Bacterial Growth In Vivo

AN IMPORTANT DETERMINANT OF THE PULMONARY CLEARANCE OF *DIPLOCOCCUS PNEUMONIAE* IN RATS

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A B S T R A C T   Lung clearance of *Diplococcus pneumoniae* was markedly reduced in rats with acute hemorrhagic pulmonary edema produced by instillation of hydrochloric acid. Bacterial clearance was enhanced in both control and acid-instilled animals by pretreatment with a bacteriostatic antibiotic, tetracycline, 30 mg/kg. From these data the contributions of bacterial multiplication and bacterial elimination to net lung bacterial clearance were estimated. In control animals the constant for exponential bacterial elimination was − 1.4283 (fractional clearance = 76% per h), and the doubling time for the pneumococcus was 170 min. In acid-instilled rats the elimination constant was − 0.5336 (fractional clearance = 41% per h), and the doubling time of the pneumococcus was 47 min, approximating the doubling time of 42 min observed with pneumococci grown in broth.

These results indicate that, in the case of pneumococci, both bacterial elimination and bacterial growth contribute to lung bacterial clearance in normal animals as well as animals with damaged lungs. In the present study changes in both parameters were required to explain the observed results in acid-instilled animals. The pulmonary pathogenicity of some bacterial species may be determined by their capacity for growth in the lung, since infection of the lung occurs when bacterial multiplication exceeds the rate of elimination of viable organisms.

INTRODUCTION

Pulmonary defense mechanisms against bacteria have been studied by exposing experimental animals to aerosols containing bacteria and observing the decrease of viable organisms remaining in the lungs with time, a process termed "lung bacterial clearance" (1–4). Decreased clearance, or a lesser decrement in the number of viable bacteria in the lung, has been demonstrated in animals with experimentally induced disease states (5–15). Bacterial multiplication in the lung has been assumed to be an insignificant factor during such studies (16), except in those studies in which the number of bacteria in the lungs increased with time (14).

The contributions of bacterial killing and in vivo bacterial multiplication to the clearance of *Diplococcus pneumoniae* from the rat lung were examined in the present study; the data indicate that multiplication of the pneumococcus significantly reduces the observed net clearance of the organism from the lungs of normal animals. Further, both decreased killing and enhanced multiplication contribute to the markedly impaired net clearance of pneumococci from rat lungs damaged by hydrochloric acid.

METHODS

White male Sprague-Dawley rats, weighing between 200 and 250 g, were lightly anesthetized with ether. In half of the animals the cervical trachea was exposed and 4.0 ml/kg of 0.1 HCl (pH 1.0) was injected into the lungs. 4 h after recovery from anesthesia the animals were exposed to the bacterial aerosol.

D. pneumoniae, type III, was grown overnight in 1,000 ml of Todd-Hewitt broth, centrifuged, washed, and resuspended in sterile distilled water. The final concentration of this slurry was 4.4×10^7–6.5×10^8 (mean 3.9×10^8) bacteria per ml. The aerosol was generated from this slurry by a Collison nebulizer in a recirculating Henderson exposure chamber. Andersen air sampler analysis of aerosols generated by this apparatus has shown that 90% of particles leaving the exposure tube are 2 µm or less in size (W. G. Johanson, Jr., unpublished observations). Two control and two

acid-instilled animals were exposed simultaneously to the aerosol for 30 min; each study consisted of eight such exposure periods with a total of 16 animals in each group.

Groups of animals were sacrificed immediately, and at 1, 2, and 4 h postexposure (T_0, T_1, T_2, T_4). Sacrifice was performed by cervical subluxation and cross-clamping of the neck to prevent agonal aspiration of oropharyngeal contents. The thorax was washed with 70% ethyl alcohol, opened aseptically, and the lungs removed at the hila. A small portion of one lung of each animal was removed for histological study. The remainder of both lungs was weighed, homogenized with a mortar and pestle in 2.0 ml of sterile water, and 0.1 ml of serial 10-fold dilutions of the homogenate was spread on blood agar plates. After 24 h incubation in 5% CO_2 in air at 37°C colonies of pneumococci were enumerated. Counts from all plates containing pneumococci were averaged to obtain a final quantitation for each animal which was corrected for dilution and expressed as the number of bacteria per both lungs.

The number of viable bacteria in the lungs of T_0 animals varied between studies due to differences in the concentration of bacteria in the aerosol and to differences in the breathing pattern of individual animals. To pool results of different studies the following normalizing procedure was performed (1). In each study bacterial counts from the lungs of the four T_0 control and four T_0 acid-instilled animals were averaged to obtain the mean initial bacterial deposition in each group. The bacterial count obtained from the lungs of each animal sacrificed at subsequent hours was divided by the respective group mean initial deposition to obtain a ratio (R) of remaining viable bacteria to the number initially deposited (R = bacterial count observed/mean initial bacterial deposition). Results of studies performed under similar conditions were combined for analysis. Values of R for individual animals within each time interval were averaged and subjected to statistical evaluation using Student's t test for unpaired samples. Probabilities of P equal to, or less than, 0.05 were considered significant.

Four studies, encompassing a total of 64 control and 64 acid-instilled animals, were performed in the manner described. Because bacterial multiplication was apparent in the lungs of the acid-instilled animals, three studies were performed in which tetracycline, 30 mg/kg, was administered intraperitoneally to a total of 48 control and 48 acid-instilled animals. The tetracycline was administered immediately after the instillation of acid, 4 h before bacterial exposure. Blood was obtained from the hearts of similarly injected but not aerosol-exposed animals at 1, 4, and 8 h after injection of tetracycline and the concentration of tetracycline in serum was determined by the technique of Simon and Yin (17).

Growth curves of the challenge organism were determined in vitro by inoculating samples of a slurry prepared for nebulization into fresh Todd-Hewitt broth. Duplicate 1-ml samples were obtained from this broth after inoculation and at 30-min intervals for 5 h. The logarithmic growth rate was determined from the change in bacterial concentration between 2 and 4 h after inoculation. Bacterial concentration was plotted against time on semilogarithmic paper and the duration of the lag phase between inoculation and logarithmic growth phase was calculated from the intercept of the extrapolated slope of log phase growth with the base line initial concentration. Growth of the organism in Todd-Hewitt broths containing tetracycline, ranging in concentration from 1 to 20 μg/ml, was evalu-

TABLE I

Viable Intrapulmonary Bacteria after Aerosol Exposure

Group	Hours postexposure			
	0	1	2	4
Control				
Number of animals	*16*	*15*	*15*	*15*
Mean*	146.0	31.6	11.3	3.9
SEM*	50.9	10.6	3.9	1.9
Acid instilled				
Number of animals	*15*	*16*	*16*	*16*
Mean	161.6	56.3	54.3	190.1
SEM	50.9	26.2	19.4	69.0
Control tetracycline				
Number of animals	*11*	*11*	*12*	*11*
Mean	65.4	23.4	2.8	0.4
SEM	16.1	8.2	0.9	0.1
Acid-instilled tetracycline				
Number of animals	*12*	*11*	*12*	*11*
Mean	78.7	20.4	14.1	8.8
SEM	17.3	6.1	3.4	3.6

* Number $\times$ 10^3.

ated in similar fashion using initial bacterial concentrations between 10^4 and 10^5 per ml.

RESULTS

Histologically, the lungs of animals which had received acid showed severe focal hemorrhagic pulmonary edema. Necrosis of airway epithelium was not seen. Lungs of control animals appeared normal. Pretreatment with tetracycline did not alter the histology of the lungs.

In the four studies performed without tetracycline pretreatment, the mean initial bacterial deposition was 1.46×10^5 in the control and 1.6×10^5 in the acid group. In the three studies performed with tetracycline pretreatment, the mean initial bacterial deposition was 6.5×10^4 in control animals and 7.9×10^4 in acid-instilled animals. Neither the differences between control and acid-instilled groups nor the differences between the tetracycline and nontetracycline groups were significant. The viable intrapulmonary bacteria found in each group at 0, 1, 2, and 4 h postexposure are shown in Table I.

The change in R with time is plotted for each group in Fig. 1. Bacterial clearance was most rapid in tetracycline-treated control animals (B); R at 4 h was significantly lower in this group than in control animals without tetracycline (A) ($P < 0.05$). Acid-instilled animals (C) showed markedly diminished clearance; differences from controls (A) were significant at 2 ($P < 0.05$) and 4 h ($P < 0.01$). Tetracycline pretreat-
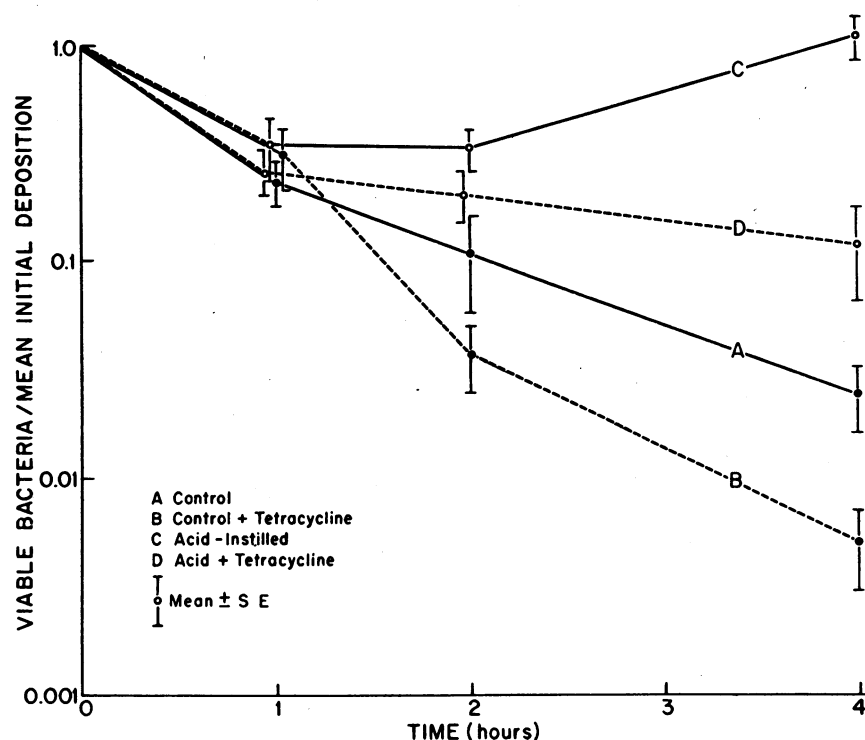
FIGURE 1 Change in the ratio of remaining viable bacteria to mean initial bacterial deposition at intervals after exposure to airborne *D. pneumoniae,* type III, in four groups of rats. Intratracheal instillation of 0.1 N HCl, 4.0 ml/kg, was performed 4 h before aerosol exposure. Tetracycline, 30 mg/kg, was administered intraperitoneally at the same time. Numbers of animals in each group were: A = 61, B = 45, C = 63, D = 46.

ment also enhanced bacterial clearance in acid-instilled animals (D); the difference between the acid-instilled, no-tetracycline group (C) and the acid-instilled tetracycline group (D) was significant at 4 h ($P < 0.01$). A comparison of the two tetracycline-treated groups (B and D) revealed significantly reduced clearance in the acid-instilled group at 2 ($P< 0.01$) and 4 h ($P < 0.05$). The bactericidal capacity of the lungs, k_2, was calculated by the least squares method on a semilogarithmic plot of R against time assuming that tetracycline prevented bacterial multiplication but did not kill organisms; k_2 was $- 1.4283$ (76% fractional elimination per h) in controls and $- 0.5336$ (41% fractional elimination per h) in acid-instilled animals (see Appendix). These slopes were significantly different ($P < 0.01$). Clearance was similar at 1 h in all groups.

The logarithmic growth phase of the challenge organism in broth reflected a doubling time of 42 min after a lag period of 60 min. The calculated in vivo bacterial growth rate, k_1, expressed as doubling time, was 170 min in control animals and 47 min in acid-instilled animals. The concentration of pneumococci suspended in broth containing tetracycline at concentrations of 5 and 10 µg/ml, did not change significantly

over a 5-h period. In broths containing 1 µg/ml bacterial multiplication occurred while bacterial killing was observed at a concentration of tetracycline of 20 µg/ml.

The concentration of tetracycline in serum of treated animals was 5.5 µg/ml at 1 h, 4.3 µg/ml at 4 h, and 2.2 µg/ml at 8 h after injection with tetracycline.

DISCUSSION

The number of viable pneumococci in the lungs of acid-instilled animals increased with time after an initial decline. This circumstance can be explained only by the occurrence of bacterial multiplication. The issues we then sought to address were the relative contributions of impaired defense mechanisms and enhanced bacterial growth to the change in net clearance and whether similar multiplication occurred in the lungs of normal animals but was obscured in the presence of intact defense mechanisms. The enhancement of net bacterial clearance by tetracycline in both control and acid-instilled animals would be consistent with the latter hypothesis, although alternative explanations for these observations are possible.

Tetracycline may have directly stimulated the antibacterial defenses of the lung. However, Martin and

Warr have shown that tetracycline in concentrations of 10 µg/ml or less inhibits leukocyte chemotaxis (18), and other investigators have shown that tetracycline neither inhibits nor stimulates phagocytosis and killing of viable organisms in vitro by leukocytes (19). It remains possible, although unlikely, that the phagocytic activity of the alveolar macrophage is enhanced by tetracycline. Although the effects of tetracycline on mucociliary transport have not been studied such transport plays a relatively minor role in the elimination of viable bacteria from the lung (9).

Tetracycline may have been bactericidal at the concentrations achieved in the lungs of treated animals. Our in vitro data indicate that the drug was bactericidal for the strain of pneumococcus which we used at a concentration of 20 µg/ml and that bacterial multiplication was not entirely prevented at a concentration of 1 µg/ml. However, at 5 and 10 µg/ml bacteriostasis was observed over a 5-h period. Similar observations have been reported with other strains of tetracycline-susceptible organisms (20). These results suggest that, at the serum concentrations which were achieved, incomplete bacteriostasis might have occurred but that bacterial killing would not be expected. If tetracycline did not completely inhibit bacterial multiplication, we would underestimate the rate of in vivo bacterial multiplication; since our calculated doubling time of 47 min in the lungs of acid-instilled animals agrees closely with the observed doubling time in broth of 42 min, it seems unlikely that a significant underestimate was made.

A major artifact may be produced in lung clearance studies if the site of deposition of airborne particles differs between test subjects (21). Differences in site of deposition probably did exist between the acid-instilled and control animals in the present study and may have contributed to the difference observed in bactericidal activity between these groups. However, unless the site of particle deposition was affected by tetracycline administration this factor would not influence the comparison of bacterial clearance within the control and acid-instilled groups. Another possible artifact might have been the initial deposition of fewer bacteria in the tetracycline-treated groups leading to more rapid clearance in these animals. However, the differences in initial deposition between the no-tetracycline and tetracycline groups among controls and acid-instilled animals were not significant. Other investigators have reported that the fractional clearance of viable bacteria is independent of initial concentration over ranges encompassed by our data (5). Further, when each study was analyzed separately, no correlation between initial deposition and fractional clearance was

found in our data. Thus, these potential artifacts in the experiment do not seem to explain our results.

We conclude that multiplication of D. pneumoniae occurred in the lungs of both control and acid-instilled animals and that such multiplication is an important determinant of net bacterial clearance of this organism. Early investigations on the mechanisms of lung bacterial clearance suggested that bacterial killing in the lung was accomplished by the alveolar macrophage after phagocytosis of the organisms (6). This concept has been generally accepted, although direct proof of the quantitative importance of this mechanism has not been presented. Since pneumococci are rapidly killed after ingestion by phagocytic cells (22), our hypothesis of bacterial multiplication implies that some organisms deposited in the lungs were not ingested for a period of time. Further, the enhanced rate of bacterial multiplication which we observed in the acid-instilled animals could be explained by a larger fraction of inhaled bacteria being protected from contact with phagocytes. Nonquantitative morphologic observations have suggested that phagocytosis of bacteria in the lung occurs rapidly (6). However, further clarification of the mechanisms and sites of bacterial killing and multiplication in the lung await the application of quantitative techniques.

Comparisons of the calculated rate constants for bacterial elimination and bacterial multiplication show that elimination was impaired and multiplication was enhanced in the acid-instilled groups. In fact, the observed net increase in the number of viable bacteria in the lungs of acid-instilled animals at 4 h can be explained only by changes in both parameters; reduced elimination or increased multiplication alone could not have produced this result (Fig. 2).

The strict validity of our formulation of the interaction between bacterial multiplication and bacterial elimination depends on the independence of the rate constants k_1 and k_2. We cannot be certain that such independence exists, although it seems more likely that each would be altered independently by various conditions within the lung than that changes in one parameter would produce changes in the other. Whereas the possible interdependence of k_1 and k_2 might impart some imprecision to the model, it does not invalidate the central conclusion of this study, that the rate of bacterial multiplication is a significant variable in lung bacterial clearance of the pneumococcus. This suggests that the pulmonary pathogenicity of some bacterial species may be due to their greater ability to multiply in the lungs and not necessarily due to their resistance to lung defenses. Further, an appreciation that alterations in the net removal of viable bacteria from the lung may be due

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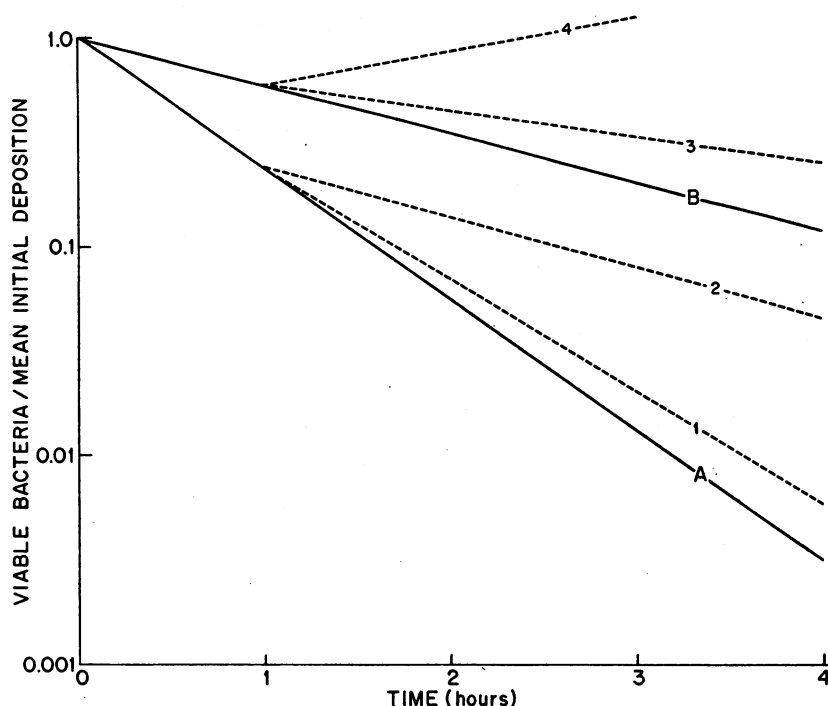
FIGURE 2 Effects of altering lung bactericidal activity and growth rate of bacteria in vivo on theoretical curves of net lung bacterial clearance (Eq. 2). A 1-h period of lag phase bacterial growth was assumed. Curve A represents the bactericidal activity of tetracycline-treated normal animals ($k_1 = 0$, $k_2 = -1.4283$): curve 1 represents the net loss of viable bacteria which would occur if bacterial doubling time was 170 min ($k_1 = 0.2441$, $k_2 = -1.4283$); curve 2 represents the net loss of viable bacteria which would occur if the bacterial doubling time was 47 min ($k_1 = 0.8824$, $k_2 = -1.4283$).

The reduced bactericidal activity observed in acid-instilled tetracycline-treated rats is represented by curve B ($k_1 = 0$, $k_2 = -0.5336$): the net loss of viable bacteria which would occur with bacterial doubling times of 170 min ($k_1 = 0.2441$, $k_2 = -0.5336$) and 47 min ($k_1 = 0.8824$, $k_2 = -0.5336$) are represented by curves 3 and 4, respectively.

to either enhanced bacterial growth or diminished lung clearance should assist further in the definition of pulmonary defense mechanisms and their alteration in disease.

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APPENDIX

After an initial lag phase an implanted bacterial population increases logarithmically at a constant rate, k_1, which is determined by nutrient and physical elements of the supporting medium and inherent properties of the bacterium. We assumed that in vivo bacterial growth occurred at rate k_1, after an initial lag phase of 60 min. It would be anticipated that k_1 would differ between species of bacteria and that k_1 for a given species might vary with local conditions in the lung.

Available data suggest that nonpathogenic bacteria deposited in the lung are eliminated exponentially with time.

We have described this process by a single logarithmic rate constant, k_2, while recognizing that the process is due to the net effects of multiple mechanisms.

Thus, we postulate that the net change with time bacterial concentration in the lungs after deposition of a bacterial aerosol is controlled by these constants which are opposite in sign, or:

$$dC/dt = (k_1 \times C) - (k_2 \times C)$$
$$dC/dt = C(k_1 - k_2) \tag{1}$$

where C = the concentration of bacteria in the lungs; t = time; k_1 = a constant representing the rate of bacterial growth; k_2 = a constant representing the rate of elimination of viable bacteria in the lung.

By rearranging and substitution we obtain:

$$C_t = C_0 e^{(k_1 - k_2)t} \tag{2}$$

where C_t = the concentration of bacteria in the lungs at time t; C_0 = the concentration of bacteria in the lungs at time 0.

Bacterial multiplication may be prevented by exposure to bacteriostatic antimicrobial agents. Thus, if $k_1 = 0$ in tetra-

cycline-treated animals:

$$C_{tet_t} = C_{tet_0}e^{-k_2 t} \qquad (3)$$

where C_{tet_t} = the concentration of bacteria in the lungs of tetracycline-treated animals at time t; C_{tet_0} = the concentration of bacteria in the lungs of tetracycline-treated animals at time 0.

By dividing Eq. 2 by Eq. 3, we obtain:

$$C_t/C_{tet_t} = e^{k_1 t} \qquad (4)$$

This relationship can be used to calculate the growth rate of bacteria in vivo or in broth.

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