

THE EFFECT OF TRIETHYLENETETRAMINE DIHYDROCHLORIDE  
ON THE *IN VIVO* SUSCEPTIBILITY OF *PSEUDOMONAS*  
*AERUGINOSA* TO GENTAMICIN

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A chelating agent, triethylenetetramine dihydrochloride (TRIEN dihydrochloride) increased the efficacy of gentamicin *in vivo* against a clinical isolate of *P. aeruginosa*, designated Ps 15. Mice which were inoculated with  $10 \times LD_{50}$  of Ps 15 and treated with doses of 2~16 mg of gentamicin per kg per day all died. However, treatment with 8 mg of gentamicin per kg body weight per day plus 30 mg of TRIEN dihydrochloride per day markedly reduced the mortality. The combined therapy also reduced the number of viable organisms that accumulated in the kidney during a 24-hour period post inoculation. When a dosage level of 8 mg of gentamicin was exceeded in the combined treatment regimen, all of the infected mice died, and a high concentration of endotoxin could be detected in the mouse sera by the limulus assay.

*Pseudomonas aeruginosa* infections continue to pose a problem to both patients and clinicians because of their high morbidity and mortality. Because of the organisms' resistance to antibiotics, effective control of *P. aeruginosa* infections has not been accomplished. It has been reported that the cell wall of *P. aeruginosa* may act as a permeability barrier for antibiotic penetration<sup>1,2</sup>. Investigators have demonstrated that treatment of *P. aeruginosa* with the chelating agent, ethylenediaminetetraacetic acid (EDTA), enhances the sensitivity of the organism to various antibiotics *in vitro*<sup>3,4</sup>. EDTA may increase the cell wall permeability by removal of divalent cations, which are involved in essential crosslinkages for cell wall stability<sup>5-8</sup>. However, due to its toxic properties, the use of EDTA in *P. aeruginosa* septicemia is contraindicated<sup>9</sup>. In contrast to EDTA, TRIEN dihydrochloride has been shown to be a chelating agent which is low in toxicity and nonteratogenic<sup>10</sup>. Furthermore, the compound has been shown to increase the susceptibility of *P. aeruginosa* to carbenicillin *in vitro*<sup>11</sup>. LIGHT and RIGGS<sup>12</sup> found that TRIEN dihydrochloride enhanced the activity of both gentamicin and carbenicillin against *P. aeruginosa in vitro*. The purpose of this study was to examine the efficacy of using TRIEN dihydrochloride to enhance the susceptibility of *P. aeruginosa* to gentamicin during a systemic infection.

### Materials and Methods

#### Culture

*Pseudomonas aeruginosa* strain Ps 15, a clinical isolate, resistant to both gentamicin and carbenicillin, was obtained from the University of Missouri Medical Center, Department of Pathology. The culture was stored on Trypticase Soy Agar (TSA;BBL) at 4°C and transferred to a fresh TSA slant every two weeks to maintain viability. *In vitro* sensitivity tests indicated that the organism was resistant to both gentamicin and TRIEN dihydrochloride; however, combinations of the two compounds inhibited the organism<sup>12</sup>. Inocula for *in vivo* testing were prepared by diluting cells from an 18-hour broth culture of Ps 15 in TSB to an optical density corresponding to the desired number of organisms derived from a standard curve.

#### Preparation and assay of TRIEN dihydrochloride

TRIEN dihydrochloride was synthesized and analyzed as previously described<sup>12)</sup>. Sensitivity of mice to the compound by the intraperitoneal route of administration was determined. Mice were given injections of TRIEN dihydrochloride in doses of 10, 12, 14 and 16 mg in groups of five mice for each dose, every 8 hours for 72 hours to examine toxicity.

#### In vivo testing

C57 Black mice (5 week old males) were injected intravenously with  $10 \times LD_{50}$  ( $2.3 \times 10^8$  cells) of *P. aeruginosa* in 0.2 ml broth. Groups of five infected mice were treated with either 2, 4, 6, 8, 10, 12, 14 or 16 mg of gentamicin per kg body weight per day. Additional groups of five mice received the above treatment regimen in combination with 30 mg of TRIEN dihydrochloride per day. A dose of 30 mg of TRIEN dihydrochloride per day (2.1 g of TRIEN per kg body weight per day) had been found to be the maximum non-toxic dose and was well tolerated by these mice in preliminary studies. The daily dosages of both gentamicin and TRIEN dihydrochloride were divided into 3 equal parts (0.2 ml each) and administered intraperitoneally beginning within 15 minutes post infection and then at 8-hour intervals for a period of two days, at which time the percent survival was determined for each group. Calcium levels were monitored during the experiment by extracting blood from the eye with a capillary pipette and analyzing the sample by atomic absorption spectrophotometry.

To examine the effect of a combination of gentamicin and TRIEN dihydrochloride on the degree of kidney infiltration, three groups of 15 mice each were injected intravenously with  $10 \times LD_{50}$  of *P. aeruginosa*. One group of infected mice was treated with sterile physiological saline as a control. Another group of mice received 8 mg of gentamicin per kg body weight per day, while a third group received the same level of gentamicin in combination with 30 mg of TRIEN dihydrochloride per day. At 2, 6, 12, 16 and 24 hours post infection, the kidneys were aseptically removed from three mice from each group and the number of bacteria in the organs per gram of wet weight was determined.

#### Limulus assay

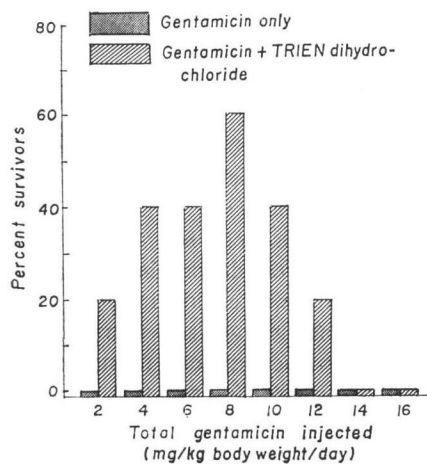
To examine endotoxin levels  $2.3 \times 10^8$  cells of *P. aeruginosa* strain Ps 15 were inoculated into three groups of six mice each. One group subsequently received 8 mg of gentamicin per kg body weight per day with 30 mg of TRIEN dihydrochloride daily. A second group was treated with 16 mg of gentamicin per kg body weight per day with 30 mg of TRIEN dihydrochloride daily, while a third group received no treatment. At 30 minutes and 16 hours post infection, three mice from each group were anesthetized, shaved and dissected aseptically to obtain cardiac blood with a 25 gauge tuberculin syringe. Contact of the needle with the skin of the mice was avoided to prevent extraneous endotoxin contamination. Cardiac blood was then placed in a sterile heparinized vial. As negative controls, blood was obtained from three healthy, uninfected mice as well as from three uninfected mice which had received 16 mg of gentamicin plus 30 mg of TRIEN dihydrochloride at each time interval. Limulus assays were performed according to the method of RIEGLE and COOPERSTOCK<sup>13)</sup>.

### Results

All mice which were infected with  $10 \times LD_{50}$  *P. aeruginosa* and then treated with only gentamicin at several dosage levels died (Fig. 1). However, administration of 30 mg of TRIEN dihydrochloride per day with various amounts of gentamicin showed a protective effect. At 48 hours post infection, the highest percent of survival (60%) occurred in mice which had been treated with TRIEN dihydrochloride in combination with 8 mg of gentamicin per kg body weight per day. With lower and higher dosages of gentamicin, the survival rate decreased.

The effect of this dosage on the number of viable organisms contained in the kidneys of mice which had been infected intravenously with  $2.3 \times 10^8$  organisms was monitored periodically for 24 hours. These data are presented in Fig. 2. Kidneys from control mice which received no antibiotic or gentamicin alone after infection contained  $> 10^7$  organisms/g. When mice were treated with 8 mg of gentami-

Fig. 1. Effect of treatment with varying concentrations of gentamicin with or without 30 mg of TRIEN dihydrochloride on the 48-hour survival of mice infected with  $10 \times LD_{50}$  *P. aeruginosa* Ps 15.



micin in combination with 30 mg of TRIEN dihydrochloride per day, cultures of kidney tissue produced significantly ( $p=0.05$ ) lower cell counts than the other two groups on all sampling occasions. A peak level of  $1.25 \times 10^6$  organisms/g was determined at 16 hours and there was a decrease to  $8.5 \times 10^5$  organisms/g at 24 hours.

To determine if the increase in mortality at the 16 mg dosage of gentamicin was due to increased endotoxin production, limulus assays were performed on sera from mice infected with Ps 15 (Table 1). Mice that were infected but not treated were found to have 3.0 ng of endotoxin/ml at 30 minutes post infection. By 16 hours the level had increased to 7.0 ng/ml. Sera from infected mice that were treated with 8 mg of gentamicin per kg body weight plus 30 mg of TRIEN dihydrochloride per day contained 2.0 ng of endotoxin/ml at 30 minutes post infection and 5.5 ng/ml at 16 hours. Sera from infected mice which had been treated with 16 mg of gentamicin per kg body weight per day and 30 mg

Fig. 2. Effect of treatment with 8 mg of gentamicin/kg body wt/day with and without 30 mg of TRIEN dihydrochloride on the average concentration of *P. aeruginosa* Ps 15 in the kidneys of infected mice at various times post infection.

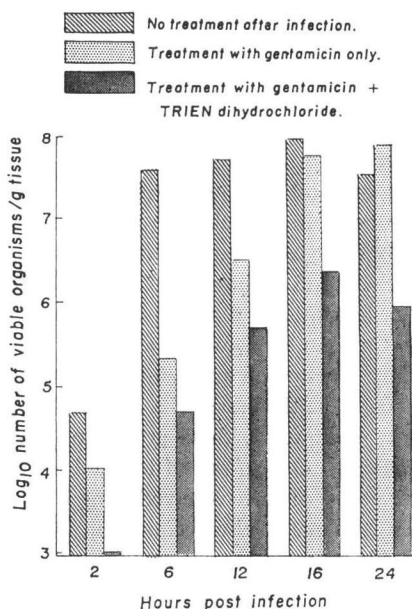


Table 1. Serum endotoxin level in *P. aeruginosa* strain Ps 15 infected mice during treatment with gentamicin plus TRIEN dihydrochloride.

		Average concentration of endotoxin post infection (ng/ml)	
		30 min.	16 hr.
Uninfected control mice	a) Untreated	<0.1	<0.1
	b) 16 mg Gentamicin/kg+30 mg TRIEN	<0.1	<0.1
Infected mice*	a) Untreated	3.0	7.0
	b) 8 mg Gentamicin/kg+30 mg TRIEN	2.0	5.5
	c) 16 mg Gentamicin/kg+30 mg TRIEN	30.0	No survivors

\* Mice injected with  $2.3 \times 10^8$  cells ( $10 \times LD_{50}$ ).

of TRIEN dihydrochloride per day contained 30.0 ng of endotoxin/ml at 30 minutes after infection. This level of endotoxin is significantly ( $p=0.05$ ) higher than the levels in the infected control and in the infected mice treated with 8 mg of gentamicin. Data could not be obtained from this group at 16 hours because there were no survivors. Less than 0.1 ng of endotoxin/ml was detected in sera from uninfected control mice and in uninfected mice which received 16 mg of gentamicin and 30 mg of TRIEN dihydrochloride, indicating that the treatment compounds had no effect on the endotoxin level and that the techniques employed in obtaining serum samples did not introduce extraneous endotoxin materials.

### Discussion

Mice treated with only gentamicin after intravenous injection of *P. aeruginosa* strain Ps 15 all succumbed to the bacterial infection. On the other hand, treatment of mice with 30 mg of TRIEN dihydrochloride in combination with gentamicin resulted in reduced mortality. In a previous study it was shown that the chelating agent acted synergistically with gentamicin in reducing the number of organisms *in vitro*<sup>12)</sup>. The proposed mechanism for this synergism was that the TRIEN dihydrochloride chelates cations either in the cell wall or in the medium which resulted in an increased permeability of the cell wall to the antibiotic. Since the *in vitro* studies were performed in broth containing 50% serum, it is assumed that the same mechanism for increasing the *in vitro* susceptibility of strain Ps 15 to gentamicin may also be operating *in vivo*. Serum calcium levels were monitored during each treatment regimen and remained within normal limits. This would strongly suggest that the chelating agent is reacting with the cell walls to increase the permeability rather than removing the cations from the medium.

Injection of different amounts of gentamicin in combination with 30 mg of TRIEN dihydrochloride daily in infected mice indicated that in this combination 8 mg of gentamicin per kg body weight per day was the optimal dosage of the antibiotic. Treatment with lower or higher amounts of gentamicin resulted in a reduction in survival of the infected mice. Because the highest dosage of gentamicin used in these studies (16 mg/kg of body weight/day) was considerably below the acute toxic level of 75 mg/kg reported by BLACK, *et al.*<sup>14)</sup>, the rapid mortality of infected mice receiving this dose was unexpected. Examination of healthy uninfected mice receiving this high dose of gentamicin alone showed that this dose was not toxic by itself nor in combination with 30 mg of TRIEN dihydrochloride. This suggested that the increased mortality was due to the interaction of the antibiotic, TRIEN dihydrochloride and the *P. aeruginosa*. Limulus assays of sera from infected mice showed that a 15-fold increase in endotoxin concentration resulted within 30 minutes after treatment with 16 mg of gentamicin per kg body wt per day in combination with 30 mg of TRIEN dihydrochloride per day as compared to the group treated with 8 mg of gentamicin in combination with the same dose of TRIEN dihydrochloride.

In a previous paper, it was reported that gentamicin plus TRIEN dihydrochloride acted rapidly on *P. aeruginosa in vitro*, resulting in a 2-log drop in viable cells in 30 minutes<sup>12)</sup>. BRYAN, *et al.*<sup>1)</sup> have shown that high broth concentrations of gentamicin cause greater accumulation of intracellular gentamicin in *P. aeruginosa*. Because *in vitro* results indicate that TRIEN dihydrochloride may increase the permeability of the organism to gentamicin, it is possible that the 16 mg dose of gentamicin caused a greater accumulation of intracellular antibiotic in the presence of TRIEN dihydrochloride. Therefore, due to the bactericidal properties of gentamicin, rapid lysis of the bacteria with subsequent release of cell wall material, may be responsible for the high level of endotoxin in the serum. However, it is possible that the higher doses of gentamicin suppressed the reticuloendothelial system, resulting in the increased levels of endotoxin. These results suggest that the groups of mice receiving the high dose of gentamicin died of endotoxic shock.

In a previous study, it was noted that the MIC of gentamicin determined in broth for strain Ps 15 was significantly lower than the MIC observed in a mixture of broth and serum<sup>12)</sup>. The inhibitory concentration of gentamicin in the serum-broth mixture (72  $\mu\text{g/ml}$ ) can not be achieved in a patient without toxic effects. However, the addition of TRIEN dihydrochloride to the serum-broth mixture

reduced the MIC of gentamicin to a level which is attainable and non-toxic in human serum. Furthermore, bacterial counts from tissue sampling from the kidneys indicated that the chelating agent causes an increased susceptibility of the bacteria to the antibiotic, resulting in a decrease in cell number presumably, before concentration could occur in the kidney. It is also important to stress that mice tolerated 2.1 g of TRIEN dihydrochloride per kg body weight per day, indicating a low toxicity of the compound. Therefore, it is suggested that TRIEN dihydrochloride if found non-toxic to humans<sup>10)</sup> may be a valuable adjunct to the usual gentamicin therapy for *P. aeruginosa* infections.

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