Multiple Gonococcal Pilin Antigenic Variants Are Produced during Experimental Human Infections

H. Steven Seifert,* Christina J. Wright,* Ann E. Jerse,‡ Myron S. Cohen,§ and Janne G. Cannon‡
**Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611; ‡Departments of Microbiology and Immunology and §Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514*

Abstract

Gonococcal pilin variation is thought to allow immune evasion and change the adherence properties of the pilus. We have examined the process of pilin antigenic variation in human volunteers inoculated with strain FA1090. Our data show that pilin variation occurred throughout the process of infection, that at each time sampled after inoculation multiple pilin variants were present, and that later pilin variants appear to be recombinants between previously expressed genes and the silent storage pilin copies. Thus, during infection a large repertoire of proteins are available to the population to help avoid immune responses, to provide pili with varying functions, and to transmit to a new host. (*J. Clin. Invest.* 1994. 93:2744–2749.)
Key words: *Neisseria gonorrhoeae* • bacterial adhesion • pili • genetic recombination • vaccines

Introduction

Neisseria gonorrhoeae (the gonococcus, Gc)[1] causes an estimated one million cases of the sexually transmitted disease gonorrhea in the United States each year. Three major problems complicate the treatment of gonorrhea. (*a*) Although antibiotic therapy is effective, the rapid rise of antibiotic resistance has produced strains that are refractory to some treatment regimes. (*b*) Many patients are asymptomatic, remain untreated, and contribute to the spread of the disease. (*c*) Finally, infection does not result in long term immunity to reinfection. Some of the basis for the lack of natural immunity to gonorrhea is now being explained at the molecular level. A single strain can express different antigenic variants of the major pilus subunit (pilin), the opacity associated protein (Opa), and the outer membrane lipooligosaccharide (LOS)—all these variations contribute to immune evasion. In this study, we examined the process of pilin antigenic variation in vivo by inoculating a laboratory isolate of Gc into human volunteers with no recorded history of gonorrhea.

The pilus is thought to contribute to pathogenesis by functioning as an adhesion. The expression of pili enhances adherence of the bacterium to a variety of eukaryotic cells in vitro including several cells derived from tissues relevant to disease (for review see Stephens) (1). In addition, pilus-dependent adherence appears to be the first step in cell invasion and transcytosis of the epithelium (2–5). A single strain of Gc has the ability to repeatedly change the primary amino acid sequence of the pilin protein. This process requires homologous recombination (6) between silent storage variant gene copies and the expression locus (7–9). The recombination reactions are usually nonreciprocal with the silent locus remaining unchanged and the expression locus receiving part of the variant coding information. Thus the expressed gene in any Gc strain represents a patchwork collection of the previous recombination reactions that occurred in that strain. This allows for numerous combinations of the variant sequences contained in the silent copies and may also contribute to the production of pilins that cannot be assembled into pili (phase variation) (10–12). The arrangement of conserved and variable DNA sequences in the pilin gene has resulted in the mini-cassette model for antigenic variation that postulated six mini-cassettes of variable pilin gene sequences (mc1 through mc6) each recombining as a genetic unit (9).

Pilin antigenic variation has been demonstrated during laboratory culture (7–10, 13–15), during experimental human infection (16), and in single strains of bacteria isolated from pairs of sexual consorts (8, 17, 18). It is assumed that by changing the antigenic properties of the pilus, the bacterial population can extend the infection of a given host and allow reinfection of a host with preexisting immune responses. In addition, changing the pilin amino acid sequences may alter the ability of Gc to adhere to eukaryotic cells (14, 19).

The pioneering studies of Kellogg et al. (20) linked changes in colonial morphology (caused by changes in pilus expression) with virulence of Gc in male volunteers. Later studies (21) demonstrated that vaccination with isolated pili can protect against challenge with organisms expressing the same pilus type, although the vaccination raised the 50% infectious dose only ~ 10-fold (22). Furthermore, this pilus-based vaccine was ineffective in protecting against gonorrhea in a field trial (23, 24). In a more recent human volunteer study three similar pilin variants of strain MS11 were isolated from urine sediments of one volunteer at a single time point before the onset of clinical signs of disease (16). Two other variant pilin sequences were detected from a second volunteer at the time clinical signs of disease were observed. Since only a single time point was analyzed from each volunteer, no conclusions could be drawn

Address all correspondence to H. Steven Seifert, Department of Microbiology-Immunology W213, Northwestern University Medical School, Chicago, IL 60611.

C. J. Wright's present address is Institute of Ecology, University of Georgia, Athens, GA 30602.

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1. *Abbreviations used in this paper:* Gc, the gonococcus; WBC, white blood cell.

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about the variability of the Gc population in these volunteers and whether antigenic variation had occurred throughout the process of infection. We have extended these observations by collecting multiple isolates of strain FA1090 at several time points during the process of infection and analyzing antigenic variation by sequence analysis. Our data show that pilin variation occurs throughout the process of infection, that multiple pilin variants are present in a single individual, and that later pilin variants appear to be recombinants of previously expressed genes.

Methods

Human infection protocol. Procedures for recruiting male subjects, obtaining informed consent, carrying out intraurethral inoculation of gonococci, and processing urethral swab and urine samples for gonococcal culture have been described (25). All protocols were reviewed and approved by the Institutional Review Board and by the General Clinical Research Center Advisory Board of the University of North Carolina at Chapel Hill.

DNA sequence analysis. The DNA sequence of the variable portions of the expressed pilin gene was determined using direct PCR amplification of the expression site and cycle sequencing (Promega, Madison, WI) as described (26). Briefly, cells were lysed by boiling in a Triton X-100–containing buffer and an aliquot containing chromosomal DNA was used in PCR. The expression locus was amplified from this crude material using a primer that binds only in the promoter region of the expression site (PILSTART = GAGATAAACGCATAAAATTTCACC) matched with a primer that binds to the Sma/Cla repeat present in all pilin loci (SP3A = CCGGAACGGACGACCCCG). The clinical samples often contained limited template for amplification and therefore required a second round of amplification using nested primers. Again an expression site specific primer (PILRBS = GGCTTTCCCCTTTCAATTAGGAG) was matched with a primer that binds to all pilin loci (SMACLA1 = CAAACCCTTAAAAGACAAGC). Either of these primer-pairs will specifically amplify the expression locus from Gc chromosomal DNA (data not shown). The DNA sequence of both strands was determined using end-labeled primers that hybridize to conserved nucleotide sequences contained within the variable regions of the expressed gene (CONSTF2 = TACCAAGACTACACCGCCCG, CYS1R = GTCCGCAGAACCATTTTACCG, CYS1F = CGGTAAAATGGTTCTGCGGAC, and PILEND = CGCTTGATTTATTTAAAATTTAAGG). Each sample was amplified at least two times to detect possible sequence changes occurring during Taq polymerization, but none were observed. However, separate amplifications of the clinical sample usually produced different pilin sequences reflecting the number of different templates contained in these samples (26). Each separate amplification product was sequenced at least two times. The DNA and protein sequences are described in Genebank accession numbers L28978-L28994.

A PCR-based assay to detect antigenic variants in single colonies. A measure of variability in the predominately expressed pilin gene contained within a Gc population was performed using two oligonucleotide primers corresponding to the hypervariable region of the pilin gene (see Fig. 2, *double underlines*). HV-1 is contained in the hypervariable region of the inoculum sequence (TCGTCGGCGCCGGTTTTGG) and HV-2 is contained in same region of the hypervariable region in all of the volunteer 1 isolates and one volunteer 2 isolate, 2-57-U17 (CAACGGTGTCGTCGTTGTCG). To detect these variant sequences, FA1090 was propagated on GCB medium and single colonies were collected with sterile filter paper into 3% tryptic soy broth with 25% glycerol. This glycerol stock was either frozen or mixed with the Triton X-100 lysing solution for direct PCR. The PCR reactions were performed on 10 μl of soluble cell lysate with PILSTART paired with each of the variable oligonucleotides. Additionally, the SP3A oligonucleotide that binds to the 3′-conserved sequences of the expression locus was used as a positive control for amplifiable pilin template. PCR was done under standard conditions (26) for 25–30 cycles and a portion of each amplification was run on an ethidium bromide-stained, agarose gel to detect product. A reaction was judged positive if the band intensity using a variable primer was similar to that of the positive control.

Results

Human volunteer infection. In this human challenge experiment, two volunteers were infected with a culture of piliated strain FA1090 by intraurethral inoculation as described (25). Urine sediments and urethral swabs were collected daily between the time of inoculation and the onset of clinical signs. Viable Gc were always present in the urine samples but the urethral swabs only contained bacteria when clinical signs of disease were noted. Viable bacteria could not be revived from frozen urine sediments, but these samples still contained sufficient Gc DNA for PCR amplification and sequence analysis. Frozen stocks of both urine and swab samples contained viable bacteria after in vitro growth.

Fig. 1 shows the time course of infection in the two volunteers. In both, a rapid decrease in the number of Gc in urine was observed after inoculation, with no colony forming units detected at 9 or 14 h (< 3 cfu/ml). The basis for the disappearance of Gc during the first 9–14 h is unknown. It is equally possible that bacterial death occurs soon after inoculation, or that the inoculated bacteria travel to places where urination cannot dislodge them. After the initial decrease, the number of bacteria cultured from urine sediments increased faster, and clinical signs of disease were observed earlier in volunteer 2 than in volunteer 1. Subsequent experiments have indicated that a shorter time between inoculation and clinical signs of disease correlates with an increase in the number of bacteria delivered into the urethra (25).

Before inoculation, urine samples from both volunteers were essentially free of white blood cells (WBCs). A large increase in the number of WBCs (essentially a neutrophil influx) occurred soon after inoculation of volunteer 1, but no such increase was observed in volunteer 2. The number of WBCs present at the two hour time point in later volunteers varied between the two extremes shown (data not shown). Since the same bacterial suspension was inoculated into these two volunteers, the variable WBC count must be due to host factors or differences in inoculation (25). The purulent exudate that is indicative of gonorrhea correlates with a high number of neutrophils present in the urine at the times representing clinical signs of disease. No bacteria or neutrophils were detected in urine soon after antibiotic treatment.

Pilin variation during infection. We determined the sequence changes in the pilin expression locus that occurred during human experimental infections (Fig. 2). The DNA sequence was determined by PCR amplification of samples isolated directly from the volunteers (urethral swabs or centrifuged urine sediments), or from colonies grown from swabs or urine sediments passaged once and frozen. Therefore, the sequences we determined were expressed in bacteria directly isolated from humans or after minimal passage in vitro.

In volunteer 1, the 2-h urine sample contained the inoculum pilin sequence, 1-2-U7 (also detected in sample 1-2-BU1 not shown) and variant pilin gene sequences (1-2-BU1′ and 1-2-BU2). After the 2-h sample, the inoculum sequence was not detected again in volunteer 1 and several different pilin variants were detected. A new variant was detected at 14 h in urine isolate 1-14-U29. Expressed genes identical (1-57-U19) or similar (1-81-S32, 1-81-U24, and 1-81-S2) to this variant

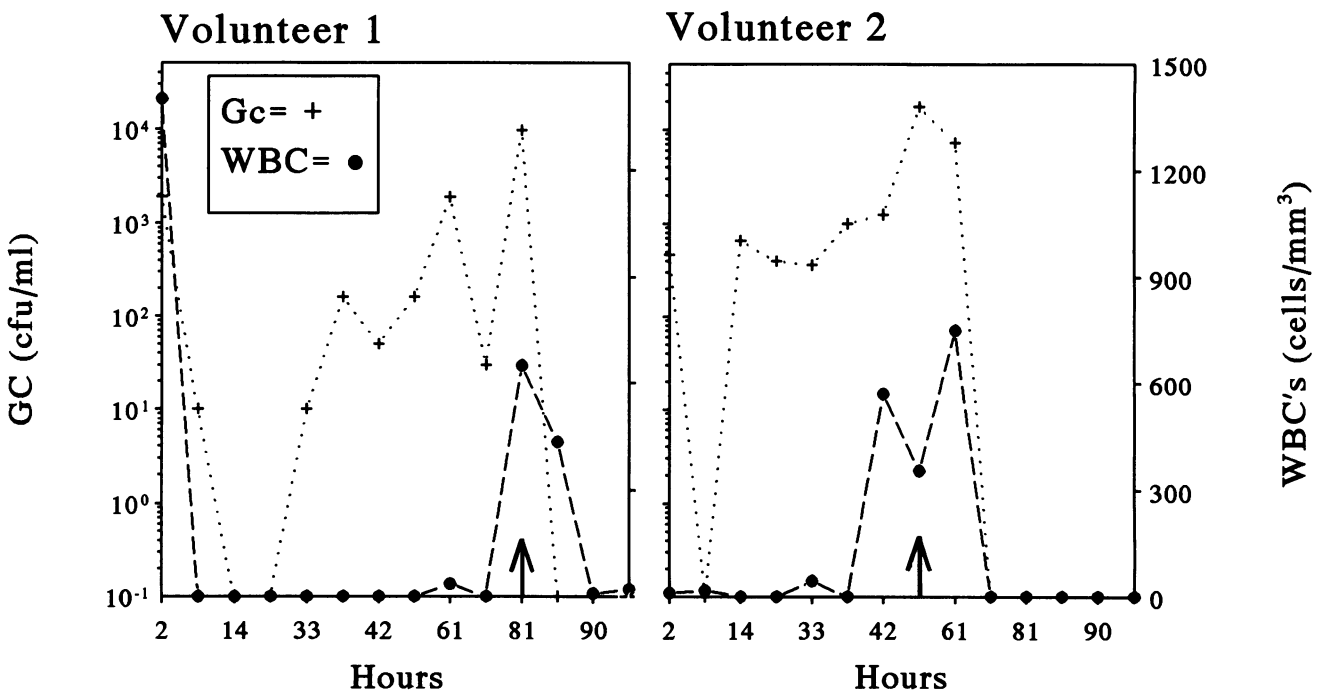

Figure 1. Time course of human volunteer infection. ~ 1.2×10^6 colony forming units (cfus) of the same culture were inoculated intraurethrally in each volunteer at time zero as described (25). At the times after inoculation, Gc cfu's or WBCs in resuspended urine sediments were quantified. Data for Gc are expressed as cfu/ml of resuspended urine sediment and WBCs as cells/mm^3 of resuspended urine sediment (note: samples with zero cfu are represented as 10^{-1} on the log scale). The arrow shows when clinical signs of disease were recorded and antibiotic treatment was administered.

were also detected at later times. The detection of the 1-81-U24 and 1-81-S2 isolates shows that both urethral swab and urine samples can contain bacteria expressing identical pilins. Other variants detected at 38 and 57 h (1-38-BU1 and 1-57-BU1) were similar to the two hour variant, 1-2-BU1, although the 3′-end of 1-2-BU1 was not determined. Additionally, a nonpiliated nonsense variant was detected in the 33-h urine sample (1-38-BU2). Thus in volunteer 1, the variant expressed on the majority of the inoculum was not detected after two hours and many new variants were observed throughout the remainder of the infection. Some of the variants appear to express pilin sequences based on genes expressed at earlier times, although it is impossible to determine if these are sequential or independent recombination events. The number of nucleotide changes supports a mechanism based on homologous recombination as previously described (6, 8, 11, 27). The most striking observation from the volunteer 1 pilin sequences is that all of the piliated variants had identical changes in the 5′-portion of the hypervariable region (detected by the variable oligonucleotide, HV-2 whose position is shown by the second double underline in Fig. 2, 1-2-BU1′). Interestingly, two sets of 3′-hypervariable sequences were present in these isolates (compare the hypervariable regions of 1-14-U29 with 1-38-BU1).

In contrast to volunteer 1, volunteer 2 retained the inoculum pilin sequence throughout the infection in both urine and swab isolates (2-2-U9, 2-14-U13, 2-42-BU1, and 2-57-S18). As in volunteer 1, the expression of a new pilin variant was detected in the 2-h urine sample (2-2-BU1′) and was maintained later in infection (2-14-BU1). Again, new pilin variants appear to be recombinants of previously expressed genes. For example, the gene expressed in the inoculum may have recombined with part of a silent locus to produce variant 2-42-U14 which then recombined with a silent copy to give 2-57-U35. The HV-2 sequence present in all of the volunteer 1 isolates was expressed in the 2-57-U17 isolate. Thus, infection of both volunteers with the same inoculum sample produced many different pilin variants suggesting that recombination occurs during infection.

Analysis of variability of the inoculum. Sequence analysis of the inoculum sample resulted in one pilin sequence yet the 2-h urine samples contained different sequence variants. We considered that these variants could have been present in the inoculum but at a level undetectable by sequence analysis. Mixing experiments with two variant pilin templates have demonstrated that a minority template must comprise at least 25% of the sample to be detected by sequence analysis (26). We therefore analyzed individual colonies grown from the inoculum sample with an oligonucleotide corresponding to the 5′-portion of the hypervariable region of in the inoculum sequence (HV-1, Fig. 2) and a second oligonucleotide that binds to the 5′-portion of the hypervariable region in most of the variant genes isolated from volunteer 1 (HV-2, Fig. 2). By matching these variable oligonucleotides with the expression site specific primer (PILRBS) in PCR, we can detect colonies that express either of these hypervariable sequences, or neither sequence. In agreement with the sequence analysis of the inoculum culture, 77% of 74 colonies grown from the frozen inoculum expressed the HV-1 sequence. However, 7% expressed the HV-2 sequence and 16% expressed a hypervariable sequence recognized by neither oligonucleotide. This analysis proves that the inoculum was more heterogeneous than the sequence analysis of the culture showed and suggested that the pilin variants isolated 2 h after inoculation may have been preexisting in the inoculum culture. Therefore, the expressed gene sequence of three colonies expressing the hypervariable sequence containing HV-2 were sequenced (Fig. 2, *Inoculum variants*). All

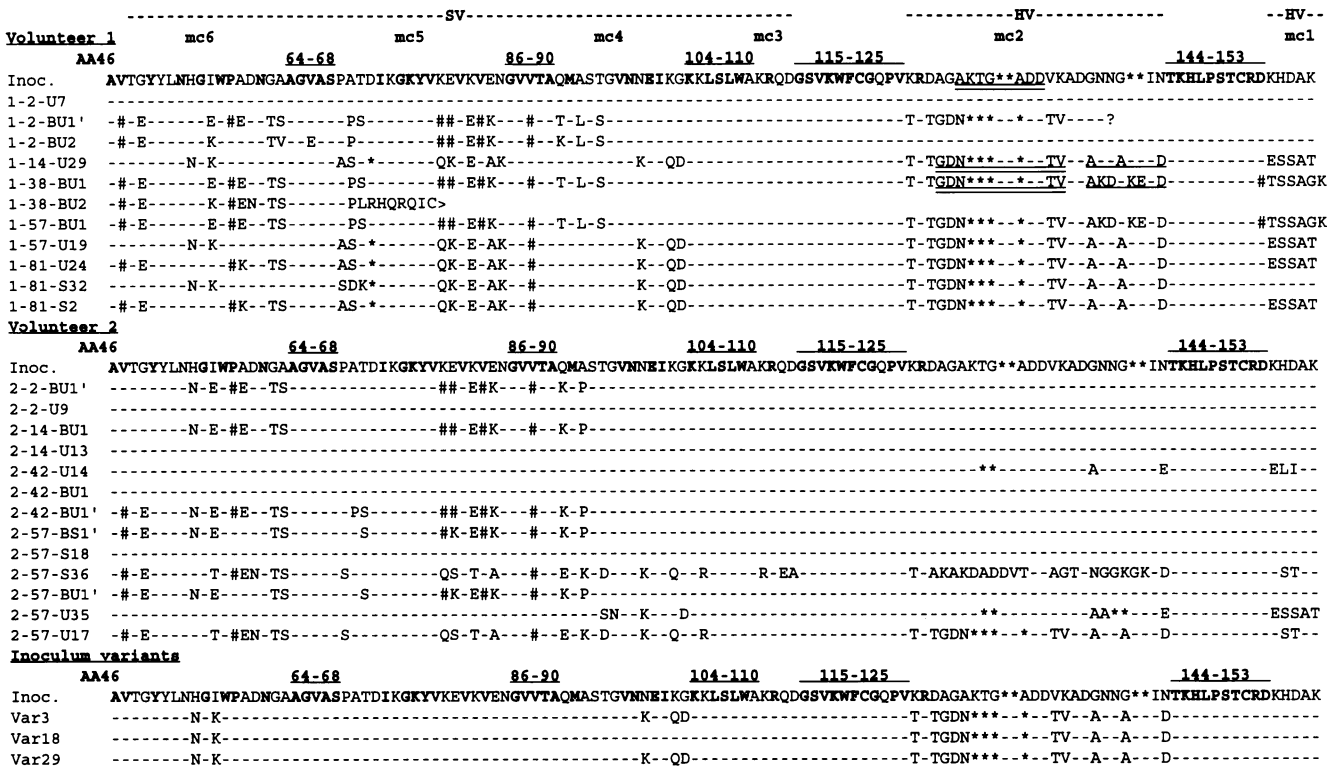

Figure 2. Predicted variable pilin amino acid sequences from FA1090 isolates. DNA sequences were determined from PCR amplified expression site DNA using two sets of primers that generate overlapping sequence of the entire variable portion of the expressed gene (GenBank numbers L28978-L28994). The predicted amino acid sequences from amino acid 46 to the end of the protein are shown. Identical sequences from the same sample at the same time point are not shown. The semivariable (SV) and hypervariable (HV) regions are indicated on the top line. The regions of the protein sequence corresponding to the DNA sequences recognized by the variable oligonucleotides are shown by the double underlines. The corresponding DNA sequences in the inoculum hypervariable region are recognized by HV-1. The same region of all volunteer 1 isolates are recognized by HV-2 (double underline in 1-2-BU1'). The apparent recombination breakpoint within the hypervariable regions of variants 1-14-U29 and 1-38-BU1 occurs between the double underline and the single underline (see Discussion). Only changes relative to the FA1090 inoculum sequence are shown for the variants with identical residues (-). (#) Silent changes in DNA sequence not reflected in the amino acid sequence; (*) deletions relative to another sequence presented in the Figure. (–) Deletions in the variant sequences that match the inoculum. (?) Regions of that gene that were not determined; (>) a nonsense codon. Amino acid residues that have never varied in an expressed gene on any pilin variant from any reported strain are indicated in bold on the FA1090 inoculum sequence. The conserved regions defined by Haas and Meyer (9) are underlined above each FA1090 inoculum sequence and variable mini-cassettes (mc) between these are indicated above the volunteer 1 sequences. The sequence expressed on the inoculum sample is presented on the top line of each section of the Figure. The DNA sequences and predicted protein sequences are identified by the following nomenclature. The volunteer from whom the sample was isolated is presented in the first position. The hour after inoculation that the sample was isolated is indicated in the second position. The third position shows if the sequence was derived from template amplified from a urine sediment or urethral swab sample without in vitro growth (*B*) and whether the isolate was from a urine or swab sample (*U* or *S*). The final number in the third position indicates the colony number or separate amplification reaction from the clinical samples. For example, 2-57-U17 was isolated from volunteer 2, at 57 hours, and was taken from colony No. 17 grown from the urine sediment. 1-2-BU1 is from the first amplification of the urine sediment isolated from volunteer 1, at 2 h. Some samples produced mixed sequences that were recognized by the double bands present at variable positions in the pilin gene sequence (26). If part of the mixed sequence matched a previously identified variant sequence from FA1090, we predicted the second sequence by "subtracting" out the known sequence. These deduced pilin variants are indicated by a prime (').

three variants were different than any isolated from the volunteers (Fig. 2). Either the pilin variants isolated in the two hour urine sample of volunteer 1 comprised an exceedingly small subpopulation of the inoculum and these were enriched by selection, or antigenic variation occurred within the two hours after inoculation.

Discussion

By sequencing the expressed pilin gene from strain FA1090 before and during the establishment of infection in human volunteers, we have observed extensive pilin antigenic variation. The sequences shown are likely to represent a subset of all variants expressed in these samples since a limited number of sequences were examined at each time point. However, the number of different variants detected shows that many different antigenic variants arise during infection. The rate of antigenic variation in vitro has not been reported and preliminary experiments suggest that the rate may be similar to rates of phase variation (Howell, B., C. J. Wright, and H. S. Seifert, unpublished observations). The highest reported rate of phase changes (6.5×10^{-4} nonpiliated phase variants/piliated cfu/generation) would result in a 2% subpopulation of pilin variants after 30 generations (6). Each specimen isolated from the volunteers contained bacteria expressing many different pilin

variants and each sample contained new variants that appeared to result from recombination between silent copies and previously expressed genes. This suggests that a higher proportion of antigenic variants are generated during growth in vivo than during growth in vitro. Additionally, variation was found in every variable portion of the pilin gene. Before this study, an analysis of pilin variation in human subjects had also reported changes in pilin sequences during infection, but did not analyze sequential isolates (16). This study extends that work to show that multiple pilin variants can be detected at different times during infection, and that the repertoire of pilin variants changes over time.

We compared the expressed FA1090 pilin sequences to those described previously (8, 16, 28). The alignment of all expressed pilin variants has led us to propose two alterations of the mini-cassette model for the pilin antigenic variation (9). The mini-cassette model suggested that each pilin gene copy contained six mini-cassettes of variable DNA/amino acid sequence (see mc1-6 above the inoculum sequence in Fig. 2). Each variable mini-cassette was surrounded by conserved DNA/amino acid sequences (see underlined numbers above the inoculum sequence in Fig. 2). This arrangement of variable and conserved mini-cassettes would suggest that each variable mini-cassette could recombine as a unit using the conserved DNA sequences as crossover points for homologous recombination. The pilin sequences reported to date show that between amino acid residues 46 and 115, many conserved amino acid residues exist within each variable mini-cassette (see bold type in the inoculum sequence). In addition, some variable amino acid residues have been found in some conserved cassettes (see normal type in the conserved regions, 106–110 and 115–125). These observations suggest that the original designation of the region between amino acids 46 and 114 as semivariable is more accurate (7, 8) since both conserved and variable residues are found throughout this portion of the gene.

A second modification to the mini-cassette model results from the analysis of the hypervariable gene sequences expressed in the volunteers. The hypervariable pilin sequence corresponding to the HV-2 oligonucleotide (Fig. 2, 1-14-U29, and 1-38-BU1, *double underline*) was found with two different neighboring hypervariable sequences (Fig. 2, 1–14-U29, and 1–38-BU1, *single underline*). Southern hybridization and DNA sequence analysis show that the HV-2 sequence is only contained in one silent copy (Snodgrass, T. L., and J. G. Cannon, unpublished observations). To produce two different hypervariable regions containing this sequence, recombination must have occurred within the hypervariable region and therefore this most variable mini-cassette does not always recombine as a unit. A greater number of pilin variants are possible if recombination can occur within a variable mini-cassette although sufficient homology must occur between the paired hypervariable sequences to allow for heteroduplex formation.

There are two main hypotheses that could explain the extent of variation observed in vivo. Either variation is stimulated by environmental conditions encountered during infection or certain pilin types have a selective advantage during infection. These hypotheses are not mutually exclusive since the rate of variation in vivo could be stimulated to increase the probability that a wide panel of different pilin variants are present for selection. How environmental conditions could influence the rate of variation is unknown. Changes in environment could increase the levels of autolysis/transformation that has been shown to be responsible for some portion of pilin recombinations (27, 28, 29). Additionally, we have recently shown that a specific DNA repeat associated with all pilin loci is required for efficient antigenic variation (30). Changes of expression of factor(s) (recombinases or nucleases) that could act at this site to promote gene conversion could also be stimulated by the conditions encountered in vivo. A better understanding of the molecular mechanisms used to produce antigenic variation will be required before the relationship between environment and rates of variation are understood. We can, however, propose three selective conditions that are likely to increase the proportion of antigenic variants in vivo. First, variants could be selected for different adherence properties. Second, variation could be selected by nonspecific immune responses. The inflammatory response elicited at the time of clinical signs could select for a subpopulation of piliated bacteria. It is interesting to note that volunteer 1 showed a transitory inflammatory response immediately after inoculation and that the inoculum pilin gene was not detected after the two hour sample. This observation suggests that infection of volunteer 1 was initiated by a single clone expressing HV-2 or that only HV-2 expressing clones survived killing by the recruited neutrophils. While no relationship between neutrophil killing and pilin variation has been previously described, this possibility will be explored further. Finally, some of the pilin variations observed could be due to specific immune responses (i.e., a secretory IgA response). We consider it unlikely that a specific immune response could develop in the first two days after inoculation, so that the antigenic variation that occurs early in infection must be independent of a specific response. While it is possible that a response was elicited by the third or fourth day, the high rates of variation observed at all times during infection prevents us from invoking a specific immune response in selecting for antigenic variation at later times. Regardless of how multiple variants are produced, each infected person contains multiple pilin variants available for transmission to a sexual partner. Even if the partner was previously infected and maintained a specific antipilus immune response, the new inoculum is likely to contain variants not recognized by the previous response. This may explain why the pilus-based vaccine (21) gave only partial protection from experimental infection, and why the same vaccine was unable to protect against natural disease (23, 24). Clearly there is sufficient variation in one individual such that the inoculum passed to a partner would carry a sufficient number of pilin variants to escape from the vaccinated response. Interestingly, the particular combination of semivariable amino acid residues expressed on FA1090 is very similar to those reported for other Gc strains (8, 16, 31). Thus, it appears that the semivariable portion of pilin has a limited number of amino acid sequence combinations that can be expressed. This observation, coupled with the suggestion that the hypervariable residues are often shared in diverse strains (32), suggests that there are limits to the sequence changes that can be accommodated without disrupting function. This raises the possibility that a multivalent pilin-based vaccine could be formulated that reacts with all of the variable pilin sequences.

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