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In Vivo Selection for *Neisseria gonorrhoeae* Opacity Protein Expression in the Absence of Human Carcinoembryonic Antigen Cell Adhesion Molecules

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The neisserial opacity (Opa) proteins are phase-variable, antigenically distinct outer membrane proteins that mediate adherence to and invasion of human cells. We previously reported that *Neisseria gonorrhoeae* Opa protein expression appeared to be selected for or induced during experimental murine genital tract infection. Here we further defined the kinetics of recovery of Opa variants from the lower genital tracts of female mice and investigated the basis for this initial observation. We found that the recovery of different Opa phenotypes from mice appears cyclical. Three phases of infection were defined. Following intravaginal inoculation with primarily Opa$^-$ gonococci, the majority of isolates recovered were Opa$^+$ (early phase). A subsequent decline in the percentage of Opa$^+$ isolates occurred in a majority of mice (middle phase) and was followed by a reemergence of Opa$^+$ variants in mice that were infected for longer than 8 days (late phase). We showed the early phase was due to selection for preexisting Opa$^+$ variants in the inoculum by constructing a chloramphenicol-resistant (Cm^r) strain and following Cm^r Opa$^+$ populations mixed with a higher percentage of Opa$^-$ variants of the wild-type (Cm^s) strain. Reciprocal experiments (Opa$^-$ Cm^r gonococci spiked with Opa$^+$ Cm^s bacteria) were consistent with selection of Opa$^+$ variants. Based on the absence in mice of human carcinoembryonic antigen cell adhesion molecules, the major class of Opa protein adherence receptors, we conclude the observed selection for Opa$^+$ variants early in infection is not likely due to a specific adherence advantage and may be due to Opa-mediated evasion of innate defenses.

Neisseria gonorrhoeae is primarily a mucosal pathogen, which most often causes urethritis in men and endocervical infections in women; the female urethra is also frequently infected. Infections can be symptomatic or asymptomatic, and ascended infection occurs in 10 to 20% of women with endocervical infection. Pelvic inflammatory disease, which can lead to fallopian tube scarring, ectopic pregnancy, and chronic pelvic pain, is the major source of morbidity and mortality associated with gonorrhea (24).

A remarkable feature of *N. gonorrhoeae* is the propensity of this organism to undergo high-frequency, reversible expression of surface molecules. The neisserial opacity (Opa) proteins are a well-characterized family of outer membrane proteins that undergo phase-variable expression via a frameshift mechanism that stems from the insertion or deletion of one or more copies of a pentameric nucleotide sequence within the coding region of each *opa* gene (47, 51). Gonococcal strains express 8 to 11 antigenically distinct Opa proteins, each the product of its own structural gene. Individual *opa* genes phase vary independently of each other at a rate of 10^{-3}/cell/generation (41), and a single gonococcus can express no Opa proteins, one Opa protein, or multiple Opa proteins simultaneously. Two surface-exposed hypervariable regions (HV_1 and HV_2) are responsible for the differences in molecular weight and antigenicity of individual Opa proteins. The Opa protein repertoire differs among strains, and *opa* gene phase variation confers genetic diversity within a strain by creating subpopulations of antigenically distinct variants. Opa variants can also differ functionally with regard to the capacity to evade host defenses (2, 6, 19) or to mediate adherence to and invasion of host cells (reviewed in reference 15).

The best-characterized function of the neisserial Opa proteins is Opa-mediated binding to human tissue culture cells, which can lead to bacterial internalization (3, 37, 40, 50, 55). Some Opa proteins also mediate uptake by phagocytes in the absence of opsonization (2, 5, 11, 19, 37, 50). Members of the carcinoembryonic antigen cell adhesion molecule (CEACAM) family serve as receptors for Opa-mediated uptake. CEACAMs are present on a variety of cells, including human epithelial and endothelial cells and professional phagocytes, and within the CEACAM family, differences exist in the expression of the various CEACAMs on different cell types and tissues (25). Some Opa proteins also utilize heparin sulfate proteoglycan (HSPG) molecules as a receptor for attachment and invasion of epithelial cells (5, 9, 11, 20, 54, 56). Opa proteins also may play a role in evasion of host complement (6, 12) and in regulation of the immune response either by stimulation of cell death in neutrophils (10) or by down-regulation of the activation and proliferation of $CD4^+$ T cells (7).

Early analyses of urethral, endocervical (26, 27, 49), and fallopian tube isolates (17) from naturally infected individuals suggested the expression of Opa proteins during infection is influenced by anatomical site and hormonal state. These reports used colony photo-opacity as the main indicator of Opa phenotype. Most notable was a survey of over 200 clinical isolates

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by James and Swanson (27), in which a majority of urethral isolates from men displayed an opaque (Opa^+) colony morphology. The resultant hypothesis that selection or induction of Opa protein expression occurs in the male urethra was later confirmed in an experimental human urethritis model using more-accurate methods of determining Opa phenotype. In these studies, Opa protein-expressing gonococci were recovered from a majority of male volunteers following intraurethral inoculation with primarily Opa^- variants (29, 52). There is less information on Opa protein expression during endocervical infection in women; interestingly, hormonally driven factors may influence Opa protein phenotype, as suggested by James and Swanson's original survey, in which the colony photo-opacity of endocervical isolates corresponded to certain phases of the menstrual cycle (26).

It is not known if selection of particular Opa phenotypes is responsible for these observations on Opa protein expression during genital tract infections in men and women. No selective forces have been identified definitively, and it is possible that induction of an increased rate of *opa* gene phase variation may occur in vivo, which might contribute to the increased recovery of Opa^+ variants following inoculation with a predominantly Opa^- population. Our laboratory previously reported that Opa protein expression appeared to be selected for in a female mouse model of genital tract infection (28), even though the murine CEACAM1 molecule is not highly related to its human counterparts (22). Here we tested the hypothesis that selection of a preexisting population of Opa^+ variants is responsible for the increased recovery of gonococci that express Opa proteins from mice; Opa expression in the murine model over an extended period of time was also characterized.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* was cultured on supplemented GC agar as described previously (29). *N. gonorrhoeae* strain FA1090 was initially isolated from a female patient with disseminated infection (13). A chloramphenicol-resistant (Cm^r) derivative of strain FA1090 ($FA1090Cm^R$) was constructed for use in selection studies by using the Neisserial Insertional Complementation System (NICS; a gift of H. S. Seifert, Northwestern University), which allows one to insert foreign genes in an intragenic region between the *aspC* and *lctP* genes that has no known function (44). NICS vector pGCC5 was digested with ClaI to release a 5.2-kb fragment containing the *aspC* and *lctP* genes and a chloramphenicol acetyltransferase (*cat*) gene, and the fragment was introduced into a piliated, Opa^- variant of *N. gonorrhoeae* strain FA1090 by allelic exchange (21). Transformants were selected on GC agar containing 0.5 μg/ml Cm. PCR amplification using primers IcatB (5′ TTAAGG GCACCAATAACTGC) and IcatC (5′ AACTGGTAGGTATGGAAGATCTC TAGA), which are located within the *cat* gene, was used to confirm the presence of the *cat* gene in the Cm^r transformants, and a stable transformant ($FA1090Cm^R$) was selected for further characterization. To confirm that integration of the *cat* gene occurred in the desired region, primers F1451A (5′ CATCCGTTCTGCTCTATACCC) and R1451B (5′ GTCCCATTCTTCAAA GTCGG), which correspond to the nucleotide sequence immediately adjacent to the Cm^r transposon insertion in pGCC5, were designed. As predicted, PCR analysis of DNA from wild-type FA1090 with F1451A and R1451B led to the production of a 400-bp fragment, and consistent with the insertion of the *cat* gene, a 1.6-kb fragment was amplified from $FA1090Cm^R$ genomic DNA. OpaI, OpaB, and Opa^- variants of strain $FA1090Cm^R$ were identified by colony suspension immunoblotting, and nonpiliated stocks of each were frozen for subsequent experiments. The predominant Opa phenotype of each frozen stock was confirmed by Western blot analysis with the broadly reactive anti-Opa antiserum AB4L and with HV_2-specific antisera (data not shown). Nonpiliated gonococci were used in all experiments to lessen the likelihood of genetic transfer of the *cat* gene to wild-type gonococci during mixed infections. Oligonucleotide primers were synthesized by the Biomedical Instrumentation Center at Uniformed Services University. PCR amplifications were completed in a Thermolyne Amplitron II thermal cycler (Barnstead/Thermolyne Corp., Dubuque, IA) by cycling 25 times at 94°C for 1 min, 59°C for 1 min, and 72°C for 2 min. Reaction mixtures consisted of 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.25 mM of each deoxynucleoside triphosphate, 200 ng of each primer, 0.5 U of *Taq* polymerase, and 200 ng of template. Chromosomal DNA was isolated via the cetyltrimethyl-ammonium bromide protocol (33). Purification of DNA fragments was performed with a QIAquick gel extraction kit, and plasmid DNA was prepared with a QIAGEN Plasmid Midi kit and a QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA).

Experimental murine infection. Female BALB/c mice (6 to 8 weeks old; National Cancer Institute) were treated with 17-β-estradiol and antibiotics to induce susceptibility to *N. gonorrhoeae* as described previously (28, 30). Bacterial suspensions composed of the desired ratio of Opa variants were prepared by first prescreening colonies for Opa phenotype by colony suspension immunoblotting. Colonies of the desired phenotype were subcultured on GC agar and incubated for 20 to 21 h; isolated colonies were then suspended in phosphate-buffered saline (PBS) and filtered to remove bacterial aggregates (28). Filtered suspensions were adjusted to an A_{600} of 0.08 (10^8 CFU/ml); aliquots of each filtered suspension were mixed to obtain the desired ratio of Opa variants, and 20-μl portions were inoculated intravaginally into mice. Vaginal mucus was quantitatively cultured on GC-VCNTS agar as described previously (28). In experiments with mixtures of $FA1090Cm^R$ and FA1090, inocula were cultured on GC agar with and without Cm selection; primary vaginal isolates were subcultured onto GC agar with Cm to determine the Cm^r phenotype. The Opa phenotypes of individual colonies isolated directly from the inoculum (96 colonies; limit of detection, 1%) and vaginal cultures (36 colonies; limit of detection, <3%) were determined by colony suspension immunoblotting. Animal experiments were conducted in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, under a protocol that was approved by the University's Institutional Animal Care and Use Committee.

Determination of Opa phenotype. Colony suspension immunoblots were used to determine the Opa phenotype of primary isolates without further in vitro passage as described previously (28, 29). Antibodies specific for OpaA, OpaB, OpaC, OpaD, OpaF, and OpaI were produced in rabbits by immunization with peptides that correspond to unique sequences within the HV_2 regions of these proteins and affinity purified. Peptide sequences were as follows: for OpaA, YLQSGKPSPIVRGSTL; for OpaB/D, AYPSDADAAVTV; for OpaC, TTEFL TAAGQDGGA; for OpaF, VITAPPTTSDGA; and for OpaI, HSAGTKPTYY DDIDSGKTKK. OpaB and OpaD express the same HV_2 sequence and therefore bind to antibodies specific for the same peptide. In some cases, OpaB and OpaD variants were distinguished by a difference in electrophoretic mobility on Western blots or by a difference in colony photo-opacity. Detection of OpaE and OpaK variants was done with monoclonal antibody (MAb) H164 (4) (a gift from Janne Cannon, University of North Carolina) as described previously (29). MAb H164 binds both the OpaE and OpaK proteins, which differ only slightly in electrophoretic mobility (29). OpaE and OpaK variants both produce transparent colonies and were not distinguished in this study. Therefore, H164-binding variants are referred to as OpaE/K variants. Remaining colony suspensions were frozen and used to confirm the phenotype of variants that expressed more than one Opa protein simultaneously as described previously (29). The phenotype of all Opa^- variants was confirmed by analyzing whole bacterial lysates prepared from subcultures of the corresponding frozen suspension by Western blotting with the broadly cross-reactive anti-Opa MAb 4B12 (1) (a gift of M. S. Blake, CBER, FDA) or polyclonal rabbit serum AB4L. AB4L was obtained against the peptide HYWGRLENTRFKTHE, which corresponds to the fourth surface-exposed Opa protein loop. This fourth loop is highly conserved among the neisserial Opa proteins, with the only difference among the Opa proteins of strain FA1090 being the presence of an asparagine residue instead of a tyrosine in the OpaB, OpaC, OpaD, and OpaF proteins. Both AB4L and MAb 4B12 recognize all known Opa proteins of strain FA1090 by Western blotting, with the exception of OpaE. MAb H164 therefore was also used to probe Western blots of presumptive Opa^- variants to confirm the lack of expression of OpaE. For all immunoblot assays, dilutions of primary antibodies were determined empirically, and bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG (Sigma). Peptide synthesis and conjugation, immunizations, and affinity purification of antibodies were performed by Bethyl Labs.

Indirect fluorescent-antibody staining. Detection of OpaI- and OpaB-expressing gonococci in vaginal samples was performed by a sequential staining procedure. Vaginal mucus was smeared in duplicate onto Teflon-printed indirect fluorescent-antibody slides (Electron Microscopy Sciences) and fixed in 100%

methanol at −20°C. Slides were blocked in PBS with 1% immunoglobulin-free bovine serum albumin (Sigma) and incubated with affinity-purified HV_2-specific antibodies against OpaI or OpaB/D (1:4,000) as the first primary antibody. Goat anti-rabbit IgG (Alexa 488 conjugate; Molecular Probes) (1:500) was used as the first secondary antibody. Slides were then incubated with a polyclonal rabbit serum raised against heat-killed *N. gonorrhoeae* strain FA1090 (second primary antibody; 1:3,000) and subsequently with goat anti-rabbit IgG conjugated to Texas Red (Sigma) (final secondary antibody; 1:800). All antisera were diluted in Ig-free bovine serum albumin, and all incubations were done for 1 h at room temperature. Slides were rinsed three times with PBS containing 0.05% Tween 20 after each incubation. Evans blue (Sigma) was used to counterstain vaginal epithelial cells by diluting a 0.5% (wt/vol) stock 1:30,000 in the final secondary antibody solution. Slides were examined with an Olympus BX60 system microscope with a BX-FLA reflected light fluorescence attachment and Olympus U-M41001, U-M41002, and U-N51006 filters. All images were obtained with a SPOT charge-coupled-device digital camera (Diagnostic Instruments, Inc.).

RESULTS

Kinetics of Opa protein expression in vivo. *N. gonorrhoeae* strain FA1090 possesses 11 different *opa* genes, which collectively encode eight antigenically distinct Opa proteins (OpaA, -B, -C, -D, -E, -K, -F, and -I) (14, 16). The expression of Opa proteins by *N. gonorrhoeae* strain FA1090 during experimental urethritis in male volunteers was previously characterized (29). More recent data from our laboratory show that expression of Opa proteins by strain FA1090 was selected for and/or induced during experimental murine infection. In these experiments, >50% of isolates recovered from four of eight BALB/c mice and three of four SLC:ddY mice expressed one or more Opa proteins within 4 days after inoculation with mostly Opa^- variants (28). Similar to what was seen with results from male volunteer studies, different Opa variants predominated in different mice. Here we further characterized this initial description of Opa protein expression in the mouse model and examined the kinetics of Opa protein expression for a longer period of time.

We first performed experiments in which we inoculated mice with defined mixtures of bacteria that consisted primarily of Opa^- (62 to 72%) and OpaI (28 to 38%) variants. Vaginal mucus samples were cultured each day, and the percentages of different Opa phenotypes isolated at each time point were determined using HV_2-specific antisera. OpaI variants were initially tested because OpaI variants are easily identified due to their very dark colony opacity phenotype and because OpaI-expressing gonococci were frequently represented among murine vaginal isolates in a previous report (28). Based on the combined results of two experiments, three distinct phases of infection were observed. An apparent selection for OpaI variants was detected between days 1 and 3 postinoculation in 9 of 10 (90%) infected mice. We refer to this phase as the early phase of infection and consider it similar to that reported previously (28). A middle phase of infection, characterized by a decrease in the percentage of Opa^+ isolates recovered, was observed between days 3 and 7 postinoculation in six of nine mice (67%) that displayed the early phase. A decrease in the total number of CFU recovered paralleled the reduction in the recovery of Opa^+ variants in the middle phase. Interestingly, the middle phase was followed by a reemergence of OpaI variants in two of three mice in which infection progressed for longer than 8 days. This stage, which we designate the late phase of infection, coincided with an increase in overall recovery of *N. gonorrhoeae*. Consistent with previous studies with female mice and with male volunteers (28, 29), variants that expressed multiple Opa proteins were frequently isolated from mice, with the highest percentages recovered in the late phase of infection. No significant change in Opa phenotype was detected when an Opa^- variant was serially cultured without single colony passage in vitro or when mixed suspensions were cultured in broth. It should also be noted that expression of multiple Opa proteins is rare during in vitro passage and that this phenotype is rapidly lost upon subculturing.

The fluctuation of Opa^+ and Opa^- populations among vaginal isolates over the course of murine infection is suggestive of a cyclical pattern of expression or selection. This cyclical pattern is illustrated in Fig. 1, which shows the kinetics of recovery of Opa^+ variants and the total CFU (Fig. 1A) and the distribution of Opa phenotypes among vaginal isolates over time (Fig. 1B) from a mouse that displayed all three phases of infection. In this experiment, the inoculum consisted of 30% Opa^+ variants. Eighty-three percent and 100% of isolates from this mouse expressed one or more Opa proteins on days 1 and 3, respectively (early phase), and most of the variants recovered in this phase were OpaI. A decrease in the percentage of Opa^+ isolates to the level present in the inoculum followed (35% on day 7); this decrease corresponded to a decline in the total number of CFU isolated (days 6 to 8) (middle phase). The percentage of OpaI variants decreased during this phase, and similar percentages of OpaF and OpaB variants were detected at this time point. An increase in the total number of CFU, which was accompanied by an increase in the percentage of Opa^+ variants (73% on day 9) (late phase), followed. Interestingly, the late phase showed a marked increase in the percentage of multiple expressers, with over 60% of isolates expressing OpaI in conjunction with OpaF or in conjunction with both OpaF and OpaB. A second decrease in the percentage of Opa^+ isolates was detected 3 days later (day 12 postinoculation); this change in Opa phenotype is reminiscent of the middle phase characterized above and is tentatively referred to as the second middle phase. The percentages of OpaI and OpaF,I variants dropped dramatically in this second middle phase, and OpaB was the predominant Opa protein expressed by the low percentage of Opa^+ variants recovered on days 12 and 13. The mouse remained infected for 4 more days; we did not continue to analyze the Opa phenotype of vaginal isolates after day 13. We therefore do not know which Opa phenotype predominated the day before infection cleared.

Preexisting populations of Opa^+ variants are selected for in vivo. To begin to dissect the factors that might be responsible for the observed pattern of Opa phenotypes described above, we focused on the early phase of infection. We hypothesized that selection of a preexisting population of OpaI variants in the inoculum was responsible for the reisolation of mainly OpaI-expressing gonococci after inoculation. An alternative hypothesis is that the increased recovery of OpaI variants was due to an increase in Opa protein expression via induction of increased *opa* gene phase variation in vivo. To distinguish between these two possibilities, we constructed a Cm^r strain of *N. gonorrhoeae* FA1090 and isolated OpaI, OpaB, and Opa^- variants of this strain to provide a means of following specific populations of gonococci over time as described in Materials and Methods.

Strain FA1090Cm^R did not exhibit a growth rate different

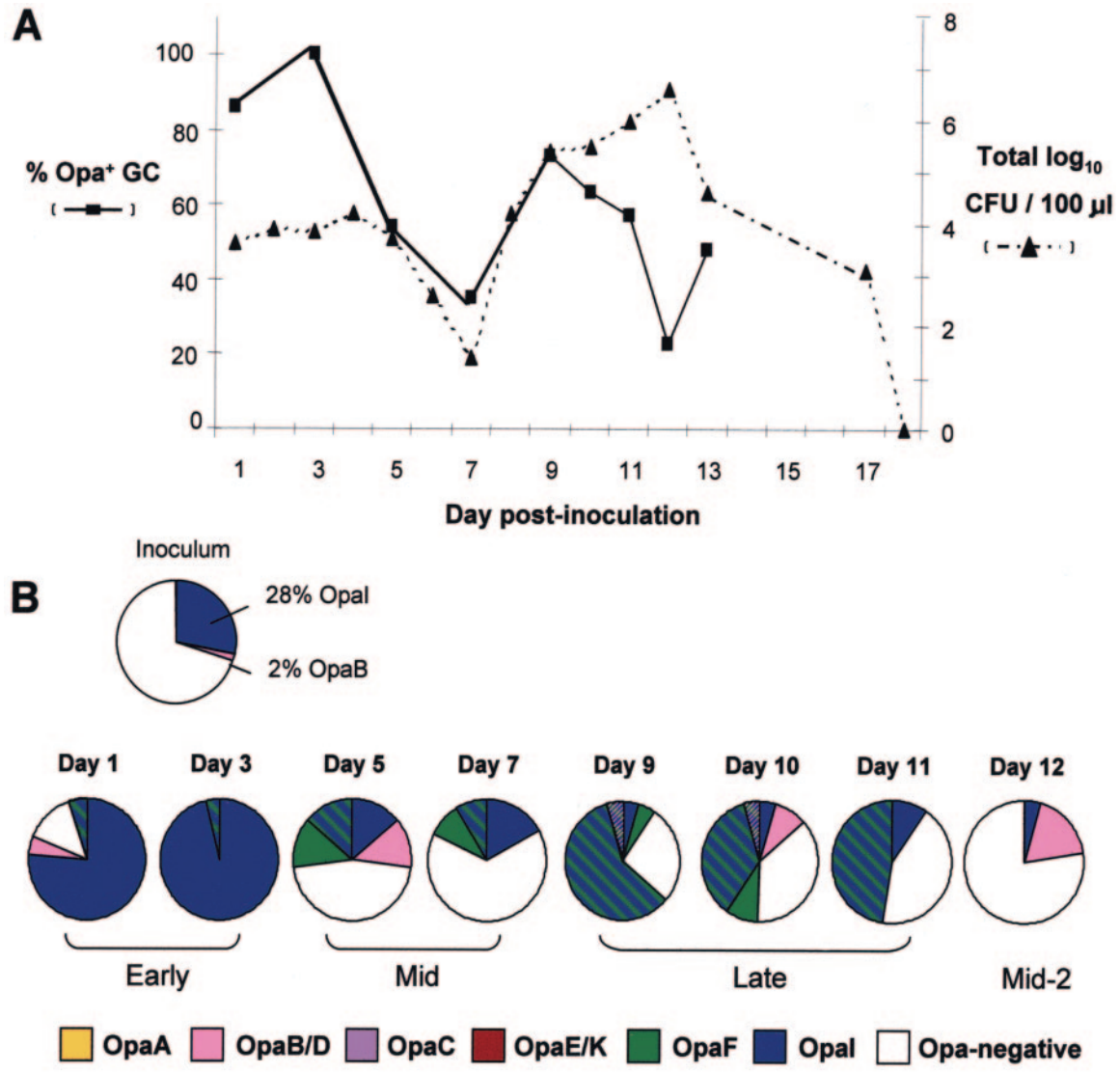

FIG. 1. Kinetics of infection and recovery of Opa phenotypes. The results from a mouse that was infected for 17 days are shown to illustrate the pattern of Opa phenotypes expressed by vaginal isolates following inoculation with predominantly OpaI and Opa$^-$ wild-type gonococci. In this experiment, the inoculum was 28% OpaI, 2% OpaB/D, and 70% Opa$^-$, with other variants present at levels of <1%. (A) The percentage of Opa$^+$ gonococci (solid line) and the total number of CFU (dotted line) recovered each day following inoculation. (B) The percentage of each Opa variant recovered from this mouse at selected time points. Gonococci that expressed more than one Opa protein simultaneously are represented by stripes. The different phases of infection as defined in the text are indicated below the pie graphs. This mouse remained culture positive for 4 more days after the second middle phase (Mid-2) was detected.

from that of the wild-type strain when cultured independently from or together with the wild-type strain in supplemented GC broth (data not shown). We next examined if strain FA1090Cm^R had an advantage in vivo over the wild-type parent strain (Cm^s) that was independent of Opa phenotype. Mice were inoculated with suspensions containing Opa$^-$ wild-type (Cm^s) gonococci spiked with a lower number of Opa$^-$ FA1090Cm^R gonococci, and the recovery of Cm^r gonococci was followed over 3 days. These control experiments were designed to mimic the experimental design of the selection experiments, in which a predominantly Opa$^-$ Cm^s population would be spiked with a lower percentage of Opa$^+$ Cm^r gonococci. In four different control experiments, there was no significant difference between the mean frequency of Cm^r gonococci recovered from mice inoculated with Opa$^-$ Cm^s and Opa$^-$ Cm^r gonococci and the frequency of Cm^r gonococci in the inoculum (Fig. 2A). From these results, we concluded that Opa$^-$ variants of strain FA1090Cm^R had no inherent growth or survival advantage and that this Cm^r strain therefore would be a suitable tool for following a specific population of gonococci during the early phases of murine infection.

We next predicted that if selection of Opa$^+$ variants was responsible for the observed early phase of infection, inoculation of mice with a defined ratio of Opa$^+$ Cm^r gonococci and Opa$^-$ Cm^s gonococci would result in the reisolation of the Opa$^+$ Cm^r population. Groups of mice were first inoculated

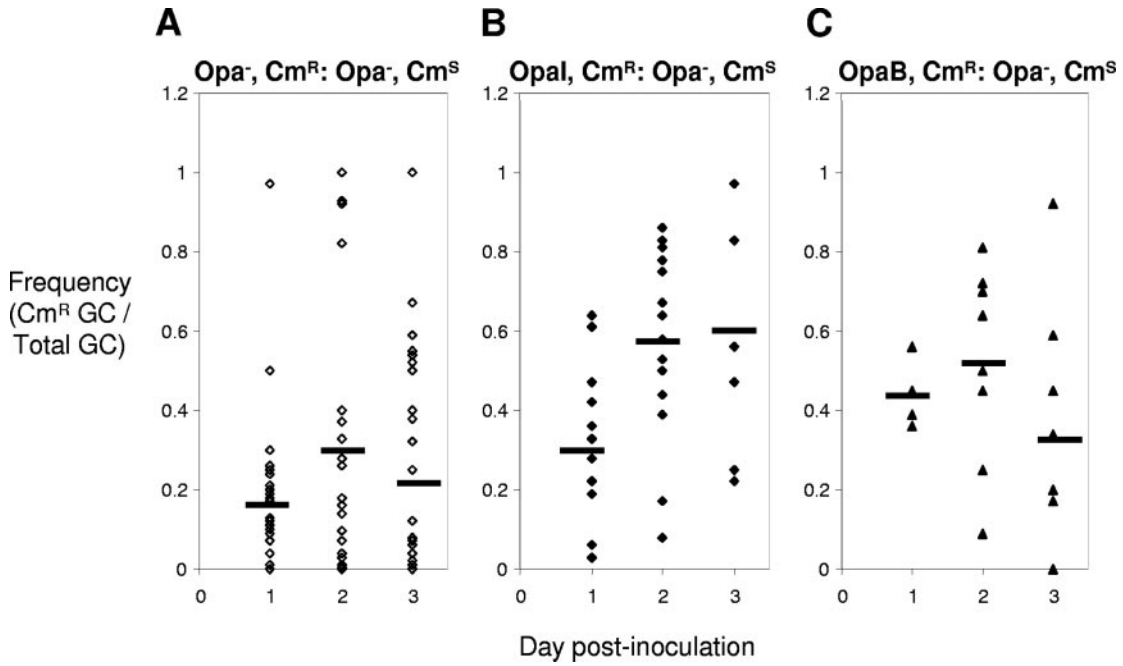

FIG. 2. Strain FA1090Cm^R has an Opa-dependent advantage in vivo over the wild-type strain. Data points correspond to the frequency of Cm^r colonies isolated from individual mice following inoculation with the following mixtures: (A) Cm^s and Cm^r Opa$^-$ variants (control group) (data are combined from four experiments [n = 5 to 9 mice per experiment; total n = 30]); (B) OpaI Cm^r and Opa$^-$ Cm^s variants (data are combined from three experiments [n = 5 or 6 mice/experiment; total n = 16]); (C) OpaB Cm^r and Opa$^-$ Cm^s variants (n = 8). The frequency of FA1090Cm^R gonococci among the total number of CFU in the inocula ranged from 0.07 to 0.19 for all experiments. The frequency of Cm^r vaginal isolates from mice inoculated with Cm^r Opa$^+$ variants (OpaI or OpaB) compared to that recovered from mice inoculated with Opa$^-$ Cm^s and Opa$^-$ Cm^r gonococci is shown (P values of <0.001 and <0.008, respectively; repeated measures analysis of variance). A mixed-model approach (SAS PROC MIXED, version 8.0) was used to accommodate animals for which data were collected on fewer than three days. The main effect of group was evaluated using Tukey's adjustment for multiple comparisons.

with suspensions of primarily Opa$^-$ Cm^s gonococci spiked with lower numbers of OpaI Cm^r gonococci. In each of three experiments, the frequency of Cm^r gonococci recovered from vaginal mucus of infected mice within 2 days postinoculation was higher than that recovered from control mice inoculated with mostly Opa$^-$ variants of both the Cm^r and Cm^s strains (Fig. 2A and B) ($P < 0.001$). The vast majority of Cm^r gonococci recovered expressed OpaI at each time point. Using our criteria for defining selection (the isolation of Cm^r OpaI variants at a percentage $\geq$3 times that of Cm^r OpaI variants in the inoculum), selection for the Cm^r OpaI population occurred in 12 of 16 (75%) mice (Table 1). The results of a representative experiment are shown in Fig. 3. It should be noted that in this experiment, infection was monitored for longer than 3 days and evidence of a middle phase of infection (decrease in the recovery of Opa$^+$ variants) was seen by day 5 postinfection in three mice that showed early selection for OpaI Cm^r gonococci (data not shown).

As discussed above, the receptor repertoire of the Opa proteins of the pathogenic *Neisseria* strains consists of members of the human CEACAM family as well as HSPG molecules. The Opa protein receptor specificity has been defined fully for gonococcal strains MS11 (5, 11, 20, 54, 56) and FA1090 (N. B. Guyer, M. C. Piriou, M. M. Hobbs, and J. G. Cannon, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. D-135, 2001). The majority of Opa proteins expressed by these strains bind to CEACAM1, -3, -5, and/or -6; only one Opa protein in each strain also binds HSPG. In the case of strain FA1090, this protein is OpaI. Therefore, to examine whether selection of Opa$^+$ gonococci occurs when mice are inoculated with an Opa variant that binds CEACAM receptors only (i.e., OpaB), eight mice were inoculated with a suspension of predominantly Opa$^-$ Cm^s gonococci spiked with lower numbers of OpaB Cm^r bacteria. As seen with the OpaI Cm^r variant, a significant increase in the frequency of Cm^r reisolates over that from

TABLE 1. Selection experiments using mixtures of OpaI and Opa$^-$ variants of FA1090Cm^R and wild-type FA1090 (Cm^s)

Expt with indicated inoculum[a]	No. of mice with selection of preexisting OpaI population/total (%)
OpaI Cm^r:Opa$^-$ Cm^s	
1	5/6 (83)
2	3/5 (60)
3	4/5 (80)
OpaI Cm^s:Opa$^-$ Cm^r	
4	0/7 (0)
5	1/8 (13)
6	3/5 (60)

[a] OpaI Cm^r:Opa$^-$ Cm^s inocula contained 8 to 18% OpaI Cm^r and 72 to 79% Opa$^-$ Cm^s variants. OpaI Cm^s:Opa$^-$ Cm^r inocula contained 5 to 24% OpaI Cm^s and 17 to 74% Opa$^-$ Cm^r variants.

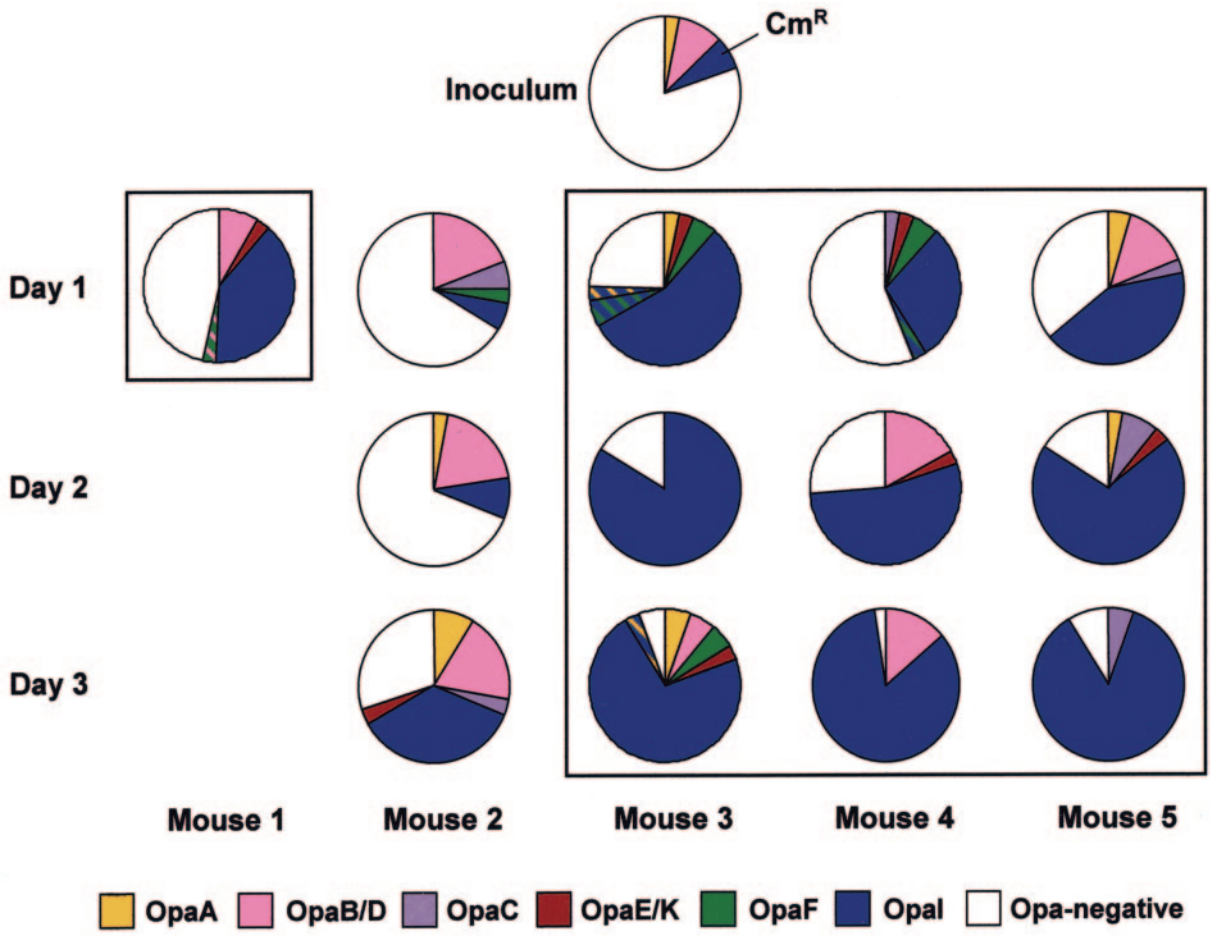

FIG. 3. Opa phenotype of isolates from mice inoculated with OpaI Cm^r and Opa^- Cm^s gonococci. In this experiment, OpaI variants were selected in four of five mice, of which the vast majority (90 to 100%) were Cm^r. Data for time points at which selection of OpaI Cm^r gonococci was observed are enclosed in black boxes. Gonococci were recovered from mouse 1 only on day 1. The criterion for selection is defined in the text.

control mice inoculated with Opa^- Cm^r and Opa^- Cm^s gonococci occurred early in infection ($P < 0.008$) (Fig. 2A and C). The majority of the Cm^r colonies expressed OpaB, and as determined by our threefold criteria for selection, selection of the OpaB Cm^r population occurred in all eight infected mice (Table 2).

Reciprocal experiments in which mice were inoculated with bacterial suspensions that contained mostly Opa^- Cm^r gonococci and lower numbers of OpaI Cm^s or OpaB Cm^s gonococci were performed. The recovery of Opa^+ gonococci was not as dramatic in these experiments; selection for OpaI variants occurred in two of three experiments (Table 1), and results from only 13% and 60% of mice in the second and third of these three experiments, respectively, met our criteria for selection. However, for all mice in which OpaI isolates predominated, those OpaI-expressing gonococci were Cm^s. None of the other mice had high percentages of Opa^+ variants of any type among their vaginal isolates. In reciprocal experiments designed to test the selection of OpaB variants, selection of OpaB Cm^s gonococci occurred in only two of seven infected mice (Table 2). Interestingly, a predominance of OpaE/K variants was isolated from the other five infected mice, the majority of which were Cm^r (Fig. 4). The percentage of OpaE/K Cm^r gonococci in the inoculum (7%) was similar to that of the OpaB Cm^s population (11%). Given that the OpaI reciprocal experiments were not as convincing, it is possible that $FA1090Cm^R$ may have a slight advantage in vivo that was not detected in the in vivo control studies shown in Fig. 2A. Alternatively, another phase-variable factor present in one population but not the other may have influenced the recovery of gonococci in the different experiments. Overall, however, results of the recipro-

TABLE 2. Selection experiments using mixtures of OpaB and Opa^- variants of $FA1090Cm^R$ and wild-type FA1090 (Cm^s)

Inoculum[a]	No. of mice with selection of preexisting OpaB population/total (%)
OpaB Cm^r:Opa^- Cm^s	8/8 (100)
OpaB Cm^s:Opa^- Cm^r	2/7 (29)[b]

[a] OpaB Cm^r:Opa^- Cm^s inoculum contained 11% OpaB Cm^r and 81% Opa^- Cm^s variants. OpaB Cm^s:Opa^- Cm^r inoculum contained 12% OpaB Cm^s and 78% Opa^- Cm^r variants.

[b] Selection for OpaE/K Cm^r variants occurred in the five mice that did not select for OpaB Cm^s gonococci.

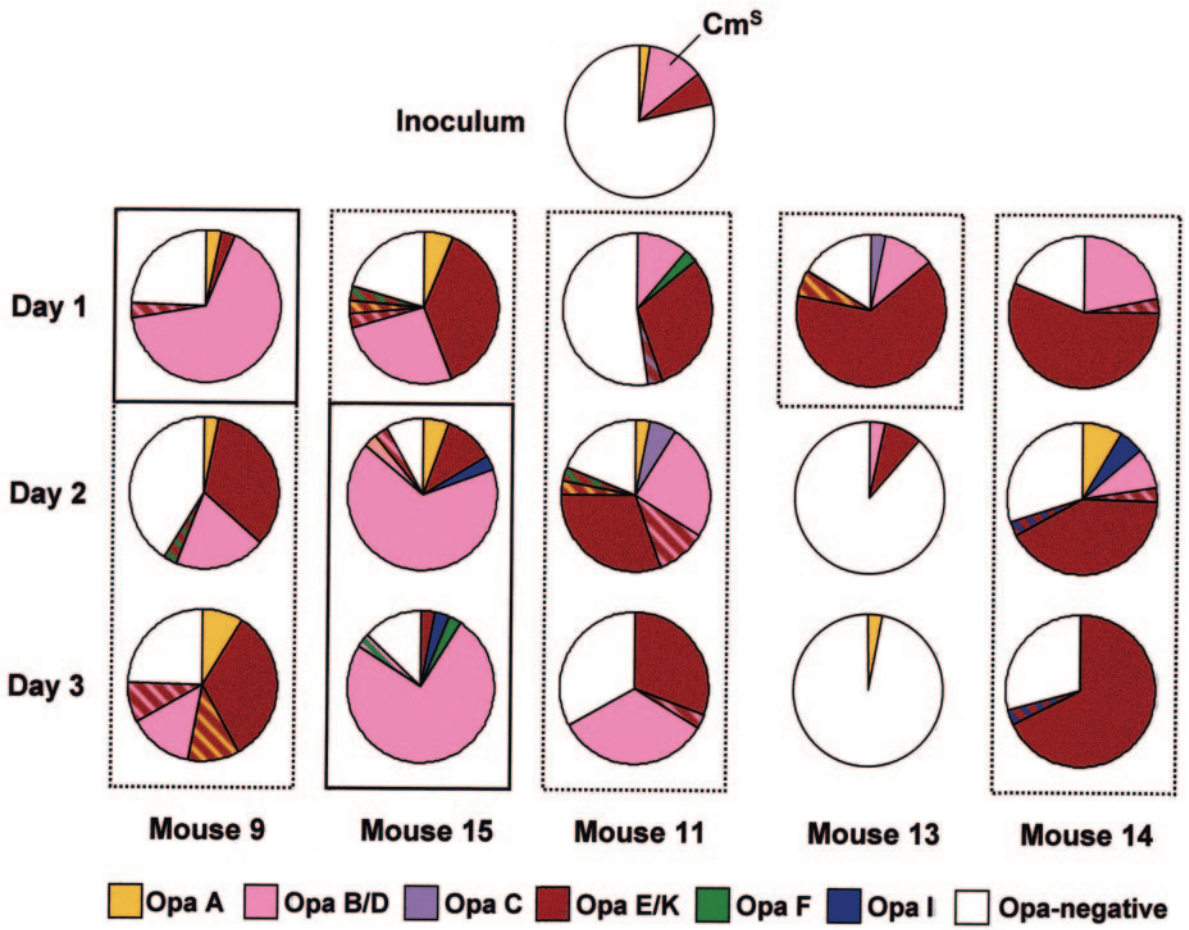

FIG. 4. Opa phenotype of isolates from mice inoculated with OpaB Cm^s and Opa^- Cm^r gonococci. Boxes highlight those time points at which selection of OpaB Cm^s gonococci (solid lines) or OpaE/K Cm^r gonococci (dashed lines) occurred. Two of seven mice in this experiment (mice 9 and 15) showed selection of OpaB Cm^s gonococci. Selection of OpaE/K Cm^r gonococci occurred in the remaining five mice; representative pie graphs for three of these five mice (mice 11, 13, and 14) are shown.

cal experiments are consistent with selection of a preexisting population of Opa^+ variants when selection for Opa protein expression occurred.

Both OpaI and OpaB variants are cell associated in vaginal smear samples. Gonococci that express Opa proteins, particularly highly opaque colony variants such as the OpaI variants of strain FA1090, are prone to aggregation. This phenomenon can skew quantitative culture results. Therefore, the Opa phenotype of gonococci in vaginal smear samples from infected mice was analyzed by a staining assay. The results were consistent with the immunoblot results in that large numbers of gonococci bound to OpaI or OpaB/D antibodies were observed in mice from which a majority of OpaI or OpaB/D variants, respectively, were recovered. Furthermore, both OpaI and OpaB variants were associated with epithelial cells during infection (Fig. 5). We often saw cell-attached gonococci that did not react with the OpaI or OpaB/D antiserum (data not shown). It is not known if these gonococci were Opa^- or if they represented small numbers of gonococci that expressed other Opa proteins.

DISCUSSION

Cumulative evidence from clinical isolates and from studies with male volunteers suggests that selection or induction of Opa protein expression occurs during urethral infection in males and that selection for Opa^+ or Opa^- phenotypes in the female genital tract is subject to hormonally regulated factors (26, 27). A high percentage of Opa^+ variants is also isolated early during experimental murine infection (28). Here we examined Opa expression in the murine model more extensively and demonstrated that selection of a preexisting population of gonococci is responsible for the early isolation of Opa^+ variants. We also observed cyclical changes in Opa phenotype after this early selection phase. The early advantage conferred by Opa proteins in the human and murine infection models is not known. In light of the role played by Opa proteins in adherence to and invasion of cultured human cells, a simple explanation may be that Opa^+ variants have a colonization advantage. This hypothesis is confounded, however, by the report that human CEACAM1, -5, -6, and -8 were not detected on primary male urethral cells, and CEACAM3 was detected on only a

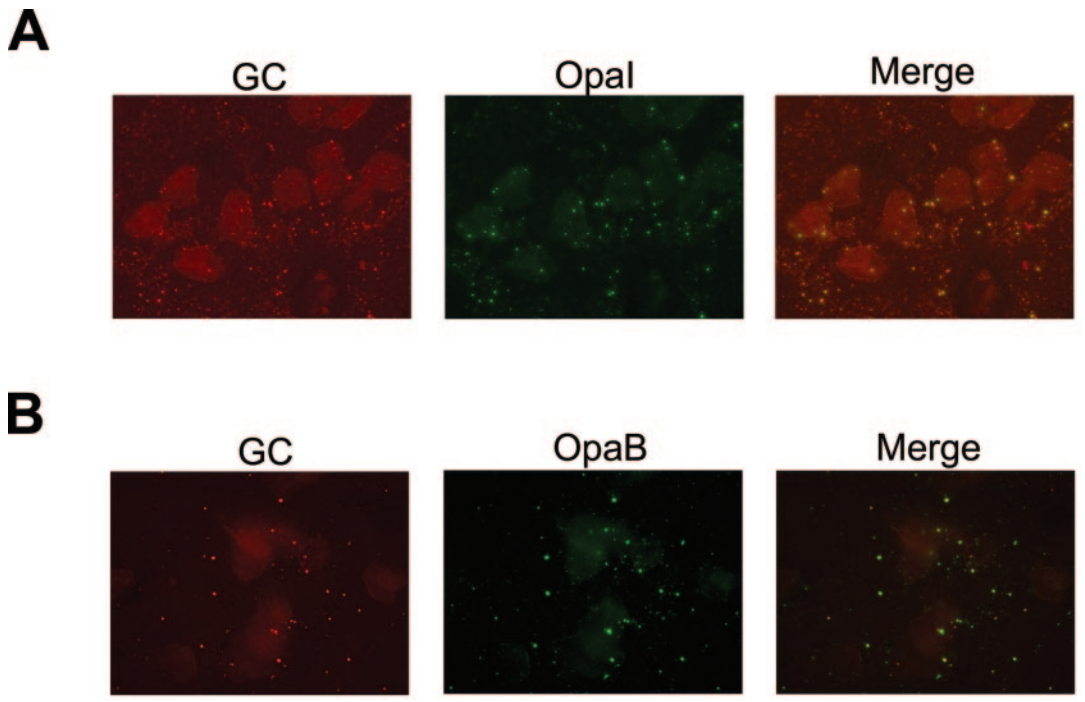

FIG. 5. Association of OpaB and OpaI variants with murine vaginal cells. Vaginal smear samples from mice infected with a mixture of OpaI Cm^r and Opa^- Cm^s gonococci (top panels) or a mixture of OpaB Cm^r and Opa^- Cm^s gonococci (bottom panels) stained sequentially with Opa-specific antibodies against OpaI (A) or OpaB/D (B) (green) and a polyclonal rabbit antiserum against whole gonococci (GC) (red) (29). Results from one mouse in each group are shown. All images were obtained at an original magnification of 40×.

small population of these cells (23). Additionally, the human CEACAM family is not expressed in mice. Mice express only one homolog of the human CEACAMs, CEACAM1 (22); this homolog is not highly related to human CEACAMs at the predicted amino acid level. In particular, murine CEACAM1 has only one of the two critical amino acids required for Opa protein binding (tyrosine-34) as delineated by Virji et al. (55). A threonine residue occurs in the place of the other critical amino acid, isoleucine-91, and none of four amino acids that enhanced Opa protein binding to human CEACAMs (glutamine-44 and -89, serine-32, and valine-39) are present (22). It therefore seems unlikely that the neisserial Opa proteins can utilize murine CEACAM1 as a cellular receptor. While HSPG-binding Opa proteins may promote colonization of mice, two non-HSPG-binding Opa variants, OpaB and OpaE/K, were also selected in our studies here. We conclude that unless murine CEACAM1 binds to Opa proteins at levels sufficient to confer selection or unidentified Opa adherence receptors exist in mice, Opa-mediated colonization or invasion is not responsible for selection of Opa^+ gonococci early during murine infection.

When discussing Opa-mediated adherence in human infection, it is important to consider that there is not yet a consensus on the expression of CEACAMs by epithelial cells of the human female genital tract. CEACAM5 was not detected on primary cervical tissue from any subject in one study (45), and although some human cell lines of female genital origin, such as ME180 (cervical carcinoma) and NC9 (cervical) cells, express CEACAM molecules on the cell surface, CEACAM molecules are not always constitutively expressed by Hec-1-B (endometrial) or HeLa (endocervical) cells (53). The failure to detect these molecules on cultured cells may be explained by the up-regulation of CEACAMs during infection (46). Currently, there exists no in vivo infection model elucidating the role of CEACAM receptor binding in selection for Opa expression in women. The gathering of information on Opa expression during endocervical infection is hindered by ethical restrictions against using female volunteers. Differences between the cellular receptors and the microenvironments of the male urethra and the endocervix caution against the extrapolation of data from male volunteers to models of endocervical infection. While many physiological aspects of the lower genital tracts of female mice and women are similar, the murine model does not mimic human infection with regard to all the cellular receptors utilized by *N. gonorrhoeae*. Genital tissue from female BALB/c mice does not express the CR3 receptor, which has been shown to be exploited by the gonococcus to mediate invasion of primary human endocervical cells via a triplex of porin, pilus, and iC3b (18). Mice also do not express human CD46, which is implicated in pilus-mediated functions (34, 38). The recent development of transgenic mice that express human CEACAMs (8) may provide a useful tool to further explore Opa protein and host interactions in vivo.

An alternative hypothesis to explain the apparent advantage conferred by Opa protein expression early in infection is that Opa proteins may confer resistance to innate host defenses. Bos et al. (6) reported that Opa^- gonococci of strain MS11 were more sensitive to the bactericidal action of normal human serum (NHS) than were their Opa^+ counterparts. Some Opa^+ variants can also bind heparin, which was shown to protect

Opa$^+$ gonococci from killing by NHS (9, 12). Whether or not these in vitro results are predictive of what occurs in vivo is not yet known; serum resistance mediated by certain lipooligosaccharide phenotypes masked Opa-mediated serum resistance (6), and the relative hierarchy of the many serum resistance mechanisms of *N. gonorrhoeae* during genital tract infection is not defined. It is highly conceivable, however, that complement may be a selective factor. Complement components exude onto mucosal surfaces during inflammation, and complement proteins were present on the surface of gonococci isolated by endocervical lavage (43). Complement is also detected in mouse uterine luminal fluid during the high-estrogen phase of the estrous cycle (31), and administration of exogenous estradiol stimulated the synthesis of C3 in the endometrium of both ovariectomized and normally cycling mice (39). From this we surmise that complement may challenge *N. gonorrhoeae* during murine infection. Unfortunately, strain FA1090 is inherently resistant to high concentrations of NHS, which complicates the study of the relative serum resistance levels of different FA1090 Opa variants in vitro. Nonetheless, studies are under way to examine the possible contribution of complement in the selection of Opa$^+$ gonococci during murine infection.

An intriguing finding from our study is that the initial selection for Opa protein expression in mice is followed by fluctuations in the recovery of Opa$^+$ and Opa$^-$ variants over time. Consistent with our observation, Kita et al. reported changes in Opa phenotype with respect to the estrous cycle in experimentally infected SLC:ddY mice (35). These observations are consistent with the reported recovery of mostly opaque isolates and of transparent isolates from women in the proliferative and luteal phases of the menstrual cycle, respectively (26, 27). In our study, the observed changes in Opa phenotype corresponded with fluctuations in the total number of gonococci recovered, with the recovery of predominantly Opa$^+$ variants associated with a higher colonization load. We therefore hypothesize that *opa* gene phase variation may help the gonococcus evade hostile hormonally driven factors in the female reproductive tract. This hypothesis was proposed by others over 20 years ago (26, 27) and is supported by clinical studies that link rates of positive culture in infected women with the proliferative (high-estrogen) phase of the menstrual cycle. Most notable was a study in 1947 by Koch (36), in which positive cultures were obtained from four hospitalized patients during the proliferative phase of their menstrual cycles; endocervical cultures from these same patients collected during the luteal (high-progesterone) phase were consistently negative, and in one untreated patient, positive cultures were obtained upon retransition into the proliferative phase. Similar findings have been reported by others (26, 32, 42).

Several hormonally driven factors may challenge the survival of *N. gonorrhoeae* in the female genital tract. Complement is produced at higher levels in the high-estrogen phase and therefore may select for Opa$^+$ gonococci in the proliferative phase of the cycle (6). Proteases present in menstrual blood might drive the selection for different Opa phenotypes, based on the increased trypsin resistance of gonococci within transparent colonies (26). Mice do not menstruate, so factors specific to menstrual blood should not contribute to Opa selection in mice. However, hematogenous factors may be present in the serum exudate that accompanies inflammation during experimental murine infection. Others reported that opaque gonococci were more resistant to progesterone in vitro (48); the progesterone concentrations used in this study were nonphysiological, however, and we have been unable to reproduce this result using defined Opa variants of strain FA1090 (Simms and Jerse, unpublished). Other explanations for the cyclical variation in Opa expression we detect in mice include evasion of a developing adaptive response; in this case, one might predict similar fluctuations in Opa phenotype would be seen in urethral isolates from men in which infection progressed past the onset of symptoms. Additionally, the gonococcus itself may be programmed to regulate *opa* gene expression in response to environmental cues. Increased *opa* gene phase variation after an initial selection phase might also occur; this could generate a predominance of Opa$^-$ variants if selective pressure later declines or is cyclical.

In summary, many host factors may select for or against Opa protein expression; whether such factors act independently or in concert is not known. Our laboratory is continuing to examine the role of selected host factors on gonococcal Opa protein expression under in vitro and in vivo conditions. The availability of mice that are genetically deficient in one or more host factors should enable us to further address these questions.

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