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Evaluation of Combination Chemotherapy in a Lightly Anesthetized Animal Model of *Pseudomonas* Pneumonia

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Gram-negative bacillary pneumonia is a major cause of morbidity and mortality in hospitalized patients. The use of synergistic combinations of aminoglycosides and beta-lactams for therapy of this infection has been recommended but remains controversial. We designed a new model of *Pseudomonas* pneumonia in a lightly sedated guinea pig by using a long-acting anesthetic to impair natural respiratory defenses. We used this model to compare the efficacy of ceftazidime and tobramycin alone and in combination in the therapy of *Pseudomonas* pneumonia. The two antibiotics were shown to be synergistic in vitro for the strain of *Pseudomonas aeruginosa* tested. Treated animals receiving both antibiotics had fewer viable bacteria remaining in lung tissues ($P < 0.05$) and exhibited a trend towards improved survival in comparison to animals receiving a single drug. In this model of *Pseudomonas* pneumonia, in vitro synergy was reflected by increased efficacy in vivo.

Therapy of gram-negative bacterial infections is currently undergoing a period of active investigation. Gram-negative bacillary organisms resistant to many antimicrobial agents have become increasingly important, especially in hospital-acquired infections (22, 25). The recent development of powerful and relatively nontoxic extended-spectrum penicillins and cephalosporins has provided the opportunity to develop new therapeutic approaches. Studies in vitro have shown these drugs to be very active against a wide range of resistant organisms. In addition, many of these drugs show synergism with aminoglycosides against these strains in vitro (13). Studies in both humans and other animals have shown these synergistic combinations to be advantageous in treating infections in hosts with impaired defenses, especially in neutropenic individuals (16, 27, 28). However, it is not yet known if there is a therapeutic advantage to using these synergistic drug combinations in patients with normal numbers of circulating polymorphonuclear leukocytes.

Two groups of patients are at high risk of developing gram-negative bacillary pneumonia: those who are neutropenic or immunosuppressed, and those who have depressed mechanisms of respiratory clearance, such as the lethargic or intubated patient. One prospective study of patients in an intensive care unit showed a 12% incidence of nosocomial gram-negative bacillary pneumonia in this population (15). By using a long-acting anesthetic agent that depresses the cough reflex, we developed a guinea pig model of pneumonia that resembles the infection in the intensive care patient. The present study utilizes this model to evaluate the potential in vivo value of combination therapy with ceftazidime and tobramycin, drugs which act synergistically in vitro against *Pseudomonas aeruginosa*, compared with therapy with either antibiotic used alone.

MATERIALS AND METHODS

Bacteria. A strain of *P. aeruginosa* (P-5) originally isolated from the blood and cerebrospinal fluid of a neutropenic patient (Claude Bernard Hospital, Paris, France) was used for these studies. The organisms were stored at −70°C, and fresh inocula were prepared for each experiment from cultures grown for 14 h in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and then rinsed twice and suspended in normal saline prior to use.

Antibiotics. The antibiotics used in these experiments were ceftazidime pentahydrate (Glaxo Co., Research Triangle Park, N.C.) and tobramycin sulfate (Eli Lilly & Co., Indianapolis, Ind.). Antibiotics were kept in powder form and were freshly diluted with saline prior to each experiment.

Animals. Male and female Hartley strain guinea pigs (350 to 450 g) were obtained from Animals West, Soqueal, Calif. Animals were housed in regulation cages with free access to food and water.

In vitro studies. The MIC of each antibiotic for the organism was determined by the tube dilution method by using a standard inoculum of 10^5 organisms in 1.0 ml of Mueller-Hinton broth supplemented with Mg^{2+} and Ca^{2+} (30). The mean of five separate determinations was used to calculate the MIC of each antibiotic.

Synergy was assessed by the checkerboard method under standard conditions previously described (18). An inoculum of 5×10^5 CFU of *P. aeruginosa* was placed in Mueller-Hinton broth supplemented with Mg^{2+} and Ca^{2+} to achieve a final volume of 1.0 ml. Synergy was considered present when growth was inhibited by concentrations of one-fourth or less the MIC for each individual drug. Time-kill studies with each antibiotic alone and in combination were also performed (18).

Concentrations of drugs for the time-kill studies were chosen to correspond with three sets of drug concentrations observed in the animal, corresponding to peak, midinterval, and trough time periods. Log-phase organisms were placed in 10 ml of supplemented Mueller-Hinton broth at 35°C to achieve an inoculum of 10^7 CFU/ml. Samples (0.1 ml) were removed at 0, 4, 8, and 24 h and quantitatively cultured by serial dilution to remove any effect of carryover of drug which might influence the viable counts. Significantly increased killing was defined as a ≥2-log decrease in bacterial numbers by the combination of antibiotics compared with that resulting from exposure to each drug alone.

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Pharmacokinetics. Preliminary drug-dosing studies were done in uninfected guinea pigs. Blood was obtained by cardiac puncture at 0.5, 1, 2, 4, and 8 h after drug administration. Serum concentrations of tobramycin and ceftazidime were measured by using standard agar dilution bioassay techniques (8). Test organisms used for these assays were *Escherichia coli* (ATCC 10536) for ceftazidime and *Staphylococcus epidermidis* (ATCC 27626) for tobramycin. Minimal detectable concentrations of antibiotics by these bioassays were 0.15 μg/ml for tobramycin and 0.015 μg/ml for ceftazidime. The log values of the antibiotic concentrations were plotted versus time, and the serum elimination-phase half-life was calculated from the slope of the line determined by the last three points.

Animals in all treatment groups had serum antibiotic concentrations measured 30 min after drug administration (peak) and just prior to a dose (trough). Also, lung tissue concentrations were measured 60 min after the first dose was administered. Lung was excised, washed with normal saline, and homogenized. Antibiotics were measured by the bioassay described above. Hemoglobin concentration in the homogenate was measured by quantitative colorimetric spectrophotometry (9). Antibiotic concentration in the lung homogenate was corrected for contamination with blood by comparison of the concentration of hemoglobin in the homogenate with that of the hemoglobin in circulating blood.

Development of model of pneumonia. Preliminary studies showed that guinea pigs only receiving short-acting anesthetics could rapidly clear a challenge of 10^8 CFU of *P. aeruginosa* given via intratracheal inoculation. As a means of reducing the ability of animals to cough and thus clear the infection, animals were sedated with an intraperitoneal injection of 1.82 g of urethane (Sigma Chemical Co., St. Louis, Mo.) per kg that had been diluted in saline. This drug produced a long-lasting (18 to 24 h), but light, anesthesia with depressed respiration, cough reflex, and movement. The animals were in a stuporous condition, responsive only to forceful stimulation or pain. The animals were evaluated every 4 h during an experiment for degree of sedation and were given urethane as needed, with the dose titrated to maintain a stuporous condition.

Urethane in doses used in these experiments had no effect on the numbers of circulating leukocytes: the mean leukocyte count was 7,520 ± 4,000 cells per mm^3 in awake animals and 7,360 ± 1,860 cells per mm^3 after urethane. Likewise, urethane did not appear to influence leukocyte migration in response to chemotactic stimuli (14). Activated complement was prepared by adding 2 mg of zymosan to 2 ml of guinea pig serum. After 30 min of incubation at 37°C, the suspension was centrifuged at 1,500 × *g* for 15 min and the supernatant was removed. The supernatant was given in a 0.2-ml intradermal injection to three sets of guinea pigs: animals sedated for 24 h with urethane, animals sedated for 1 h with urethane, and control animals. All animals also received 0.2-ml injections of normal saline as a control. Animals were sacrificed 3 h after injection, and skin samples were blindly read and scored for leukocyte infiltration. Equal numbers of leukocytes were seen at the sites of activated complement injection in all three sets of animals. Thus, urethane had no apparent effect on leukocyte migration.

Animals were infected by the method of Pennington and Ehrie (23). Each guinea pig's trachea was exposed by a vertical midline incision. A 0.5-ml portion of a bacterial suspension containing 5×10^7 CFU of *P. aeruginosa* was injected intratracheally with a syringe with a 25-gauge needle. Following inoculation, incisions were closed with stainless-steel wound clips, and the animals were gently shaken for 15 s to help distribute the inoculum in the lungs.

Sedated animals infected in this fashion developed a bilateral hemorrhagic pneumonia, which proved to be fatal in all animals within 48 h. Control animals had no mortality from urethane alone or from intratracheal injection of $<10^6$ CFU of *P. aeruginosa*.

Treatment regimens. Therapy was initiated 14 h after inoculation. Preliminary work had shown that *Pseudomonas* titers in the lungs decreased immediately after inoculation by approximately 1 log; however, by 14 h the bacterial titers were $>\log_{10} 7$ CFU per lung. Sections of the lungs at 14 h showed interstitial edema and a polymorphonuclear reaction (Fig. 1).

Each experiment utilized 30 guinea pigs, of which 2 were sacrificed at 14 h to document the establishment of pneumonia. Remaining animals were divided into one control and three treatment groups. During development of the model, animals were treated with various doses of tobramycin (1, 2.5, or 5 mg/kg every 4 h) to show that therapeutic response is dose related. After the validity of the model was verified, treatment groups received ceftazidime alone (150 mg/kg every 8 h), tobramycin alone (5 mg/kg every 4 h), or a combination of ceftazidime and tobramycin given at these same doses. These doses were selected because they provided serum levels in the same range as when these drugs are used in humans. All antibiotics were given as intramuscular injections in the flank for a total treatment period of 36 h.

Evaluation of therapy. Animals alive at 36 h after therapy was begun were sacrificed with a 1.0-ml intraperitoneal injection of sodium pentobarbital. Lungs were aseptically removed, rinsed with sterile saline, weighed, and added to 20 ml of sterile saline.

A homogenizer (Polytron; Brinkmann Instruments, Inc., Westbury, N.Y.) was used to create a suspension of lung tissue. This homogenate was quantitatively cultured on blood agar plates. After overnight incubation at 37°C, viable bacteria were counted and expressed as $\log_{10}$ CFU per lung. A volume of 0.05 ml of homogenate from animals that had received tobramycin was also cultured onto plates containing 5 μg of tobramycin per ml, and the plates were incubated for 48 h to detect the emergence of drug-resistant subpopulations.

Statistics. Comparisons between lung titers from treatment groups were made by using Student's *t* test with the Bonferroni correction factor (11).

RESULTS

In vitro studies. The MICs of ceftazidime and tobramycin for *P. aeruginosa* P-5 were 4 and 1 μg/ml, respectively. The two antibiotics showed synergy by the checkerboard method, as the organism was inhibited by a combination of 0.25 μg of tobramycin and 1.0 μg of ceftazidime per ml. Time-kill studies were done at three different antibiotic concentrations: those reflecting peak concentrations (ceftazidime, 128 μg/ml; tobramycin, 8 μg/ml); intermediate concentrations (ceftazidime, 32 μg/ml; tobramycin, 4 μg/ml); and trough concentrations (ceftazidime, 8 μg/ml; tobramycin, 1 μg/ml) (Fig. 2). Significantly increased killing was detected only at the trough concentrations of antibiotics; at the peak and intermediate concentrations, the combination was only as effective as tobramycin alone.

Serum concentrations and half-lives of tobramycin and ceftazidime are shown in Table 1. In 11 animals studied, the mean ceftazidime concentration in lung tissue was 129 ± 40

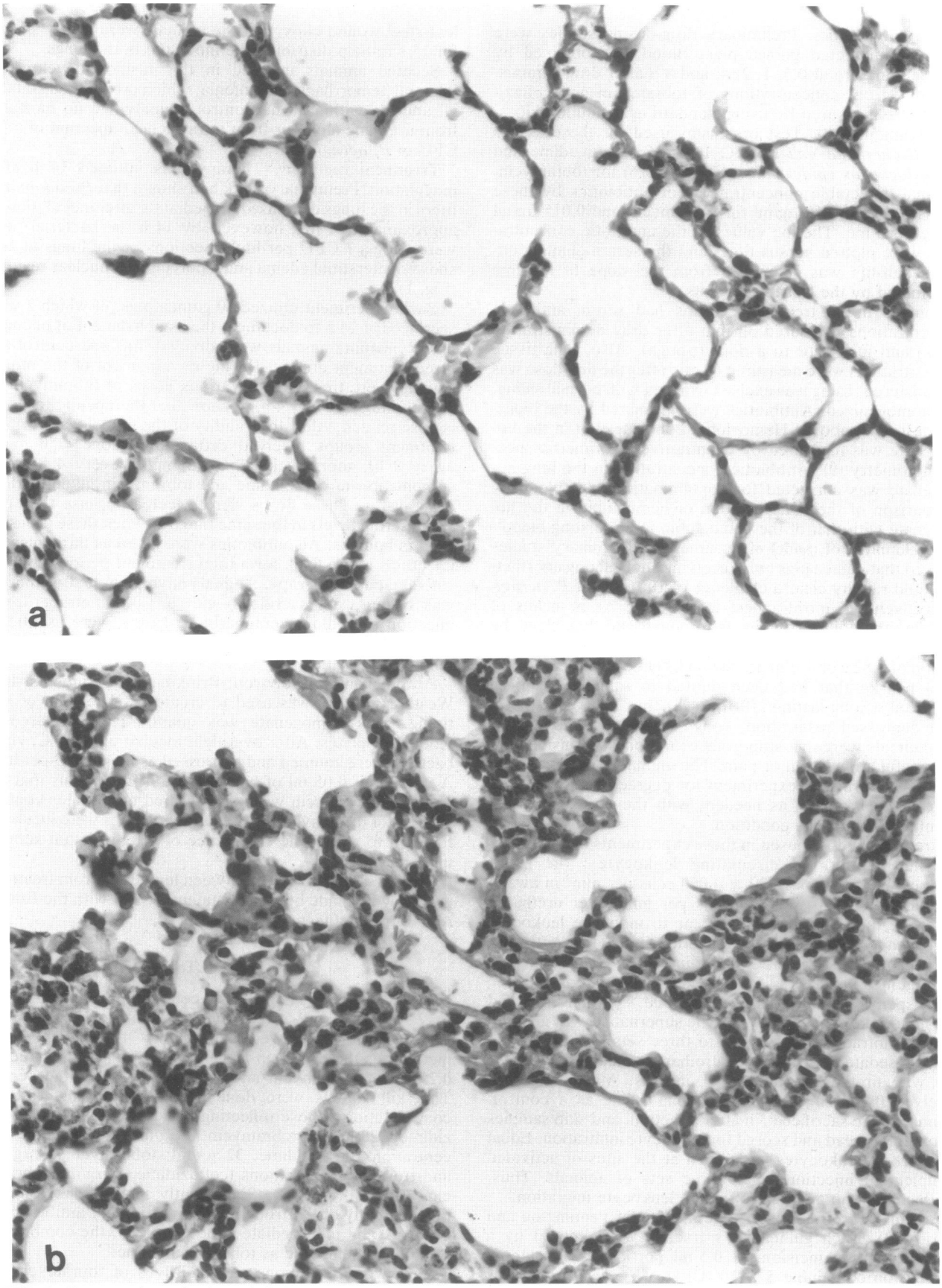

FIG. 1. Cross sections of guinea pig lung. (a) normal; (b) 14 h after inoculation with *P. aeruginosa*. An intense inflammatory reaction can be seen in panel b. Magnification, ×250.

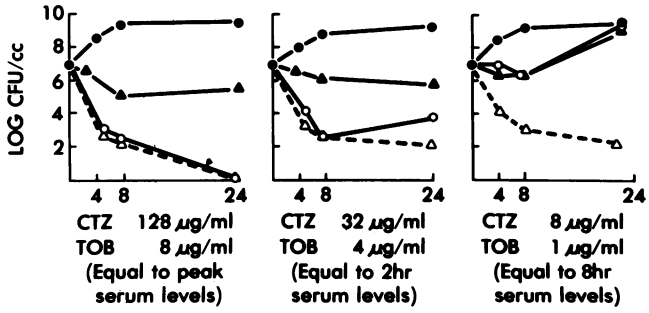

FIG. 2. Time-kill curves of *P. aeruginosa* with test antibiotics at three drug concentrations. A total of 10^7 CFU were incubated for 24 h with ceftazidime (CTZ), tobramycin (TOB), and combined antibiotics at peak drug (left), midinterval (center), and trough (right) concentrations. Significantly increased killing is demonstrated only at trough concentrations. Symbols: ●, control; ▲, ceftazidime; ○, tobramycin; △, ceftazidime and tobramycin.

μg/ml, almost 90% of the serum level. Tobramycin was not detectable in the lung tissue of the 10 animals assayed.

Efficacy of therapy. Animals received a mean inoculum of $\log_{10}$ 7.43 CFU of *P. aeruginosa*. Six animals were sacrificed at the start of therapy, 14 h after inoculation, to document the presence of a well-established pneumonia. These animals were found to have a bilateral hemorrhagic pneumonia and titers of $\log_{10}$ 7.92 ± 0.64 CFU of *P. aeruginosa* per lung. All untreated animals died within 48 h of inoculation, with high titers of *P. aeruginosa* in their lungs ($\log_{10}$ 8.3 ± 0.64 CFU per lung). All treatment groups demonstrated marked diminution in numbers of bacteria and improved survival in comparison to untreated animals. Animals receiving a low dose of tobramycin during the model development (1 and 2.5 mg/kg) had a <2-log reduction in bacteria. The animals receiving both antibiotics had significantly fewer *P. aeruginosa* at the end of therapy than those in either of the two groups receiving either antibiotic alone ($P < 0.5$) (Fig. 3). Animals receiving tobramycin had $\log_{10}$ 6.4 ± 0.64 CFU per lung with 64% (7 of 11) survival; animals receiving ceftazidime had $\log_{10}$ 5.66 ± 0.82 CFU per lung with 73% (11 of 15) survival. Animals receiving combined antibiotic therapy had $\log_{10}$ 4.50 ± 1.37 CFU per lung with 82% (9 of 11) survival. While survival appeared to be improved in the group receiving combined antibiotics, survival differences between groups did not achieve statistical significance. Tobramycin-resistant strains of *P. aeruginosa* were not isolated at autopsy.

DISCUSSION

Animal models of gram-negative bacillary pneumonia have been difficult to develop because of the ability of a healthy animal to clear the infecting organisms rapidly (4, 23, 29). It has been shown that the granulocyte plays a key role in the clearance of *P. aeruginosa* and other gram-negative organisms (26). Although several groups have rendered animals neutropenic to overcome this rapid clearance and thereby establish a gram-negative bacterial pneumonia (23, 27), results of studies in these neutropenic animals may not be relevant to results in the host with normal circulating granulocytes.

We have developed a model of *P. aeruginosa* pneumonia which allows therapeutic studies in animals with impaired respiratory defense mechanisms. Rendering the animal lethargic by a long-acting, relatively light anesthetic inhibits the animal's cough reflex. Initiation of therapy is delayed for 14 h until a pneumonia is well established. Other animal models of gram-negative pneumonia have depended upon initiation of therapy soon after bacteria are instilled, often as early as 15 min after instillation of bacteria (24). When therapy is initiated near the time of inoculation, results may not accurately reflect results in the patient in whom therapy is not started until after an infection is well established.

We have used this model to address the question of whether or not antimicrobial synergism in vitro is reflected in improved efficacy in vivo. This question had been difficult to answer in humans because of the large number of variables in complex and acutely ill patients. Several groups have attempted to correlate the outcome of illness in patients with gram-negative bacterial infections to the antibiotic regimens used. Klastersky et al. have reported two large series in which patients who received synergistic combinations of drugs seemed to have a more favorable outcome (16, 17). Patients in these studies either were neutropenic or had received cytotoxic therapy. Bodey and his group, however, found no advantage to combination therapy in 26 neutropenic patients with gram-negative bacterial infections (5), and a recent retrospective review of 100 cases of *Pseudomonas* bacteremia also showed no improvement in outcome in those receiving synergistic combinations of drugs as compared to patients receiving single drug therapy or combinations of antibiotics not found to be synergistic in vitro (2).

Because of the confusion resulting from analysis of data from patients, animal models are useful in determining the potential value of such synergistic antibiotic combinations. Several investigators have demonstrated increased efficacy with synergistic combinations of drugs in treating gram-negative bacterial infections in neutropenic animals. Scott

TABLE 1. Pharmacokinetics of ceftazidime and tobramycin in 15 guinea pigs

Antibiotic	Dose (mg/kg)	Serum concn (μg/ml)		Half-life (min)
		Peak (30 min)	Trough (prior to dose)	
Tobramycin	5 (every 4 h)	8.5 ± 3.5	2.2 ± 1.0	30
Ceftazidime	150 (every 8 h)	143.6 ± 35	4.9 ± 3.1	65

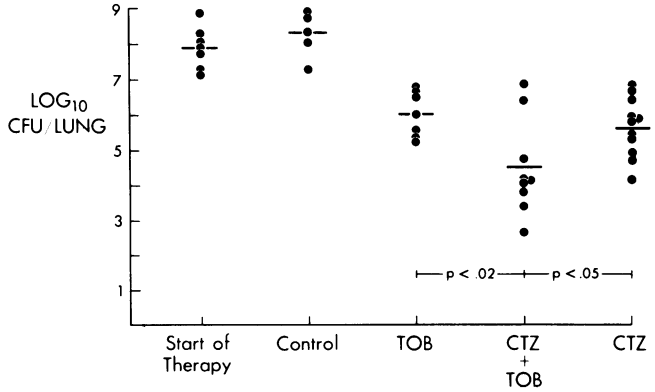

FIG. 3. Number of *P. aeruginosa* in guinea pigs treated with tobramycin (TOB), ceftazidime (CTZ), or both antibiotics. Each mark represents a single animal.

and Robson showed the combination of carbenicillin and gentamicin to be more effective than either drug alone in the therapy of *Pseudomonas* bacteremia in neutropenic rats (28). More recently, Chadwick and co-workers found the combination of amikacin and imipenem to be more effective than either drug alone in a similar model of *Pseudomonas* sepsis in neutropenic rats (6). This laboratory recently reported a new model of *Pseudomonas* pneumonia in neutropenic guinea pigs (27). In these neutropenic animals combined therapy was shown to be more effective than individual agents if a variety of beta-lactams and aminoglycosides that were synergistic in vitro were used. Results from studies in animals with normal circulating granulocytes have been mixed. In studies by Andriole, rats given a lethal intraperitoneal inoculum of *Pseudomonas* sp. had improved survival when treated with synergistic combinations of antibiotics as compared to those treated with aminoglycosides or beta-lactams given alone (1). Other investigators have failed to demonstrate an advantage to synergistic drug combinations in experimental sepsis, pneumonia, and osteomyelitis in animals with normal host defenses (7, 20, 24).

In this experiment we studied ceftazidime and tobramycin in the therapy of *P. aeruginosa* pneumonia. Ceftazidime was chosen because of its superior activity against *P. aeruginosa* among extended-spectrum cephalosporins (12). The strain of *P. aeruginosa* used was found to be fairly representative in its susceptibilities to both of these antibiotics (19). Both checkerboard and time-kill curves were used to test for drug interactions, as these two tests sometimes provide conflicting results (21). Although ceftazidime and tobramycin exhibited significantly increased inhibition of the P-5 strain by both methods, it is interesting to note that only at drug concentrations reflecting trough levels did ceftazidime and tobramycin display significantly increased killing in the time-kill studies. At higher antibiotic concentrations tobramycin alone was as effective as combined therapy. This finding may be relevant as only small amounts of tobramycin were present in lung tissue in the treated animals.

The trend towards improved survival and decreased bacterial counts in the lungs of the animals receiving combined therapy supports the concept that synergy in vitro may be reflected by improved efficacy in vivo in treating *Pseudomonas* pneumonia in the nonneutropenic host. This accelerated in vivo bacterial clearance could not be explained by the elimination of aminoglycoside-resistant bacteria by the beta-lactam drug, as has been suggested by Gerber et al. (10), since we failed to isolate aminoglycoside-resistant organisms from animals treated with tobramycin alone. It is possible, however, that 36 h is too short for aminoglycoside resistance to develop. Bayer and co-workers found the emergence of both aminoglycoside and beta-lactam resistance in animals treated for *Pseudomonas* endocarditis, but resistance developed only after 1 week of combined therapy (3).

The improved outcome in the animals receiving combined antibiotics gives support to those who recommend two drugs in the therapy of *P. aeruginosa* pneumonia. This dual therapy may be particularly valuable when the two drugs are shown to be synergistic in vitro. Further studies with this animal model, varying both the infecting organism and the antibiotics tested, are needed to verify this generalization.

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