Multiple Gonococcal Opacity Proteins Are Expressed during Experimental Urethral Infection in the Male

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Summary

The opacity (Opa) proteins of *Neisseria gonorrhoeae* are a family of outer membrane proteins demonstrating phase and antigenic variation. *N. gonorrhoeae* strain FA0190 has 11 *opa* loci that encode at least 8 antigenically distinct Opa proteins. To determine if expression of one Opa protein or a subset of them is favored during gonococcal infection, we inoculated Opa-negative variants of strain FA1090 intraurethrally into male volunteers. The Opa phenotype of gonococci isolated from urine and urethral swab cultures from nine infected subjects was determined. Opa proteins were expressed in a large proportion of the reisolates from the infected subjects. Gonococci cultured from urine or urethral swab samples from six of the subjects were uniformly Opa positive, with the predominant Opa variants differing among subjects. Three different Opa proteins were represented as the predominant type in at least one subject each. In three subjects, there was more heterogeneity in Opa phenotype of the reisolates, including the presence of Opa-negative variants. An increase in the proportion of isolates expressing multiple Opa proteins occurred over time in most subjects. Passage of the inoculum in vitro did not result in similar changes in Opa expression. There was no detectable difference in infectivity of an Opa-negative variant and one expressing an Opa protein (OpaF) that was highly represented in reisolates from the original nine subjects. Reisolates from three infected volunteers inoculated with the OpaF variant showed continued expression of OpaF alone or in conjunction with other Opa proteins. These results demonstrate that there is strong selection for expression of one or more Opa proteins by strain FA1090 in vivo, but that no single protein is preferentially expressed during early infection in the male urethra.

Neisseria gonorrhoeae is the causative agent of the sexually transmitted disease gonorrhea. Humans are the exclusive host of *N. gonorrhoeae*; although some animal models have been described, their usefulness in studying pathogenesis has been limited by their lack of similarity to human mucosal disease (1). *N. gonorrhoeae* infection of men, unlike that of women, rarely leads to complications (2), and experimental infection of adult men has been used to study potential virulence factors and to evaluate candidate vaccines (3–8).

The gonococcus has a number of surface components demonstrating phase and antigenic variation. Reversible, high-frequency switches in expression result in turning on or off the synthesis of a component (phase variation) or in changing from one expressed antigenic version of a component to another (antigenic variation). These variable components include the opacity (Opa)[1] proteins of the outer membrane (previously designated Protein II). Opa proteins are heat modifiable and range from 25 to 30 kD in molecular mass. Gonococci can express a number of antigenically different Opa proteins; a single bacterium can express none of the proteins, one protein, or multiple Opa proteins. Expression of different Opa proteins affects the photo-opacity of gonococcal colonies, with Opa variants of a strain ranging from transparent to deeply opaque in colony phenotype (reviewed in reference 9).

Individual Opa proteins are encoded by separate structural genes. Each gene oscillates between expression and nonexpression at an in vitro rate of $\sim 10^3$/cell per generation by a frameshifting mechanism involving insertions or deletions of copies of a repetitive DNA sequence (10–13). Because of this high rate of variation, gonococcal populations cultured in the laboratory consist of mixtures of different Opa variants. The *opa* genes contain both conserved and variable regions, including two hypervariable (HV) regions encoding structurally and antigenically variable portions of the pro-

[1] *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; FSM, freezer storage medium; HV, hypervariable; LOS, lipooligosaccharide; Opa, opacity.

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teins that are surface exposed (9, 10, 14–16). The HV sequence repertoires are not the same in all gonococcal strains, although some HV regions are present in multiple strains (14, 15, 17).

Opa proteins are expressed in vivo, and variation in Opa expression occurs during both natural and experimental gonococcal infection (6, 18, 19). Altering the expression and antigenic type of these immunodominant surface components may aid the organisms in evading the host immune response. In addition, different Opa proteins may have different functions. This latter view has emerged from recent studies on the ability of Opa proteins to mediate adherence to and invasion of different host cell types in vitro, including epithelial cells and neutrophils (20–24). These studies have led to the suggestion that the expression of different Opa proteins may influence tissue tropism in vivo, so that particular Opa proteins may be important in the establishment or maintenance of gonococcal infection (20, 23).

Experimental urethral infection of male volunteers with well-characterized gonococcal strains provides an opportunity to examine the role in infection of variable surface components such as Opa proteins. If expression of one Opa protein or a subset of them is required during early infection of the male urethra, then one would predict that the same Opa protein(s) would be expressed by organisms reisolated from different men infected with the same gonococcal strain. In a previous study by Swanson et al. (6), three men were experimentally infected with an Opa-negative variant of strain MS11; reisolated gonococci were Opa positive, with the same Opa proteins expressed by the predominant reisolates from all three subjects. However, since the Opa protein repertoires of different strains may differ, it is difficult to generalize from results with one strain. We previously characterized the Opa proteins and genes encoding them in gonococcal strain FA1090 (14, 25, 26). The *opa* genes of strains FA1090 and MS11 show little similarity in the HV regions (14, 15). In this study, we infected subjects with Opa-negative or Opa-positive variants of strain FA1090 and characterized the Opa phenotypes of reisolated gonococci to determine if expression of one or more of the Opa proteins of this strain is associated with the ability of the organisms to establish infection.

Materials and Methods

Bacterial Strain and Culture Conditions. *N. gonorrhoeae* strain FA1090, which was isolated from a patient with disseminated gonococcal infection, has been described, as have GC agar and broth culture media and conditions (8).

Determination of Opa Phenotype. Strain FA1090 has 11 *opa* loci encoding at least eight different Opa proteins (the *opaB* and *opaF* genes are duplicated) (14, 27). Expression of the Opa proteins is associated with differences in colony opacity when colonies are viewed with a dissecting microscope: Opa-negative, OpaB, and OpaE/K: transparent; OpaC and OpaF: slightly opaque; OpaA, OpaD, and OpaI: deeply opaque. Opa expression was determined by colony blotting or Western blotting with mAbs specific for Opa proteins of FA1090 (25, 26). mAb H.138 binds OpaA. mAb H.4 binds OpaB and OpaD, which can be distinguished by a difference in electrophoretic mobility on SDS-PAGE. mAb H.157 binds OpaC. In theory, this mAb should also bind the product of the *opaJ* locus (14), but we have never detected expression of OpaJ and believe the *opaJ* gene is nonfunctional (our unpublished data). mAb H.164 binds OpaE and OpaK, which can be distinguished only by a slight difference in electrophoretic mobility. We did not analyze all H.164-positive variants by Western blotting; they are therefore designated as OpaE/K, indicating that OpaE and/or OpaK is expressed. mAb H.156 binds to OpaF. OpaI is not recognized by any of the aforementioned mAbs; its expression was identified by the characteristic colony morphology of OpaI-expressing variants or by Western blotting with mAb 4B12, which recognizes most Opa proteins (27a).

Colony immunoblots were performed by a modification of a previously described technique (26). Suspensions of individual colonies were spotted onto multiple nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) and probed with each of the Opa-specific mAbs. Bound antibody was detected with ^{125}I-Protein A (26) or with goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO), followed by enhanced chemiluminescence substrate (ECL; Amersham International, Amersham, UK).

For Western blotting, bacteria harvested from agar plates and solubilized in loading buffer for 5 min at 100°C were run on 11% polyacrylamide gels as described, except that SDS was not present in the gel (28). Separated proteins were transferred electrophoretically to nitrocellulose or PVDF membranes (Gelman Sciences, Ann Arbor, MI) and probed with mAbs (29), followed by goat anti-mouse IgG horseradish peroxidase conjugate and ECL substrate, before autoradiography. mAb H.5 (specific for protein I of FA1090) was included in each blot as a control and to provide a reference point for the migration of the different Opa proteins.

To analyze Opa phenotype of colonies cultured from infected subjects, individual colonies from the primary isolation plates were suspended in microtiter plate wells containing freezer storage medium (FSM) (8). Aliquots of the suspensions were dotted onto nitrocellulose membranes, and binding of the Opa-specific mAbs was determined by immunoblotting. The remaining suspensions were frozen at −70°C. If a colony suspension bound more than one mAb, the frozen stock was cultured and several colonies were tested by immunoblot and Western blotting to distinguish variants expressing more than one Opa protein from those that were mixtures of multiple single Opa-expressing variants.

Determination of Pilin Phenotype. Pilation of colony variants was assessed by the characteristic colony morphology and competence for genetic transformation (30). For each variant used to inoculate volunteers, the DNA sequence of the pilin expression locus (*pilE*) was determined as described (31).

Determination of Lipooligosaccharide (LOS) Phenotype. LOS phenotype was determined by SDS-PAGE of proteinase K–treated cell lysates and Western blotting with LOS-specific mAbs 3F11, 1-1-M, and 6B4 as previously described (7, 32, 33).

Preparation of the Bacterial Inoculum. Piliated colony variants with defined expression of LOS and Opa proteins were stored at −70°C. 2 d before each trial, the desired frozen stock was subcultured to GC agar. 24 h later, individual colonies with appropriate colony opacity were suspended in FSM, dotted onto nitrocellulose filters, and tested by immunoblot with Opa-specific mAbs. Aliquots of each suspension were also inoculated onto fresh GC agar plates. After 22–24 h of incubation, plates inoculated with colonies that were confirmed to be of the correct Opa phenotype by immunoblot were inspected visually, using a dissecting microscope to identify piliated colonies with the desired opacity for the inoculum suspension. Procedures for preparation of the suspension, filtering to

remove clumped gonococci, and quantitation of the number of organisms inoculated are described elsewhere (8). The Opa phenotype of at least 100 colonies cultured from the final inoculum suspension was determined by immunoblotting.

Inoculation of Volunteers and Specimen Processing. Procedures for recruiting male subjects, obtaining informed consent, and intraurethal inoculation of gonococci have been described (8). Subjects were treated with antibiotics when they developed signs of infection, or at the end of the 6-d trial. Protocols were reviewed by the Institutional Review Board and the General Clinical Research Center Advisory Board of the University of North Carolina at Chapel Hill. Subjects were compensated for their participation. Urine samples were collected 2 h after inoculation and at least daily thereafter; urethral swab samples were obtained when urethral discharge occurred. Specimens were processed and plated on culture media as described (8).

Results

Infection of Volunteers with Opa-negative Gonococci. Gonococcal strain FA1090 can express at least eight different Opa proteins (OpaA, B, C, D, E, F, I, and K), either singly or in different combinations (Fig. 1) (14, 25, 26). To determine if particular Opa proteins of strain FA1090 were preferentially expressed during infection, 15 male volunteers were inoculated intraurethrally with piliated, predominantly Opa-negative variants of this strain in five separate trials. The inoculum size varied from 10^5 to 10^6 CFU, with the percentage of Opa-negative variants ranging from 79 to 99%. The remainder of the inoculum consisted of Opa-positive variants. Each inoculum undoubtedly contained all of the different Opa-positive types, although an Opa type that was present at <0.8% of the population would not have been detected, based on the number of inoculum colonies analyzed. Nine subjects became infected, seven of whom developed signs of acute urethritis before treatment. Signs of infection developed from 33 to 106 h after inoculation. Two subjects (volunteers 3 and 9) had positive urine cultures but were treated before a urethral discharge appeared.

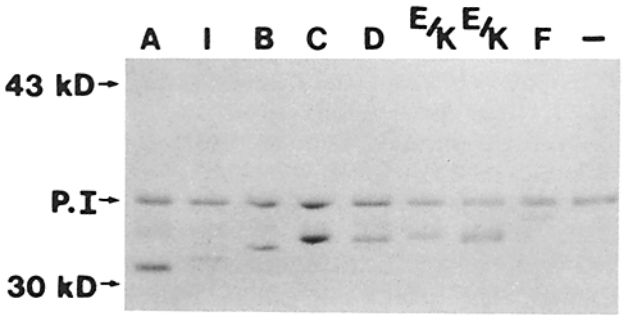

Figure 1. The Opa protein repertoire of strain FA1090. Outer membrane proteins from variants expressing the different Opa proteins of this strain were subjected to SDS-PAGE and Coomassie blue staining. The Opa protein expressed by each variant is indicated above the lane containing it. The two lanes marked E/K contain variants expressing the two different proteins, OpaE and OpaK, that bind mAb H.164; we have not determined formally which of the two is the product of the *opaE* or the *opaK* gene, and have therefore used the E/K designation for both. The arrows indicate the position of Protein I and of molecular mass standards.

In all subjects, only a small percentage (0.003–0.02%) of the number of organisms inoculated was recovered as viable CFU in urine samples obtained 2 h after inoculation. In subjects who subsequently developed signs of infection, the positive 2-h culture was followed by a period of variable length during which few or no gonococci were recovered from urine cultures. The number of gonococci cultured from urine samples then increased by at least 10-fold; we have used the term "first positive culture" to describe samples obtained at this time, which preceded the development of signs of infection.

For each infected subject, gonococci cultured from urine or urethral swab samples were characterized for Opa protein expression. This analysis was done directly on 24–30-h-old colonies growing on the primary isolation plates, without additional in vitro passage, using mAbs specific for the FA1090 Opa proteins in colony blotting and Western blotting procedures. Table 1 shows the Opa phenotypes of colonies cultured from the inoculum used in each trial, and of colonies isolated from urine cultures. For each subject, results are shown for the urine culture that was done 2 h after inoculation, for the first subsequent urine culture that was positive for isolation of gonococci, and for the last urine culture before treatment. Control experiments showed no difference in relative survival when variants expressing each of the different Opa proteins were incubated in GC broth or in urine from uninfected male volunteers, and no difference in plating efficiency on GC agar.

The distribution of Opa phenotypes in all of the 2-h urine culture samples was essentially the same as in the inoculum, with a predominance of Opa-negative colony variants. However, Opa proteins were expressed by the population of gonococci recovered from the first positive cultures and all subsequent cultures. For six subjects (volunteers 1, 3, 8, 9, 13, and 16), all of the colonies analyzed from the first positive culture expressed at least one Opa protein. For each of these subjects, the gonococci isolated from the first positive culture were quite homogenous in Opa protein expression, consisting primarily of one or two Opa types. Different Opa variants predominated in different subjects, even in subjects who received the same inoculum: OpaF in volunteers 1 and 8; OpaB in volunteers 3, 9, and 16; OpaI in volunteer 13.

In three subjects (2, 11, and 21), the first positive cultures yielded a mixture of Opa phenotypes, including Opa-negative variants (15–57%). Variants expressing OpaE/K, OpaC, OpaF, or OpaB were represented at substantial proportions in these mixed populations. Infection developed rapidly in these three subjects, with the first positive cultures obtained <24 h after inoculation. In contrast, infection took longer to develop (2–5 d until the first positive culture) in the six subjects who yielded a more homogeneous, uniformly Opa-positive population of gonococcal variants.

For five of the subjects who developed urethral discharge (volunteers 1, 2, 8, 13, and 16), we analyzed colonies from cultures of urethral discharge as well as urine. Gram stains of urethral discharge samples showed that both neutrophil-

Table 1. *Opa Phenotypes of Inocula and Urine Isolates from Subjects Inoculated with Predominantly Opa-Negative Variants*

Vol.	Day of first culture/day of treatment	Opa phenotype (% of colonies tested)			
		Inoculum	2-h culture	First positive culture	Last culture
1	2/4	Opa- (78)*	Opa- (85)	F (98)	F,I (60)
		E/K (8)	E/K (6)		F (25)
		F (6)	F (6)		F,C,I (10)
		A (2)			
		B (2)			
		C (2)			
		D (2)			
		B,E/K (1)			
		A,D (1)			
2	1/3	Same	Opa- (78)	E/K (31)	F (57)
			F (9)	F (27)	B,F (11)
			E/K (6)	D (15)	C (6)
				Opa- (15)	E/K,F (6)
				B (5)	E/K,A (6)
					F, I or C (6)
3	3/4	Same	ND	B (69)	B (48)‡
				D (28)	B,I (45)
8	3/5	Opa- (89)*	ND	F (48)	F,B,E/K (64)
		B (7)		B (24)	F,I (14)
		E/K (2)		F,B (21)	F,B (11)
		F (1)		F,B,E/K (7)	F,A,B,E/K (7)
		C (1)			
9	5/6	Opa- (98)*	ND	B (88)	B (64)
		B (1)		B,I (9)	B,I (23)
		E/K (1)			

continued

associated and -free gonococci were present, but that gonococci were rarely associated with epithelial cells. The percentages of different Opa variants in these cultures were similar to those in the urine cultures obtained at the same time (data not shown).

For six subjects (1, 2, 3, 8, 16, and 21), the fraction of reisolated colonies expressing multiple Opa proteins increased over time. The Opa protein(s) that predominated in the population obtained from the first positive urine culture were generally also expressed by the reisolates from later time points in infection, with simultaneous expression of additional proteins in those later samples (Table 1). Variants expressing two or three Opa proteins were isolated frequently, and a few variants expressing four or five Opa proteins simultaneously were recovered from cultures obtained late in the course of infection. In addition to the time points presented in Table 1, we analyzed similar numbers of colonies from one to five intermediate time points for each subject, with similar results. For three subjects (9, 11, and 13), there was little change in the proportion of the colonies expressing multiple Opa proteins during the course of infection.

The selection for Opa protein expression that occurred in vivo was not duplicated when variants were passaged in vitro. The Opa-negative inoculum variants were subjected to daily passage on GC agar for 5 d, incubation for 5 d on a GC agar plate before passage to a fresh plate, or growth in GC broth before plating on GC agar. No change in the percentage of Opa-negative colonies in the population occurred after these growth regimens (data not shown). When reisolates expressing three, four, or five Opa proteins were passaged on GC agar, they were difficult to maintain, rapidly switching off expression of one or more of the proteins.

A compilation of the number of colonies expressing each of the Opa proteins among the reisolates analyzed from all

Table 1. *(continued)*

Vol.	Day of first culture/day of treatment	Opa phenotype (% of colonies tested)			
		Inoculum	2-h culture	First positive culture	Last culture
11	1/2	Same	Opa- (100)	Opa- (57)	E/K (28)
				C (24)	Opa- (25)
				E/K (10)	C (23)
				F (10)	B (8)
					I (6)
13	2/3	Opa- (99)	Opa- (100)	I (62)	I (75)
		E/K (1)		B,E/K (38)	I,C (6)
					I,E/K (6)
					D,E/K (6)
16	3/5	Same	Opa- (86)	B (70)	B (66)‡
			B (11)	B,I (22)	B,E/K (13)
				I (9)	B,I (9)
					I (6)
21	1/4	Opa- (94)	Opa- (90)	E/K (30)§	E/K (58)§
		B (3)	C (7)	B (30)	E/K,B (28)
		A (1)		Opa- (15)	E/K,F (8)
		E/K (1)		B,I (10)	E/K,I (6)
				C (10)	

Opa phenotype was determined for 120–140 colonies for each inoculum and for 20–103 colonies from each urine culture. Data are presented for the inocula used in each trial, for colonies cultured from a urine sample obtained 2 h after inoculation, for the colonies from the first positive culture, and for the last culture that was done before treatment. Subjects who received the same inoculum in a single trial are separated by single horizontal spaces; the double spaces separate independent trials, in which different inoculum suspensions of Opa-negative variants were used. For the inocula, the Opa phenotypes are presented for the entire group of colonies analyzed; for the colonies cultured from urine specimens, variants that were detected at levels of 4% of the population or less are not listed.

* Percentage of OpaI variants in the inoculum was not determined.

‡ 3% of colonies from the last culture for volunteers 3 and 16 were Opa negative.

§ OpaE/K variants isolated from this subject were analyzed by Western blotting; all expressed the faster migrating species of this pair of proteins recognized by mAb H.164.

nine subjects (n = 1717; samples including and subsequent to the first positive cultures) showed that variants expressing each of the eight Opa protein were recovered, although the Opa types were not equally represented. Variants expressing OpaB, OpaF, OpaE/K, and OpaI were isolated most often (698, 649, 420, and 408 colonies, respectively), whereas a smaller proportion expressed OpaA, OpaD, or OpaC (29, 85, and 68 colonies, respectively). (In this analysis, colonies expressing multiple Opa proteins were tallied in the categories corresponding to each of the proteins expressed, so the sum of the numbers listed exceeds 1717.) OpaA was expressed only in combination with one or more other Opa proteins; the remaining proteins appeared singly or in combination with other proteins with nearly equal frequency (data not shown).

Infection of Volunteers with OpaF-expressing Gonococci. The strong selection for Opa protein expression in vivo suggested that inoculation with a variant expressing a suitable Opa protein might facilitate infection when compared with an Opa-negative variant. We used a variant expressing OpaF, which was one of the Opa proteins expressed most frequently by reisolates from the subjects inoculated with Opa-negative variants. To minimize differences other than Opa expression, OpaF-expressing variant A36 was isolated directly from Opa-negative variant A21 in two in vitro passages. We examined expression of two other variable surface components, pilin and LOS, in the two variants. The DNA sequence of pilin expression locus (*pilE*) was the same at both, except for two single-base differences that would result in nonconservative amino acid substitutions at positions 54 and 56 of the pilin protein. Both variants produced the same three LOS species, one of which bound mAbs 3F11 and 6B4, which recognize LOS with a lacto-*N*-tetraose moiety. The second LOS species bound mAb 1-1-M, which recognizes a terminal *N*-acetyl-

Table 2. *Comparison of the Infectivity of Opa-negative Variant A21 and OpaF Variant A36*

Inoculum		No. infected/no. challenged in separate trials	Total no. infected/no. challenged
CFU	Opa phenotype*		
10^6	Opa-	0/1	6/7
		2/2‡	
		4/4	
10^4	Opa-	0/1	1/3
		1/2	
10^6	OpaF	4/4	4/4
10^4	OpaF	1/2‡	1/2

* The percentage of the predominant Opa variant present in the inoculum ranged from 83 to 90% for variant A36 and from 90 to 98% for variant A21.
‡ These inocula were compared in a single trial.

galactosamine moiety (33). The third species did not bind any of the three tested mAbs (data not shown).

To compare infections caused by variants A36 and A21, we carried out trials with the two variants of inocula of 10^6 and 10^4 CFU (Table 2). With an inoculum of 10^6 CFU, four of four subjects became infected with the OpaF variant; six of seven were infected with the Opa-negative variant. With 10^4 CFU, one of two subjects developed infection after inoculation of the OpaF variant; one of three became infected after inoculation of the Opa-negative variant. There was no difference in the length of time until the onset of signs of infection in subjects infected with the two variants (mean of 38 h for the OpaF variant and 33 h for the Opa-negative variant). Thus, we detected no major difference in the virulence of variants A21 and A36 in this model of infection.

We characterized the Opa phenotypes of reisolated colonies from urine cultures for three subjects who became infected after inoculation of 10^6 CFU of OpaF variant A36

Table 3. *Opa Phenotypes of the Inoculum and of Urine Isolates from Subjects Inoculated with a Predominantly OpaF-expressing Variant*

Vol.	Day of first culture/day of treatment	Opa phenotype			
		Inoculum	2-h culture	First positive culture	Last culture
25	1/2	F (90)	F (90)	F,B (51)	F (35)*
		Opa- (10)	Opa- (10)	F (32)	F,I (29)
				F,I (11)	F,B (26)
				F,B,A (6)	F,E (10)
26	ND/2	Same	F (79)	ND	F (93)*
			Opa- (13)		F,I (7)
			F,A (4)		
			F,B (4)		
27	1/2	Same	F (80)	F (97)	F (86)
			Opa- (16)	F,C (3)	F,C (7)
			F,I (4)		F,B (3)
					F,C,I (3)

All three subjects were inoculated in a single trial, using the same inoculum suspension. Opa phenotype was determined for 90 colonies cultured from the inoculum suspension and for 20–35 colonies from each urine culture.
* The identification of variants expressing multiple Opa proteins was based solely on colony immunoblot results on the primary reisolate colonies.

(Table 3). Organisms cultured from 2-h urine specimens showed a similar distribution of Opa phenotypes to the inoculum (90% OpaF, 10% Opa negative). All of the colonies obtained from subsequent cultures expressed OpaF, either singly or in combination with one or more additional Opa proteins. Colonies cultured from urethral discharge samples were similar to those from urine samples in Opa phenotype (data not shown).

Discussion

Appealing aspects of the human challenge model are that the natural human host is being studied and that the portal of entry is the same as in urogenital gonorrhea. Disadvantages of the model include the small numbers of subjects that can be studied, the inability to use invasive procedures to sample infected tissues, and the artificial nature of the inoculation procedure. In addition, the ethical necessity to treat the subjects immediately upon development of signs or symptoms of infection means that this model is limited to early stages of infection. Nonetheless, previous studies of the expression of pili, LOS, and Opa proteins during experimental infection have provided crucial insights into gonococcal virulence (5–7). In this study, our objective was to extend the previous work of Swanson et al. (6) on strain MS11 by examining variation in Opa protein expression during experimental infection with Opa-negative and Opa-positive variants of strain FA1090.

Opa-positive variants were abundant among gonococci isolated from subjects infected with Opa-negative variants of strain FA1090. For six of the nine subjects, all of the colonies isolated from the first positive urine cultures expressed one or more Opa proteins. Even for the remaining three subjects, whose isolates showed more heterogeneity in Opa protein expression, the Opa-expressing fraction of the gonococcal population (43–85%) from the first positive urine culture was increased substantially relative to that of the inoculum. We do not know if the emergence of Opa-expressing organisms reflects selection for a subpopulation of Opa-positive variants present in the inocula, or if induction of Opa-negative variants to produce Opa-expressing progeny occurred within the environment of the urethra. Phase variation of Opa protein expression probably occurred during the course of infection, based on the patterns of appearance of variants expressing multiple Opa proteins. However, it is not possible to determine from our data if the rate of phase variation in vivo was the same as the rate that has been measured in vitro (11).

The increase in the proportion of reisolates expressing multiple Opa proteins as infection progressed in six of the subjects reinforces the conclusion that there is strong selection in vivo for expression of Opa proteins, particularly since it was difficult to maintain the multiple expression state of the reisolates when they were passaged in vitro. We do not know what selective advantage is responsible for the preferential survival or proliferation of gonococci expressing multiple Opa proteins. Perhaps it is not the specific identities of the expressed Opa proteins, but rather an increased amount of total Opa protein on the bacterial surface, resulting from several *opa* genes being expressed simultaneously, that is important to the success of the gonococcus.

Relatively homogeneous populations of Opa variants, composed mostly of one or two types, emerged in most of the infected subjects. In contrast to our original prediction, we did not isolate the same Opa variants from all of the infected subjects, even those subjects receiving the same inoculum. OpaB variants predominated in colonies cultured from three subjects, OpaF in two subjects and OpaI and OpaE/K in one subject each. When the number of colonies expressing each Opa protein was totaled, gonococci expressing OpaB, OpaF, OpaE/K, and OpaI were isolated most frequently, whereas variants expressing OpaA, D, or C occurred rarely. The proteins expressed by the more abundant Opa variants may share some property that is important for establishing infection. However, we believe that there are alternative factors contributing to their increased frequency of isolation. OpaB, E/K, and F variants were the Opa-positive variants present in the greatest numbers in the inocula, presumably because their relatively transparent colony phenotype made it more difficult to screen them out in the final stages of inoculum preparation. However, OpaI variants, which have a deeply opaque colony phenotype, were also abundant among the reisolates, so the correlation between greater numbers in the inoculum and greater frequency of reisolation of Opa variants was not complete. Another factor contributing to the disproportionate recovery of variants expressing OpaB or OpaF was probably the duplication of the *opaB* and *opaF* genes in the FA1090 chromosome, making it twice as likely that a gene encoding OpaB or OpaF would be expressing the protein (14). Similarly, expression of either of two proteins contributed to the OpaE/K category.

A model that is consistent with our results is that the success of one Opa-expressing variant over another in a given individual is a random event. If most of the organisms inoculated into the urethra fail to survive, and if organisms expressing at least one Opa protein are the ones most likely to persist (for unknown reasons), then the resulting population of gonococci would be derived from a few Opa-positive bacteria and would therefore be relatively homogeneous in Opa protein expression. The identity of the expressed Opa protein(s) would depend on which variants had a serendipitous survival of the initial clearance or killing mechanisms, and thus on the relative abundance of different Opa variants in the inoculum. The three subjects who developed infection most rapidly demonstrated the most heterogeneity in Opa expression of the reisolates, including Opa-negative variants. We hypothesize that a greater proportion of the inoculum survived in those subjects, so that it took less time for the proliferation of gonococci to reach the necessary level for a positive urine culture. The resulting population would be derived from a larger number of progenitors and would therefore be more heterogeneous.

There are other possible explanations for the emergence of different Opa variants in different individuals. Genetically determined differences in host cell receptors might allow preferential adherence of one Opa variant over another in

different individuals. Alternatively, preexisting antibody recognizing Opa proteins could be responsible for selective killing of Opa variants. We found no evidence for Opa-specific IgG in preinfection sera (our unpublished data), although serum IgG levels may not reflect the potential for immunoselection at the mucosal surface.

We do not believe that changes during sample processing or in vitro growth contributed significantly to the Opa phenotypes we detected. The similarity of Opa phenotypes in the inoculum and in colonies cultured from urine samples collected 2 h after inoculation argues that the procedures for inoculation and for culturing gonococci from urine samples did not cause artifactual shifts in Opa phenotypes. We analyzed colonies directly from the primary isolation plates, without additional subculture. Also, we found no evidence for differential survival of Opa variants in urine or during growth on the different culture media, and observed little or no shift in the Opa phenotypes of cultures grown under different conditions in vitro, including prolonged incubation of colonies.

An additional procedural issue concerns whether organisms cultured from urine samples represent those that were actually causing infection in the urethra. Tightly adherent bacteria or ones that have invaded epithelial cells might not be recovered from urine samples. We characterized bacteria cultured from urethral swab samples containing neutrophils and rare epithelial cells. The Opa profile of variants cultured from the swab samples was the same as those from urine samples taken at the same time. Preliminary immunofluorescence experiments with samples from two volunteers showed that the Opa phenotype of neutrophil-associated gonococci in urethral discharge samples was the same as the predominant Opa phenotype of variants cultured from urine samples from those subjects (our unpublished data).

The results of our experiments with Opa-negative inocula of strain FA1090 show both similarities to and differences from those obtained by Swanson et al. (6) with strain MS11. In both studies, there was strong selection for expression of one or more Opa proteins by the gonococci isolated from urine samples from infected volunteers. However, the same Opa proteins (OpaC and OpaA) predominated in reisolates from all three subjects infected with strain MS11, whereas different Opa proteins (OpaB, F, I, or E/K) were expressed by reisolates from nine subjects infected with Opa-negative variants of strain FA1090. (In evaluating the results obtained with strains MS11 and FA1090, it is important to remember that Opa protein nomenclature is unrelated in the two strains, so that OpaA of MS11 is not the same protein as OpaA of FA1090.) These differences could be a consequence of strain differences between FA1090 and MS11, although the time course and features of experimental gonorrhea caused by the two strains are similar (5–7, 8). Alternatively, our access to samples from a larger number of infected subjects may have revealed variability in Opa expression that was also present but was not detected in the earlier study with strain MS11.

The patterns of Opa expression in reisolates from subjects infected with strains MS11 or FA1090 show additional differences when considered in light of recent in vitro studies. For strain MS11, the OpaC and OpaA variants that were most abundant in reisolates from infected subjects have subsequently been shown to be associated with attachment to and invasion of epithelial cells, and with lack of attachment to neutrophils (20–23). The interactions of some Opa variants of strain FA1090 with neutrophils have been characterized, although epithelial cell invasion studies have not been done with this strain. In contrast to the results obtained with strain MS11, the OpaB and OpaF variants of strain FA1090 that were present in the greatest numbers in the reisolates from infected subjects expressed Opa proteins that are believed to promote association with neutrophils in vitro (34–36). It is difficult to resolve these apparent differences in the in vitro behavior of the Opa variants isolated from subjects experimentally infected with strains MS11 or FA1090. Perhaps the attributes of Opa variants that have been assessed in in vitro cell culture systems are not in fact the ones that are most relevant in this model of in vivo infection. Further investigations on the interactions of Opa variants of strain FA1090 with neutrophils and other cell types may help to resolve this issue.

There is an apparent paradox in the results we obtained when comparing the infectivity of the Opa-negative and OpaF-expressing variants. Given the strong selection for Opa protein expression in vivo, we anticipated that an inoculum containing primarily Opa-positive variants would infect more men or be associated with more rapid development of infection than an equivalent inoculum of an Opa-negative variant. However, there was no major difference in the ability of the OpaF-expressing and Opa-negative variants to cause infection, in terms of either the number of subjects infected or the time until onset of signs of infection. We would probably have not been able to detect subtle differences in infectivity of the two variants, due to the limited number of subjects available for this analysis. If expression of Opa proteins, including OpaF, is advantageous for gonococci of strain FA1090, then why was infection not more efficient with a preselected Opa-positive population? One possibility is that Opa protein expression is selected at an early stage of infection but becomes essential only later in infection. Alternatively, there may be a threshold number of Opa-positive organisms that is sufficient to establish infection. At an inoculum of 10^4 CFU of Opa-negative variant A21, there were up to 10^3 Opa-positive organisms inoculated. Perhaps that number was sufficient to cause infection in some subjects. Rather than carry out additional infection experiments with wild type gonococcal populations that will always contain mixtures of Opa variants, we believe that the best approach to resolving these questions is to construct mutants of strain FA1090 that are unable to express some or all of the Opa proteins. Such mutants should make it possible to determine if the strong selection for Opa protein expression in vivo is a reflection of an essential role for the members of this protein family in the establishment or maintenance of gonococcal infection.

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