Isolation and Characterization of a Rough Colony Type of *Neisseria gonorrhoeae*

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A new colony type of *Neisseria gonorrhoeae* was detected in the primary cultures from 8 of 180 men with gonococcal urethritis. This colony type contrasts with those previously described by having a rough and irregular surface. In six of the eight cases, the rough form predominated. The distinctive morphology of the rough colony variant could be maintained indefinitely by selective subculture. By electron microscopy, organisms taken from rough colonies of each of the eight isolates were piliated. Antimicrobial susceptibilities of type 1 and rough clones derived from the same patients were identical for ampicillin, penicillin, tetracycline, and spectinomycin. After inoculation of rough colonies into subcutaneous chambers in mice and guinea pigs, type 1 colonies predominated in cultures of material obtained from the chambers. This new piliated colony type of *N. gonorrhoeae* may provide an opportunity to investigate factors other than pili that contribute to gonococcal virulence.

In spite of the frequency with which *Neisseria gonorrhoeae* causes human disease, our understanding of the factors responsible for gonococcal virulence is incomplete. In 1963, Kellogg et al. (9) described four distinct colonial types of *N. gonorrhoeae*. Types 1 and 2 predominated in fresh clinical isolates, but after repeated nonselective subcultures colony types 3 and 4 became predominant. These workers showed that, after 720 selective transfers, type 1 and 2 colonies were virulent for human volunteers, whereas type 3 and 4 colonies remained avirulent after 440 transfers (8). Soon after this, investigators in two laboratories reported the presence of pili (fimbriae) on the surface of type 1 and 2 gonococci and their absence on type 3 and 4 gonococci (7, 15).

In 1972, we observed a gonococcal isolate that did not conform to the usual colonial morphology. On the primary culture, the colonies were small, rough, and irregular. This appearance persisted even after laboratory passage on several different media. As a result of this finding, we have studied the colonial morphology of *N. gonorrhoeae* isolated from 180 men with gonococcal urethritis. The distinctive rough colonial form was observed on primary isolation in eight cases. This rough colony type possessed pili and could be maintained in vitro by selective passage for indefinite periods.

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MATERIALS AND METHODS

Study population and initial isolations. All men with urethritis attending the DeKalb County Venereal Disease Clinic between December, 1973, and February, 1974, were studied. After a specimen was obtained from the urethra for Gram staining, a small, cotton-tipped swab was used to inoculate selective and nonselective culture media. (For this study, only the colonies on the nonselective medium were examined.) This medium contained GC agar base (Baltimore Biological Laboratory, Cockeysville, Md. [BBL]) enriched with the chemically defined IsoVitalX (BBL) supplement, 10 ml/liter, without hemoglobin or antibiotics. After 20 h of incubation at 35°C under candle extinction, culture plates were examined at ×25 magnification under a Cycloptic dissecting microscope (American Optical Corp., Buffalo, N.Y.) outfitted with a matte-ground opal mirror.

Bacteriological techniques. All isolates of *N. gonorrhoeae* were identified on the basis of Gram stain, oxidase reaction, and sugar fermentation pattern by using the modified rapid technique (2, 10). For the rough isolates, additional sugar fermentation tests utilizing cystine Trypticase agar and Mueller-Hinton agar (BBL) were performed. Direct fluorescent antibody testing of gonococci from rough colonies was done with the GONO-tect (FA) system (Abbott Laboratories, North Chicago, Ill.). Fluorescent antibody testing, electron microscopy, and selective subcultures were carried out by selecting individual colonies with the dissecting microscope.

For inoculation of plates for determination of sugar fermentation patterns, saline agglutination, and antimicrobial susceptibility testing, this was not feasible, due to the large numbers of organisms required; however, serial selective subcultures were done to achieve maximal stabilization of colonial types prior to testing. Saline agglutinability (9) was determined by inoculating two loopfuls of a 20-h culture into a tube containing 2 ml of 0.85% NaCl, followed by gentle shaking and observation for clumping. The colonial morphology of rough isolates was also examined after 20 h of incubation on a medium containing GC medium base (Difco Laboratories, Detroit, Mich.) and a defined supplement (9).

Electron microscopy of whole mounts. After 20 h of incubation on GC agar base with IsoVitaleX, individual colonies were suspended in 0.85% NaCl. A drop of this suspension was placed on a carbon-reinforced, Formvar-coated copper grid, blotted dry with filter paper, stained with 2% uranyl acetate (pH 3.8), and washed with sterile distilled water. The samples were examined for the presence of pili with a Philips model 200 transmission electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N.Y.).

Antimicrobial susceptibility testing. Minimal inhibitory concentrations were determined for ampicillin, penicillin, tetracycline, and spectinomycin. For these studies, rough and type 1 clones derived from the same isolate were tested simultaneously. After 24 h of incubation under CO₂ at 35°C on chocolate agar (Difco) supplemented with 1% IsoVitaleX, a suspension of organisms was made in Mueller-Hinton broth and adjusted to 10⁷ colony-forming units/ml with a light-scattering photometer (AutoBac 1, Chas. Pfizer & Co., Inc., New York, N.Y.). This suspension was then diluted to 10⁶ colony-forming units/ml in Mueller-Hinton broth and inoculated onto supplemented, antibiotic-containing chocolate agar plates with a Steers replicating device. After the surfaces of the plates had dried, they were incubated at 35°C under candle extinction for 24 h. The minimal inhibitory concentration was read as the lowest concentration of antimicrobial agent at which no growth occurred. A faint haze due to inoculum was ignored. To assure that the colonial type present on the antibiotic-containing plates was the same as the type originally inoculated, subcultures were made onto a translucent, antibiotic-free medium from the plate containing the highest concentration of antibiotic that still permitted growth.

Animal inoculations. Type 1 and rough colonies derived from the same primary isolates were inoculated into subcutaneous chambers in guinea pigs and mice (1, 13). Droptainer bottles (Dougherty Brothers Co., Buena, N.J.) with additional openings were surgically implanted into the dorsolateral flank of adult Hartley strain guinea pigs. Silicone rubber tubing chambers with an internal diameter of 3.2 mm (New Brunswick Scientific Co., New Brunswick, N.J.) were implanted dorsolaterally in adult, pathogen-free ICR Swiss mice. For all animals, chamber insertion was carried out at least 2 weeks prior to bacterial challenge.

For chamber inoculation, 20-h cultures of *N. gon-*

orrhoeae were used. Cells were suspended in Eagle minimal essential medium containing Earle balanced salt solution. The suspension was adjusted to an optical density of 0.3 at 530 nm (approximately 10⁸ colony-forming units/ml), and 0.1 ml of this suspension was injected percutaneously into the chamber. Infection was documented by the periodic removal of 0.05 ml of chamber fluid. This fluid was inoculated onto GC agar base medium. *N. gonorrhoeae* was identified as indicated above.

RESULTS

Occurrence and characterization of the rough colony form of *N. gonorrhoeae*. Colony type 1 or 2 predominated in most of the primary isolates (Table 1). In one case, an intermediate form that has previously been designated by Kellogg et al. as type 21 (8) was predominant. However, in 8 of the 180 primary cultures from men with urethritis, a distinctive rough colony form was observed. In six of these cases, the rough form was the predominant colony type in the primary isolate, with scattered type 1 and 2 colonies also present. In the other two cases, 90% of the colonies were type 1, and the remaining 10% were rough. Figure 1 shows type 1, type 2, and rough colonies selectively propagated from isolate 263. The irregularity of the surface of the rough colony is apparent. The rough colonies varied in size according to the isolate. Some were similar in size to type 1 colonies, and others (e.g., no. 161) were considerably smaller.

All rough colony forms gave typical reactions for *N. gonorrhoeae* in the direct fluorescent antibody procedure and in sugar fermentation tests. To determine if the supplement used in the medium was a factor in the colonial appearance, rough colonies of each strain were subcultured onto a medium containing the defined supplement described by Kellogg (9). With each strain, the rough colonial form was unchanged on this medium. If excessively moist culture plates were used, the distinction between type 1 and 2 colonies became difficult; however, the rough form remained rough regardless of the moisture content of the medium.

TABLE 1. Predominant colony types of *N. gonorrhoeae* in primary isolates from 180 men with gonococcal urethritis

Colony type	No.
1	121
2	41
1 and 2	9
21 ^a	1
Rough	6
1 and rough	2

^a Intermediate form.

FIG. 1. *Gonococcal colonies on translucent agar medium. (A, B, and C) Type 1, type 2, and rough colonies, respectively, selectively propagated from isolate 263. (D) Rough colonies from isolate 161, with a larger smooth colony contiguous to one of the rough colonies* ($\times 50$).

Saline agglutination. Rough colonies from each of the eight rough isolates agglutinated in saline. Control samples of types 1 and 2 also agglutinated; controls of types 3 and 4 did not agglutinate.

Colonial morphology after selective and nonselective transfer. The rough colonial variant from each of the eight isolates could easily be maintained by selective subculture. If, however, transfers were made nonselectively, the typical smooth type 3 and 4 colonies became predominant after about six passages.

Serial transfers of each rough isolate were made to separate colony types 1 to 4 in addition to the rough form. For each isolate, all of these colony types were separated and identified. In two cases (isolates 9 and 264) the type 3 colonies appeared markedly granular and irregular.

Clinical correlates of colonial morphology. The eight men whose cultures yielded rough colonies were all black men with symptomatic urethritis and positive Gram-stained smears of urethral exudate. Since 114 of the total of 180 isolates were from black men, the occurrence of the rough colony form only in blacks failed to

reach statistical significance (χ^2 , $0.05 < P < 0.1$). Only one of the men had taken antibiotics prior to coming to the clinic. The duration of symptoms, physical findings, and history of past infections were not significantly different from the overall clinic population.

Presence of pili on rough colonies. By electron microscopic examination, pili were demonstrated on organisms taken from rough colonies from each of the eight isolates. Pili were not present on the large, granular type 3 colonies derived from isolates 9 and 264.

Antimicrobial susceptibility of rough colonies. Minimal inhibitory concentrations of type 1 and rough colonies derived from the same primary isolates were not significantly different for ampicillin, penicillin, tetracycline, and spectinomycin.

Experimental infection with type 1 and rough colonies. Both type 1 and rough colonies from each isolate tested produced an infection in subcutaneous chambers in guinea pigs. After inoculation of type 1 colonies, the predominant colony type recovered from the animal was always type 1, with occasional rough colonies

observed. After inoculation of rough colonies, however, the major colony type recovered from the chamber after 1 week was rough in only two of eight isolates; after 4 weeks, type 1 colonies predominated in the cultures from every chamber (Table 2).

In the mouse chambers, infection was more difficult to establish with the same inoculum. Only four of the eight isolates grew in this system. With these four isolates, both type 1 and rough colonies produced growth. Even after inoculation of rough colonies, however, the predominant colony type recovered was type 1 for three of four isolates.

DISCUSSION

In this study we have identified a new colonial form of *N. gonorrhoeae* which occurred as the predominant colony type in primary isolates from 6 of 180 men with gonococcal urethritis, and which was present in smaller numbers in two additional cases. We have designated these colonies as "rough" variants because their most dramatic morphological features are the highly granular appearance of the surface and the irregularity of the periphery. The diameter of the colonies after 20 h of incubation under standard conditions varies from 0.3 to 0.6 mm, which is similar to type 1 colonies. The rough form could easily be maintained in vitro by using selective transfer. On nonselective passage, large, granular type 3 colonies and pale type 4 colonies became predominant within six transfers. Typical type 1 and 2 colonial forms could be isolated by selective subculture from each of the rough isolates.

Following the initial description of four gonococcal colony types in 1963 by Kellogg et al. (9), workers in Denmark identified a fifth colony

type in a laboratory strain (6). Their type 5 differs from the rough colony forms in several important respects: (i) type 5 is larger and often exhibits concentric rings; (ii) type 5 is non-piliated, whereas the rough forms are pilated; and (iii) type 5 usually arises in laboratory strains, rather than in clinical isolates (unpublished observations).

In a study of colonial morphology in primary isolates (14), Sparling and Yobs noted small, rough colonies in about 6% of cases, which they speculated might have resulted from excessive plate moisture. Other observers noted "untypable" forms in some clinical cases, but these were not further characterized (6). Unusual colonial forms also occur when antibiotics are included in the culture media (5), but these changes revert after subculture on antibiotic-free media. The rough form that we have described maintained its characteristic morphology on translucent culture media containing different supplements on both moist and dry plates. Recently, a variant of type 1 colonies (type 1') has been described in two laboratory stains (4). It differs from typical type 1 colonies only in being darker and slightly granular and is apparently identical to the dark type 1 colony described by Brown and Kraus (3).

An awareness of the rough colony type will assist in the laboratory diagnosis of gonococcal infections. An initial step in the identification of *N. gonorrhoeae* after growth on translucent media is the colony appearance as seen with a dissecting microscope outfitted with a matte-ground opal mirror (11). The rough colony type differs considerably from previously described gonococcal colony types, and recognition of *N. gonorrhoeae* in the primary culture could be missed if the observer were not familiar with the rough colony type.

N. gonorrhoeae colony type definition has been valuable in studies of virulence. Shortly after four gonococcal colony types were described, two of the four (types 1 and 2) were found to be virulent in human inoculation studies (9). These same two colony types predominated in primary cultures from patients with gonococcal urethritis (9, 14), cervicitis (14), proctitis (11), and pharyngitis (17). The other two colony types (types 3 and 4), developed during in vitro passage of the gonococcus and after 69 passes, were unable to induce gonococcal urethritis (9). Type 1 and 2 colonies, and not type 3 and 4 colonies, possess extracellular structures known as pili or fimbriae (7, 15). Pili, therefore, correlate with virulence, although a cause-and-effect relationship remains to be established. Pili may function in the adherence of gonococci to epithelial cells.

TABLE 2. Predominant colony type recovered from animal chambers 1 and 4 weeks after inoculation of rough colonies

Isolate	Animal system			
	Guinea pig		Mouse	
	1 week	4 weeks	1 week	4 weeks
9	Rough	1	1 > rough	1
047	1	1	NG ^a	NG
161	1	1	Rough	ND ^b
204	1	1	NG	NG
263	1	1	NG	NG
264	Rough	1	NG	NG
394	1	1	1	NG
405	1	NG	1	ND

^a NG, No growth.

^b ND, Chamber extruded.

The finding of the rough gonococcal colony type in the primary culture from certain patients with gonococcal urethritis suggests that this colony type, in addition to types 1 and 2, is virulent for humans. The rough colony type can also infect the small animal subcutaneous chamber model. Despite these associations of the rough colony type with virulence, type 1, rather than rough, colonies were much more likely to be found in the primary culture of men with urethritis, and type 1 colonies were also much more likely to be found in cultures from small animals infected with either the rough or type 1 colony type. This latter finding is evidence that type 1 colonies have a distinct advantage in the persistence of gonococcal infection in small animals. Since both type 1 and rough colonies have pili, factors other than the presence of pili must be involved in the *in vivo* replacement of rough by type 1 colonies. The possibility that factors other than pili are important in gonococcal virulence has also been suggested by the recent finding that a large inoculum of two pilated *N. gonorrhoeae* isolates were avirulent in chimpanzees that subsequently developed gonococcal urethritis with a smaller inoculum of a third *N. gonorrhoeae* isolate (12). Factors other than pili are also important in the interaction of the gonococcus with leukocytes (16). Further studies that identify surface structures, biochemical reactions, or antigens of type 1 colonies that are different or lacking on rough colonies could uncover other *N. gonorrhoeae* virulence correlates.

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