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# Effect of Subinhibitory Concentrations of Antibiotics on the Adhesion of *Streptococcus pyogenes* to Pharyngeal Epithelial Cells

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The hydrophobicity and charge of the cell surface of M protein-positive ($M^+$) and the less virulent M protein-negative ($M^-$) strains of type 12 *Streptococcus pyogenes* have been studied, respectively, by hydrophobic interaction chromatography and free zone electrophoresis. The $M^+$ strain had a more hydrophobic and a more negatively charged surface than the $M^-$ strain. When the $M^+$ strain was cultivated in the presence of sub-minimum inhibitory concentrations of different antiobiotics, its hydrophobicity either decreased or did not change. The $M^+$ organisms adhered to pharyngeal epithelial cells more avidly than $M^+$; however, cultivation of both strains with sub-minimum inhibitory concentrations of penicillin and rifampin led to a decrease in adhesion. Tetracycline caused a decrease in adhesion for the $M^+$ strain only, whereas cephalothin and polymyxin (to which the strains are resistant) did not affect adhesion or hydrophobicity of the $M^+$ organisms. The negative surface charge of the $M^+$ bacteria increased considerably upon exposure to rifampin and penicillin, and the $M^-$ bacteria exhibited small or no change. The contributions of these changes to suppression of infections are discussed.

The activity of an antibiotic is usually expressed in terms of capacity to either kill or inhibit the growth of an organism in vitro. Concentrations of antimicrobial agents below those that effect complete inhibition of growth (sub-minimum inhibitory concentrations [sub-MICs]) may produce morphological or ultrastructural changes, as observed by light or electron microscopy, or reductions in the culture populations (4). It has recently been shown that streptococci cultivated in the presence of sub-MICs of certain antibiotics have a decreased ability to produce toxins as well as some surface components (5). Other reports indicate that *Escherichia coli* and other *Enterobacteriaceae* lose certain surface substances responsible for their colonization of a host when cultivated under sub-MIC conditions (8, 9, 13).

Data obtained in our laboratory with *Streptococcus pyogenes* indicate that M protein-positive ($M^+$) and M protein-negative ($M^-$) strains have a different surface charge and hydrophobicity (11). The influence of exposure to various antibiotics on these qualities and how this affects the ability of the bacteria to adhere to human pharyngeal epithelial cells (11) have been examined in the studies recorded below.

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## MATERIALS AND METHODS

**Chemicals.** Phenyl-Sepharose (lot no. 11144) was obtained from Pharmacia Fine Chemicals AB, Uppsala. The following antibiotics were used: bacitracin (Sigma, St. Louis, Mo.), benzylpenicillin (KABI, Stockholm), cephalothin (Keflin; Eli Lilly Co., Indianapolis, Ind.), pristinamycin (S.P.E.C.I.A., Paris, France), rifampin (Lepetit Sp.A, Milano, Italy), tetracycline (Pfizer, New York, N.Y.), polymyxin B (Novo Industri A/S, Copenhagen, Denmark), and erythromycin (Abboticin; Abbot, Chicago, Ill.). All chemicals were of analytical grade.

**Bacterial strains.** *S. pyogenes* type 12, NY5 strain lacking M protein ($M^-$) and E14/51RB/21/2 strain containing M protein ($M^+$) were grown in Todd-Hewitt broth for 18 h at 37°C. The bacteria were harvested by centrifugation, washed three times, and resuspended in phosphate-buffered saline.

**Antibiotic susceptibility tests.** All strains were tested for antibiotic susceptibility by using the agar dilution method (4) on PDM-ASM agar (AB Biodisk, Solna, Sweden) supplemented with 5% defibrinated horse blood (4). Bacterial inocula of $10^5$ to $10^6$ colony-forming units per ml were used.

**Antibiotic treatment.** One colony of each $M^+$ and $M^-$ strain was isolated from blood agar plates and incubated in 5 ml of Todd-Hewitt broth for 4 h.

Cultures were then inoculated into 50 ml of Todd-Hewitt broth containing various sub-MICs of antibiotics (Table 1) and incubated for 18 h at 37°C. The bacteria were harvested by centrifugation, washed three times, and resuspended in phosphate-buffered saline.

**Hydrophobic interaction chromatography.** Hydrophobic interaction chromatography was performed on phenyl-Sepharose as described previously using the $M^+$ and $M^-$ strains after treatment with antibiotics (11). Nontreated bacteria also were used as controls. Briefly, 100 μl of bacterial suspension was applied to the gel bed equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The number of adsorbed bacteria was estimated by comparison of the adsorption of the eluant at 600 nm at a 1-cm pathlength with an appropriately diluted 100-μl volume of the original suspension. The results were expressed as percentage of bacteria attached to phenyl-Sepharose.

**Adhesion test.** Pharyngeal epithelial cells were obtained by scraping the pharynx of healthy volunteers with a wooden spatula after anaesthetization with xylocaine (Astra, Södertälje, Sweden). The cells were suspended in Ham-F-12 medium and washed once in the same medium; $10^5$ epithelial cells and $10^8$ bacteria in a total volume of 1 ml were then incubated for 30 min at 37°C. The cells were then washed three times in Ham medium to remove any nonadhering bacteria, placed on glass slides, and stained with methylene blue. Control cells not incubated with bacteria were included in each experiment. The results represent the mean number of bacteria adhering to each of 50 cells after subtraction of number of bacteria present on control cells incubated without streptococci. The results were analyzed statistically with the Student *t* test for each type of bacteria.

**Free zone electrophoresis.** Experiments were performed in a Hjertén apparatus as previously described (11). Migration velocities of $M^+$ and $M^-$ strains cultivated in the presence of penicillin or rifampin were measured. All runs were done at 14°C in 0.1 M sodium phosphate buffer (pH 7.4) at 800 V, giving a current of 7 mA.

## RESULTS

Table 1 shows that most of the antibiotics used in this study effected a concentration-related decrease in the surface hydrophobicity of the $M^+$ strain of *S. pyogenes*. In the case of erythromycin, cephalothin, and polymyxin these decreases may not be significant. The responses of the $M^-$ strain to sub-MICs of the various antibiotics were variable: an increase in some cases, a decrease or no change in hydrophobicity in others (Table 1).

Table 2 shows that exposure to all antibiotics other than cephalothin resulted in reduced adhesion of the $M^+$ strain to epithelial cells. Penicillin and rifampin had marked effects on adhesion of the $M^-$ strain.

Table 3 shows that the $M^+$ strain has a more negative surface charge than the $M^-$ strain. After cultivation in the presence of rifampin the negative charge of the $M^+$ strain increased much more than that of the $M^-$ strain. Penicillin increased the negative surface charge only of the $M^+$ strain.

TABLE 1. *Influence of antibiotics on adsorption of S. pyogenes type 12 $M^+$ and $M^-$ strains to phenyl-Sepharose*[a]

| Antibiotic | Test concn (μg/ml) | MIC (μg/ml) | Adsorption (%)[b] | |
|---|---|---|---|---|
| | | | $M^+$ | $M^-$ |
| Control (without antibiotics) | 0.0 | | 100 | 72 |
| Bacitracin | 0.005 | 2 | 96 | 74 |
| | 0.05 | | 96 | 74 |
| | 0.5 | | 42 | 96 |
| Penicillin | 0.0005 | 0.008 | 100 | 81 |
| | 0.001 | | 82 | 97 |
| Pristinamycin | 0.01 | 0.64 | 100 | 69 |
| | 0.05 | | 99 | 97 |
| | 0.1 | | 36 | 97 |
| Tetracycline | 0.1 | 0.125 | 93 | 84 |
| | 0.2 | | 93 | 85 |
| | 1.0 | | 90 | 85 |
| Rifampin | 0.01 | 0.16 | 36 | 36 |
| | 0.05 | | 14 | 90 |
| | 0.1 | | 12 | 45 |
| Erythromycin | 0.0001 | 0.016 | 97 | 73 |
| | 0.001 | | 97 | 73 |
| Cephalothin | 0.01 | 0.125 | 98 | 31 |
| | 0.1 | | 98 | 85 |
| Polymyxin | 0.2 | 64 | 97 | 74 |
| | 2.0 | | 97 | 75 |
| | 20.0 | | 96 | 73 |

[a] Bacteria were cultivated in Todd-Hewitt broth supplemented with antibiotics overnight.

[b] Percent bacteria adsorbed to phenyl-Sepharose equilibrated and eluted with 0.01 M phosphate buffer at pH 7.0.

TABLE 2. *Adherence of S. pyogenes to pharyngeal epithelial cells*[a]

| Bacteria treated with: | No. of bacteria per epithelial cell | |
|---|---|---|
| | $M^+$ | $M^-$ |
| Control (without antibiotics) | 40 | 23 |
| Penicillin | 23 | 18 |
| Tetracycline | 22 | 23 |
| Rifampin | 16 | 16 |
| Cephalothin | 37 | 24 |
| Polymyxin | 41 | 24 |

[a] Streptococci were cultivated overnight in Todd-Hewitt broth supplemented with antibiotics at concentrations 10 times lower than the MIC (given in Table 1).

## DISCUSSION

The observations recorded in this report indicate that some antibiotics affect the $M^+$ bacteria not only by killing them but also by de-

TABLE 3. *Effect of antibiotics on electrophoretic mobility of S. pyogenes*

| Culture conditions | Mobility ($\times 10^5$) ($cm^2 s^{-1} V^{-1}$) | |
|---|---|---|
| | $M^+$ strain | $M^-$ strain |
| Control | −1.6 | −1.2 |
| Penicillin[a] | −4.0 | −1.2 |
| Rifampin[a] | −6.0 | −2.1 |

[a] Bacteria cultivated in Todd-Hewitt broth supplemented with 0.01 μg of penicillin per ml or 0.1 μg of rifampin per ml at 37°C overnight.

creasing their cell surface hydrophobicity and increasing the negative electric charge, leading to a diminished interaction between the bacteria and the epithelial cells, which may contribute to a suppression of infections. We wish to stress, however, that this hypothesis to explain part of the action of antibiotics should not be considered as generally valid since we also found that bacitracin and pristinamycin increased the hydrophobicity of the bacteria but had no effect on the adhesion to the pharyngeal epithelial cells.

Our results are in agreement with earlier studies by Ellen and Gibbons, which showed that streptococcal strains rich in M protein adhere more avidly to pharyngeal epithelial cells (and tend to be more virulent) (3). The ability of *S. pyogenes* to adhere to pharyngeal epithelial cells was claimed, primarily, to be associated with the presence of M protein. However, other studies demonstrated that $M^-$ streptococci adhere to buccal epithelial cells as efficiently as $M^+$ strains and that lipoteichoic acid could be the main surface structure involved in epithelial binding via specific cell receptors (2). Recently it has been found that the C polysaccharide also contributes to the binding and that the adhesion of group A-variant streptococci is much less than that of group A streptococci (D. De Marzo and G. A. Botta, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 19th and Chemother. 11th, Boston, Mass., abstr. no. 332, 1979).

The cultivation of streptococci in the presence of sub-MICs of antibiotics alters their structure and toxigenicity, resulting in a reduction of their pathogenic potential (5). M antigen formation is diminished by growth in the presence of lincosamines, erythromycin, and chloramphenicol (5). Other studies have shown that incubation of streptococci with penicillin induces excretion of lipoteichoic acid from the bacterial cells, followed by loss of ability to adhere to buccal epithelial cells (1). The possibility that other surface structures such as the M protein could be affected prior to lipoteichoic acid excretion was not investigated. It also has been shown that certain antibiotics are responsible for preferentially affecting inhibition of synthesis or excretion of various surface substances in these bacteria. The mechanism of the observed phenomena is not clear. However, the original observations that autolytic enzymes in the cell wall, for example murein hydrolases in pneumococci, are essential for cell death or lysis, and that penicillin induces secretion of surface polysaccharides and lipids, have also been observed in other organisms (10).

It was shown by Hill et al. (6) that cell surface net charge densities are higher on M protein-rich strains than on M-negative strains, in agreement with our data (Table 3), and that, for example, the mobility of trypsin-treated M protein-positive strains becomes similar to, although still higher than, that of M protein-negative strains.

Four recent reports, published when this study was in its final stage, showed the effect of sub-MICs of different antibiotics on the attachment of *E. coli* and other gram-negative bacteria to epithelial cells (7–9, 14). Interestingly, one of these studies showed a decrease in the adhesion of different enterobacteria to buccal cells with all five of the antibiotics tested, including cell wall inhibitors such as cephalothin, protein synthesis inhibitors such as chloramphenicol, and two aminoglycoside antibiotics (9). In another study on *E. coli* isolated from urinary tract infections, subinhibitory concentrations of ampicillin decreased epithelial cell attachment, whereas chloramphenicol and nitrofurantoin had no effect (8). A study of the effect of 12 different antibiotics on the adhesiveness of *E. coli* to human embryonic intestinal cells showed that tetracycline, clindamycin, and trimethoprim-sulfamethoxazole reduce adhesiveness, whereas nalidixic acid enhances it. Several nonpenicillin or cephalosporin antibiotics, for example chloramphenicol and streptomycin, did not affect this process.

Further studies are being carried out to investigate the relative importance of the cell surface antigens and M protein in the attachment to different epithelial cells. These studies were initiated after the original observations of Ellen and Gibbons (3) showed that M-positive strains and M-negative variants have different binding properties to epithelial cells isolated from buccal and pharyngeal surfaces. Our further studies also include investigations of the effect of blocking M protein surface receptors with $\beta_2$-microglobulin and fibrinogen (L. Björk, S. Tylewska, T. Wadström, M. Söderström, and G. Kronvall, in press).

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