cytokines (Il-6, Il-8, TNF- $\alpha$ ) in the urine of experimentally challenged males (1,2). An in vitro tissue culture monolayer stimulation assay (3) has been developed to examine the induction of cytokine production by virulent gonococci and by gonococcal antigen extracts. *N. gonorrhoeae* has previously been shown to invade the ME180 (ATCC HTB 33) cell line (4), which is derived from a human cervical epidermoid carcinoma. In the present studies, we stimulated ME180 monolayers, grown in 24-well tissue culture plates, with live *N. gonorrhoeae* MS11mkC or antigen extracts [lipooligosaccharide (LOS), pili or outer membrane complex (OMC)] derived from Pil<sup>+</sup>Opa<sup>+</sup> MS11mkC. Kinetics of Il-6 and Il-8 production were measured in the culture supernatant by testing samples taken at 2, 5 or 24 h following addition of the stimulant. Pil<sup>+</sup>Opa<sup>+</sup> and Pil<sup>+</sup>Opa<sup>-</sup> phenotypic variants of MS11mkC, added to 48 h monolayers at about 50:1 bacteria:cell ratio, both stimulated about equal levels of Il-6 and Il-8 production. Cytokine levels reached a peak at the 24 h time point. The OMC (Pil<sup>+</sup>Opa<sup>+</sup>) was also able to induce Il-6 and Il-8 production when added at a concentration as low as 1  $\mu$ g/ml. At 5  $\mu$ g/ml, Il-6 and Il-8 were detected as early as the 2 h time point. The most rapid rise was seen between 2 and 5 h; levels continued to rise up to 24 h. Pili alone (10  $\mu$ g/ml) was also able to stimulate Il-6 and Il-8, although its activity was reduced compared to OMC (10  $\mu$ g/ml). LOS (10  $\mu$ g/ml) used alone was unable to stimulate any cytokine production. A low level activity was seen when LOS and pili were used together (both at 10  $\mu$ g/ml), but the activity was not greater than that of pili alone.

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## **Differential antibody and cytokine responses in male volunteers experimentally infected with *Neisseria gonorrhoeae* MS11mkC**

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The effect of challenge dose size and duration of gonococcal infection upon cytokine (1) and antibody responses was studied in three groups of male volunteers experimentally challenged intraurethrally (2) with *Neisseria gonorrhoeae* strain MS11mkC. The three groups were challenged with 7000 cfu, 5700 cfu and 57,000 cfu, respectively, and infection rates were 5/10, 5/10 and 14/15. Group 1 was treated as soon as infection was diagnosed, Group 2 at 48 hours post diagnosis and Group 3 at times varying from 24 to 99 hours post diagnosis. All infected volunteers developed IL-8 detectable in the urine while the rate of positive urine IL-6 responses rose with increased time of infection and size of challenge dose. Time of onset of urine cytokine increase (24-48 hours after challenge) did not vary among the three groups; responses waned within the same period, approximately 48 hours, after antibiotic treatment. Peripheral blood IgG, IgA and IgM antibody secreting cell (ASC) responses against MS11mkC LOS, pili and outer membrane complex (OMC) were measured in order to assess B cell responses following infection in the urethral mucosa. ASC responses were detected in only 2 of 5 infected volunteers in Group 1; in Groups 2 and 3, the longer duration of infection resulted in positive responses in 4 of 5 and 13 of 14 infected volunteers, respectively. In contrast to responses to infection at other mucosal sites in which ASC responses peak around 6-10 days (3), ASC responses in these studies did not peak until 10-12 days after challenge. The highest numbers of ASC were detected in Group 3, and the responses were most often detected with the OMC antigen, a native vesicular extract (4) incorporating both pili and LOS. Serum antibody responses against LOS, pili or OMC were detected at low rates (<50% in almost all cases) with IgG against LOS the only response consistently seen in all three groups.

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## **Cellular immune response to *Neisseria gonorrhoeae* proteins in patients with urogenital gonorrhea**

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Urogenital infection with *Neisseria gonorrhoeae* is a mucosal disease. Little is known about the immune response to this sexually transmitted disease. The anti-gonococcal humoral immune response has been investigated in patients with gonorrhea and it has been demonstrated that serum antibodies to certain *N. gonorrhoeae* outer membrane proteins correlates with immune protection from reinfection (1). However, there is a paucity of data examining the cellular immune response to *N. gonorrhoeae*. Particularly, whether T cell anti-gonococcal reactivity is present in individuals infected with gonorrhea and is it associated with protection from disease. In this preliminary study, the cellular immune response to gonococcal infections has begun to be examined.

The proliferative response of peripheral blood mononuclear cells (PBMC), isolated from *N. gonorrhoeae* infected individuals was measured, as determined by <sup>3</sup>H-thymidine incorporation assay (2), to the gonococcal proteins protein IA (PIA), protein IB (PIB) or GroES, an 11 kDa heat shock protein (obtained from Dr. William Shafer, Emory University School of Medicine). In 44% (8/18) of infected individuals we observed antigen-specific proliferation to both PIA and PIB (Stimulation Index (SI) >2). However, no antigen-specific proliferative response could be detected when the PBMC were stimulated with either GroES (SI, 1.3±0.6) or a non-relevant control antigen GST (SI, 1.1±0.6).

We also examined the cytokine profile of PIB stimulated PBMC using intracellular staining and FACS analysis (3). A significant percentage of T cells from gonococcal infected individuals produced IL-4 upon 7 days of incubation with PIB. Eleven to 37% of CD4<sup>+</sup> T cells were IL-4<sup>+</sup>. Interestingly, we also observed that 15 to 39% of PIB stimulated CD8<sup>+</sup> T cells were also IL-4<sup>+</sup>. Much smaller percentages of T cells when incubated with media alone produced IL-4 (CD4<sup>+</sup> 7% and CD8<sup>+</sup> 6%). No significant staining for IL-2, IFN $\gamma$  or TNF $\alpha$  was observed in either PIB activated CD4<sup>+</sup> or CD8<sup>+</sup> T cells.

These preliminary data indicate that in some individuals, a portion of the cellular immune response to *N. gonorrhoeae* is directed against Protein I (Por). Furthermore, our results suggest that infected individuals mount a Th<sub>2</sub> type response to *N. gonorrhoeae*. These findings correlate with previous data demonstrating that individuals with mucosal *N. gonorrhoeae* infection develop antibodies that recognize the major gonococcal outer membrane proteins, especially the porins. Even though anti-gonococcal T cell reactivity alone might not be protective, these T cells could offer help to B cells producing the potentially protective anti-gonococcal antibodies.

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## **Intracellular *Neisseria gonorrhoeae* bind host pyruvate kinase via their Opa proteins**

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Gonococci bind to and invade human epithelial cells and likely reside within the cytoplasm (1). One family of outer membrane proteins involved in gonococcal adhesion and invasion are the Opa (Opacity-associated) proteins (2). In these studies, we looked for host cell proteins that could interact with gonococcal Opa proteins, and determined if such interactions occurred in vivo. Using CLONTECH's MATCHMAKER yeast two-hybrid system, we identified 5 plasmids containing HeLa cell cDNAs coding for potential Opa-Interacting Proteins (OIPs). One of these, OIP3, is human Pyruvate Kinase (PK) subtype M2.

PK is both a glycolytic enzyme, converting phosphoenolpyruvate (PEP) to pyruvate, and a cytoplasmic thyroid hormone (triiodothyronine, T3) binding protein (3). PK exists in two forms within a cell: monomers and homotetramers. PK monomers are relatively inactive as glycolytic enzymes, but can avidly bind T3, serving as cytoplasmic receptors for T3 (3). PK homotetramers are formed from PK monomers and are active as glycolytic enzymes, but cannot bind T3. The in vivo monomer-homotetramer interconversion is regulated through glucose metabolism via intracellular fructose 1,6- bisphosphate (F1,6P2) concentrations (4). T3 also stimulates transcription of the PK subtype M1 gene (5). Thus, PK is a key enzyme in regulating cellular ADP, ATP, and pyruvate, and mediates cellular metabolic effects induced by T3 (6).

In an attempt to confirm the yeast two-hybrid results, we investigated the ability of Opa(+) and Opa(-) gonococci, and of Opa(+) and Opa(-) *E. coli*, to bind commercially available rabbit muscle PK subtype M1 in vitro by employing a standard pyruvate kinase assay. Rabbit PK subtype M1 is 96% similar and 93% identical to human PK subtype M2 at the amino acid level over the entire length of the protein, and 90% similar and 84% identical at the amino acids corresponding with OIP3 (A366-P531). Opa(+) bacteria bound substantially more PK subtype M1 than did Opa(-) bacteria. Observations were dose-dependent for bacteria and PK concentrations. These in vitro binding studies indicate that bound PK retains its enzymatic activity.

To determine if Opa binds PK in vivo, rabbit antiserum was raised against recombinant human PK subtype M2 and used to determine if PK surrounds *N. gonorrhoeae* within host cells. Opa-expressing gonococci were allowed to invade ME-180 human cervical epithelial cells for 4 hours, the cells were fixed and probed with anti-PK antiserum followed by fluoresceinated anti-rabbit antibody, and visualized via confocal fluorescent microscopy. These in vivo studies revealed that intracellular, but not extracellular, gonococci bind PK.

Gonococci are thought to use only three carbon sources - glucose, pyruvate, and lactate (7). Intracellularly, there is little available glucose, since glucose is present mainly as glucose-6-phosphate. Pyruvate, on the other hand, can be continuously produced intracellularly. It appears

that intracellular gonococci bind active PK, and use it to supply themselves with pyruvate. Additionally, this interaction may enable intracellular gonococci to interact with the T3 metabolic pathway.

These results suggest that (a) the yeast two-hybrid system can be used successfully to investigate host-parasite protein-protein interactions, and (b) gonococci can bind a metabolic enzyme (PK subtype M2) via their Opa proteins for the purpose of gaining access to a carbon source or growth substrate (pyruvate), and/or to alter host cell metabolism to their advantage.

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