Effect of Triethylenetetramine Dihydrochloride on the Antibiotic Susceptibility of *Pseudomonas aeruginosa*

B. LIGHT AND H. G. RIGGS, JR.*

Department of Microbiology, University of Missouri School of Medicine, Columbia, Missouri 65201

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A chelating agent, triethylenetetramine dihydrochloride, interacted synergistically in vitro with both gentamicin and carbenicillin against a clinical isolate of *Pseudomonas aeruginosa* designated Ps 15. The minimal inhibitory concentrations of carbenicillin and gentamicin for Ps 15 in a 50% serum-Trypticase soy broth were 250 and 72.9 μg/ml, respectively. However, addition of triethylenetetramine dihydrochloride to the 50% serum-Trypticase soy broth reduced the minimal inhibitory concentration of both antibiotics approximately 10-fold. A comparison of the growth of Ps 15 in 50% serum-Trypticase soy broth containing either of the antibiotics showed that a rapid decrease in the percentage of survivors only occurred when the chelating agent was present.

Recently, *Pseudomonas aeruginosa* has become a major problem for both patients and clinicians. Its increased prevalence in morbidity and mortality has been greatly complicated by its resistance to several antibiotic agents. Investigators have searched for both intrinsic and extrinsic factors which may be responsible for this antibiotic resistance (3, 4, 10, 12, 16). Many strains of *P. aeruginosa* may possess a cell wall which acts as a permeability barrier for antibiotic penetration (13). Studies have shown that the chelating agent, ethylenediaminetetraacetic acid (EDTA), increases the susceptibility of *P. aeruginosa* to various antibiotics in vitro (1, 17). EDTA has been shown to increase cell wall permeability by removal of calcium, which is involved in essential cross-linkages and cell wall integrity (5, 7-9, 14). However, due to the toxic properties of EDTA, its use in systemic therapy of *P. aeruginosa* infections has been limited (2). In contrast to EDTA, triethylenetetramine dihydrochloride (TRIEN dihydrochloride) has been shown to be a chelating agent of low toxicity (6). In addition, the compound has been noted to enhance the activity of carbenicillin against *P. aeruginosa* (15). The purpose of this study was to determine the efficacy of TRIEN dihydrochloride in enhancing the susceptibility of *P. aeruginosa* to carbenicillin and gentamicin.

**MATERIALS AND METHODS**

Culture. *P. aeruginosa* strain Ps 15 is a clinical isolate obtained from the University of Missouri Medical Center, Department of Pathology. The culture was stored on Trypticase soy agar (TSA; Baltimore Biological Laboratory) at 4°C and transferred to a fresh TSA slant every 2 weeks to maintain viability.

**Preparation of TRIEN dihydrochloride.** TRIEN dihydrochloride was prepared by the method of Dixon et al. (6). The crystalline product was analyzed by melting point determination and high-voltage paper electrophoresis of starting material and end product. Paper electrophoresis was performed in a formic acid solvent (pH 1.7) at 1,200 V for 30 min on no. 1 Whatman cellulose paper. The paper was subsequently stained with ninhydrin solution and dried in an 80°C oven for 15 min. Electrophoresis of the starting material TRIEN resulted in three distinct components, whereas the product, TRIEN dihydrochloride, only demonstrated one component. The melting point of the product was 115°C.

**Effect of TRIEN dihydrochloride and serum on the MICs of gentamicin and carbenicillin.** Minimal inhibitory concentrations (MICs) of gentamicin and carbenicillin for *P. aeruginosa* strain Ps 15 were determined by the tube dilution method. Strain Ps 15 was grown for 18 h in Trypticase soy broth (TSB) in a shaking water bath at 37°C. From a 1:1,000 dilution of this culture, 0.05 ml (containing 10^6 to 10^7 colony-forming units per ml) was inoculated into sets of tubes containing 1.0 ml of serial twofold dilutions of either carbenicillin or gentamicin prepared in TSB with and without 0.0025 M TRIEN dihydrochloride. This concentration of TRIEN dihydrochloride was used because results from growth in various concentrations of the reagent in TSB indicated that 0.0025 M was non-inhibitory. This experiment was also performed in a broth composed of 75% TSB and 25% human serum (vol/vol), designated TSB-25S, to determine the effect of the chelating agent on MICs when measured in broth containing serum.

In a similar manner this experiment was performed in TSB containing 50% serum (vol/vol), which was designated TSB-50S. The concentration of TRIEN dihydrochloride was increased to 0.010 M in these experiments because at lower concentrations when serum was added to the growth medium, there was no effect on the MIC of the antibiotic. This higher con-
centration of TRIEN dihydrochloride, although inhibitory to Ps 15 in TSB alone, does not affect the growth of the organisms in TSB-50S.

Each group of tubes was allowed to incubate at 37°C for 18 h. The MIC was then determined for carbenicillin and gentamicin under each of the conditions described.

**Effect of carbenicillin and gentamicin in combination with TRIEN dihydrochloride on growth.** A 1-ml amount of an overnight broth culture of *P. aeruginosa* strain Ps 15 was inoculated into tubes containing 10 ml of TSB-50S with one of the following: 0.0100 M TRIEN dihydrochloride; 10 μg of gentamicin per ml; 50 μg of carbenicillin per ml; 10 μg of gentamicin per ml plus 50 μg of carbenicillin per ml; 0.0100 M TRIEN dihydrochloride plus 10 μg of gentamicin per ml; 0.0100 M TRIEN dihydrochloride plus 50 μg of gentamicin per ml; or 0.0100 M TRIEN dihydrochloride plus 10 μg of gentamicin per ml and 50 μg of carbenicillin per ml. These tubes were placed in a shaking water bath at 37°C, and 0.1-ml samples were removed periodically, diluted, and plated on TSA to determine the viable cell counts under each condition.

Interaction of TRIEN dihydrochloride with carbenicillin or gentamicin. To determine the synergistic effect of TRIEN dihydrochloride in combination with either carbenicillin or gentamicin against *P. aeruginosa* strain Ps 15, a “checkerboard titration” was performed by the method of Lacey (11). The MIC of TRIEN dihydrochloride was determined by inoculating 0.05 ml of a 1:1,000 dilution, which contained 10^6 to 10^8 colony-forming units per ml, of an 18-h culture of strain Ps 15 into 1.0 ml of TSB containing various concentrations of TRIEN dihydrochloride. Six series of tubes containing 1.0 ml of serial twofold dilutions of carbenicillin in TSB were also prepared. To a series of these tubes, one of the following concentrations of TRIEN dihydrochloride was added: 0.0005, 0.0010, 0.0015, 0.0020, or 0.0025 M. A control series contained no TRIEN dihydrochloride. A similar titration was also carried out for gentamicin and TRIEN dihydrochloride. All tubes were inoculated with the above diluted culture of strain Ps 15 and incubated for 18 h at 37°C on a shaker.

**Effect of calcium on the MIC of gentamicin.** From a concentrated stock solution of calcium chloride, samples were added to sterile TSB (which normally contains 4 mg of calcium per 100 ml) to increase the final calcium concentration to 6, 8, 10, and 12 mg/100 ml. Serial twofold dilutions of gentamicin were made in groups of tubes containing the adjusted concentration of calcium and into TSB. These tubes were then inoculated with 0.05 ml of an 18-h culture of strain Ps 15 that had been diluted 1,000-fold. All tubes were then placed on a shaker at 37°C for 18 h.

**RESULTS**

The data in Table 1 indicate that in TSB, 0.0025 M TRIEN dihydrochloride was effective in reducing the MIC of gentamicin against strain Ps 15 from 7.8 to 1.0 μg/ml. A similar result was noted for carbenicillin as the MIC decreased from 37.5 to 15.0 μg/ml.

As increasing concentrations of serum were added to TSB, the MIC of gentamicin became larger. From a value of 7.8 μg/ml in TSB, it increased to 50 μg/ml in TSB-25S and to 72.9 μg/ml in TSB-50S. On the other hand, the concentration of serum did not affect the MIC of carbenicillin. This suggests that some serum factor is inhibitory to the action of gentamicin against strain Ps 15.

Concomitantly with the increase in MICs after the addition of serum, there was a decrease in effectiveness of 0.0025 M TRIEN dihydrochloride to lower the MICs. At this concentration of the chelating agent, there was no change in the MIC for gentamicin; and the MIC for carbenicillin was only lowered from 37.5 to 8.52 μg/ml. However, when the concentration of TRIEN dihydrochloride was increased to 0.0100 M, the MIC of gentamicin was reduced from 72.9 to 5.9 μg/ml and from 250.0 to 26.0 μg/ml for carbenicillin. These results indicate that although serum contains some factor inhibitory to gentamicin activity, TRIEN dihydrochloride may be able to reverse this inhibitory effect as well as enhance the effect of carbenicillin. These data were shown to be statistically significant at α = 0.05, as determined by the least significant difference test.

<table>
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<tr>
<th>Determination</th>
<th>Avg. MIC (μg/ml)</th>
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<tr>
<td></td>
<td>TSB</td>
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<tr>
<td>Gentamicin</td>
<td>7.8</td>
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<tr>
<td>Gentamicin + 0.0025 M TRIEN dihydrochloride</td>
<td>1.0</td>
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<tr>
<td>Gentamicin + 0.0100 M TRIEN dihydrochloride</td>
<td>NG*</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>37.5</td>
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<tr>
<td>Carbenicillin + 0.0025 M TRIEN dihydrochloride</td>
<td>15.0</td>
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<tr>
<td>Carbenicillin + 0.0100 M TRIEN dihydrochloride</td>
<td>NG</td>
</tr>
</tbody>
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* ND, Not determined.
* NG, 0.0100 M TRIEN dihydrochloride inhibits the growth of strain Ps 15 in TSB.
A comparison of growth of strain Ps 15 in carbenicillin or gentamicin with and without TRIEN dihydrochloride showed that a large decrease in cell counts only occurred when TRIEN dihydrochloride was added to broth containing either or both of the antibiotics (Fig. 1). The greatest decrease in viable cells occurred when gentamicin was used in combination with TRIEN dihydrochloride. When carbenicillin, gentamicin, and TRIEN dihydrochloride were all used in combination, the organisms were destroyed at the same rate as with only gentamicin and TRIEN dihydrochloride. This indicates an apparent lack of synergistic activity between gentamicin and carbenicillin in this strain. It was also demonstrated that gentamicin plus TRIEN dihydrochloride acted at a rapid rate, causing a 2-log decrease in viable cells in about 30 min.

Isobolograms describing the MICs of combinations of TRIEN dihydrochloride with either carbenicillin or gentamicin indicated a synergistic activity between TRIEN dihydrochloride and both antibiotics (Fig. 2 and 3). In both figures a straight line connecting the MICs of carbenicillin alone (250 μg/ml) or of gentamicin alone (7.75 μg/ml) with the MIC of TRIEN dihydrochloride alone (0.0055 M) defines the line of equivalence, which would describe the additive inhibitory effect of combinations of each antibiotic and TRIEN dihydrochloride. However, graphs of the actual MICs of combinations of various concentrations of TRIEN dihydrochloride and either antibiotic resulted in concave lines, indicating inhibition by concentrations lower than those expected by the additive interaction of the two compounds.

The minimal concentration of gentamicin necessary to inhibit *P. aeruginosa* strain Ps 15 was determined in TSB containing various concentrations of calcium. The results indicated that with increasing concentrations of calcium, a concomitant increase in MIC occurs (Table 2). TSB containing 4 mg of calcium per 100 ml required

![Figure 1. Effect of antibiotics and of antibiotics with 0.0100 M TRIEN dihydrochloride on the growth of *P. aeruginosa* strain Ps 15 in TSB-50S. Symbols: ●, 0.0100 M TRIEN dihydrochloride; ○, 10 μg of gentamicin per ml; △, 50 μg of carbenicillin per ml; □, 10 μg of gentamicin per ml + 50 μg of carbenicillin per ml; ▲, 10 μg of gentamicin per ml + 0.0100 M TRIEN dihydrochloride; ●, 50 μg of carbenicillin per ml + 0.0100 M TRIEN dihydrochloride; ■, 10 μg of gentamicin per ml + 50 μg of carbenicillin per ml + 0.0100 M TRIEN dihydrochloride.](image-url)
only 5.85 μg of gentamicin per ml to inhibit the growth of the organism, whereas TSB containing 12 mg of calcium per 100 ml required 93.75 μg of gentamicin per ml to inhibit growth. Because the MIC (62.5 μg/ml) determined in TSB containing 10 mg of calcium per 100 ml approximated the MIC (72.9 μg/ml) obtained in TSB-50S determined previously (Table 1), the chelating agent was added to this solution in an attempt to negate the effect of calcium. Addition of TRIEN dihydrochloride resulted in a decrease in MIC of gentamicin from 62.5 to 15.6 μg/mL.

**DISCUSSION**

Results from this study showed that the susceptibility of *P. aeruginosa* strain Ps 15 to gentamicin can be altered by varying the concentration of divalent cations in the media. Furthermore, it was demonstrated in vitro that gentamicin and carbenicillin resistance could be reversed by addition of the chelating agent TRIEN dihydrochloride to the media.

The MIC of gentamicin determined in media containing increasing levels of calcium required

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<tr>
<th>Calcium concn in TSB (mg/100 ml)</th>
<th>Gentamicin MIC (μg/ml)</th>
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<tr>
<td>4</td>
<td>5.85</td>
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<td>6</td>
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<td>8</td>
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<td>10</td>
<td>62.50</td>
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<td>12</td>
<td>93.75</td>
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higher concentrations of gentamicin to inhibit strain Ps 15. In TSB, which contains 4 mg of calcium per 100 ml, 7.8 μg of gentamicin per ml inhibited the organism. However, in TSB-50S (containing 7 mg of calcium per 100 ml) the MIC increased 10-fold. This phenomenon could also be demonstrated by adding calcium chloride directly to TSB. These results agree with the findings of Zimelis and Jackson (18), who noted that the presence of divalent cations was antagonistic to the action of aminoglycosides against P. aeruginosa. They speculated that divalent cations stiffen the lipoprotein membrane and prevent disruption of the membrane.

Reversal of the calcium antagonism could be accomplished by the addition of TRIEN dihydrochloride. The addition of TRIEN dihydrochloride to a final concentration of 0.0025 M in TSB lowered the MIC of gentamicin eightfold. However, this same level of TRIEN dihydrochloride did not affect the MICs which were determined in TSB-50S. Four times the concentration of TRIEN dihydrochloride used in the TSB experiments could be added to TSB-50S without causing any toxic effects to strain Ps 15. When the concentration of TRIEN dihydrochloride in TSB-50S was increased to 0.0100 M, the MIC decreased 12-fold. There are two possible explanations that may account for this phenomenon: (i) Sufficient chelating agent must be present in the medium to bind divalent cations in the medium as well as in the cell wall of the organism. Loss of these cations from the cell wall could alter the structure. Rogers et al. (14) showed that incubation of P. aeruginosa in EDTA caused the release of protein and lipopolysaccharide from the cell wall. It is suggested that TRIEN dihydrochloride may be acting in a manner similar to that of EDTA by causing an increase in cell permeability, thus allowing gentamicin easier access to its site of action in the cell; or (ii) it is possible that TRIEN dihydrochloride binds divalent cations in the medium only, thus preventing incorporation of the cations into the cell wall. This may ultimately prevent stabilization of the cell wall in new cells.

In contrast to the results obtained with gentamicin, increasing concentrations of calcium in the media had no effect on the MIC of carbenicillin against strain Ps 15. However, the addition of TRIEN dihydrochloride to either TSB or TSB-50S lowered the MIC of carbenicillin against strain Ps 15. Because altering the calcium level in the media did not affect the MIC of carbenicillin, it is suggested that chelation of divalent cations at the cell wall may be an important mechanism to increase the susceptibility of strain Ps 15 to carbenicillin. Barrett and Asscher (1) demonstrated that EDTA lowered the resistance of P. aeruginosa to carbenicillin. They proposed that removal of protein and lipopolysaccharide from the cell wall by EDTA treatment increased the permeability of the cell wall to carbenicillin. Recently, it was demonstrated that TRIEN dihydrochloride increased the susceptibility of P. aeruginosa to carbenicillin both in vitro and in vivo (15). It was reported that the addition of calcium and magnesium, in their chloride forms, to brain heart infusion broth containing 25% serum had no effect on the MIC of carbenicillin. This suggested that the chelating agent may act by binding divalent cations in the cell wall lipopolysaccharide rather than in the media to increase the permeability of the bacteria to the antibiotic.

Viability studies in TSB and in TSB-50S have established that the concentration of TRIEN dihydrochloride used in this study did not alter the growth of strain Ps 15. Therefore, the decrease in MICs of carbenicillin and gentamicin during treatment with TRIEN dihydrochloride cannot be attributed to any toxic effect of TRIEN dihydrochloride.

Isobolograms, showing the MIC of antibiotic at varying concentrations of TRIEN dihydrochloride, indicate that a synergistic interaction exists between the chelating agent and either gentamicin or carbenicillin. The interaction of TRIEN dihydrochloride and antibiotic was examined further by comparing the growth of strain Ps 15 in TSB-50S with and without TRIEN dihydrochloride. The addition of TRIEN dihydrochloride to TSB-50S containing either gentamicin or carbenicillin resulted in a large decrease in viable cell counts as compared to TSB-50S containing only antibiotic. Furthermore, when gentamicin, carbenicillin, and TRIEN dihydrochloride and antibiotic was exquisitely, strain Ps 15 was destroyed at a rate similar to that when only gentamicin and TRIEN dihydrochloride were used. These results suggest that a lack of synergistic activity exists between carbenicillin and gentamicin for strain Ps 15.

It is important to stress that the action of TRIEN dihydrochloride is extremely rapid, resulting in a 2-log drop in viable cells in only 30 min after its addition to the broth. The results of this study show that TRIEN dihydrochloride is a potentiator of antibiotic activity. Furthermore, due to its low toxicity (6) it is suggested that this compound has possibilities of being a valuable adjunct to the usual antibiotic therapy for P. aeruginosa infections.

ACKNOWLEDGMENTS

We express appreciation to Pfizer Inc. for the carbenicillin and to the Schering Corp. for the gentamicin which were used in these studies.
LITERATURE CITED


