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Treatment of Respiratory *Klebsiella pneumoniae* Infection in Mice with Aerosols of Kanamycin

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Aerosols of kanamycin resulted in greater survival of mice challenged with respiratory *Klebsiella pneumoniae* than the same dosage given intramuscularly. Determinations of viable bacteria in the blood and lungs revealed that aerosolized kanamycin was most effective when infection was confined to the lungs. After the organisms had spread to other areas, however, aerosol therapy was still more effective than intramuscular therapy, but only one-half the infected mice survived.

Antimicrobial therapy by means of aerosols has had varying popularity since it inception in 1942, when Barach et al. (1) demonstrated that guinea pigs were protected against infection with *Mycobacterium tuberculosis* by inhalation of sodium glucosulfone. After a great number of trials of aerosols of penicillin after World War II, interest in this mode of treatment declined until the 1960s, when the discovery of antibiotics that were poorly absorbed into the circulation from the lung led to renewed investigations (2, 10). Three antibacterial agents are currently of greatest interest for aerosol use: kanamycin, polymyxin B, and gentamicin (10). The rationale for the use of aerosols of these antibiotics has been that they could be used for extended periods to treat chronic obstructive lung disease with little fear of systemic toxicity. In recent years, the increased incidence and high mortality of pneumonias of gram-negative bacterial origin have placed added emphasis on the aminoglycoside group of antibiotics (3, 8, 12, 14).

The objectives of this investigation have been to establish a model for study of aerosol therapy of gram-negative bacillary pneumonia, to define the conditions under which it may be efficacious, and to provide the basic information for future systematic studies.

METHODS AND MATERIALS

Test organism. The A-D strain of (type 1) *Klebsiella pneumoniae* was grown on Trypticase soy agar for 16 to 18 h at 37 C. Cells were washed from the agar surface with 1% sterile peptone water containing 20% glycerol, distributed in 0.25-ml aliquots, and stored at −70 C. The virulence was maintained by intraperitoneal passage in mice at 6-week intervals. For experimental use, 0.25 ml of frozen seed was thawed, inoculated into a tube of 5.0 ml of Trypticase soy broth and incubated at 37 C for 16 h. The culture was adjusted on a spectrophotometer to an optical density of 0.40 at 550 nm against a Trypticase soy broth blank and then diluted 1:10 with Trypticase soy broth. The resulting suspension contained 5×10^7 to 6×10^7 viable cells/ml.

Test animals. Three-week-old, female, white ICR mice [Bla:(ICR)] were used in all experiments. They were housed in plastic cages and fed commercial mouse pellets and water ad libitum.

Aerosol exposure system and assays. The system employed for exposure of mice to organisms and antibiotic consisted of a Collison atomizer and Henderson-type exposure tube (6) with a plastic exposure box attached. For exposure, 20 mice were placed in a wire-mesh cage (10.5 by 12.5 by 22 cm), and as many as four cages were then placed in the exposure box. Aerosol sampling was accomplished with all-glass impingers (6) containing 20 ml of heart infusion broth (for sampling *K. pneumoniae*) or distilled water (for sampling antibiotic). Inhaled dosages of *Klebsiella* or kanamycin were calculated on a basis of inhalation of 25 ml of presented dose per min (4).

Particle size determinations. Aerosol particle diameters were determind by the single-stage impactor method as described by Malligo and Idoine (9). Determinations revealed that 50% of the bacteria disseminated was contained in particles <2.2 μm in diameter and 90% was in particles <5.0 μm. Fifty percent of the kanamycin (150 mg/ml) was contained in particles <3.5 μm and 90% was in particles <8.5 μm in diameter.

Assay procedures. Bacterial concentrations in impingers and tissues were enumerated by routine dilution and plating procedures. Kanamycin concentrations were determined by a modification of the method described for the assay of gentamicin by Winters et al. (15). Specifically, spores of *Bacillus subtilis* (ATCC 6633), contained in melted agar (Pen-

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assay agar no. 3, Difco), were layered on a base layer of agar and allowed to harden. Holes 3 mm in diameter were punched in the agar spaced about 1-cm apart and were filled with either standard or unknown solutions. (Laboratory standards were obtained from Bristol Laboratories, Syracuse, N.Y.) Zones of inhibition were measured after 16-h incubation at 37 C. Standard curves of inhibition versus concentration were plotted with concentrations ranging from 0.6 to 25.0 μg/ml, and test values were then determined from the standard curve.

Kanamycin solution. Kanamycin sulfate powder containing 78% kanamycin base (Kantrex, Bristol Laboratories, Syracuse, N.Y.) was dissolved in sterile water containing 2.2% sodium citrate. Sufficient concentrated sulfuric acid was added to adjust the pH to 4.5, and the final concentration was then adjusted to 150 mg of base per ml. For assay, all dilutions were prepared in 2.2% sodium citrate adjusted to pH 7.8 with sulfuric acid.

RESULTS

Experimental model. After preliminary studies, an experimental model was developed in which mice were given aerosol doses of 10^4 to 2×10^4 *K. pneumoniae*. This dose was lethal for 95% of the mice with a median time to death of 3.5 days. A few deaths were observed at 47 h, approximately 50% were dead by 72 h, and 90% were dead by 96 h.

Groups of five mice were killed by cervical dislocation at selected intervals after exposure, and their tissues were examined for histological changes. Representative sections are shown in Fig. 1. At 24 to 48 h small discrete inflammatory foci of polymorphonuclear neutrophils were seen throughout the lung (Fig. 1A). Concurrent bacteriological determination indicated that bacteria were present in the blood of approximately 67% of the mice, but only in small numbers (<100 organisms/ml).

At 48 to 72 h the foci became enlarged and/or coalescent to involve large portions of pulmonary lobes. Infiltrates still consisted largely of neutrophils. Vacuolated spaces within foci were found on close examination to be bacterial colonies, surrounded by capsular material (Fig. 1B and C).

By 54 to 72 h many mice exhibited extensive neutrophilic infiltration in the pleura and mediastinum caused by bacterial infection of the pleural cavity (Fig. 1D). The pleural membrane was invaded or ruptured at the site of a microabscess in the lung, resulting in pleuritis (Fig. 1D). Foci of bacteria and neutrophils in other organs such as liver, kidney, and spleen indicated an extensive bacteremia, an observation supported by isolation of bacteria from the blood. At 96 h and thereafter, only a few mice survived (~10%) and these manifested minimal histological change, principally small foci of macrophages admixed with neutrophils. However, most of the mice had died by this time, and any survivors may originally have had some innate resistance.

Initial therapy experiments. Initial screening of the response of infected mice to kanamycin therapy was carried out with doses of 5 mg/kg of body weight administered in aerosols or intramuscularly (i.m.). Comparison of the two routes of administration was carried out at 6, 24, and 30 h after infection. The concentration of kanamycin employed in the Collison nebulizer was 150 mg/ml, and exposures lasted for 40 min. Mice were injected i.m. with 0.1 ml of a 1,000-μg/ml solution. Uninfected mice were also treated with antibiotic; no deaths or clinical illness were observed in these groups through 21 days. At every time period the aerosol route of therapy was more effective (Fig. 2) than the i.m. route when survival rates were compared by the χ^2 test (Yates' correction).

Multiple therapy experiments. To investigate the effect of multiple dose therapy, two experiments were performed involving the administration of 10 mg of kanamycin per kg daily for three successive days by either aerosol or i.m. routes. Groups of five mice from each group were killed daily, and the concentration of viable *Klebsiella* in lung homogenates and in blood was determined. Therapy was initiated 24 h after bacterial infection in the first experiment and 48 h after infection in the second. A combined therapy group was included in the second experiment in which one-half of the daily 10-mg/kg dose was administered by aerosol and one-half by i.m. inoculation. Each experiment was replicated twice. Data are presented for both studies in Table 1; concentrations of viable organism after therapy are presented in Fig. 3. In both studies the aerosol treatment was more effective than the i.m. treatment. When initiation of therapy was delayed until 48 h, when the infection was widely disseminated, only one-half of the mice survived. In contrast, 92% of aerosol-treated mice survived when therapy was initiated at 24 h. The combined i.m. and aerosol therapy did not offer a significant advantage over the aerosol alone, i.e., 58% versus 50% survival. The viable *Klebsiella* concentrations (Fig. 3) indicate that the aerosol treatment cleared bacteria from the lungs more rapidly than did i.m. treatment.

Death patterns. The average geometric mean time to death and percentage of deaths calculated for nine experiments involving the administration of antibiotic doses ranging from

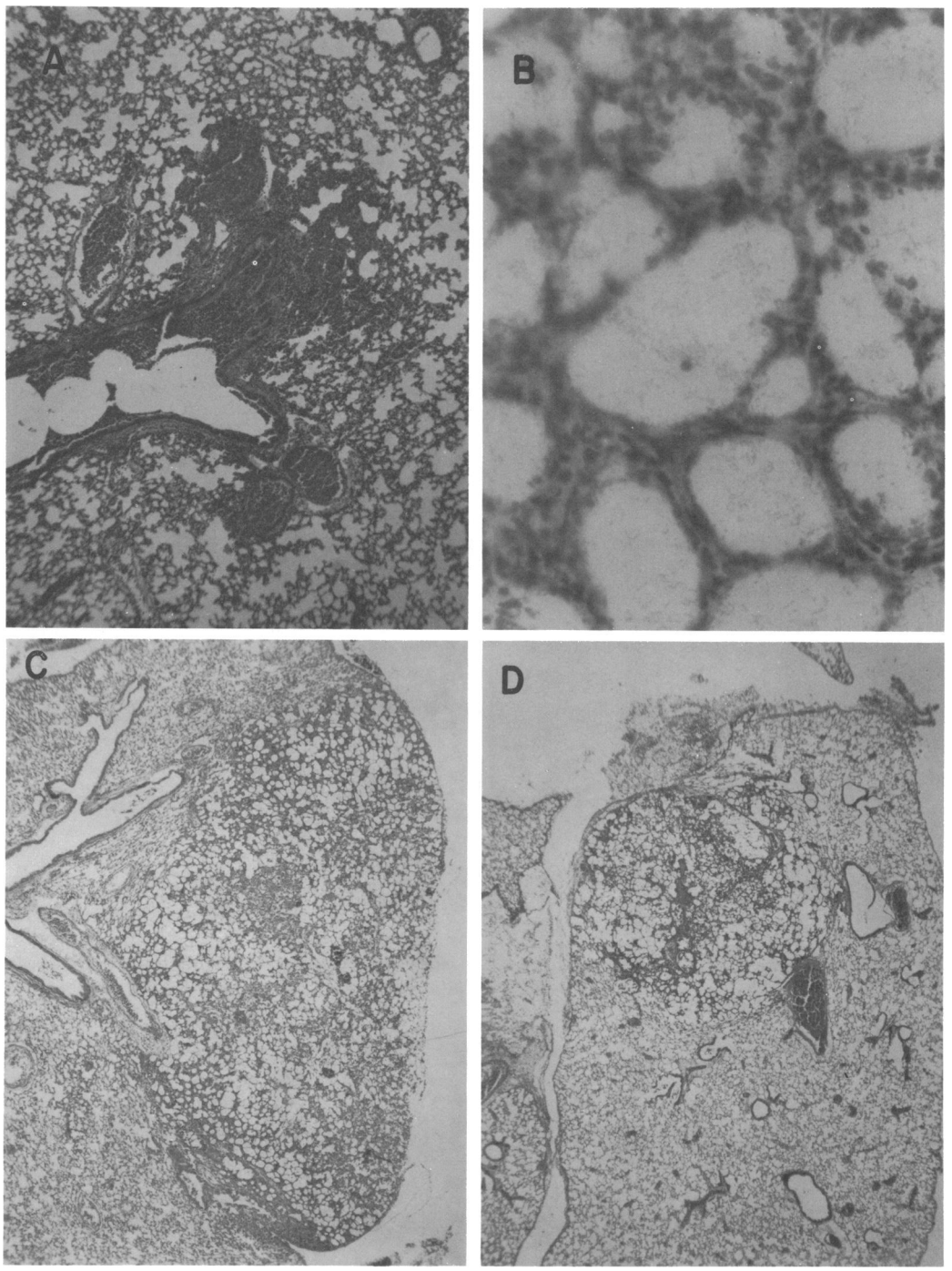

FIG. 1. *Histological changes in Klebsiella-infected mice. (A) Lung section 24 h after exposure showing polymorphonuclear neutrophil (PMN) infiltrate in bronchiole and pulmonary alveoli and focal bronchopneumonia, ×63. (B) Section at 48 h showing bacteria in pulmonary alveoli, ×720. (C) Lung section at 48 h showing PMN infiltration extending to the pleura, ×25. (D) Extension of a microabscess through pleural membrane at 54 h, ×25.*

5 to 20 mg/kg and times of administration varying from a single dose at 6 h to multiple doses at 48, 72, and 96 h are presented in Table 2. In each of the experiments the mean time to death of animals that died in the aerosol-treated groups was no different from organism controls but was lower than the i.m. treated group, even though significantly fewer mice died after aerosol than after i.m. treatment, i.e., 35% versus 60%. Intramuscular therapy significantly increased survival time of *Klebsiella*-infected mice but was the less effective of the two methods of therapy based on survival.

DISCUSSION

The results obtained in this study indicate that aerosols of kanamycin are superior to i.m. injection of the same drug for the treatment of *K. pneumoniae* in mice. Therapy was most effective when initiated very early in the disease process. The greater efficacy of aerosol therapy on numbers of bacteria in the blood indicates that the bacteremia at 24 h results from dissemination from the foci in the lungs and can be prevented or cleared by reduction of the original focus of infection, in this case, the lungs. In marked contrast, when the initiation of therapy was delayed until 48 h, the clearance of bacteria from blood occurred more rapidly after i.m. than after aerosol therapy. The resurgence in numbers of bacteria in blood and their persistence in lungs after 72 h, even in the presence of continuing therapy, suggests that extrapulmonary foci may be responsible for the bacteremia when therapy is delayed until 48 h. The persistence in the lung after 48 h of therapy probably results from microabscesses and/or pleuritis. The failure of combined routes of therapy to improve survival suggests either that the bacteria were disseminated into areas such as the pleural cavity where the blood supply was not sufficient to deliver significant amounts of kanamycin or that vascular necrosis and edema in the lungs prevented access of antibiotic both from the airways and the circulation.

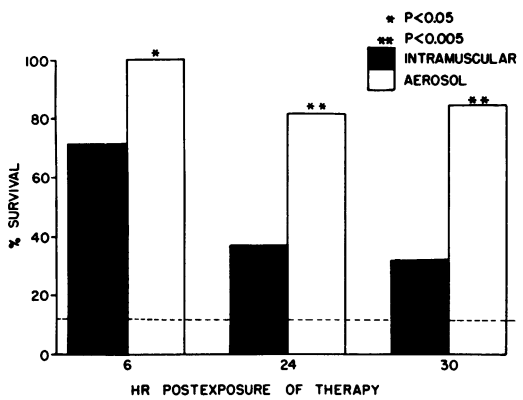

FIG. 2. *Effect of time of initiation of kanamycin therapy administered i.m. or as small-particle aerosols on survival rates of K. pneumoniae-infected mice. (Dashed line represents untreated bacterial control survival rate.)*

The great efficacy of a single, relatively low dose of aerosol-administered kanamycin is explained by persistence of the antibiotic in lung tissue. This persistence has been reported in mice by Prokhorova (11) and in rats by Teske and Miller (13). The effective aerosol dose was even lower than reported, since not all of the material inhaled was retained and that which was retained was widely distributed in the respiratory tract and was not all deposited in areas of infection (5).

The reason for reduced time-to-death after aerosol therapy is not known at present. One may speculate that sudden lysis of bacteria after treatment releases endotoxin or other substances that subsequently lead to the death of

TABLE 1. *Effect of multiple dose therapy*

Therapy[a]	Time of therapy (h postexposure)	Response	
		Survivors/ total	% Survivors[b]
None (organism control)	24, 48, 72	3/50	6
Aerosol		46/50	92 } $P < 0.005$
Intramuscular		35/50	70 } $P < 0.025$
None	48, 72, 96	7/91	8
Aerosol		38/76	50 } $P < 0.005$
Intramuscular		18/77	23 $P < 0.005$
Combined		14/24	58 } $P < 0.005$

[a] Treatment consisted of daily doses of 10 mg/kg; total, 30 mg/kg.
[b] Analysis by χ^2 test, Yates' correction.

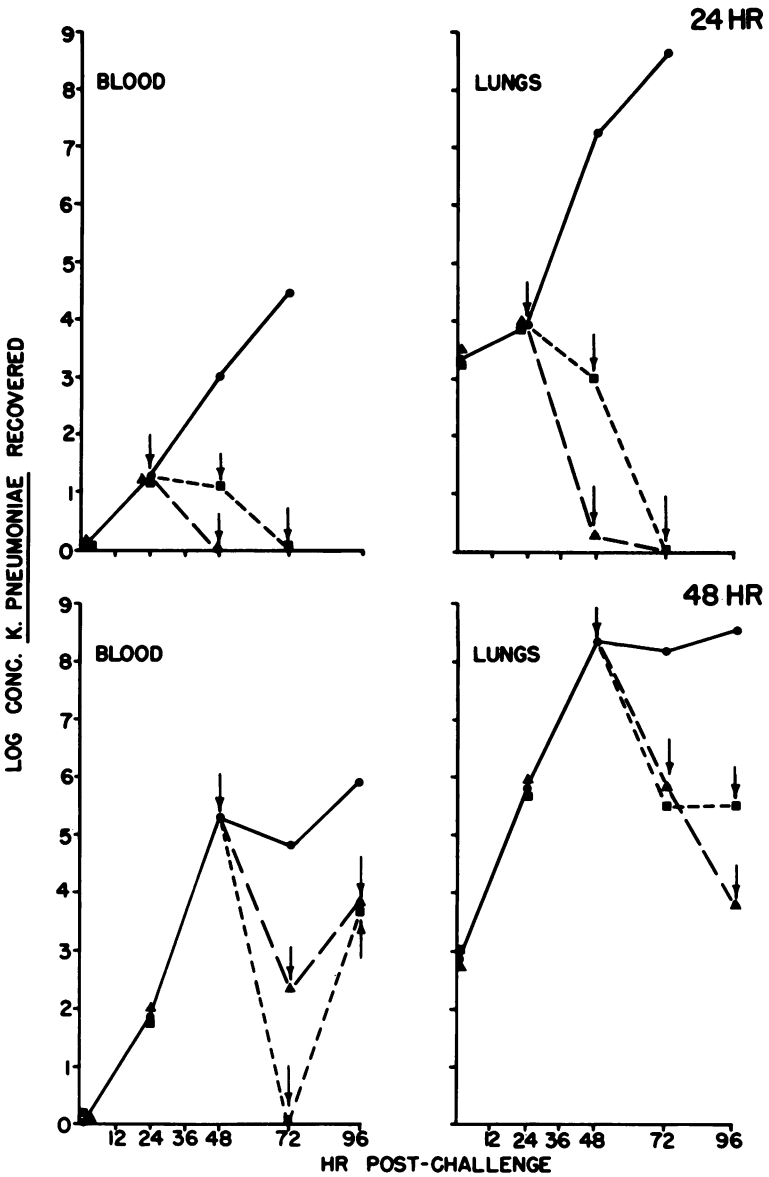

FIG. 3. *Lung and blood bacterial levels after multiple dose therapy beginning at 24 or 48 h. Symbols: Untreated controls, ●; aerosol-treated mice, ▲; i.m. treated mice, ■. Arrows indicate time of treatment.*

TABLE 2. *Effect of route of therapy on geometric mean time to death*

Route of therapy	MTD[a] (days)		SEM[b]	Death rate (%)		SEM
None (organism control)	$P < 0.005$	3.30	0.20	$P < 0.005$	89.8	2.16
Aerosol		3.39 $P < 0.005$	0.13		34.8 $P < 0.025$	7.42
i.m.		5.17	0.41		58.2	5.55

[a] MTD, Mean time to death; mean of nine experiments.
[b] SEM, Standard error of the mean.

the host. In any case, the principal advantage of aerosol treatment seems to be that small doses are more effective by aerosol than by the i.m. route; therefore, the risk of concomitant toxicity is greatly reduced. The lack of nephrotoxicity after aerosol administration of high doses of another aminoglycoside, gentamicin, has been reported (7).

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