

## EXPRESSION OF OUTER MEMBRANE PROTEIN II BY GONOCOCCI IN EXPERIMENTAL GONORRHEA

By JOHN SWANSON, OSMAR BARRERA, JAMES SOLA,  
AND JOHN BOSLEGO\*

*From the Laboratory of Microbial Structure and Function, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana 59840; and the \*Department of Bacterial Diseases, Walter Reed Army Institute of Research, U. S. Army Research and Development Command, Walter Reed Army Medical Center, Washington, DC 20307*

*Neisseria gonorrhoeae* infects mucosae of humans whose urogenital epithelia are most commonly involved. The infectious aptitudes of these bacteria are likely dictated by their surface components which, except for pili, have poorly defined roles in pathogenesis. Pilus<sup>+</sup> gonococci infect the human male urethra, whereas pilus<sup>-</sup> variants do not (1, 2, Boslego, J., J. M. Koomey, and J. Swanson, unpublished results). Pilus expression promotes adherence of gonococci to human cells in vitro (3-6), and analogous, pilus-mediated adherence in vivo to mucosal epithelium likely accounts for correlation between pilus<sup>+</sup> phenotype of gonococci and their virulence.

Expression of outer membrane protein II by gonococci markedly influences their surface properties, as deduced from the colonial opacity and intercellular adherence differences of protein II<sup>-</sup> vs. protein II<sup>+</sup> organisms (7); however, the pathogenic relevance of protein II production is not understood. Variants within a given strain synthesize several distinct protein II moieties. Expression of protein II moieties is variable for gonococci in vivo among different individuals or in separate sites of an individual (8-11). Protein II<sup>+</sup> gonococci are isolated regularly from a male's urethra and frequently from the cervix of an infected female, depending on menstrual timing, use of oral contraceptives, etc. (8, 10). But protein II<sup>-</sup> organisms populate an infected female's fallopian tubes, regardless of which protein II phenotypes reside in her cervix (11). These observations say that protein II expression relates to gonococcal pathogenicity, but they do not tell how. Certain protein II species promote attachment of gonococci to tissue culture cells (5, 12-14). Colonization of HeLa cells by gonococci is also enhanced by the diminished detachment that is displayed by organisms that express particular protein II species (15). Interactions between gonococci and peripheral blood neutrophils in vitro are also influenced by elaboration of certain protein II species (16-18).

The protein II repertoire expressed by a given strain consists of several structurally related polypeptides whose common and unique portions are membrane buried and surface exposed, respectively (19-25). This repertoire is encoded by a multi-

J. Sola was sponsored by the National Institutes of Health summer student program. Address correspondence to J. Swanson, Laboratory of Microbial Structure and Function, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT 59804.

gene family whose individual members undergo on/off expression changes at high frequencies and can be coexpressed (19–22, 26–28). Although each P.II is quantitatively a major component of the outer membrane when produced, P.II synthesis seems mostly irrelevant to gonococcal growth and survival *in vitro*.

The present study examines the protein II phenotypes of gonococci shed by men who had received intraurethral instillations of predominantly pilus-positive protein II<sup>-</sup> gonococci. Nearly all gonococci reisolated from urine and semen were protein II<sup>+</sup>. These reisolates expressed five different protein II species, a fraction of the protein II repertoire of strain MS11<sub>mk</sub>.

### Materials and Methods

**Gonococci.** Input gonococci were strain MS11<sub>mk</sub> (29) that had been repeatedly passaged on solid medium by serial selection of individual P<sup>+</sup>O<sup>-</sup> colonies that contained pilus<sup>+</sup> protein II<sup>-</sup> organisms (30). A stock was made by suspending the outgrowth of 10 such colonies in 1% skim milk and by freezing (-70°C) 1-ml portions. Aliquants of the frozen stock were propagated *in vitro* on several occasions to assess the colonial characteristics of their population. Selected colonies were passaged once and the respective outgrowths were examined by EM, SDS-PAGE, and immunoblotting to define their protein II and pilus phenotypes. Gonococci for intraurethral instillations were propagated from the frozen stock onto plain clear solid medium at 35.5° in 5% CO<sub>2</sub> for 20–22 h (22). Desired colonies were lifted from the medium on filter paper fragments and were suspended in PBS to an estimated concentration that was subsequently defined by plating aliquants and counting the resultant colonies. 1-ml vol of suspensions containing different numbers of organisms (10<sup>4</sup>–10<sup>8</sup>) were instilled into the urethrae of male subjects who did not urinate for 2 h afterwards. Their urine specimens were collected at specified post-instillation intervals, centrifuged to concentrate the gonococci, plated onto plain clear medium, and also plated onto the same medium containing vancomycin, colistin, and nystatin (V-C-N, Baltimore Biological Laboratories, Baltimore, MD, according to manufacturer's instructions) to inhibit growth of organisms other than gonococci. The remainder of the concentrated specimens were frozen at -70°C in 1:1 (vol/vol) heat-inactivated horse serum (56°C, 0.5 h), trypticase soy broth (Baltimore Biological Laboratories). After transfer on dry ice to Hamilton, MT, thawed aliquants of frozen specimens were plated on clear solid medium and on V-C-N medium. Colony opacity was a reliable guide to protein II phenotype for MS11<sub>mk</sub> organisms grown on plain solid clear medium; nonopaque colonies contained P.II<sup>-</sup> organisms while opaque colonies indicated P.II<sup>+</sup> variants. But on V-C-N medium, colonies of P.II<sup>-</sup> and P.II<sup>+</sup> organisms both appeared opaque. P.II<sup>-</sup> and P.II<sup>+</sup> variants could be differentiated on this medium by the darker colors of the latter, but their differences were subtle. When such colonies were transferred from V-C-N to plain medium, their opacities again matched protein II constitution. Accordingly, each colony reisolated on V-C-N was replated onto plain medium to define the opacity phenotype of its outgrowth whose protein II constitution was defined by SDS-PAGE and immunoblotting with αP.II mAb. Frozen stocks of these passaged reisolates were prepared in 1% skim milk.

**Male Subjects.** Guidelines for human experimentation of the Walter Reed Army Institute of Research and the U. S. Department of Health and Human Services (Wash. DC) were followed in this study. The study protocol was approved by the Human Use Review Board of the Walter Reed Army Institute of Research and by the Office of the Surgeon General, U. S. Army. Adult male volunteers who had no history of previous gonocorrheal infections and had given informed consent were the experimental subjects. They were housed at, the U. S. Army Medical Research Institute for Infectious Diseases at Ft. Detrick Frederick, MD, for the duration of the study. After receiving gonococci intraurethally, each subject was questioned frequently to assess his developing dysuria or other symptoms of urethritis. When and if urethral discomfort and purulent discharge appeared, penicillin was administered to the infected subject. After antibiotic administration, several subsequent urine samples were

<sup>1</sup> Abbreviation used in this paper: V-C-N, vancomycin, colistin, and nystatin.

cultured to document the absence of infecting gonococci whose sensitivity to penicillin had been established before the study. Urine from each subject was also cultured at 4 and 8 d after termination of the study to ensure eradication of gonococcal infection.

**Immunoblotting.** Gonococci were lysed, subjected to SDS-PAGE, and transferred to a solid matrix (HAHY membranes; Millipore Continental Water Systems, Bedford, MA). These blots were probed with an  $\alpha$ P.II mAb that recognizes all protein II species (McAb 9B12/C11, the generous gift of Milan Blake, The Rockefeller University, New York, NY) and then with  $^{125}$ I-labeled protein A. These procedures have been described in detail before (22, 29–31).

## Results

**Protein II Repertoire in Strain MS11.** Nine protein II species have been distinguished in strain MS11<sub>mk</sub> by their unique electrophoretic mobilities (Fig. 1). Only five of these (IIa, IIc, IIf, IIh, and IIi) were identified among input and reisolated organisms (Table I). The four other protein II species (IIb, IIe, IIg, and IIi) were found subsequently among P.II<sup>+</sup> variants of P.II<sup>-</sup> organisms that arose in vitro from protein II<sup>+</sup> reisolates.

**Input Gonococci.** Gonococci used for intraurethral instillation almost uniformly (98.85%) displayed nonopaque colony phenotype; the remaining 1.15% were opaque colony variants (Table I, *Input*). When 40 such opaque colonies were examined, all but one were P.IIa<sup>+</sup> and the lone exception was P.IIc<sup>+</sup>. Among the O<sup>+</sup> variants that arose on passage of one P<sup>2+</sup> O<sup>-</sup> colony of the input stock, most (83/87, 95.4%) were P.IIa<sup>+</sup>, three were IIc<sup>+</sup> and one was IIf<sup>+</sup> (Table I, *In vitro*) P.IIi<sup>+</sup> variants were absent, but they arose occasionally upon passage of other similar O<sup>-</sup> colonies. P.IIa<sup>+</sup> variants consistently predominated in several such assessments of the input stock (data not shown).

**Reisolated Gonococci.** Only protein II<sup>-</sup>, input-like organisms were reisolated from each man's "wash-out" urine (2 h after intraurethral challenge). But protein II<sup>+</sup> variants dominated (96%) in all subsequent urine samples from the three infected men (Table I, *In vivo*). One or more reisolates produced each of five different protein II species (IIa, IIc, IIf, IIh, IIi) (Figs. 1 and 2). P.IIc was most common and was expressed by 50% of all reisolated gonococci. Proteins IIa and IIc were each produced by gonococci recovered from all three men while P.IIi occurred in gonococci ob-

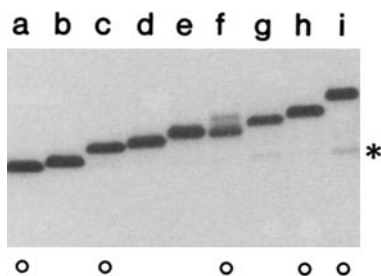


FIGURE 1. Protein II repertoire of strain MS11<sub>mk</sub> gonococci. Nine protein II species are identifiable in strain MS11<sub>mk</sub> by their distinctive electrophoretic mobilities. This immunoblot was probed with an  $\alpha$  protein II mAb after electrophoretic separation of whole cell lysates of P.II<sup>+</sup> gonococci whose expressed P.II is noted (a, P.IIa; etc.). The relative migration of each protein II and its characteristic colony opacity (1+ to 4+) is as follows: P.IIa, 29.5 (3+); P.IIb, 30 (1+); P.IIc, 31.2 (3+); P.IId, 31.5 (2+); P.IIe, 32 (2+); P.IIf, 32 (4+); P.IIg, 33 (1+); P.IIh, 34 (2+); and P.IIi, 35.5 (4+). Only P.IIa, P.IIc, P.IIf, P.IIh, and P.IIi (O) were produced by reisolated gonococci. P.IIf is split into two bands in this immunoblot, characteristic for lysates of P.IIf<sup>+</sup> organisms that are heated repeatedly. P.IIa<sup>+</sup> variants arise at high frequency when this strain is grown in vitro; although the P.IIb<sup>+</sup>, P.IIc<sup>+</sup>, and other P.II<sup>+</sup> gonococcal populations depicted here consist mainly of organisms that express the particular protein II noted, they also contain a number of organisms that express P.IIa (\*).

TABLE I  
Occurrences of Protein II<sup>+</sup> Variants in Strain MS11<sub>mk</sub>

Protein II phenotype	Input	In vitro	In vivo
II <sup>-</sup>	3,438 (98.85%)	7,167 (98.8%)	8 (4.3%)
IIa <sup>+</sup>	39 (1.12%)	83 (1.145%)	9 (4.9%)
IIb <sup>+</sup>			
IIc <sup>+</sup>	1 (0.03%)	3 (0.04%)	92 (50.3%)
IIac <sup>+</sup>			15 (8.2%)
IIcf <sup>+</sup>			1 (0.55%)
IIci <sup>+</sup>			3 (1.6%)
IIid <sup>+</sup>			
IIe <sup>+</sup>			
IIf <sup>+</sup>		1 (0.014%)	5 (2.7%)
IIaf <sup>+</sup>			38 (20.8%)
IIg <sup>+</sup>			
IIh <sup>+</sup>			1 (0.55%)
IIah <sup>+</sup>			2 (1.1%)
IIi <sup>+</sup>			4 (2.2%)
IIai <sup>+</sup>			3 (1.6%)
II <sup>+</sup>	40 (1.15%)	87 (1.2%)	173 (94.5%)
Total	3,478	7,250	183

Results are shown for an aliquot of the input preparation used for intraurethral instillation (Input), for progeny of a single P<sup>2+</sup>O<sup>-</sup> colony that was serially passaged (In vitro), and for all gonococci reisolated from male subjects C, D, and E (not including those in initial "wash-out" urines) (In vivo). All the protein II species recognized in MS11<sub>mk</sub> are listed as are the combinations of two different P.II moieties found among the reisolates. P.IIi<sup>+</sup> organisms were not found in the progeny of the singly colony examined here (\*), but were identified as variants of input-like organisms in other, similar assessments. The P.II<sup>-</sup> colonies shown in Input and In vitro were scored visually with a dissecting microscope and were not examined by immunoblotting; all others were tentatively classified by their colony morphologies and were then defined by SDS-PAGE and immunoblotting.

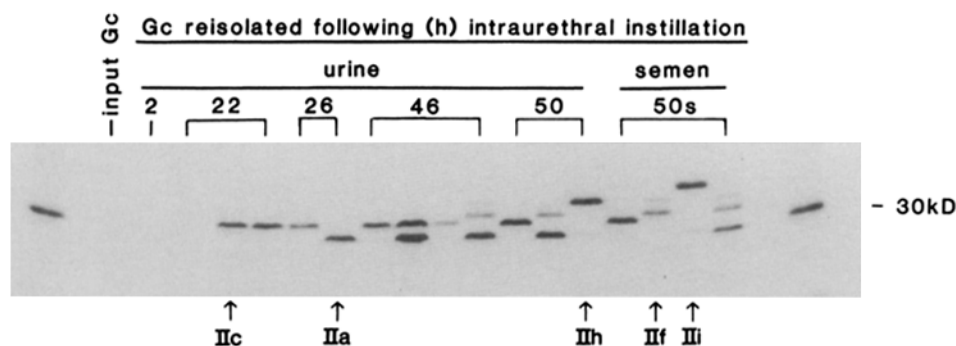


FIGURE 2. Input and representative gonococci reisolated from subject D. Input gonococci (*Gc*) were protein II<sup>-</sup> as were organisms reisolated 2 h after intraurethral instillation. At 22 h after instillation, a few P.II<sup>-</sup> organisms were recovered, but most reisolates from this and subsequent urine samples displayed protein II<sup>+</sup> phenotypes. Early in this infection the majority of the reisolates were P.IIc<sup>+</sup>; P.IIa<sup>+</sup>, P.IIac<sup>+</sup>, P.IIaf<sup>+</sup>, P.IIi<sup>+</sup>, etc.; variants occurred later (Table II).

tained from two men; P.IIf<sup>+</sup> and P.IIh<sup>+</sup> variants were reisolated from only one (subject D). All reisolated gonococci were pilus<sup>+</sup>, and many had pilins that differed from input gonococci in electrophoretic mobility or immunoblotting with a panel of mAbs (not shown), as noted before (30).

Subject D received 10<sup>8</sup> gonococci, exhibited signs and symptoms of acute urethritis within 24 h, and received penicillin after 50 h. 113 colonies were reisolated from his urine or semen (Table II) and nearly all contained protein II<sup>+</sup> gonococci. His first (2-h) post-instillation urine contained only protein II<sup>-</sup>, input-like gonococci (Fig. 2). P.IIc<sup>+</sup> variants predominated in his 22-, 26-, and 46-h urine specimens, while P.IIf<sup>+</sup> and P.IIaf<sup>+</sup> variants abounded in both the 50-h urine and a corresponding semen specimen (50S; Fig. 2 and Table II) collected just before penicillin administration. That semen specimen contained gonococci with nine different P.II phenotypes; most were P.IIc<sup>+</sup> or P.IIaf<sup>+</sup>. The remainder included a few each of P.II<sup>-</sup> and six other protein II<sup>+</sup> phenotypes.

Most gonococci that were reisolated from subject E were P.IIc<sup>+</sup> plus a few other protein II<sup>+</sup> phenotypes (Table II). For subject F, very few organisms were reisolated from one urine, and they were mostly P.IIc<sup>+</sup>.

TABLE II  
*Protein II Phenotypes of Gonococci Reisolated from the  
Three Experimentally Infected Males*

Subject	Protein II phenotype	Hours after intraurethral instillation							
		2	22	26	46	50	50S	64	98
D (10 <sup>8</sup> )	II <sup>-</sup>	5	1		3		4		
	IIa <sup>+</sup>			1	2		3		
	IIc <sup>+</sup>		6	2	9	3	19		
	IIf <sup>+</sup>					1	4		
	IIh <sup>+</sup>					1			
	IIi <sup>+</sup>						2		
	IIac <sup>+</sup>		1		4		3		
	IIaf <sup>+</sup>				5	7	26		
	IIag <sup>+</sup>						2		
	IIcf <sup>+</sup>						1		
	E (10 <sup>8</sup> )	II <sup>-</sup>	4						
IIa <sup>+</sup>								2	
IIc <sup>+</sup>					15			2	29
IIi <sup>+</sup>					1				1
IIac <sup>+</sup>					1			1	2
IIai <sup>+</sup>					2				1
IIci <sup>+</sup>					2				1
IIci <sup>+</sup>					1				1
C (10 <sup>4</sup> )	IIa <sup>+</sup>				1				
	IIc <sup>+</sup>				7				
	IIac <sup>+</sup>				3				

Subjects D, E, and C each received intraurethral instillations of gonococci (10<sup>4</sup> or 10<sup>8</sup>, as noted). Their first post-instillation urines were collected after 2 h, with subsequent urine samples collected at the times indicated. A semen specimen was also obtained 50 h after gonococcal instillation from Subject D (50S).

### Discussion

Male volunteers developed typical gonorrhea when they received intraurethral instillations of mostly pilus<sup>+</sup> protein II<sup>-</sup> gonococci. The organisms shed in their urine and semen were all pilus<sup>+</sup>; virtually all were protein II<sup>+</sup>. These *in vivo* protein II<sup>+</sup> variants produced one or two of five different protein II species, with P.IIc expressed most frequently. In contrast, P.IIa<sup>+</sup> were the most common variants of input gonococci passaged *in vitro*, where P.IIc<sup>+</sup> variants were rarely spawned. Nine different protein II phenotypes, including P.II<sup>-</sup>, were seen among organisms reisolated from one man; P.IIc<sup>+</sup> variants predominated early in his infection while P.IIc<sup>+</sup> and P.IIaf<sup>+</sup> organisms prevailed later. The results strongly suggest that protein II expression is a key item for gonococci residing in the male urethra, but they do not point to a defineable pathogenic function for these outer membrane proteins. Instead, they raise several questions, including those that follow.

Does the population of gonococci shed in urine and semen accurately reflect the organisms that reside in the infected male's lower genitourinary tract? Or do they comprise a special subpopulation that is easily flushed from the urethra, while organisms of different phenotypes are retained on and, perhaps, within urethral epithelial cells? Do the P.IIc<sup>+</sup> organisms in early urines represent loosely adhering, readily shed forms, while P.II<sup>-</sup> and P.IIaf<sup>+</sup> variants are intracellular and tightly adherent to urethral cells, respectively, and thereby occur in later urines and semen? Clear answers would require invasive techniques, such as urethral mucosal biopsy. Here, we omitted even gentler procedures, such as collecting specimens with intraurethral swabs to avoid factitious damage to urethral epithelium that might influence the infectious scenario and inflammatory responses. If a discrete population of gonococci, such as P.II<sup>-</sup> phenotypes, were to either attain intracellular locales or adhere very tightly to the urethral mucosa, they would likely be underrepresented in urinary and semen fluids before urethral inflammation and exfoliation of epithelial cells. But gonococcus-laden epithelial cells would likely exfoliate if damaged directly by bacteria or by the host's inflammatory response, would be shed in the later urine, and would lyse on solid growth medium to release their intimately associated bacteria. Ejaculation and urination differ markedly in their rheological characteristics, and ejaculation could detach organisms other than those flushed out by urination; this might explain the spate of P.IIaf<sup>+</sup> variants in semen.

Are P.II<sup>-</sup> gonococci *per se* pathogenic? The paucity of P.II<sup>-</sup> phenotypes among reisolated gonococci suggests that they are not and that urethral infections were generated by the P.II<sup>+</sup> gonococci that partially comprise or are spawned by input organisms. However, P.II<sup>-</sup> phenotypes "reappeared" in appreciable numbers in subject D late in his infection (46 and 50 h). Such protein II<sup>-</sup> reisolates could be surviving protein II<sup>-</sup> input organisms, or they might have arisen *in vivo* from P.II<sup>+</sup> variants. Primer extension analysis of the late-appearing P.II<sup>-</sup> reisolates revealed that not all of their P.II transcripts had the same number of pentameric (CTTCT) repeats as the analogous transcripts in P.II<sup>-</sup> input organisms (data not shown). When a P.II gene switches off-on-off, its transcripts typically have different numbers of CTTCT repeats in the two off, out-of-frame configurations (Swanson, J., unpublished results). Because the analogous P.II transcripts of input vs. reisolated P.II<sup>-</sup> gonococci differed in their numbers of repeats, these P.II<sup>-</sup> organisms appear to be genealogically separated by an intervening P.II<sup>+</sup> stage. That observation suggests

that P.II<sup>-</sup> reisolates that appeared in later urine specimens arose from P.II<sup>+</sup> parents. However, P.II transcripts can undergo "silent" changes in their CTTCT repeat region, switching from one off out-of-frame configuration to another without a discernible on in-frame phase (unpublished results). That finding supports a notion that P.II<sup>-</sup> organisms, if they were unable to switch to P.II<sup>+</sup>, would not cause urethritis despite their pilus<sup>+</sup> nature. But the experiment cannot be done, currently, because the appropriate, irreversibly P.II<sup>+</sup> and P.II<sup>-</sup> mutants are not available.

What is the role(s) of a protein II on gonococci? Protein II<sup>+</sup> in vitro exhibit striking aggregation, and this might enhance colonization in vivo if it promotes the retention of newly multiplied organisms at infected mucosal loci. In addition, several studies have demonstrated heightened attachment of protein II<sup>+</sup> gonococci to eukaryotic cells in vitro (12-15). One report suggests that, after their attachment to HeLa cells, some P.II<sup>+</sup> variants are detached more readily than others (15). The early occurrence of P.IIc<sup>+</sup> cells suggests that they might be such variants; but they do not seem to detach from tissue culture cells in vitro more readily than P.IIa<sup>+</sup> organisms (Swanson, J., unpublished data). Such phenomena undoubtedly mirror the direct interactions between gonococci and eukaryotic cells and those between gonococci; the latter may generate various-sized aggregates of gonococci that behave differently to the fluid and peristaltic dynamics in the urethra.

What accounts for disparate frequencies of occurrence for P.IIa<sup>+</sup> and P.IIc<sup>+</sup> variants in vivo vs. in vitro? The relative paucity of P.IIa<sup>+</sup> phenotypes among the reisolates is not due to their lacking the gene for P.IIa, as shown clearly by the abundant P.IIa<sup>+</sup> phenotypes that arise from other P.II<sup>+</sup> reisolates when they are serially propagated in vitro (data not shown). Rather, it must reflect either the preferred expression or the enhanced survival of variants that express a protein II other than P.IIa. Relatively little is known about either the comparative survival abilities of different P.II variants in vitro (12) or their differential expression of their P.II repertoire. Although all protein II genes are switched on-off by gain or loss of pentameric repeats in its leader peptide-encoding portion (26, 27), different *P.II* genes switch on-off at very different frequencies among cells in a given strain (19, 32, Swanson, J., unpublished results). Factors that promote these sequence changes in one or another protein II gene have not been identified, nor is the mechanism defined; but P.II switching occurs in recombination-deficient gonococci (Swanson, J., and J. M. Koomey, unpublished results).

### Summary

Gonorrheal urethritis was induced in three males by intraurethral instillation of predominantly pilus<sup>+</sup> protein II<sup>-</sup> gonococci. Virtually all gonococci reisolated from the infected men exhibited protein II<sup>+</sup> phenotype. The reisolated gonococci expressed five distinct outer membrane protein II species. Protein IIc<sup>+</sup> organisms predominated in urines of all three subjects, but variants expressing this particular protein II were rarely spawned in vitro by input organisms. Protein IIc<sup>+</sup> gonococci appeared early in one man's infection; they were joined later by variants that displayed eight other protein II phenotypes, including protein II<sup>-</sup>. These results show that input protein II<sup>-</sup> gonococci are supplanted by protein IIc<sup>+</sup> variants during incipient gonorrheal urethritis. As infection progresses, a broader variety of protein II<sup>+</sup> variants appears.

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