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Antimicrobial Interference with Bacterial Mechanisms of Pathogenicity: Effect of Sub-MIC Azithromycin on Gonococcal Piliation and Attachment to Human Epithelial Cells

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The effects of subinhibitory concentrations of azithromycin (CP-62,993) on the piliation and attachment properties of *Neisseria gonorrhoeae* were examined. Subinhibitory concentrations of azithromycin significantly reduced the percentage of gonococci that expressed assembled pili on their surfaces by decreasing pilin subunit synthesis and substantially decreased gonococcal adherence to human mucosal cells.

Concentrations of antibiotics lower than those necessary to inhibit growth (sub-MICs) can alter the ability of microorganisms to adhere to epithelial cells, change microbial toxin and enzyme production, and increase microbial susceptibility to host defense mechanisms (19). To evaluate the possible effect of azithromycin on the virulent phenotype of *Neisseria gonorrhoeae*, we studied the effect of sub-MIC azithromycin on the percent piliation of three strains of gonococci and the ability of such sub-MIC-treated gonococci to attach to human cells.

Piliated (T1) clones of *N. gonorrhoeae* 2686, R10 (12, 17, 20), and LOG (a fallopian tube clinical isolate) were grown on GCIso agar as previously described (12, 20). Agar dilution MIC determinations on a series of GCIso plates containing twofold dilutions (concentration range, 2 to 0.015625 μg/ml) of azithromycin (Pfizer Inc., Groton, Conn.) were performed (5, 20). The MICs were 0.0625, 0.125, and 0.5 μg/ml for strains 2686, R10, and LOG, respectively. Microorganisms were harvested from control (no azithromycin) and one-half-, one-fourth-, and one-eighth-MIC plates for studies of piliation and attachment properties.

Negative-staining electron microscopy as described by McGee et al. (11) was utilized to identify piliated organisms. Piliation was judged according to the presence (one or more) or total absence of pili emanating from the surface of the diplococcal pair. Strain R10 control and sub-MIC-treated groups were also examined with gold immunoprobe electron microscopy (16, 17) to detect shortened pili and surface-exposed pilin subunits that could still mediate attachment but might be easily overlooked by negative-staining electron microscopy (anti-R10 pilin antibody was provided by Gary K. Schoolnik). For both methods, 50 diplococcal pairs were examined for each treatment group, and the observer was blinded to the identity of the groups being scored. Chi-square analysis or Fisher's exact test were utilized for the comparison of piliation of sub-MIC-treated groups with that of controls, as appropriate for the data (15). Sub-MIC azithromycin appeared to decrease the percentage of piliated organisms in a significant manner (Table 1) for each of the three strains ($P < 0.030$). The gold immunoprobe studies revealed no evidence of significant organism surface labeling that was not associated with morphologic pili (Fig. 1), which resulted in similar percent piliation figures for immunogold and negative-staining techniques.

TABLE 1. Azithromycin effects on piliation of three strains of *N. gonorrhoeae*

Strain	% Piliation ± SD (no. piliated/no. nonpiliated), significance with:			
	Control[a]	1/8 MIC	1/4 MIC	1/2 MIC
2868	97 ± 6 (193/200)	76 ± 25 (114/150), $P \leq 10^{-6}$[b]	70 ± 29 (139/200), $P \leq 10^{-6}$	27 ± 17 (54/200), $P \leq 10^{-6}$
LOG	100 ± 0 (150/150)	97 ± 6 (145/150), $P = 0.03$[c]	75 ± 26 (112/150), $P < 10^{-8}$	93 ± 9 (139/150), $P = 0.0004$
R10	98 ± 2 (196/200)	93 ± 13 (186/200), $P = 0.0136$[c]	54 ± 38 (107/200), $P < 10^{-8}$	81 ± 27 (81/100), $P = 6.071 \times 10^{-7}$

[a] No azithromycin.
[b] Chi-square analysis was used for this strain category.
[c] Fisher's exact test was used for this strain category. Results represent data from three or more experiments for each strain. *P* values are for comparisons with the control.

To assess sub-MIC azithromycin effects on the pilin subunit content of gonococci, equal volumes of solubilized R10 microorganisms (24) containing equivalent numbers of bacteria per unit of volume from each treatment group were analyzed by Western immunoblot techniques (2, 8, 25) with polyclonal rabbit antibody with a specificity for R10 pilin (17). Immunoreactive bands were identified (Fig. 2) at approximately 21.9 and 19.9 kDa. The one-eighth- and one-

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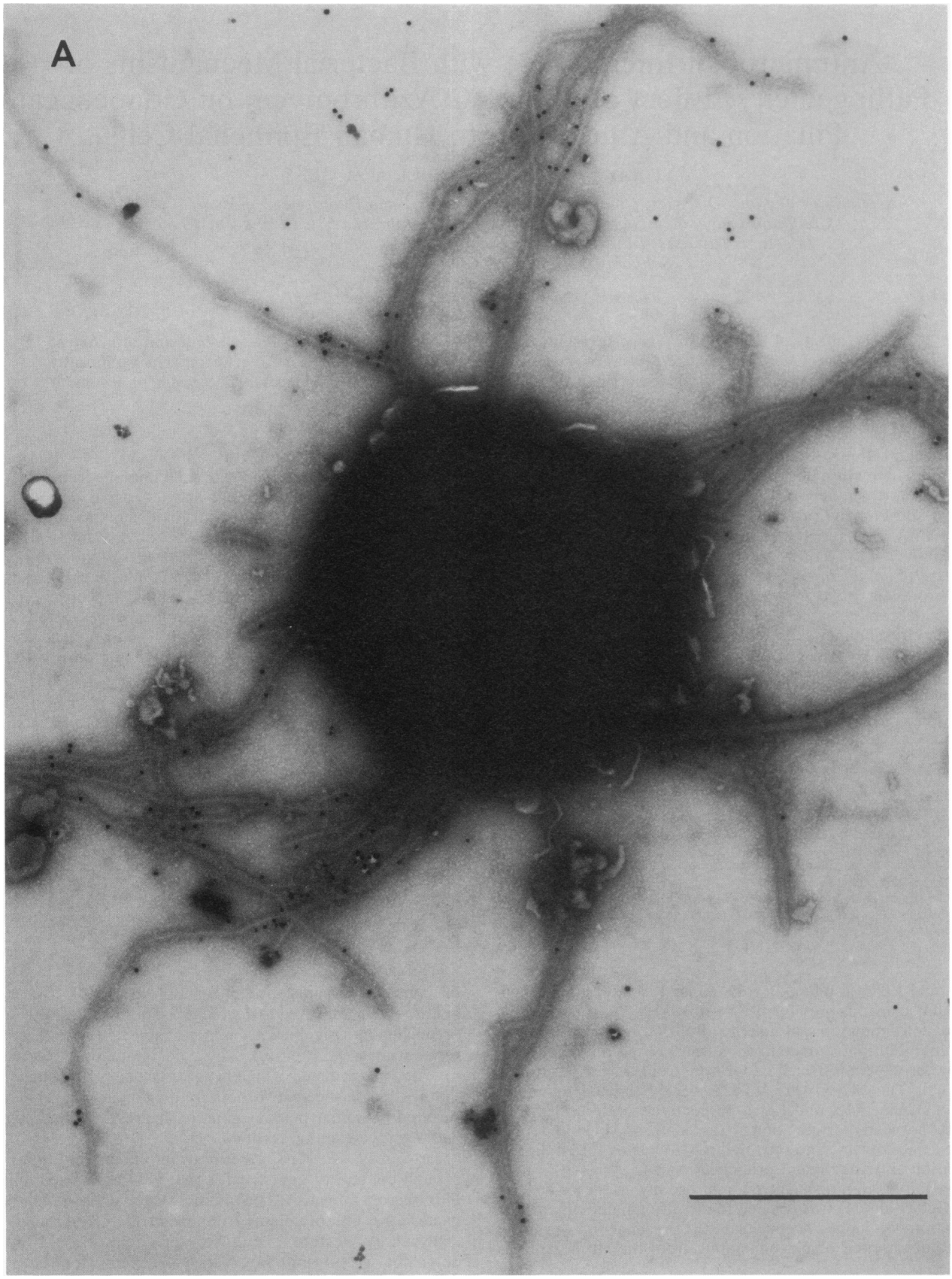
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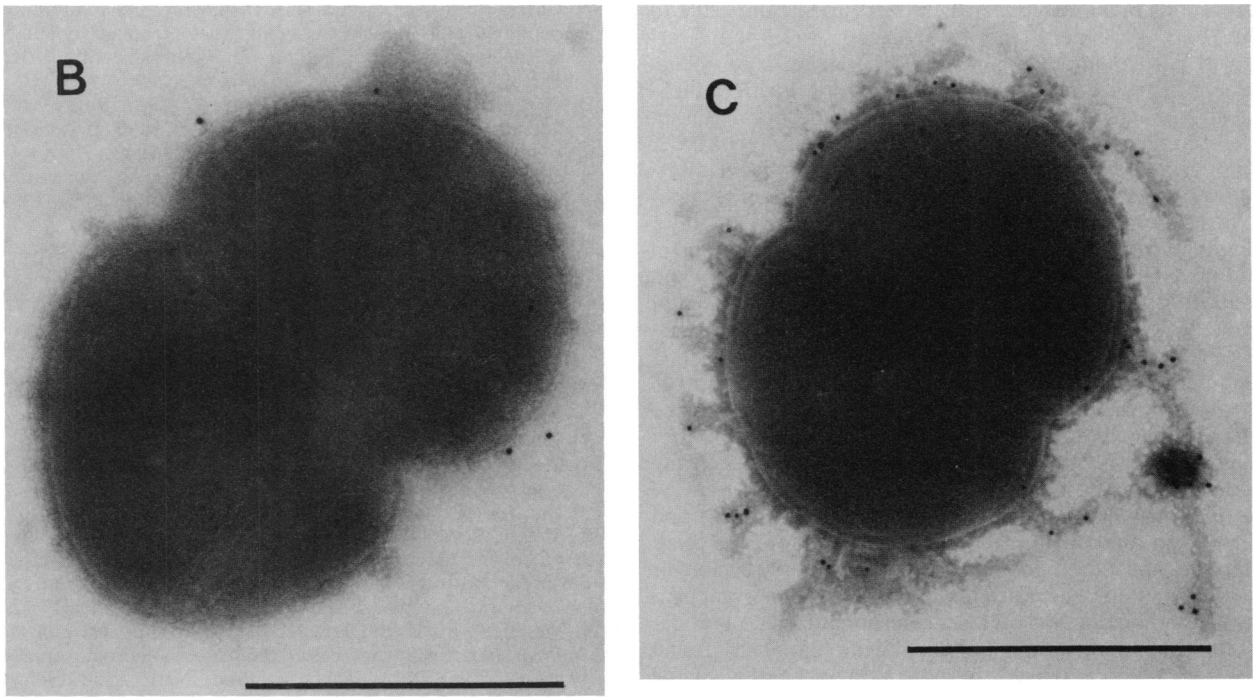

FIG. 1. Azithromycin effects on piliation of gonococci. (A) Typical appearance of gold immunoprobe-labeled R10 control organism. Note the immunoprobes armed with rabbit anti-R10 pilus antibody clearly demonstrating the pilus structure radiating outward from the surface of the organism. Ninety-eight percent of the control organisms possessed pili. (Bar = 1 μm.) (B) Typical appearance of R10 organism that was grown in the presence of sub-MIC azithromycin and lost all piliation. Note the lack of surface-associated gold probes, which indicates a paucity of surface-accessible pilin subunits. (Bar = 1 μm.) (C) Typical appearance of abnormal pilus morphology that was seen only in the presence of sub-MIC azithromycin. This electron micrograph also illustrates the decrease in number of pili per organism that was seen among sub-MIC-treated organisms that managed to maintain some piliation. (Bar = 1 μm.)

fourth-MIC lanes showed a progressive attenuation in the 21.9-kDa pilin band compared with the control, and the one-half-MIC lane showed an increase in the density of the band compared with the other sub-MIC lanes, although it was still less dense than in the control lane. There was a correlation of the relative pilin subunit content at one-eighth, one-fourth, and one-half MIC (as judged by Western blot) with percent piliation (as seen by electron microscopy). The

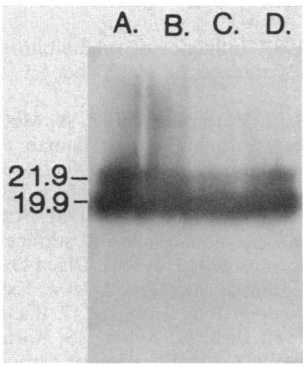

FIG. 2. Western blot study of the effect of sub-MIC azithromycin on pilin subunit production. The primary antibody is rabbit anti-R10 pilus. Each lane represents whole-organism lysates of strain R10 grown in the presence of various concentrations of azithromycin. Each lane represents equivalent numbers of bacteria. Lanes: A, control; B, one-eighth MIC; C, one-fourth MIC; D, one-half MIC. Note the reduced intensity of the 21.9-kDa pilin band in the sub-MIC lanes compared with the band in the control.

19.9-kDa pilin band did not appear to change in relation to sub-MIC azithromycin. It is not clear whether the 19.9-kDa band represents a simultaneously expressed second pilin moiety from a second expression locus (13), a second pilin in a heterogeneous cell population (9), or a constitutively present shortened version (S pilin) of the 21.9-kDa pilin (7).

To determine whether a sub-MIC-induced reduction in piliation resulted in altered gonococcal adherence to human cells, a buccal-cell adherence assay was performed as previously described (20, 21). Because the sub-MIC azithromycin effect on piliation was most pronounced for strain 2686, sub-MIC effects on adherence were assessed in this strain alone. The ability of one-fourth- and one-half-MIC-exposed gonococci and controls to attach to human buccal cells was quantified by calculating the mean fraction of gonococcal inoculum attached for each group (20, 21). Analysis of variance and the Walker-Duncan adaptive procedure were utilized for pairwise comparison of the means (15). The gonococcal cell/buccal cell ratio was very consistent be-

TABLE 2. Azithromycin effects on the adherence of gonococcal strain 2686 to human buccal cells

Expt no.	Mean attachment fraction with:		
	Control[a]	1/4 MIC	1/2 MIC[b]
1	0.7535	0.6986	0.2529
2	0.0436	0.0267	0.0188

[a] No azithromycin.

[b] Attachment fractions in the one-half-MIC category were significantly lower in both experiments ($P < 0.05$) than fractions with the controls.

tween groups in a given experiment but varied from 220:1 to 1,500:1 between experiments, which accounts for the divergence in percent inoculum attached between experiments (Table 2). There was a significant reduction in the attachment of one-half-MIC-exposed gonococci to buccal epithelial cells compared with attachment of controls ($P < 0.05$). A similar trend which was not statistically significant ($P > 0.05$) was observed in the one-fourth-MIC-exposed groups.

Exposure of gonococci to sub-MIC antibiotics can cause thickening of cross walls between organisms, alterations in peptidoglycan, changes in outer membrane proteins, and decreased piliation associated with reduced attachment characteristics (6, 10, 20). Attachment of gonococci is mediated primarily by pili (12) but is also affected by protein II (1) and perhaps other adhesins (23). The effects of sub-MIC azithromycin which we observed are most similar to the effects of sub-MIC tetracycline on gonococci (20). The similarity might be predicted, since both agents act on the ribosome to decrease protein synthesis, although tetracycline binds primarily to the 30S ribosomal subunit (4) and azithromycin binds to the 50S subunit (14). Stephens et al. found that exposure of gonococci to sub-MIC tetracycline or penicillin resulted in decreased numbers of pili per organism, a lower percentage of piliated microorganisms, and decreased attachment to human buccal cells (20). They found that tetracycline decreased the pilin subunit content of the outer membrane by reducing pilin synthesis, in contrast to penicillin, which merely affected pilus anchoring. Stephens et al. ascribed the decreased adherence of tetracycline-exposed gonococci to decreased piliation, but they also observed a shift in the migration pattern of protein II on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sub-MIC-treated organisms (20). We observed no alterations in the migration pattern of gonococcal pilin subunits or major outer membrane proteins on Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-organism lysates (data not shown), which suggests that azithromycin's alteration of gonococcal adherence results solely from decreased piliation. A definitive explanation for the relatively selective inhibition of pilin synthesis by sub-MIC azithromycin or tetracycline is lacking. One theory states that certain proteins, such as exoenzymes and exotoxins, are synthesized by peripherally localized membrane-associated ribosomes that are more susceptible to inhibition by low concentrations of antibiotics than are cytoplasmic ribosomes (3, 18, 22).

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