Combined Action of Chloramphenicol and Ampicillin on Chloramphenicol-Resistant *Haemophilus influenzae*

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The interaction of ampicillin and chloramphenicol on three ampicillin-susceptible, chloramphenicol-resistant strains of *Haemophilus influenzae* was studied by checkerboard testing with subcultures, time-kill experiments, and a disk method. In all three strains there was inhibition of the bactericidal action of ampicillin by chloramphenicol at concentrations close to the MIC (10 μg/ml). This chloramphenicol concentration was close to that which might be achieved in cerebrospinal fluid during treatment for meningitis and was in the bactericidal range for chloramphenicol-susceptible organisms. It is suggested however that in the initial treatment of meningitis caused by ampicillin-susceptible, chloramphenicol-resistant strains, inhibition of the action of ampicillin by chloramphenicol may represent a clinical risk.

Combined therapy with chloramphenicol and ampicillin is standard initial therapy for *Haemophilus influenzae* type b meningitis. Results of previous studies (10, 22, 28) have indicated that at certain concentrations chloramphenicol may inhibit the bactericidal action of ampicillin. This investigation was undertaken to determine whether, in the case of chloramphenicol-resistant organisms, this antibiotic might interfere with the action of ampicillin.

**MATERIALS AND METHODS**

**Bacterial strains.** All four *H. influenzae* strains (A, B, C, D) used in this study were ampicillin susceptible by disk testing and β-lactamase negative by the nitrocefin method. The chloramphenicol-susceptible (Chl<sup>s</sup>) strain (A) was one of eight strains described previously (22). The three chloramphenicol-resistant (Chl<sup>r</sup>) strains (B, C, D) were isolated from blood culture, nasopharynx, and eyes, respectively. The MIC of all three strains to chloramphenicol was 10 μg/ml with the medium described below with an inoculum of 10<sup>5</sup>/ml, and all three strains produced a chloramphenicol-inactivating enzyme (33). Strains B and C were type b; strain D was nontypable.

**Experimental methods.** The growth medium used in all experiments was brain heart infusion broth (Oxoid Ltd., London, England) with 2% Fildes extract (Difco Laboratories, Detroit, Mich.). For the checkerboard experiments an overnight broth culture was diluted to give a suspension containing 10<sup>9</sup> to 10<sup>10</sup> CFU/ml. An accurate viable count of the inoculum was determined in each experiment. This starting culture was divided into 1-ml volumes, and antibiotic solutions were added in 0.01-ml volumes to provide the required concentrations. The cultures were then incubated overnight, and a 10-μl sample from each tube was inoculated onto a chocolate agar plate and was allowed to dry and spread. The plates were incubated for 48 h. The surviving colonies were counted, and the number of colonies representing a 99.9% kill was determined from previously published tables (25).

Checkerboard titration results were analyzed as follows (15):

\[
\Sigma_{FBC} = \frac{\text{MBC combination}}{\text{MBC alone}} + \frac{\text{MBC combination}}{\text{MBC alone}}b
\]

where a and b are ampicillin and chloramphenicol, respectively, and Σ_{FBC} is the fractional bactericidal concentration. An Σ_{FBC} of >4 indicated antagonism. The MBC combination was the highest MBC recorded for each antibiotic in the presence of the other antibiotic.

For the time-kill experiments an overnight culture was adjusted in growth medium to a density close to 10<sup>9</sup> CFU/ml, and the tubes were set up as described above for the checkerboard experiment. Viable counts were performed on chocolate agar plates spread with penicillinase at the times indicated in the tables and figures.

**Agar diffusion method to demonstrate antagonism.** The agar diffusion method to demonstrate antagonism was similar, in principle, to the cellophane transfer method (14). A thin layer of agar was prepared by pouring 5 ml of hot Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar onto sterile Whatman no. 52 filter paper in a 10-cm-diameter petri dish. After the agar was allowed to set, it could be handled as a thin layer held together by the filter paper. This agar membrane was transferred to the surface of a chocolate agar plate and flooded with a suspension of *H. influenzae* with a density of approximately 10<sup>9</sup>/ml. The plate was then allowed to dry. Two 7-mm-diameter antibiotic disks (chloramphenicol, 10 μg; ampicillin, 10 μg) were placed on the surface, with a 1.5-cm distance from center to center of each disk. The plate was then incubated at 37°C overnight, after which time the expected zones of inhibition could be seen. After this incubation the disks were removed and the whole agar membrane was transferred to another chocolate agar plate which had been previously spread with 1,000 U of penicillinase (Difco). A further 24 h of incubation in the absence of antibiotic revealed areas in which inhibition without killing had taken place, and a pattern of interference between the antibiotics could be seen (see below).

* Corresponding author.
TABLE 1. Checkerboard titrations

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ampicillin MIC (µg/ml)</th>
<th>Ampicillin MBC (µg/ml)</th>
<th>Chloramphenicol MIC (µg/ml)</th>
<th>Chloramphenicol MBC (µg/ml)</th>
<th>ΣFBC (µg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.08</td>
<td>0.3</td>
<td>0.6</td>
<td>2.5</td>
<td>17.1</td>
</tr>
<tr>
<td>B</td>
<td>0.15</td>
<td>1.2</td>
<td>10</td>
<td>40</td>
<td>4.6</td>
</tr>
<tr>
<td>C</td>
<td>0.08</td>
<td>0.3</td>
<td>10</td>
<td>20</td>
<td>17.6</td>
</tr>
<tr>
<td>D</td>
<td>0.15</td>
<td>0.3</td>
<td>10</td>
<td>40</td>
<td>17.6</td>
</tr>
</tbody>
</table>

* ΣFBC, Fractional bactericidal concentration (see text).

RESULTS

Checkerboard titration of ampicillin and chloramphenicol.

The results of the checkerboard titration are shown in Table 1. All four strains showed antagonism between the two antibiotics, indicated by a fractional bactericidal concentration of >4.

Killing experiments. The effect of an ampicillin-chloramphenicol combination on the Chl² strain and on all three Chl strains is shown in Table 2 and Fig. 1. The kill achieved by ampicillin alone was reduced by the addition of chloramphenicol at concentrations close to the MIC. As the concentration of chloramphenicol increased beyond the MIC, the kill achieved by the combination increased and correlated closely with that achieved by chloramphenicol alone.

Disk diffusion tests. Figure 2 shows the results obtained by the agar transfer method. After overnight incubation, zones of inhibition could be seen for both the chloramphenicol and the ampicillin disks. After the second incubation there was confluent growth up to the edge of the chloramphenicol disk, indicating failure to kill; and this zone of failure to kill around the chloramphenicol disk extended into the zone of ampicillin killing, indicating that chloramphenicol interfered with the killing action of ampicillin. The result illustrated in Fig. 2 was obtained with strain B, and similar results were obtained reproducibly with all Chl strains.

DISCUSSION

After the introduction of chloramphenicol, it was generally regarded as the treatment of choice for H. influenzae meningitis (29) until ampicillin became available. Resistance to ampicillin was first described in H. influenzae in 1974 (7), and the prevalence of resistant strains rapidly increased to the point at which it was not safe to continue to rely on ampicillin as the initial treatment for H. influenzae type b meningitis. It was then recommended that physicians use chloramphenicol and ampicillin in combination for the initial treatment of this infection (11), and from that time until the present combined therapy has been practiced widely.

It is therefore relevant to examine the combined action of chloramphenicol and ampicillin on H. influenzae in vitro. Previous investigators have reached conflicting conclusions. Results of one early study, in which a rather imprecise method was used, reported interstrain variability (24). Synergy between ampicillin and chloramphenicol on H. influenzae has been described in checkerboard experiments (13), but the results were only evaluated by visual inspection of the tubes after overnight growth without subculture, which is often necessary to demonstrate antagonism between antibiotics. Results reported by others (10, 12, 22, 28, 30) from growth experiments indicate that the antibacterial action of ampicillin may sometimes be inhibited by chloramphenicol, and in some of these studies (10, 22, 28) there is an indication that the degree of antagonism may be related to the chloramphenicol concentration. The isolation in our laboratory of three strains of H. influenzae resistant to chloramphenicol provided an opportunity to investigate further the interaction of these two antibiotics.

We have demonstrated here that in the case of our Chl strains, inhibition of the bactericidal effect of ampicillin by chloramphenicol can occur, and that this effect is maximal at concentrations of chloramphenicol close to the MIC (10 µg/ml) (Tables 1 and 2 and Fig. 1). This conclusion is supported qualitatively by the results obtained by the agar diffusion method (Fig. 2).

Chloramphenicol antagonized the early killing produced by ampicillin in both the Chl and Chl² strains. The late killing observed in combination only with the Chl² strain was probably due to ampicillin for the following reasons. After 12 h, a delayed growth occurred in the Chl² strains exposed to chloramphenicol alone. Since replication could occur under these conditions the cells would be susceptible to killing by ampicillin (Fig. 1). This phenomenon may be due to enzymatic inactivation of the chloramphenicol by the Chl² strains following prolonged incubation, as it did not occur with the Chl strain for which the chloramphenicol alone was bactericidal at 20 h.

On the basis of these results and those from a previous study (22), we propose the following hypothesis to explain our results on the interaction of these two antibiotics on H. influenzae. As the concentration of chloramphenicol is in-

TABLE 2. Kill achieved by combinations of ampicillin and chloramphenicol

<table>
<thead>
<tr>
<th>Chloramphenicol concn (µg/ml)</th>
<th>A (Chl²)</th>
<th>B (Chl)</th>
<th>C (Chl²)</th>
<th>D (Chl²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (5 µg/ml)</td>
<td>No ampicillin</td>
<td>Ampicillin (5 µg/ml)</td>
<td>No ampicillin</td>
<td>Ampicillin (5 µg/ml)</td>
</tr>
<tr>
<td>0</td>
<td>3.3</td>
<td>G</td>
<td>2.8</td>
<td>G</td>
</tr>
<tr>
<td>0.6</td>
<td>1.7</td>
<td>G</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td></td>
<td>0.9</td>
<td>G</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.5</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3.6</td>
<td>3.6</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>20</td>
<td>1.7</td>
<td>1.4</td>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>40</td>
<td>1.9</td>
<td>1.9</td>
<td>4.0</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Incubation was for 7 h.

* G, Bacterial growth occurred with no killing.
creased from zero toward the MIC there is progressively increasing interference with the bactericidal action of ampicillin which is greatest at or close to the chloramphenicol MIC. As the concentration of chloramphenicol is increased beyond the MIC, the chloramphenicol bactericidal effect increases so that the inhibitory action on ampicillin is masked. There is therefore a critical antagonistic concentration of chloramphenicol at the MIC, at which the bactericidal effect of ampicillin is maximally inhibited but at which the chloramphenicol is not bactericidal.

Results of an early study (18) showed that in *Klebsiella pneumoniae*, chloramphenicol at a concentration of 1 μg/ml was more antagonistic to penicillin than at a concentration of 25 μg/ml. A study of gram-negative bacilli other than *H. influenzae* has shown antagonism between chloramphenicol and ampicillin in those strains in which the chloramphenicol was only bacteriostatic (1). The results of both these studies provide additional indirect evidence in support of our hypothesis, but these results are for other organisms.

Chloramphenicol levels in the range of 5 to 10 μg/ml are commonly achieved in cerebrospinal fluid by using current therapeutic regimes (27, 34, 38). These levels are in the bactericidal range for Chl strains but would be in the critical antagonistic concentration range for Chl strains. In this
The isolation of Chl' Amp^a *H. influenzae*, both type b and nontypable, has also been reported from many parts of the world (2-4, 6, 8, 9, 12, 17, 20, 21, 23, 26, 31, 37, 39). In one of these studies (8), there was a description of a poor initial response, in a case of meningitis, to an adequate dose of ampicillin in the presence of chloramphenicol, despite the fact that the organism was susceptible to ampicillin. This might be explained by our findings in this study.

With regard to the Chl' Amp^a strains, our results suggest that ampicillin alone is adequate therapy but that the presence of even high levels of chloramphenicol in blood and cerebrospinal fluid represents a danger, and we seriously question the assumption that ampicillin is always effective against Chl' organisms when used in combination with chloramphenicol. Chloramphenicol has served well as therapy for *H. influenzae* type b meningitis for many years, and at present chloramphenicol resistance is not sufficiently common to justify its replacement. Currently, it is appropriate, however, to draw attention to the risk indicated in this study and to point out the possibility that combined therapy might fail with Chl' Amp^a strains owing to the phenomenon described here.

**LITERATURE CITED**


**FIG. 2. (A) Incubation of agar membrane overnight showing growth inhibition produced by a 10-μg chloramphenicol disk (C) and a 10-μg ampicillin disk (labeled A). Strain B (Amp^a Chl^a). (B) Further incubation after transfer of agar membrane to new plate. The bactericidal action of the ampicillin was inhibited by chloramphenicol (arrow).**

situation, with combined therapy it is possible that neither chloramphenicol nor ampicillin would be active therapeutically, and the result could be therapeutic failure. The antagonistic effect that we describe is less evident at 20 h than at 12 h in the time-kill experiments (Fig. 1), but the short-term nature of the antagonistic effect demonstrated in vitro does not reduce its potential clinical significance for two reasons. First, in meningitis the action of antimicrobial therapy in the first few hours of treatment is in many cases a critical factor in the prognosis. Second, although it is possible that under our experimental conditions there may be late inactivation of chloramphenicol, permitting bactericidal activity by ampicillin, in the clinical situation the levels of chloramphenicol in tissue and cerebrospinal fluid are probably maintained by repeated doses at levels sufficient to inhibit the bactericidal activity of ampicillin.

Double resistance to both ampicillin and chloramphenicol has been reported previously (5, 6, 16, 19, 32, 35, 36). In this situation neither chloramphenicol nor ampicillin is a suitable drug to use for treatment.
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