Effect of Carbon Dioxide on Testing of Susceptibilities of Respiratory Tract Pathogens to Macrolide and Azalide Antimicrobial Agents

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The in vitro activities of erythromycin, azithromycin, and clarithromycin against 178 clinical isolates from the lower respiratory tract of patients with chronic obstructive pulmonary disease were determined by an agar dilution method. The plates were incubated in air alone or in 5% carbon dioxide. The MICs measured in air alone were lower for most isolates than those measured in 5% carbon dioxide, illustrating the “pH effect” of incubation in carbon dioxide. Testing of isolates in 5% carbon dioxide on pH-adjusted medium (pH 8.4) resulted in MICs of one or two doubling dilutions lower than those obtained on agar with a neutral pH. A bioassay of the three agents incubated in air and in 5% carbon dioxide resulted in a significant loss of activity of all three agents in the carbon dioxide-enriched atmosphere. However, this loss-of-activity effect was significantly reduced when the bioassay medium was adjusted to pH 8.4 prior to incubation in 5% carbon dioxide.

Materials and Methods

Bacteria and growth conditions. Seventeen isolates of S. pneumoniae, 51 isolates of H. influenzae, 48 isolates of M. catarrhalis, and 62 isolates of H. parainfluenzae recovered from the sputum of patients with chronic obstructive pulmonary disease were used throughout. Two quality control strains were used, H. influenzae NCTC 8466 and S. pneumoniae NCTC 7406. The indicator strain Sarcina lutea was used in microbiological plate assay experiments. The liquid medium for all strains was brain heart infusion broth (Unipath, Basingstoke, United Kingdom) supplemented with NAD (Sigma) at a final concentration of 10 μg/ml and hemin, at a final concentration of 10 μg/ml. The solid medium was Iso-sensitest agar (Unipath) supplemented with 5% lysose horse blood and 10 μg of NAD per ml.

Antimicrobial agents. The following antibiotics were obtained from their respective manufacturers and made up and used according to the manufacturers’ instructions: erythromycin (Abbott), azithromycin (Pfizer), and clarithromycin (Abbott).

Growth conditions. All isolates were grown overnight in 5% carbon dioxide in liquid medium to give a viable count of ~106 CFU/ml. Three strains of S. pneumoniae, and three strains of H. influenzae were inoculated into duplicate brain heart infusion broths (supplemented with hemin and NAD for H. influenzae strains): one broth was incubated in 5% carbon dioxide and the other in ambient air. After overnight incubation, viable counts were obtained; there was no significant reduction in the numbers of viable cells after overnight incubation in air compared with 5% carbon dioxide, with similar numbers (5 × 105 CFU/ml) for both incubation atmospheres for all strains. However, when grown in air, 26 clinical isolates of H. influenzae (n = 19) and S. pneumoniae (n = 7) grew poorly or did not grow at all, indicating a requirement for an atmosphere that is enriched with carbon dioxide.

Determinations of susceptibility. The MIC of each antibiotic for each strain was determined by the agar doubling dilution method on Iso-sensitest agar supplemented with 5% lysose horse blood and NAD. Two sets of plates containing doubling dilutions of antibiotic were inoculated by transferring 106 CFU/ml. One set of plates was incubated overnight in air and the other in 5% carbon dioxide. All isolates were inoculated onto an antibiotic-free plate to confirm growth. After 24 h incubation, the lowest concentration of antibiotic that inhibited growth was defined as the MIC; single colonies or hazy growth were ignored. The MIC was expressed in micrograms per milliliter, and antibiotics that inhibited growth were defined as the MIC; single colonies or hazy growth were ignored. The MIC was expressed in micrograms per milliliter, and antibiograms per milliliter, and geometric means were calculated for the four bacterial species against each antimicrobial agent and compared with the breakpoint concentrations recommended by National Committee for Clinical Laboratory Standards (NCCLS) and British Society of Antimicrobial Chemotherapy (BSAC) guidelines (9). These experiments were repeated on two further separate occasions. The MICs of the macrolides for 24 clinical isolates of H. influenzae and H. parainfluenzae were also determined in parallel with pH-adjusted medium (the pH was adjusted to 8.4 by the addition of filter-sterilized 1 M sodium hydroxide to the agar before the addition of antibiotic) and Iso-sensitest medium, pH 7.2. Both sets of plates were incubated in 5% carbon dioxide.
Microbiological assay of antimicrobial activity. To determine the stability of each agent in carbon dioxide and air, a bioassay was performed. Antibiotic agar no. 11 (150 ml) (Unipath) was prepared according to the manufacturer's instructions. Biocultived colonies were punched into agar with a no. 4 cork borer. One set of standard concentrations of each of the three agents was prepared (10, 5, 2.5, 1.25, and 0.6 μg/ml) and aliquoted into the wells of two sets of agar plates. One set of plates was incubated in air, and an identical set was incubated overnight in 5% carbon dioxide. Bioassays were performed for each agent in parallel and in triplicate on 3 separate days. The diameters of the zones of inhibition (in millimeters) were measured after 18 h of incubation. Bioassays were also carried out in pH-adjusted medium to quantify any difference in the stability of the three agents caused by a change in pH. pH adjustment was achieved by aseptic addition of sterile 1 M sodium hydroxide until a pH of 8.4 was reached. These plates were then incubated in 5% carbon dioxide; an identical set was incubated in air overnight.

RESULTS

Antimicrobial activity. For S. pneumoniae isolates, all MICs were well below the NCCLS- and BSAC-recommended breakpoint concentrations (Table 1). For erythromycin, there was no difference between the MIC50s and MIC90s of all three agents. The reduction in the in vitro activity was caused by a change in pH. For azithromycin and clarithromycin; geometric mean data reinforce this interpretation, with values in air being much lower. Although the majority of H. parainfluenzae isolates were resistant to erythromycin irrespective of incubation in air or carbon dioxide, for the other agents there were marked differences in the MICs and geometric means obtained in air versus carbon dioxide (Table 1). As for H. influenzae, many isolates tested in carbon dioxide were apparently resistant.

For the 24 strains tested on pH-adjusted medium, the MICs were consistently one or two doubling dilutions lower on the more alkaline medium than on agar at pH 7.2 when both sets of plates were incubated in 5% carbon dioxide (Table 2). Microbiological assay. After overnight incubation in air, the zone diameter of a 10-μg/ml standard of all three agents was unchanged (Fig. 1). However, overnight incubation in 5% carbon dioxide resulted in a substantial reduction in the potency of all three agents. The reduction in the in vitro activity was greater than that for some isolates.

<table>
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<tr>
<th>Antibiotic</th>
<th>Recommended breakpoint concentration</th>
<th>Microorganism</th>
<th>MIC of indicated antibiotic for:</th>
<th>MIC of indicated antibiotic for:</th>
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<tr>
<td></td>
<td>NCCLS</td>
<td>M. catarrhalis</td>
<td>S, sensitive; I, intermediate; R, resistant.</td>
<td>S, sensitive; I, intermediate; R, resistant.</td>
</tr>
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<td>Erythromycin</td>
<td>0.5 16 8 1 1</td>
<td>Air</td>
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<td>0.06 0.06 0.06 0.06 0.06</td>
</tr>
<tr>
<td></td>
<td>CO2</td>
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<td>0.05 0.05 0.05 0.05 0.05</td>
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<tr>
<td>Clarithromycin</td>
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<tr>
<td>Azithromycin</td>
<td>1 4 2 1</td>
<td>Air</td>
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TABLE 1. Susceptibilities of isolates to macrolides in air and CO2.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC of indicated antibiotic for:</th>
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</tr>
</tbody>
</table>

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most marked for azithromycin; when measured after overnight incubation in carbon dioxide, the 10-μg/ml standard was found to contain <0.6 μg of active compound per ml. For erythromycin, overnight incubation in carbon dioxide reduced the 10-μg/ml standard to 1.25 μg/ml. For clarithromycin, the reduction was from 10 to 1.8 μg/ml after overnight incubation in carbon dioxide. For the 5- and 2.5-μg/ml concentrations, there were no measurable zones of inhibition after incubation in carbon dioxide.

Overnight incubation of pH-adjusted agar in 5% carbon dioxide resulted in a less marked reduction in the potency of all three agents. For both clarithromycin and erythromycin, the 10-μg/ml standard, when measured after overnight incubation, was found to contain 5.4 and 5.0 μg/ml, respectively; similar percent reductions in activity were observed for both the 5.0- and 2.5-μg/ml standards. The reduction in the in vitro activity was again most marked for azithromycin; when measured after overnight incubation in carbon dioxide, the 10-μg/ml standard was found to contain only 1.0 μg/ml. For the 5.0-μg/ml standard, only 0.6 μg of active compound per ml remained.

**DISCUSSION**

These data show that the activities of azithromycin, clarithromycin, and erythromycin are dramatically affected by the incubation atmosphere, most likely due to an effect of carbon dioxide upon the agent. Some microbiology laboratories perform sensitivity testing of bacterial respiratory tract isolates in an atmosphere containing increased carbon dioxide, potentially leading to overestimation of the MICs of macrolides and an incorrect designation of resistance. The decrease in activity of these agents observed in carbon dioxide is therefore of major importance. Other studies have shown that incubation in carbon dioxide leads to a marked decrease in the pH of the medium (1, 5), with these agents becoming less active as the pH becomes more acidic. However, for testing of macrolides against organisms that require incubation in a carbon dioxide atmosphere for growth, the pH of the growth medium can be adjusted to 8.4 after autoclaving, thus compensating for (although not completely eliminating) the reduction in pH which occurs during incubation and allowing for the testing of bac-

**FIG. 1.** Effects of incubation of bioassays in air (□), 5% CO₂ (■), and pH-adjusted medium with CO₂ (□□) upon the microbiological activities of erythromycin, clarithromycin, and azithromycin.
terial species requiring carbon dioxide (1, 4). It is important to understand, however, that isolates recovered from the lower respiratory tract of patients with chronic obstructive pulmonary disease may be subjected in vivo to relatively high partial pressures of carbon dioxide in the lung. It has therefore been suggested that the activity of clarithromycin against isolates of H. influenzae may be significantly compromised in respiratory tract infections (6). In light of the data obtained in this study, it is recommended that susceptibility testing of macrolides for bacterial isolates from the lower respiratory tract, or those requiring carbon dioxide, be carried out wherever possible with pH-adjusted medium and incubation in carbon dioxide. Clinical trials of these agents with the emphasis on clinical outcome are critical for determining the significance of the in vitro findings of this study.

REFERENCES