Effect of Antibiotics on Adherence of *Haemophilus influenzae* Type b

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During antibiotic therapy for serious *Haemophilus influenzae* type b infections in children, respiratory mucosal colonization with this organism is suppressed but not eradicated. To define possible mechanisms contributing to this suppression, the ability of six antibiotics to influence the adherence of *H. influenzae* type b to human epithelial cells was investigated. In assays in which the organisms were grown in broth containing 0.5 × the MIC of rifampin, ampicillin, clindamycin, chloramphenicol, lincomycin, or trimethoprim-sulfamethoxazole, all drugs except rifampin significantly reduced bacterial adherence. In assays in which nonreplicating organisms were exposed to the antibiotics, all six drugs reduced the adherence of the bacteria. In assays in which the epithelial cells were exposed to the antibiotics, all drugs reduced bacterial adherence. In addition, the presence of ampicillin, chloramphenicol, lincomycin, or trimethoprim-sulfamethoxazole appeared to facilitate the release of organisms adherent to epithelial cells. Thus, antibiotics appear to inhibit adherence of *H. influenzae* type b to human epithelial cells and may interfere with bacterial or epithelial cell binding sites. These observations may explain the suppression of *H. influenzae* type b mucosal colonization that occurs during antibiotic treatment of patients with systemic *H. influenzae* type b infections.

*Haemophilus influenzae* type b, a common cause of serious childhood bacterial infections, including meningitis, epiglottitis, septic arthritis, pneumonia, and cellulitis, colonizes the mucosa of the upper respiratory tract as the initial event in the establishment of infection (11). Attempts to prevent serious *H. influenzae* type b infections in children have included the use of antibiotics to eradicate nasopharyngeal colonization with this organism. Although a number of orally administered antibiotics, including ampicillin (1, 4), cefaclor (7, 21), trimethoprim-sulfamethoxazole (20), and erythromycin-sulfisoxazole (7), have failed to eradicate mucosal colonization, rifampin has been shown to be effective (5, 7).

Although *H. influenzae* type b is often cultured from the nasopharynges of children with serious systemic *H. influenzae* type b infections before the initiation of intravenous antibiotic therapy, the organism often is not isolated during therapy (2). However, after discontinuation of antibiotic therapy that successfully treated infections with ampicillin (10) or chloramphenicol (15), the organism frequently may again be recovered on throat or nasopharyngeal swabs. Thus, the organism appears to be suppressed but not eradicated in the respiratory tract by antibiotics that are successful in treating invasive, systemic infections.

Recent studies have shown that *H. influenzae* type b attaches to respiratory mucosal epithelial cells by means of pili (6, 13), which are protein appendages that extend from the surface of the organism. Pili on the surface of *Escherichia coli* or *Neisseria gonorrhoeae* bind to specific receptors on epithelial cells, and this binding may be inhibited by competing substances such as α-D-mannose (8), pilus-specific antibodies (19), and subinhibitory concentrations of antibiotics (12, 14, 18).

Several mechanisms may contribute to antibiotic inhibition of adherence, including (i) decreased production of pili by an antibiotic-mediated decrease in protein synthesis, (ii) direct antibiotic interference with binding sites on the pilus, (iii) direct antibiotic interference with the receptor sites of the epithelial cell, and (iv) release of adherent organisms from epithelial cells.

Using in vitro adherence assays with an *H. influenzae* type b organism that was shown to possess pili and to adhere to human epithelial cells, we investigated the effect of subinhibitory concentrations of antibiotics on the adherence of *H. influenzae* type b, altering the conditions of the assays to explore several of these possible mechanisms.

**MATERIALS AND METHODS**

**Bacteria.** *H. influenzae* type b M43, isolated from the nasopharynx of a child with *H. influenzae* type b meningitis, was previously shown to possess pili, to adhere to human buccal epithelial cells (BEC), and to agglutinate human erythrocytes (3, 6). Samples of this organism in mid-log growth phase were stored in Levinthal broth (brain heart infusion broth containing lysed horse erythrocytes and β-NAD as growth factors) at −70°C until use in the adherence assays.

**Antibiotic susceptibility testing.** Preparations of rifampin, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole for antibiotic susceptibility testing were obtained from Carl Pierson, University of Michigan Medical Center Microbiology Laboratory. Preparations of clindamycin and lincomycin were obtained from The Upjohn Co., Kalamazoo, Mich.

Antimicrobial susceptibility testing was performed by a broth microdilution method (16) in Levinthal broth with an inoculum of 10⁵ CFU of *H. influenzae* type b M43. The following MICs of the antibiotics for *H. influenzae* type b
M43 were determined: rifampin, 0.5 µg/ml; ampicillin, 0.25 µg/ml; clindamycin, 8.0 µg/ml; chloramphenicol, 2.0 µg/ml; lincomycin, 128.0 µg/ml; and trimethoprim, 0.5 µg/ml, and sulfamethoxazole, 8.5 µg/ml.

**Adherence assay.** The adherence assay used in our studies was previously described (3). In brief, washed late-log-phase *H. influenzae* type b cells suspended in phosphate-buffered saline (pH 7.5) with 0.1% gelatin were mixed with washed human BEC in a ratio of 1,000 bacteria to one epithelial cell. After incubation for 30 min, nonadherent bacteria were removed from the epithelial cell suspensions by washing over 8.0-µm-pore-size nitrocellulose membrane filters (Millipore Corp., Bedford, Mass.). The cells were transferred to microscope slides, and the slides were stained with Gram stain.

Fifty consecutive epithelial cells that were adequately stained and not folded or overlapping were evaluated by light microscopy. Organisms that were morphologically identical to *H. influenzae* type b and adherent to the epithelial cells were counted, and the mean number of organisms per epithelial cell was determined. Control preparations used epithelial cells incubated with buffer containing no bacteria. The number of nonadherent organisms seen in the background on the microscopic preparations between the epithelial cells was assessed, and slides showing >10 nonadherent organisms were not analyzed.

**Effect of drugs on adherence.** The effect of drugs on adherence was tested in four types of assay systems to evaluate the various mechanisms by which adherence could be inhibited. Experiments in each assay type included control preparations without antibiotics, with which the test preparations were compared.

**Assay 1.** To investigate the role of the antibiotics on pilus formation, *H. influenzae* type b M43 was grown in the presence of subinhibitory concentrations of the six antibiotics. A 0.3-ml sample of an *H. influenzae* type b broth culture, grown overnight at 37°C to stationary phase, was added to 3 ml of Levinthal broth containing 0.5 × the MIC of the antibiotic to be tested and incubated at 37°C for 4.5 h to late-log phase. The bacteria were then washed in phosphate-buffered saline with 0.1% gelatin, and the bacterial concentrations of each antibiotic-treated preparation were estimated by spectrophotometry and confirmed by colony counts of serially diluted samples. The bacterial samples were diluted to the desired concentration and tested in the adherence assay.

**Assay 2.** To investigate the role of the antibiotics in interfering with binding sites on the *H. influenzae* type b surface, nonreplicating bacteria suspended in buffer were exposed to the antibiotics before being used in the adherence assay. The organisms were grown to late-log phase in Levinthal broth at 37°C, washed, suspended in phosphate-buffered saline with 0.1% gelatin containing a test antibiotic at a concentration of 0.5 × the MIC for *H. influenzae* type b M43, and incubated for 30 min at room temperature. The antibiotic-exposed bacteria were then tested in the adherence assay.

**Assay 3.** To investigate the role of the antibiotics in interfering with receptor sites on mucosal cell surfaces, BEC were exposed to the antibiotics before being used in the adherence assay. The epithelial cells were washed, suspended in phosphate-buffered saline with 0.1% gelatin containing 0.5 × the MIC of the antibiotic to be tested, and incubated at room temperature for 30 min. The antibiotic-exposed epithelial cells were then washed and tested in the adherence assay.

**Assay 4.** To test the competitive interaction of the antibiotics with organisms that are adherent to mucosal cells, epithelial cells with adherent *H. influenzae* type b were exposed to antibiotics. The BEC and bacteria were mixed and incubated in the standard adherence assay. Subsequently, 0.5 × the MIC of the antibiotic to be tested was added to the BEC preparation and incubated at room temperature for 30 min, and the nonadherent organisms were removed by washing and filtering. The number of the remaining adherent organisms per epithelial cell was determined by microscopic examination of the Gram-stained cells.

**Statistical comparisons.** We expressed the effect of the various drugs on the adherence of *H. influenzae* type b to the BEC in several ways. We determined the mean number of bacteria per epithelial cell, as well as the mean percent of BEC with greater than five adherent bacteria, and we calculated the concordance between these two numbers. The mean number of adherent bacteria in the presence of each antibiotic was compared with the number in the absence of the antibiotic by Student’s *t* test. The mean values represent the results of either four or five experiments under identical conditions. In addition, we calculated the percent inhibition of adherence by the antibiotics with the formula, 

\[
\text{percent inhibition} = 100 \times \left(1 - \left(\frac{\text{mean number of } H.\ influenzae \text{ type b on BEC with drug}}{\text{mean number of } H.\ influenzae \text{ type b on BEC without drug}}\right)\right)
\]

**RESULTS**

**Assay 1. Effect of antibiotics on adherence on *H. influenzae* type b grown in 0.5 × the MIC.** All drugs except rifampin reduced bacterial adherence when *H. influenzae* type b M43 was grown in broth containing 0.5 × the MIC of each antibiotic. The mean numbers of organisms adherent to each BEC and the mean percentages of BEC with greater than five adherent *H. influenzae* type b bacteria are shown in Table 1. The concordance between these two methods of expressing results was 83.3%. The percentages of inhibition [mean (95% confidence limits)] of *H. influenzae* type b adherence, after the organism was grown in the presence of the antibiotics, were as follows: rifampin, 38.0% (15.9 to 58.1%); ampicillin, 70.7% (51.5 to 89.9%); clindamycin, 82.9% (72.7 to 93.1%); chloramphenicol, 88.8% (80.1 to 97.5%); lincomycin, 94.2% (90.0 to 98.5%); and trimethoprim-sulfamethoxazole, 62.3% (35.1 to 90.5%).

**Assay 2. Effect of antibiotics on adherence of nonreplicating *H. influenzae* type b exposed to 0.5 × the MIC.** All drugs except ampicillin reduced bacterial adherence after *H. influenzae* type b...
TABLE 2. Inhibition of *H. influenzae* type b adherence by exposure of organisms to various antibiotics at 0.5 × MIC

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of Hib/% BEC (mean [95% confidence limits])</th>
<th>% BEC with &gt;5 Hib per cell (mean [95% confidence limits])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.7 (10.9-16.5) 74.7 (58.5-90.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>3.6 (0-8.0) 22.0 (6.0-38.0)</td>
<td>0.5</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>6.0 (1-13.0) 33.0 (18.0-48.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>4.8 (0.78-8.9) 26.0 (4.2-47.8)</td>
<td>1.4</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.0 (0.4-1.6) 4.0 (0.12-12.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>3.5 (1-2.5-8) 14.0 (0.31-31.0)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* *H. influenzae* type b.

* P < 0.05 compared with the control.

Assay variability. The coefficient of variation of the mean number of *H. influenzae* type b M43 cells adhering to each epithelial cell in control specimens was 22.7% (*n* = 26); the coefficient of variation of the percentage of BEC with greater than five *H. influenzae* type b bacteria was 23.7% (*n* = 25).

**DISCUSSION**

In evaluating the possible role of antibiotics in the inhibition of *H. influenzae* type b adherence to human BEC, we considered four possible mechanisms of inhibition: decreased production of pili, direct antibiotic interference with binding sites on the pili, direct antibiotic interference with the receptor sites on the epithelial cells, and release of adherent organisms from epithelial cells. Because these experiments were conducted with subinhibitory levels of the antibiotics and because the organisms were viable after exposure to the drugs, the decreased adherence we observed was not a function of the bactericidal effects of the antibiotics.

Other investigators have postulated that the growth of bacteria in the presence of antibiotics whose bactericidal effect is mediated by decreased protein synthesis depresses the formation of the bacterial pili even with subinhibitory levels of the drugs. After growth in media containing subinhibitory levels of antibiotics, both *N. meningitidis* and *E. coli* have been shown by electron microscopic examination to lose pili (9, 17). In support of this mechanism, in our assay 1, *H. influenzae* type b M43 grown in the presence of 0.5 × the MIC of ampicillin, clindamycin, chloramphenicol, lincomycin, or trimethoprim-sulfamethoxazole showed significantly decreased adherence to human BEC. However, when the organisms were exposed to these same antibiotics in a buffer solution that did not support bacterial replication, a similar inhibitory effect was seen. Also, *H. influenzae* type b grown in ampicillin, an antibiotic that interferes with bacterial cell wall synthesis without interrupting bacterial protein synthesis, displayed decreased adherence, whereas

TABLE 3. Inhibition of *H. influenzae* type b adherence by exposure of BEC to various antibiotics at 0.5 × MIC

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of Hib/% BEC (mean [95% confidence limits])</th>
<th>% BEC with &gt;5 Hib per cell (mean [95% confidence limits])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.8 (14.7-32.9) 91.0 (86.0-96.0)</td>
<td>3.0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>20.0 (11.2-28.7) 86.0 (83.4-86.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>9.1 (1-14-6.7) 41.0 (7-74.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>14.2 (10.1-18.4) 71.0 (59-82.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>7.2 (3.9-12.4) 43.0 (12.8-59.2)</td>
<td>1.0</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>11.2 (4.5-17.9) 59.0 (44.8-73.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>3.0 (1.8-4.1) 13.3 (1.7-24.9)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* *H. influenzae* type b.

* P < 0.05 compared with the control.

**TABLE 4. Release of adherent *H. influenzae* type b from BEC after incubation with various antibiotics at 0.5 × MIC**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of Hib/% BEC (mean [95% confidence limits])</th>
<th>% BEC with &gt;5 Hib per cell (mean [95% confidence limits])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.9 (12.2-19.6) 87.2 (79.5-94.9)</td>
<td>3.0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>9.6 (6.7-12.6) 64.0 (51.9-76.1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>5.8 (0.7-8.4) 31.2 (4.4-58.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>14.6 (9.7-17.0) 74.4 (59.8-89.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>7.6 (1.2-10.8) 42.2 (7.3-77.5)</td>
<td>1.0</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>7.5 (1.9-10.2) 32.0 (5.5-58.5)</td>
<td>1.0</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>2.6 (0.9-3.5) 27.0 (0.0-56.3)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* *H. influenzae* type b.

* P < 0.05 compared with the control.
organisms grown in rifampin, an antibiotic that blocks bacterial protein synthesis, showed no decrease in adherence. Thus, in addition to decreased pilus production, steric hindrance of the bacterial binding sites by antibiotic molecules may be another mechanism of adherence inhibition. On the basis of our results, we cannot speculate on the specificity of this interaction at the bacterial binding sites.

The observation that epithelial cells pretreated with antibiotics bound H. influenzae type b less well than did untreated cells suggests that binding sites on epithelial cells are also blocked by antibiotics. In addition, the exposure of epithelial cells with adherent H. influenzae type b to antibiotics led to a decrease in the number of adherent organisms. The biologic phenomena responsible for this apparent release of adherent organisms are unclear. Thus, our data suggest that any of the postulated mechanisms operate in the inhibition of H. influenzae type b adherence by antibiotics.

Because the drugs were not removed after exposure to nonreplicating bacteria in assay 2, the inhibition of the adherence that occurred may have been the result of the drugs interacting with the epithelial cells during the adherence assay incubation step rather than a result of the drugs binding to the bacteria. However, we noted that the adherence was dramatically inhibited by exposing nonreplicating H. influenzae type b to rifampin, clindamycin, and lincomycin but was not inhibited by exposing epithelial cells to these drugs, suggesting that the drugs interact with the bacterial binding sites but not with the epithelial cell receptors.

In choosing a standard amount of drug to be used in each type of experiment, we selected 0.5 × the MIC to avoid levels that would kill the organism. Because our test organism was very resistant to lincomycin (MIC, 128.0 μg/ml), the concentration of this drug in our assays was considerably higher than that of the other drugs, and this could explain the profound inhibitory effect observed with lincomycin. However, a marked inhibitory effect was observed with chloramphenicol, and a somewhat lesser effect was observed with clindamycin. Both drugs had lower MICs (2.0 and 8.0 μg/ml, respectively) than that of lincomycin and thus were present in the assays at lower concentrations.

Two methods of expressing results (the mean number of organisms per epithelial cell and the mean number of epithelial cells with greater than five adherent bacteria) were examined. Both methods reflect the number, avidity, and affinity of binding sites on both the bacteria and the epithelial cells. Results expressed in these two ways show a high degree of concordance, suggesting that the methods measured the same phenomena.

The results from the adherence assay show a considerable degree of variability, possibly because of differing cell types among the epithelial cells, day-to-day differences in the intraoral environment of the epithelial cells, and other, unrecognized factors. Thus, the statistically insignificant inhibitory effect of ampicillin in assay 2 and of chloramphenicol in assay 4 may have been related to this high variability and to the relatively low number of trials (n = 4), as suggested by the wide 95% confidence levels.

In summary, antibiotics appear to inhibit the adherence of H. influenzae type b to human epithelial cells, and this inhibition appears to be independent of the antibiotic killing of the actively metabolizing bacteria. The inhibition of adherence may be the result of several effects of the antibiotics, including decreased production of the pilus protein as a result of depressed bacterial protein synthesis, as has been previously described. Our data suggest that steric hindrance of adherence at the bacterial or epithelial cell binding sites may be another mechanism. This decreased adherence of H. influenzae type b in the presence of antibiotics may explain the suppression of mucosal colonization observed during antibiotic therapy of systemic H. influenzae type b infections. The inhibitory effect of rifampin, a drug that successfully eradicates mucosal colonization, was not greater than that seen with other drugs, such as ampicillin and chloramphenicol, which suppress but do not eradicate colonization.

ACKNOWLEDGMENT

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LITERATURE CITED


