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AEROSOL AND INTRAMUSCULAR PROPHYLAXIS OF RESPIRATORY 'KLEBSIELL--ETC(U)
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Aerosol and Intramuscular Prophylaxis of Respiratory Klebsiella pneumoniae Infection in Mice and Squirrel Monkeys.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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SUMMARY

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 Aerosol administration and intramuscular injection were compared for kanamycin prophylaxis against respiratory Klebsiella pneumoniae infection in both mice and squirrel monkeys. Mice challenged with an LD₉₀ of Klebsiella 0.5, 4, 24, and 48 and 72 hours after aerosol treatment with 27 mg/kg of kanamycin were significantly better protected at all time periods than were mice given 450 mg/kg by IM injection. Squirrel monkeys given 11.25 mg/kg by aerosol were completely protected against Klebsiella challenge at 6 and 24 hours whereas only 1 of 8 monkeys injected IM with the same dose survived at 6 hours and none survived at 24 hours. Antibiotic clearance curves suggested that kanamycin remained in the lungs at higher concentrations and for a longer time after aerosol than after IM treatment.

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Gram-negative bacterial pneumonia is an important medical problem in both man and animals. For example, a number of reports have been published in recent years indicating that the incidence of fatal pneumonia due to Klebsiella pneumoniae has increased.¹⁻⁵

We have shown that aerosols of kanamycin are more effective for the treatment of respiratory K. pneumoniae infection in mice than were the same doses of antibiotic given IM.⁶ The greater therapeutic efficacy of aerosols of antibiotic was apparently due to persistence in the lungs as reported by Prokhorova⁷ and Teske and Miller.⁸ Therefore, we also speculated that the administration of single doses of antibiotic by aerosol might be more effective prophylactically than the same dose injected IM. This report presents the results of experiments designed to investigate this possibility both in mice and in a recently developed model using the squirrel monkey.⁹

Materials and methods

Test Organism--Techniques for growing, storing and enhancing the virulence of the A-D strain of type 1 K. pneumoniae have been described previously.⁶ Inocula for infection of experimental animals were prepared as previously described.¹⁰

Test Animals--Monkeys: Healthy, juvenile, male squirrel monkeys (Saimiri sciureus) weighing from 0.5 to 1.0 kg were used. They were housed individually in wire-bar cages and were allowed free access to commercial monkey food and water. Their diet was supplemented with fresh fruit several times weekly.

Mice: 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.

Infection Techniques--Mice were infected with aerosols in a modified Henderson apparatus, and aerosol sampling was accomplished with all-glass impingers, filled with 20 ml of heart infusion broth.⁶ Estimation of viable bacterial concentration was accomplished by routine procedures of dilution and spreading on trypticase-soy agar plate. These values were then employed for inhaled dosage estimation by previously described calculations.⁶ Infection of monkeys was carried out by the intratracheal (IT) inoculation¹¹ of 7000 K. pneumoniae organisms.

Treatment Techniques--All kanamycin solutions were prepared from 78% kanamycin base^a dissolved in 0.085 M sodium citrate solution adjusted to pH 4.5 with concentrated sulphuric acid.

Aerosol treatment of mice with antibiotics was accomplished with the same Henderson apparatus that was used for infecting the animals.

^aBristol Laboratories, Syracuse, N.Y.

All calculations of inhaled aerosol dose were adjusted to allow for 50% retention of the drug in the lungs as previously described.⁶

Aerosol treatment of monkeys required fabrication of a special exposure box. This box (Fig 1) was constructed of acrylic plastic and was divided by a partition into two compartments each 32 cm wide by 25 cm deep by 40 cm high. Perch, exercise bars, and a tray for collection of urine and feces are shown. Four monkeys could thus be exposed to aerosols simultaneously. Aerosols were disseminated into the mixing tube at the top and exhausted through ports near the bottom of each compartment. Ports on the side of the box provided access for collection of aerosol samples to determine aerosol concentration of antibiotic. A port on the top of the box, fitted with a pressure gauge, permitted measurement of pressure differential between the box and the safety cabinet in which it was housed. This pressure differential was equal to 0.5 inch of water so that the exposure box was maintained at a lower pressure than the safety cabinet thus preventing escape of aerosols.

Kanamycin was disseminated into the system from a Collison nebulizer¹² at a rate of 0.22 ml/min. The aerosol particles were entrained in air flowing at a rate of about 40 liters/min. One-half this volume was directed into each exposure compartment. Figure 2 shows the entire aerosol-treatment system including the apparatus required to maintain an equal airflow to the two compartments as well as to equilibrate the air-balance during dissemination and sampling.

All-glass impingers containing 20 ml of distilled water were used for sampling the kanamycin aerosols at the middle of each

exposure period.

Kanamycin Assay--Kanamycin concentrations in impinger fluids and tissues were estimated by microbiological assay as previously described.⁶

Experimental Designs--Mice. The initial step consisted of a dose-response experiment to estimate the dose of K. pneumoniae that would kill 90% of challenged mice (LD₉₀). The LD₉₀ dose was calculated from response data by the method of Litchfield and Wilcoxon.¹³

The second phase of the study involved estimation of the dosage of kanamycin given once by aerosol or IM injection that would prevent mortality in 90% of infected animals (ED₉₀) that had been given an LD₉₀ dose of Klebsiella 6 hours earlier. The 6-hour period was arbitrarily chosen because the results of earlier studies indicated that aerosol therapy was completely effective at this time. The method of Litchfield and Wilcoxon¹³ was used to calculate the ED₉₀ values from the percent survival of mice after treatment with graded doses of antibiotic.

Prophylaxis was investigated by first administering an ED₉₀ dose of kanamycin to mice by aerosol or IM injection. Then, at 0.5, 4, 24, 48 and 72 hours, the treated mice were challenged with aerosols of K. pneumoniae. Untreated mice were also infected as controls. This experiment was repeated for a total of four replications. Statistical analysis of the differences between treatment groups was accomplished by Fisher's exact test.¹⁴

The rate of clearance of kanamycin from blood and lungs of IM or aerosol-treated mice was determined by killing 3 mice at each of several selected times and making 25% w/v (lung) or v/v (blood) homogenates in citrate-sulphate buffer, pH 4.5. Tissue was homogenized

with a Polytron.^b All homogenates were frozen at -30 C and later were assayed by previously reported procedures.⁶

Monkeys: Since the number of monkeys available was relatively small in comparison to the number of mice employed, many of the preliminary investigations that were carried out in mice were omitted. The IT challenge dose of K. pneumoniae was 7×10^3 . This value was obtained from previously published data.¹⁰ Twelve monkeys were treated by aerosol and 12 by IM injection. At 6 hours, 8 monkeys from each treatment group as well as 4 untreated controls were challenged with K. pneumoniae. The remaining 4 monkeys in each group and 4 more untreated controls were then challenged at 24 hours.

To determine kanamycin clearance patterns, 4 monkeys were given 15 mg/kg by aerosol and 4 received the same dose IM. Two untreated monkeys served as controls. At 6 hours, 2 monkeys from each group and 1 control were bled and then killed with pentobarbital given IV. Homogenates of blood and lungs were prepared and frozen in the same manner as the mouse tissues. The remaining monkeys were killed at 24 hours and samples were taken. Assay of antibiotic was carried out as previously described. Minimum inhibitory and bactericidal concentrations (MIC, MBC) were determined as described by Anderson.¹⁵

^bPolytronTM, Brinkmann Instruments, Westbury, N.Y.

Results

Minimum Inhibitory Concentration (MIC)--The MIC of kanamycin for this strain of Klebsiella was determined to be 0.625 $\mu\text{g/ml}$. The minimum bactericidal concentration (MBC) was 1.25 $\mu\text{g/ml}$.

Mice--The number of K. pneumoniae organisms producing a LD_{90} after aerosol exposure was determined by giving aerosol doses ranging from 8×10^1 to 8×10^4 cells to 5 groups of 15 mice each. The LD_{90} calculated by extrapolation from the response curve was 1.2×10^5 organisms (approximately 35 LD_{50}). This number of bacteria was employed as a challenge dose in all subsequent experiments.

In order to determine the dosage of kanamycin to use in further studies the ED_{90} therapeutic dose was calculated. One hundred thirty-five mice were given 35 LD_{50} of K. pneumoniae by aerosol. Six hours later, 4 groups of 15 mice each were injected IM with 0.1 ml of kanamycin solution ranging in concentration from 1.0 to 20 mg/kg of body weight. Similarly, 4 groups of 15 mice each were given from 1.0 to 15.0 mg/kg by aerosol. Fifteen mice were reserved as infected controls; all died. Calculations from dose responses are shown in Table 1. As expected from previous work,⁶ the aerosol route proved much more effective than the IM. The ED_{90} values for the aerosol and IM routes were 27 and 450 mg/kg respectively. These ED_{90} doses were then employed in all subsequent studies.

The prophylactic efficacy of aerosolized kanamycin was then compared to IM injection by administering an ED_{90} dosage of antibiotic to 40 mice for each route. At selected times after the administration of antibiotics groups of 10 mice were challenged with an ED_{90} of K. pneumoniae by aerosol. Ten untreated mice were infected at each time

period as controls. The survival of mice in 4 replicate experiments is shown graphically in Figure 3. All untreated mice died in this experiment. The difference in survival between the 2 routes of antibiotic administration was striking; IM injection protected 83% of the mice against challenge at 0.5 hour, 58% at 4 hours, and less than 10% thereafter; whereas, 100% of the mice were protected at 0.5 and 4 hours after aerosol treatment. Significant protection by aerosol persisted throughout the experiment.

Since kanamycin was more effective prophylactically by aerosol than IM, an experiment was carried out to determine the rate of clearance of antibiotic from blood and lungs. Mice were given an ED_{90} dose of kanamycin by aerosol or IM routes and were killed in groups of 3 at selected intervals ranging from 0.5 to 72 hours after treatment. The concentration of antibiotic in the blood of the IM treated mice killed at 30 minutes averaged 20 $\mu\text{g}/\text{ml}$. No kanamycin was detected in the blood of any other mice. The amount of kanamycin recovered from the lungs of the two groups is shown in Figure 4. Significantly more kanamycin was found in the lungs of the aerosol-treated group than in the IM-treated mice at every time period except at 0.5 hours.

Monkeys--The dose of kanamycin employed by both routes was 11.25 mg/kg. This dose was chosen arbitrarily and was based upon an assessment of the therapeutic efficacy of aerosols for monkeys (unpublished data). Monkeys were exposed by aerosol or injected IM with 11.25 mg/kg and challenged with 7000 K. pneumoniae 6 or 24 hours later. Four untreated controls were challenged at each time. The results of this trial (Table 2) show that aerosol prophylaxis was clearly

superior to IM injection at both time periods. In fact, IM prophylaxis had almost no effect.

In a kanamycin clearance experiment similar to that carried out in mice, the concentration of antibiotic in blood and lungs was determined at 6 and 24 hours; these were the times at which Klebsiella challenge had been carried out. No antibiotic was detected in the blood of any animal at any time. The lung clearance curves are given in Figure 5. Although aerosolized kanamycin was present in much larger quantities and was cleared more slowly than the injected material, it is of interest that a relatively large amount (21 μ g) was present in the lungs of IM treated monkeys at 6 hours. Despite this concentration, 7 of 8 monkeys died.

Discussion

Aerosols of kanamycin were significantly more effective than IM injection in preventing subsequent respiratory K. pneumoniae infection in both mice and squirrel monkeys. These results seem to be related to the concentration of kanamycin in the lungs. Correlation coefficients (r) calculated on the regression of log percent survival of mice vs. log kanamycin concentrations were 0.80 for both routes of challenge. The r could not be calculated for monkey survival data, but the high concentration of kanamycin in lungs of aerosol-treated monkeys is readily apparent. It is interesting, however, that the concentration of kanamycin in the lungs of IM-treated monkeys at 6 hours was 20 to 30 times the amount required to inhibit replication in vitro (MIC = 0.625 μ g/ml), but 7 of 8 monkeys died. A possible explanation may be that the site of persistence of

IM administered kanamycin in the lungs was different than the site of deposition of organisms. In contrast to monkeys, significant protection was seen when mice were challenged 4 hours after IM treatment despite the relatively low tissue concentrations of antibiotic detected at that time.

Persistence of aerosolized kanamycin in the lungs has been shown in mice⁷ and rats;¹⁶ our data seem to extend this list to squirrel monkeys. It has been of interest to us to determine whether the bound kanamycin was active in vivo or whether it dissociated and thereby became active only during the procedures associated with assay. Our prophylaxis data indicate that it is active in vivo in mice and monkeys even though it is bound, presumably to nucleic acid as reported by Potter et al.¹⁷

Our data suggest that single-dose aerosols of kanamycin will protect against subsequent respiratory infection. We have not yet attempted a multiple (i.e., more than one treatment by one route) or combined (i.e., IM and aerosol) regimen, nor have we investigated the problem of superinfection that so often complicates prophylaxis. Nevertheless, it appears that aerosolized antibiotic administration with aminoglycosides may be useful for the prophylaxis of infection in animals and man. Prophylaxis would be especially useful in patients at risk because of preexisting illness.

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TABLE 1--Response of Mice Treated with Kanamycin by the Aerosol or
IM Routes 6 Hours after Infection with Klebsiella pneumoniae

Route of therapy	ED ₅₀ (mg/kg)	95% Confidence limits	ED ₉₀ (mg/kg)
IM	63.0	37.3-106.5	450
Aerosol	1.4	0.6- 3.6	27

* Challenge dose of K. pneumoniae was 1.2×10^5 organisms (35 LD₅₀)

TABLE 2--Kanamycin Prophylaxis on K. pneumoniae Infection of Squirrel
Monkeys

Route of kanamycin administration*	Controls Dead/Total	Response to challenge at			
		6 hours		24 hours	
		Dead/Total	P**	Dead/Total	P
None	8/8	-	-	-	-
IM	-	7/8	0.005	4/4	0.05
Aerosol	-	0/8		0/4	

* Dose by both routes was 11.25 mg/kg.

** χ^2 test (Yate's correction) aerosol vs. IM response.

Figure Legends

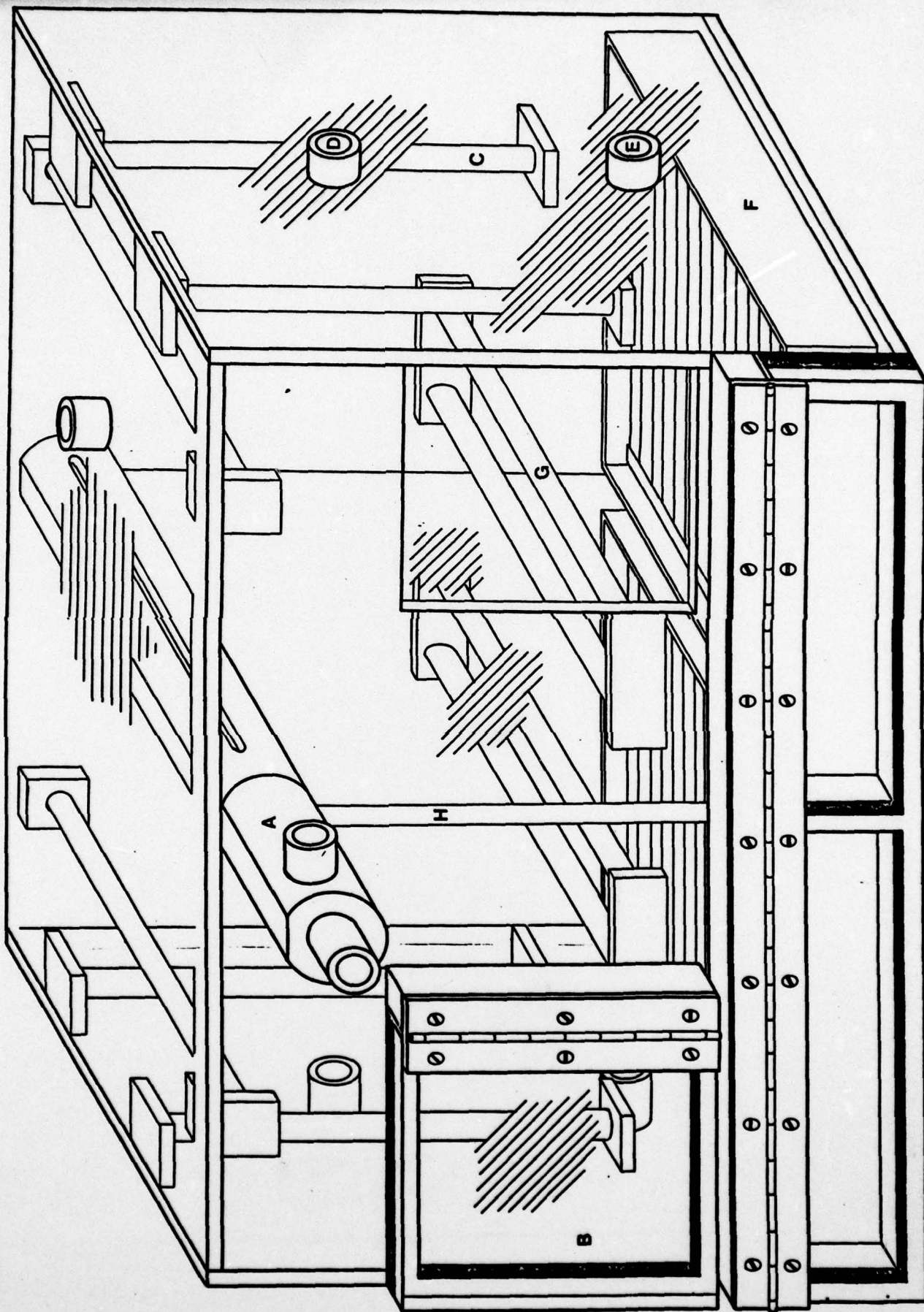
Fig 1--Box for the exposure of monkeys to aerosols of antibiotics (right door removed for clarity). A - Aerosol sampling tube; B - left door; C - exercise bar; D - sampling port; E - exhaust port; F - refuse tray; G - perch; H - partition.

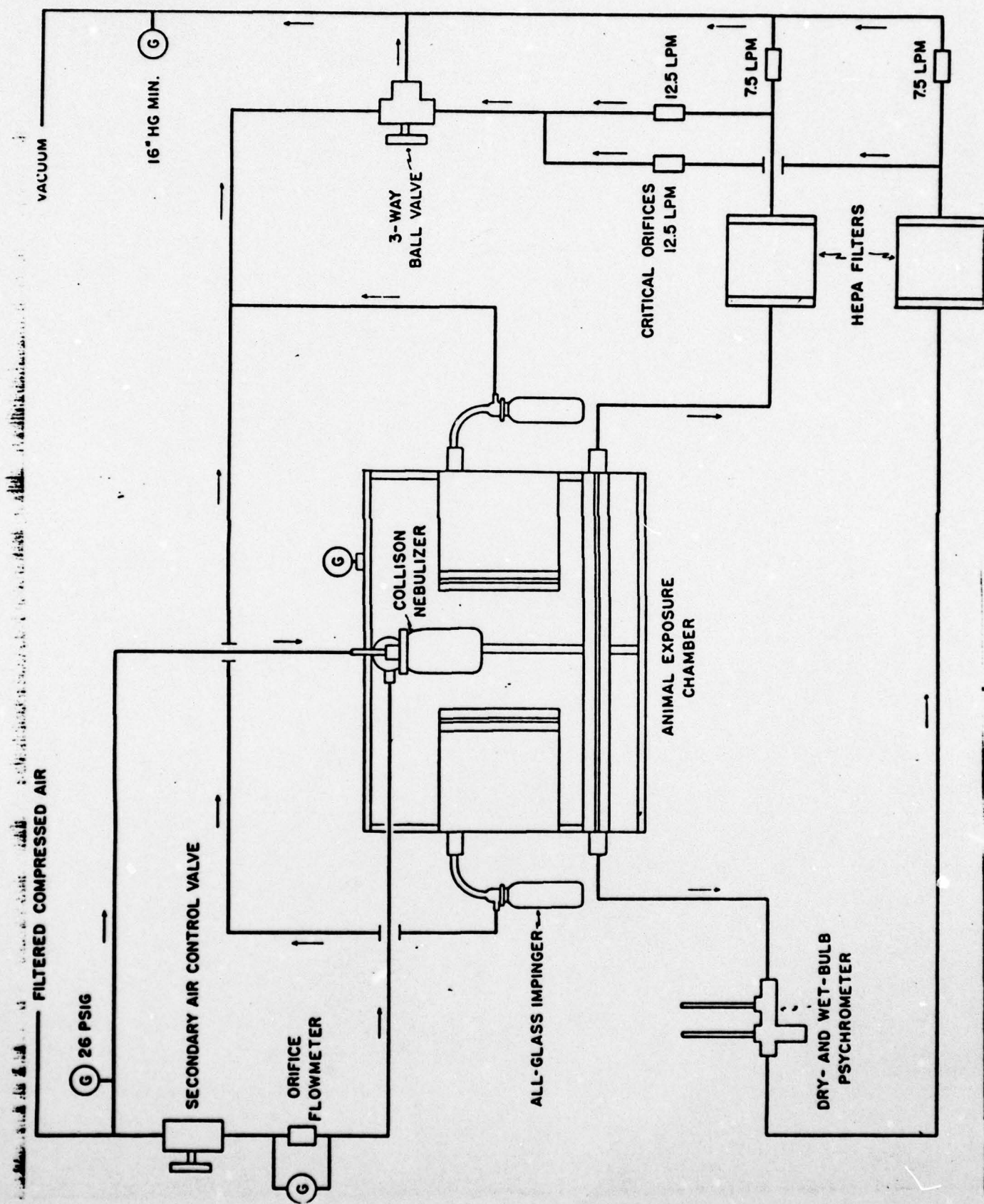
Fig 2--Schematic diagram of aerosol therapy system.

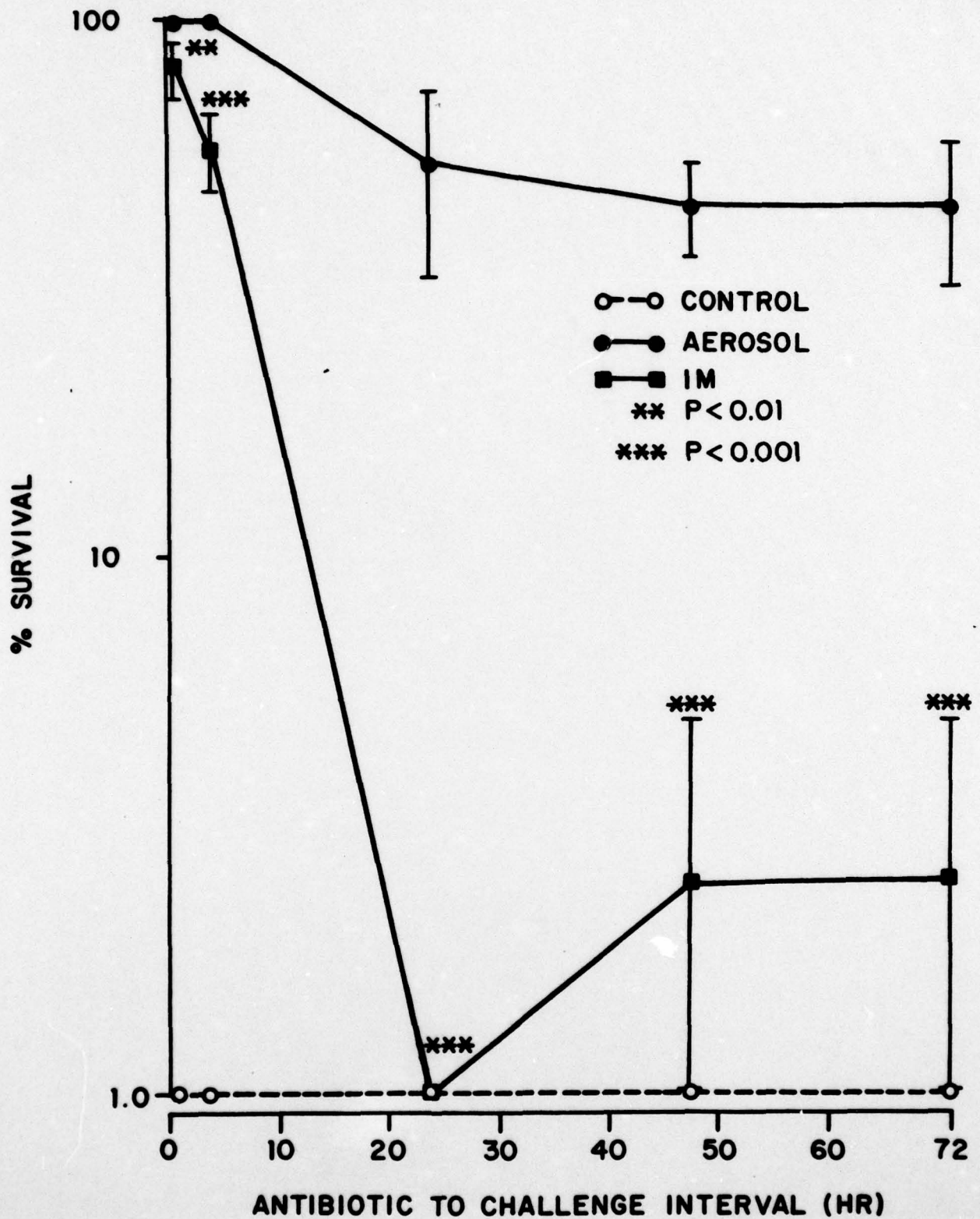
Fig 3--Survival of mice exposed to Klebsiella pneumoniae at selected intervals after administration of kanamycin. Asterisks represent rounded probability levels as determined by Fisher's exact test.

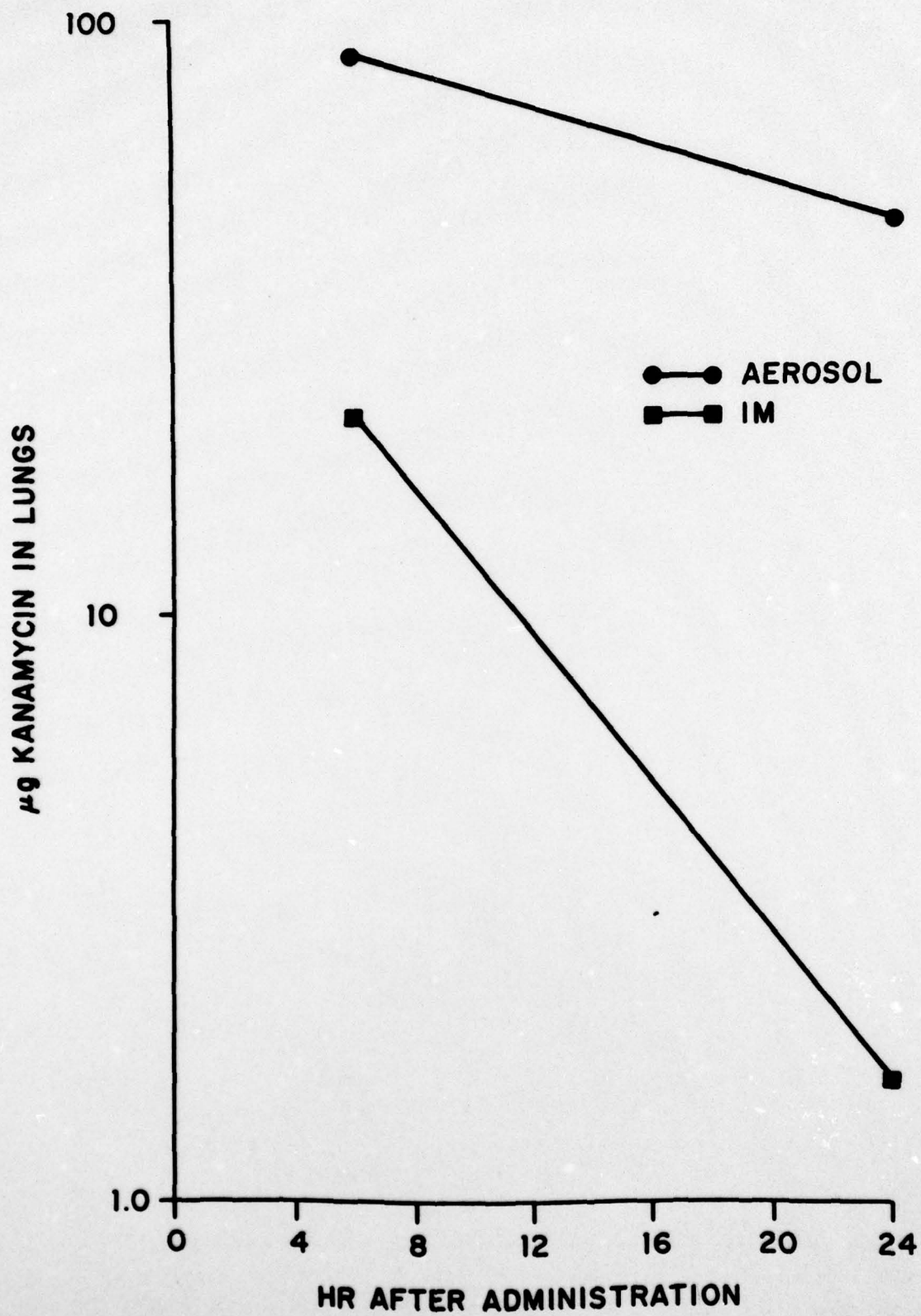
Fig 4--Concentration of kanamycin in lungs of mice at selected intervals after IM or aerosol exposure.

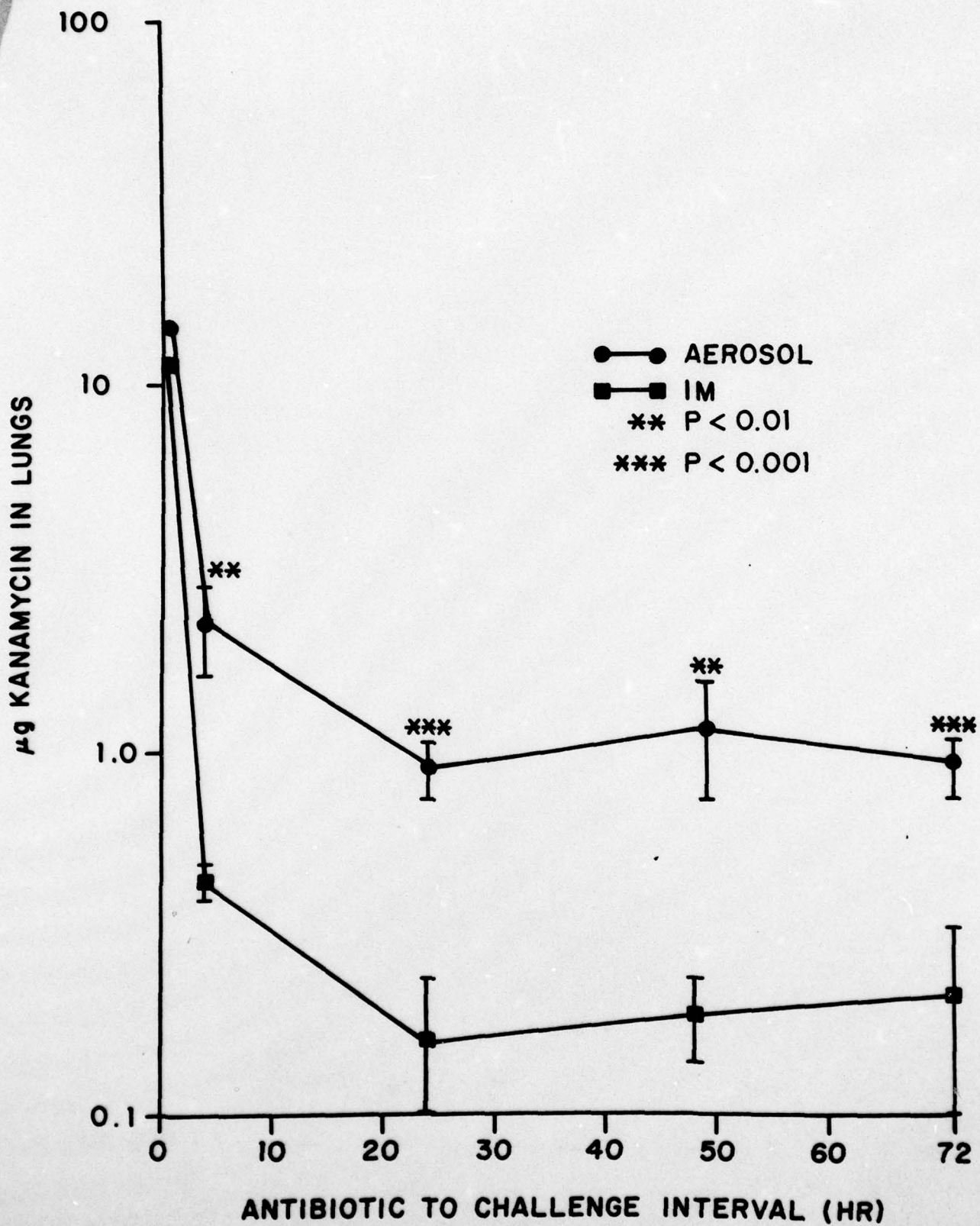
Fig 5--Concentration of kanamycin in the lungs of squirrel monkeys at 6 and 24 hours after aerosol or IM exposure.











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