

## Loss of Pili and Decreased Attachment to Human Cells by *Neisseria meningitidis* and *Neisseria gonorrhoeae* Exposed to Subinhibitory Concentrations of Antibiotics

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Recent evidence has suggested that surface structures of pathogenic bacteria, which are important in attachment to human mucosal surfaces, may be absent on bacteria grown in the presence of subinhibitory concentrations of antibiotics. We studied the effect of tetracycline and penicillin on meningococcal and gonococcal pili. Subinhibitory concentrations of tetracycline and penicillin were found to markedly reduce the number of pili per meningococcus or gonococcus and the percentage of meningococci or gonococci with pili, as determined by negative-staining electron microscopy. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane preparations suggested that tetracycline decreased expression of pili by inhibiting synthesis of pilin subunits. In contrast, pilin subunit synthesis was unaltered by penicillin, suggesting a defect in assembly of pilin subunits or in anchoring of assembled pili. The decrease in the number of pili that occurred with subinhibitory concentrations of both tetracycline and penicillin was accompanied by a marked decrease in the ability of the organisms to attach to human cells. Gonococci or meningococci removed from the influence of subinhibitory concentrations of the antibiotics regained piliation, and attachment returned to levels near those of controls. The expression of meningococcal and gonococcal pili may be affected by factors that influence synthesis of pilin subunits or factors that interfere with the assembly and anchoring of pili in the outer membrane.

The ability of bacteria to attach to mucosal surfaces depends upon or is highly correlated with their ability to cause disease. This is especially true with pathogenic *Neisseria* spp. Pili facilitate the attachment of gonococci to human fallopian tube mucosa (19), and piliated gonococci of colony types 1 and 2 produce gonorrhea in human volunteers, whereas nonpiliated gonococci do not (14). Although there are no comparable data regarding the ability of piliated meningococci to cause disease, isolates of *Neisseria meningitidis* from the blood or cerebrospinal fluid have pili on primary isolation (6, 29), piliated meningococci attach to human cells in greater numbers than nonpiliated meningococci (5, 25, 29), and piliated meningococci attach in greatest numbers to cells from the nasopharynx, the presumed site of meningococcal invasion (25, 28, 29).

Recent evidence has suggested that antibiotics in concentrations that fail to inhibit bacterial growth nonetheless may have marked effects on the structural characteristics of the bacteria. Little attention, however, has been directed to the effect of these structural changes on pathogenicity. It is possible that the structural changes produced by subinhibitory concentrations of antibiotics may alter virulence without significantly altering growth of the microorganisms. To assess the possible effect of subinhibitory concentrations of antibiotics on virulence of pathogenic *Neisseria* spp., we studied the effect of selected antibiotics on surface structures, pili, that are important mediators of meningococcal and gonococcal attachment to human cells. Four major

effects were demonstrated: (i) subinhibitory concentrations of penicillin and tetracycline inhibited expression of assembled pili on the surface of meningococci and gonococci; (ii) attachment of meningococci and gonococci to human cells was significantly reduced by growth of the organisms with subinhibitory concentrations of tetracycline and penicillin; (iii) tetracycline inhibited synthesis of pilin subunits, whereas penicillin appeared to cause loss of pili by blocking assembly or anchoring of pili; and (iv) with removal of meningococci and gonococci from environments containing subinhibitory concentrations of the antibiotics, the degree of piliation and attachment returned to levels near those of controls.

### MATERIALS AND METHODS

**Media.** Solid media for cultivating microorganisms and performing colony counts consisted of chocolate agar plus 1% (vol/vol) IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and gonococcal agar base (Difco Laboratories, Detroit, Mich.) plus 2% (vol/vol) IsoVitaleX (GC Iso agar). The medium used for tube dilution sensitivities was GC broth plus 2% (vol/vol) IsoVitaleX. The medium used for suspending microorganisms was Eagle minimal essential medium containing Earle salts, L-glutamine (GIBCO Laboratories, Grand Island, N.Y.) and 0.05 M N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid buffer (pH 7.45).

**Microorganisms.** Colony type 1 (T1, piliated) *Neisseria gonorrhoeae* strains 2686, F62, and FA5 were used. Three piliated strains of *N. meningitidis*, 269B, 1643, and 34NP, were also employed. Isogenic transparent and opaque clones of each strain were derived by single-colony passage. The

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source and characteristics of these strains have been previously reported (19, 30).

**Electron microscopic techniques.** Pili were identified by negative-staining electron microscopy (20), and the negatively stained preparations were used to determine the percentage of meningococci or gonococci with pili and the mean number of pili per diplococcus as previously described (20).

**Assessment of antimicrobial effects on pili.** The minimal inhibitory concentration (MIC) of tetracycline or penicillin for each clone of the three piliated gonococcal or meningococcal isolates was determined by using twofold dilutions of the antibiotic in GC broth with 2% IsoVitaleX. These solutions were inoculated with a suspension of organisms that yielded a final concentration of  $\sim 2 \times 10^5$  CFU per ml, and the mixture was incubated for 20 h in 3% CO<sub>2</sub> at 36°C with gentle shaking. Tetracycline (crystalline tetracycline; Sigma Chemical Co., St. Louis, Mo.) and penicillin (penicillin G; Sigma or Ely Lilly & Co. Indianapolis, Ind.) were chosen because they act on gonococci and meningococci at different sites. After 20 h of growth, the effect of subinhibitory concentrations (1/2 MIC or less) of the antibiotics on the number of pili per diplococci and on the percentage of diplococci with pili was determined by electron microscopic examination of negatively stained preparations as previously described (20). These data were compared with the numbers and percentages of pili found on gonococci or meningococci incubated under the same conditions, but in the absence of antibiotics.

To assess the effects of subinhibitory concentrations of antibiotics on colony phenotype, similar studies were performed with an agar plate dilution method (10). Initial colony type was confirmed visually with a stereomicroscope at the time of inoculum preparation, and plates that contained <95% of colonies of the desired phenotype were not used. The designations of gonococcal colony types (T1, T4, opaque, transparent) and meningococcal colony types (opaque, transparent) were based on characteristics previously described (19, 30). GCISO agar plates containing no antibiotic or twofold dilutions of penicillin and tetracycline were prepared and inoculated with 0.001 ml of a suspension containing  $\sim 10^6$  CFU per ml. The percentage of colonies remaining as the original colony type was determined by classifying at least 100 colonies from antibiotic-containing and control plates after 20 h of growth.

**Assessment of antimicrobial effects on pilin subunits.** Outer membrane preparations from piliated clones of *N. gonorrhoeae* strain F62 were obtained as previously described (30). Briefly, GCISO agar plates were inoculated with T1, transparent, or opaque clones of gonococcal strain F62. GCISO agar plates containing 1/2 MIC of either tetracycline or penicillin were inoculated in parallel experiments. GCISO plates were incubated in a humid ambient atmosphere with increased (3%) CO<sub>2</sub> for 20 h at 36°C. Gonococci were harvested from the plates with a glass rod and suspended in 20 ml of 0.1 M phosphate buffer (pH 7.4). This suspension was centrifuged at  $12,100 \times g$  for 30 min at 4°C. The supernatant was removed and discarded. The pellet was suspended in 20 ml of 0.1 M Tris buffer containing 0.2 M NaCl and 0.02% sodium azide (pH 8.0). The suspension was incubated in a shaking water bath for 90 min at 43°C. The supernatant from two low-speed centrifugations ( $7,000 \times g$  for 10 min,  $22,000 \times g$  for 20 min) was ultracentrifuged at  $175,000 \times g$  for 90 min. The pellet was suspended in 1 ml of the 0.1 M Tris-sodium azide buffer described above. The protein concentration of the outer membrane preparations was determined by the Coomassie blue method of Bradford

(3) and adjusted by dilution to give a final protein concentration of 1.0 mg/ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a multiphor slab gel apparatus (LKB Instruments, Rockville, Md.), 10% horizontal thin-layer acrylamide gels, and a modification of the Tris-glycine buffer system of Laemmli (30). Protein samples were prepared by diluting 20  $\mu$ l 1:1 with sample buffer containing 0.1 M Tris-hydrochloride (pH 6.8), 2% (wt/vol) SDS, 2% (vol/vol) 2-mercaptoethanol, 20% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue. The samples were heated at 56°C for 30 min or boiled at 100°C for 3 min. Electrophoresis in Tris-glycine buffer with 0.1% SDS was carried out at a constant current of 30 mA. On completion of the electrophoresis the gel was removed and fixed overnight in a solution of 50% methanol and distilled water. The gels were stained for protein by a modification of the silver staining method of Wray et al. (34). The locations of pilin subunits in these gels were determined by molecular weight (17,000 to 22,000), by reaction on Western transfer blot (33) with antibody (kindly supplied by Gary Schoolnik, Stanford University) to a region of the pilin subunit common to gonococcal pili (26), and by the absence of the bands in preparations from isogenic clones of T4 colonies.

**Quantitation of meningococcal and gonococcal attachment to human cells.** An assay for quantitation of attachment of microorganisms to human epithelial cells was developed by us as previously described (29). Careful attention was directed at developing a method of inoculum preparation that minimized clumping of organisms. In most inocula prepared by this method and examined by phase microscopy, 97% or more of the bacterial units (particles containing one or more bacteria; comparable to CFU) contained one to three bacteria, and none of the units contained more than six bacteria. An inoculum was not used if, in 100 units counted, more than 4% of the units contained four to six diplococci or if any unit contained more than six diplococci. Because inoculum colony counts of the same optical density varied between  $1 \times 10^7$  and  $5 \times 10^7$  CFU/ml from day to day, the results of the attachment assay were expressed as the percentage of the inoculum attached. This percentage was calculated from the number of gonococci attached per cell relative to the number available to attach per cell when the latter ratio was approximately 100:1.

**Statistical analysis.** The results were derived from three or more experiments with each variable. The significance of differences between the means of two variables was determined by using Student's *t*-test or Student's *t*-test with paired values and a two-tailed hypothesis.

## RESULTS

**Effect of subinhibitory concentrations of tetracycline and penicillin on growth and colony phenotype of meningococci and gonococci.** Colonies of meningococci and gonococci grown on agar media containing 1/2 MIC of tetracycline were slightly smaller than colonies grown on media without antibiotics. Colony size did not appear to be affected by 1/4 MIC or less of tetracycline and 1/2 MIC or less of penicillin. Growth curves in broth with gonococcal strain F62 (T1 OP) were performed without or with subinhibitory concentrations (1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 MIC) of penicillin and tetracycline. These subinhibitory concentrations of penicillin and tetracycline did not significantly alter growth.

For each of the meningococcal and gonococcal strains, the degree of opaque or transparent colony type or rate of transition from one colony type to another were not affected

by penicillin or tetracycline. The percentage of T4 colonies of the gonococcal strains increased slightly (change of mean percentage from < 5% to 12.9%) ( $P = 0.06$ ) with growth on 1/2 MIC of tetracycline. Similar percentages of T4 colonies were seen with growth on 1/2 MIC of penicillin and when each isogenic opaque or transparent gonococcal clone was examined separately. Thus, subinhibitory concentrations of tetracycline and penicillin had little effect on growth or colony phenotype of meningococci and gonococci.

**Effect of subinhibitory concentrations of tetracycline and penicillin on number of pili per meningococcus or gonococcus.** The electron microscopic appearance of meningococci on primary isolation from clinical specimens and in control cultures is shown in Fig. 1A. Note the multiple pili that emanate from the surface of the organisms. Gonococci on primary isolation from the urethra, cervix, or pharynx have a similar appearance. In contrast, the electron micrograph shown in Fig. 1B demonstrates a portion of the surface of a meningococcus grown in 1/4 MIC of tetracycline. Note the marked decrease in the number of pili which was typical of such preparations.

The effect of subinhibitory concentrations of antibiotics (1/2 MIC) on the number of pili per diplococcus of three meningococcal and three gonococcal strains is shown in Table 1. Meningococci and gonococci possess approximately 20 pili per diplococcus. When grown in agar or in broth containing 1/2 MIC of tetracycline or penicillin, piliated

meningococci and gonococci lost a significant number of pili per diplococcus. Similar results were obtained when each strain or each clone was examined separately and when the piliated meningococcal and gonococcal strains were grown with 1/4 MIC of penicillin or tetracycline. However, concentrations of penicillin less than 1/64 MIC and concentrations of tetracycline less than 1/32 MIC had no effect on the number or the electron microscopic characteristics of pili. Those endpoints of subinhibitory antibiotic effects were similar regardless of the susceptibility (MIC) of the strain to the antibiotics.

Subinhibitory concentrations of penicillin and tetracycline that decreased the number of pili also had a discernible effect on the electron microscopic characteristics of the assembled pili seen on the surface of the diplococci and on the outer membrane and outer membrane blebs. The pili were often bent, curved toward the cell surface, and twisted when compared with control preparations. Also in treated preparations, the outer membrane was irregular in appearance (Fig. 1B), and the number of outer membrane blebs observed was increased.

Thus, subinhibitory concentrations of tetracycline and penicillin well below concentrations that affect growth decreased the number of pili on meningococci and gonococci. Further, these subinhibitory concentrations also affected the electron microscopic characteristics of the remaining pili.

**Effect of subinhibitory concentrations of tetracycline and penicillin on the percentage of gonococci and meningococci with pili.** Gonococci grown with 1/2 MIC of tetracycline and meningococci grown with 1/2 MIC of penicillin showed not only a decrease in the number of pili per organism, but also a decrease in the percentage of organisms with pili (Table 2). The reduction in the percentage of meningococci with pili seen with 1/2 MIC tetracycline (Table 2) did not achieve statistical significance. Thus, we found that 1/2 MIC of tetracycline and penicillin reduced the proportion of gonococci and, to a lesser degree, the proportion of meningococci that were piliated, in addition to causing a marked decrease in the number of pili on those organisms that maintained at least some piliation.

**Effect of subinhibitory concentrations of penicillin and tetracycline on gonococcal pilin subunits.** To determine whether the reduction in the percentage and number of pili seen with subinhibitory concentrations of antibiotics was associated with a change in pilin subunits in the outer membrane, we examined the effect of 1/2 MIC of tetracycline and penicillin on pilin subunits of opaque and transparent isogenic clones of *N. gonorrhoeae* strain F62. Pilin subunits of 21,500 molecular weight was present in SDS-PAGE of outer membrane preparations from opaque colonies of untreated piliated (T1) gonococci (Fig. 2). In contrast, pilin subunits were absent or markedly decreased in SDS-PAGE of outer membrane preparations of equal protein concentration from opaque clones of opaque piliated gonococci exposed to 1/2 MIC of tetracycline. Pilin subunits in SDS-PAGE of outer membrane preparations of gonococci exposed to 1/2 MIC of penicillin had identical molecular weights and similar concentrations to that seen in outer membrane preparations from untreated organisms. Similar results were obtained when isogenic transparent clones of piliated (T1) colonies of strain F62 were exposed to 1/2 MIC of tetracycline and penicillin, despite a shift in pilin subunit molecular weight to 20,500. Although both penicillin and tetracycline decreased the number and percentage of attached pili on meningococci or gonococci, only tetracycline appeared to alter production of pilin subunits.

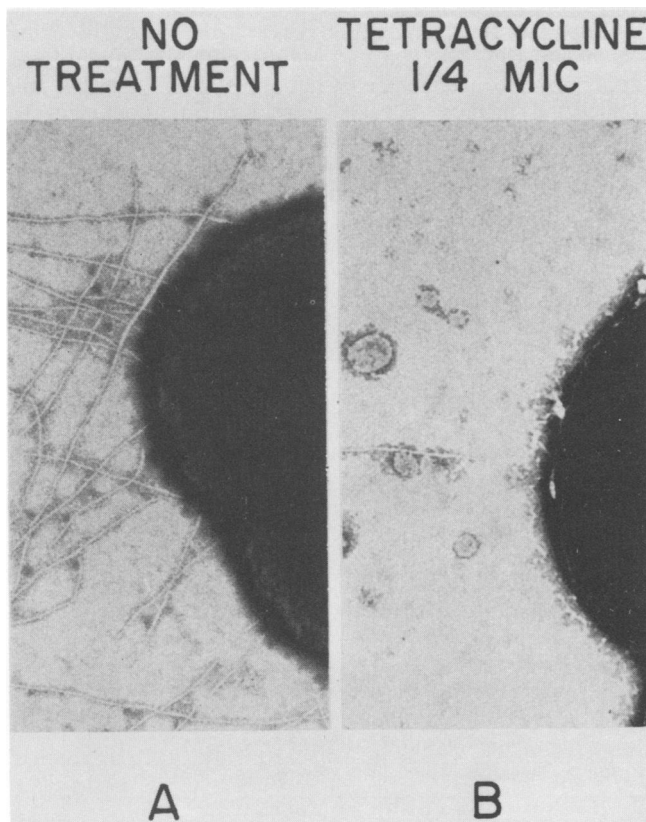


FIG. 1. Transmission electron micrographs of piliated meningococci before and after exposure to subinhibitory concentrations of tetracycline. (A) Piliated meningococci of strain 269B from control preparations. (B) Piliated meningococci of strain 269B grown with 1/4 MIC of tetracycline. The loss of a significant number of pili per organism is evident and was typical for such preparations. Preparations were negatively stained with 3% phosphotungstic acid (pH 7.2) ( $\times 8,212$ ).

TABLE 1. Effect of subinhibitory concentrations of tetracycline or penicillin on number of pili per diplococcus among three strains of *N. meningitidis* and three strains of *N. gonorrhoeae*<sup>a</sup>

Strains	Treatment	Mean no. of pili per diplococcus <sup>b</sup> ± SE			
		agar	P	broth	P
Meningococci	No antibiotic	20.8 ± 1.9		16.0 ± 3.2	
	½ MIC of tetracycline	9.5 ± 3.5	<0.05	7.0 ± 1.5	<0.05
	½ MIC of penicillin	5.7 ± 2.2	<0.005	1.5 ± 0.5	<0.05
Gonococci	No antibiotic	26.3 ± 1.2		21.5 ± 1.0	
	½ MIC of tetracycline	6.0 ± 3.1	<0.001	7.7 ± 5.3	<0.05
	½ MIC of penicillin	10.5 ± 3.2	<0.001	ND <sup>c</sup>	

<sup>a</sup> All comparisons of means of antibiotic and control groups were by Student's *t* test (two sided).

<sup>b</sup> The mean number of pili per diplococcus of the three meningococcal or gonococcal strains was computed for each medium at the same passage.

<sup>c</sup> ND, Not determined.

Tetracycline also appeared to alter the amount of protein I (PI) and the electrophoretic migration characteristics of protein II (PII) (Fig. 2). These changes were not seen with penicillin.

**Effect of subinhibitory concentrations of antibiotics on attachment of meningococci and gonococci to human cells.** Piliated gonococci or meningococci grown in 1/2 MIC of tetracycline attached to human buccal epithelial cells significantly less than gonococci or meningococci that had no treatment ( $P < 0.001$ ) (Fig. 3). Similar results were obtained with 1/2 MIC of penicillin. Brief exposure during the attachment assay of piliated gonococci or meningococci (grown in drug-free media) to the same subinhibitory concentration of tetracycline and penicillin did not significantly affect attachment. In addition, the washing of gonococci grown in the subinhibitory concentration of tetracycline or penicillin did not restore the suppressed attachment activity. Thus, subinhibitory concentrations of antibiotics needed to be present during the period of active bacterial growth and synthesis of pili to affect binding activity.

**Reversal of effects of subinhibitory concentrations of antibiotics on meningococci and gonococci upon subculture to drug-free media.** To determine whether the subinhibitory concentrations of tetracycline and penicillin selected mutants defective in adhering ability, we removed gonococci grown in subinhibitory concentrations of antibiotics to drug-free media for 18 h. The mean number of pili per diplococcus on meningococci and gonococci removed from the effects of subinhibitory concentrations of tetracycline (1/2 MIC) was significantly increased to levels near those of the control (Fig. 4). Likewise, the percentage of piliated gonococci removed from the influence of 1/2 MIC of tetracycline and the attachment of these organisms to human buccal epithelial cells also returned to levels not significantly different from those of the control. Similar results were seen with 1/2 MIC of penicillin. These data indicate that the effects of subinhibi-

tory concentrations of penicillin and tetracycline on pili and attachment are reversible and that a mutant strain defective in adhering ability was not selected.

## DISCUSSION

Attachment of *N. meningitidis* and *N. gonorrhoeae* to human mucosal surfaces appears to be the first step in the pathogenesis of meningococcal and gonococcal infections (19, 29). This attachment involves interaction between gonococcal and meningococcal surface structures (ligands) and human cell surface components (receptors) (4). Information about bacterial ligands responsible for attachment of bacteria to host cell receptors has been successfully applied in the development of vaccines that prevent disease by blocking attachment (13, 27). Thus, it may be critical to understand the nature and mechanism of action of meningococcal and gonococcal ligands that are important in attachment to human mucosal surfaces to develop more effective vaccines.

Studies of interactions between antibiotics and bacteria have focused upon killing or decrease of growth of bacteria. As reviewed by Eisenstein et al. (8), subinhibitory concentrations of several antibiotics also cause inhibition or enhancement of various surface components of bacteria. For example, streptomycin and neomycin increase phospholipid on *Serratia marcescens* (1); in *Escherichia coli*, kanamycin and rifampin inhibit cytoplasmic proteins, whereas tetracycline inhibits outer membrane proteins (12); and clindamycin potentiates opsonization and phagocytosis of *Streptococcus pyogenes* by removal of surface M-protein (11). Other studies have shown that antibiotics induce changes in *E. coli* surface morphology (15), increase the susceptibility of *Proteus mirabilis* to the bactericidal action of serum (31), affect cross-wall thickness in *N. gonorrhoeae* (17) and penicillinase production in *Staphylococcus aureus* (22), and interfere with genetic transfer in *N. gonorrhoeae*, which may involve pili (2).

TABLE 2. Effect of subinhibitory concentrations of tetracycline or penicillin on the percentage of diplococci with pili among three strains of *N. meningitidis* and three strains of *N. gonorrhoeae*<sup>a</sup>

Strains	Treatment	Mean % diplococci with pili <sup>b</sup> ± SE			
		Agar	P	Broth	P
Meningococci	No antibiotic	95 ± 2.2		95 ± 3.5	
	½ MIC of tetracycline	60 ± 20	<0.1	70 ± 20	<0.2
	½ MIC of penicillin	75 ± 2.9	<0.005	5 ± 0	<0.001
Gonococci	No antibiotic	95 ± 2.2		67.5 ± 5.12	
	½ MIC of tetracycline	52.5 ± 15.5	<0.025	7.0 ± 3.74	<0.001
	½ MIC of penicillin	78.8 ± 2.4	<0.001	ND <sup>c</sup>	

<sup>a</sup> All comparisons of means of antibiotic and control groups were by Student's *t*-test (two sided).

<sup>b</sup> The mean percentage of diplococci with pili of the three meningococcal and gonococcal strains was computed for each medium at the same passage.

<sup>c</sup> ND, Not determined.

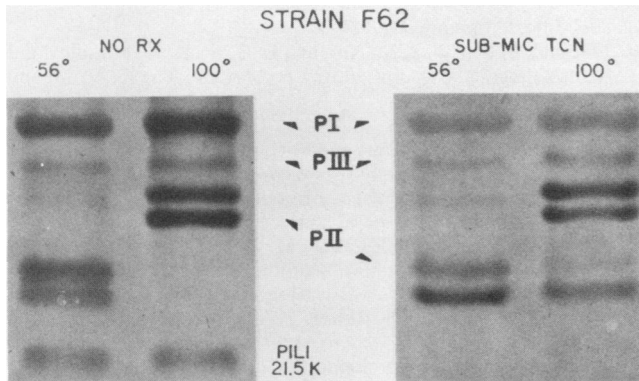


FIG. 2. SDS-PAGE profile (10% [wt/vol] total acrylamide, Wray et al. [34] silver stain) comparing the outer membrane proteins of *N. gonorrhoeae* (strain F62, opaque colony type) grown on medium containing no antibiotics (NO RX) or subinhibitory concentrations of tetracycline (SUB-MIC TCN). Pilin subunits of 21,500 molecular weight were present in the preparation of gonococci grown on medium without antibiotics. Samples were heated at 56°C for 30 min or boiled at 100°C for 3 min. Pilin subunits were not found or were found in decreased concentrations among outer membrane preparations of gonococci grown on medium containing 1/2 MIC tetracycline. Tetracycline also appeared to alter the amount of protein I (PI) and the electrophoretic migration characteristics of protein II (PII). These changes in pilin subunits and outer membrane proteins were not seen with penicillin.

Eisenstein et al. (8) and Svanborg-Eden et al. (32) have shown that antibiotic suppression of mannose-sensitive pili on *E. coli* resulted in decreased attachment to human cells. Edlemann and Gallant (7) studied changes in flagellin, the protomeric subunit of flagella, isolated from *E. coli* that had been grown in subinhibitory concentrations of streptomycin. Streptomycin produced misreading of the CGU and CGC arginine codons and caused illegitimate incorporation of cysteine in the flagellin. Eisenstein et al. (9) found that growth of a streptomycin-resistant *E. coli* strain in subinhibitory concentrations of streptomycin resulted in the production of structurally altered nonfunctional type 1 (mannose-sensitive) pili. They suggest that the aberrant fimbrial protein was caused by misreading of mRNA.

Thus, subinhibitory concentrations of antibiotics with

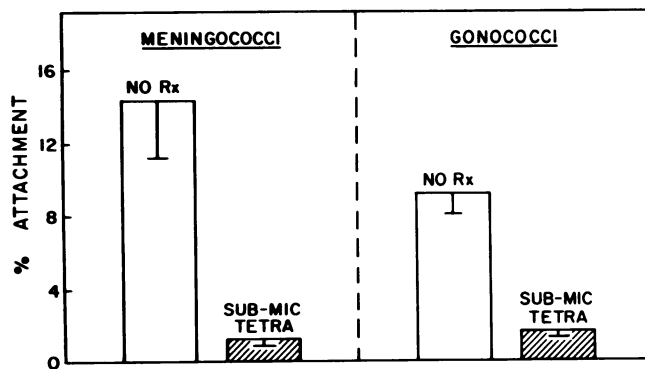


FIG. 3. Effect of subinhibitory concentrations of tetracycline on attachment of meningococci and gonococci to human buccal epithelial cells. As shown by the cross-hatched columns, piliated meningococci, (strain 269B, opaque colony type) and gonococci (strain F62, T1, opaque colony type) grown in subinhibitory concentrations (1/2 MIC) of tetracycline (SUB-MIC TETRA) attached significantly less than organisms that had no treatment (NO Rx) ( $P < 0.001$ ).

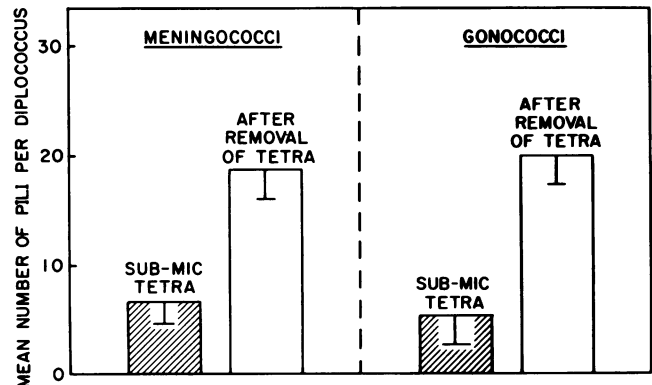


FIG. 4. Reversal of the effects of subinhibitory concentrations of tetracycline (SUB-MIC TETRA) on meningococcal and gonococcal pili upon subculture to drug-free medium. As noted in the open columns, the number of pili on meningococci (strain 269B, opaque colony type) and gonococci (strain F62, T1, opaque colony type) removed from the effects of subinhibitory concentrations (1/2 MIC) of tetracycline was significantly increased to levels near those of controls. The attachment of these organisms to human buccal epithelial cells (data not shown) also returned to levels not significantly different from those of controls.

defined mechanisms of action may be useful probes to study the surface ligands of meningococci and gonococci that are involved in attachment to human mucosal surfaces. Recently, Salit (23) found that subinhibitory concentrations of several antibiotics reduced piliation of meningococci and altered certain cell-associated proteins. Kristiansen et al. (16) noted a remarkable decrease in *in vitro* piliation and adherence to human buccal epithelial cells after exposure to lincomycin and was able to decrease meningococcal colonization in carriers by giving subinhibitory concentrations of lincomycin. In our study, both tetracycline and penicillin in concentrations below the MIC decreased the number and percentage of meningococci and gonococci with pili. This was true for piliated meningococci and gonococci of both opaque and transparent colony phenotypes. The differences in the molecular weight of pilin subunits between isogenic opaque and transparent gonococcal clones noted in our study and previously by others (24) did not influence this loss of piliation.

Despite a marked effect of subinhibitory concentrations of antibiotics on the number of pili and the percentage of gonococci with pili, the percentage of T4 colonies increased only slightly. McGee et al. (18) have shown no consistent relationship of pili to colonial morphology among meningococci and nonpathogenic species of *Neisseria*. Factors other than pili may be responsible for gonococcal T1 colony type; whether these same factors, either alone or in conjunction with pili, are also responsible for gonococcal virulence warrants further investigation.

The mechanisms of loss of piliation caused by subinhibitory concentrations of tetracycline appeared to differ from that caused by subinhibitory concentrations of penicillin. Meningococci and gonococci exposed to inhibitory concentrations of tetracycline produced few pili, and the production of pilin subunits was markedly decreased. In contrast, meningococci and gonococci exposed to subinhibitory concentrations of penicillin produced pilin subunits. These data suggest that subinhibitory concentrations of penicillin may cause a defect in assembly or anchoring of pili in the outer membrane, whereas subinhibitory concentrations of tetracy-

cline inhibit the expression of pili by decreasing the production of pilin subunits. In view of its well-defined mode of action, tetracycline probably inhibits pilin subunit synthesis at the level of the ribosome. Alternatively, tetracycline might indirectly influence chromosomal rearrangements, which appear to be the on-off switch for pilus production (21). The decrease in number and percentage of pili induced by either antibiotic was associated with decreased attachment of the treated meningococci and gonococci to human cells. In addition, tetracycline appeared to cause changes in the SDS-PAGE characteristics of the major outer membrane porin protein (PI) and the surface exposed (PII) proteins. The significance of these changes is unclear and will require further study.

In summary, attachment of pathogenic *Neisseria* spp. to mucosal surfaces appears to be the first step in tissue invasion or damage. Attachment is mediated by pili and possibly other surface components. Our study suggests that subinhibitory concentrations of antibiotics selected for a specific mechanism of action can be used to indicate the components of bacteria that are important in attachment to human cells and can be used to study synthesis and assembly of these components. Further studies with selected antibiotics may help determine the expression, structure, and function of surface components on pathogenic *Neisseria* spp. that are important in attachment to human mucosal surfaces.

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