Lung Bacterial Clearance in Murine Pneumococcal Pneumonia

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We studied the bactericidal capacity of the rat lung during the development of pneumococcal pneumonia. Pneumonia was produced in a lower lobe by the intrabronchial instillation of 10^4 Streptococcus pneumoniae cells in buffer. Lung bacterial counts progressively increased, reaching 10^7 per lung within 48 h, and the increase was associated with localized atelectasis and consolidation. Bacterial multiplication was inhibited with tetracycline at various intervals after infection, and the subsequent clearance of pneumococci was determined. Viable pneumococci were rapidly killed by lung defenses if bacterial multiplication was inhibited within 12 h of the onset of infection. No change occurred in the bacterial population if tetracycline was delayed until 24 h after infection, indicating that pneumococcal killing by lung defenses had ceased. This effect could be reproduced with the addition of pneumococcal capsular polysaccharide to the inoculum, which produced a dose-related inhibition of pneumococcal clearance. The clearance of S. epidermidis was not impaired in the presence of pneumococcal pneumonia or by administration of exogenous capsular polysaccharide. These data indicate that pneumococcal pneumonia causes a marked impairment in lung antipneumococcal defenses within 24 h of the onset of infection. This acquired defect in antibacterial defenses may be due to the accumulation of pneumococcal capsular material in the lungs of infected animals.

Pneumococcal pneumonia has been a subject of investigation throughout this century. Early studies established that respiratory tract infection with Streptococcus pneumoniae proceeded from the upper tract to the lungs directly, not via the bloodstream (1, 8, 27, 28). The events which occur when pneumococci encounter the defense mechanisms of the lungs were examined by Wood and co-workers in a series of experiments beginning 30 years ago (30–35). Their findings indicated that pneumococci could be phagocytosed on the alveolar surface in the absence of opsonizing antibody (34) and that pneumococcal capsular polysaccharide inhibited such phagocytosis (33). Penicillin and sulfonamides, only recently available at that time, were shown to effectively eliminate pneumococci at the advancing edge of experimental pneumonia where bacterial proliferation was rapid but were ineffective against organisms in the more central areas of consolidation (30–32, 35). Persistence of pneumococci in the latter areas was attributed to the metabolic inactivity of the organisms, which protected them from the effects of the antimicrobial agents.

Quantitative aspects of lung antibacterial mechanisms were studied by Kass, Green, and co-workers in a series of experiments some years later (14, 15, 26). Their experiments incorporated several important features: "non-pathogenic" bacteria were used so that defenses could be studied without the confounding variables associated with virulent species; the bacterial challenge was delivered by aerosol rather than by intrabronchial instillation, to distribute organisms evenly within the lung; quantitative methods were used throughout; and sophisticated statistical techniques were applied in evaluating the results (26). With this approach it was shown that the lung is capable of inactivating enormous numbers of bacteria within a few hours. The rate of bacterial activation was independent of the number of organisms deposited within a range of 10^3 to 10^6 organisms per lung, and only about 10% of the initial inoculum remained viable 4 h after aerosol exposure (14). By use of radiolabeled bacteria, it was shown that bacterial inactivation was accomplished predominantly in situ in the

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lungs (13), and the alveolar macrophage was identified as the major antibacterial mechanism under these circumstances (10, 15). Similarly, techniques have been used by many investigators to show impaired lung antibacterial defenses under a variety of conditions (11, 18, 19, 21, 25). However, in only rare experiments has the bacterial population of the lung been shown to increase after aerosol challenge, and pneumonia does not ordinarily occur in these models.

Another dimension was added to this technique by Johanson et al. (22) to reexamine the Wood model of pneumococcal pneumonia. Our results indicate that during the early hours of pneumonia lung defenses are capable of eliminating viable pneumococci rapidly, although not as rapidly as the organisms multiply. However, within 24 h the ability of the pneumonic lung to kill pneumococci ceases. This defect does not extend to other organisms and appears to be due to the accumulation of pneumococcal capsular polysaccharide in the lung.

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MATERIALS AND METHODS

Development of the model of pneumococcal pneumonia. Sprague-Dawley white male rats, weighing 150 to 250 g, were used for all experiments. S. pneumoniae, type III (ATCC 6303), was grown to log phase in Todd-Hewitt broth. Concentrations of pneumococci were determined spectrophotometrically, and log-phase organisms were diluted in phosphate-buffered saline, pH 7.2 (PBS) to achieve a final concentration of 10⁶ pneumococci in 0.05 ml of buffer. Virulence of the organism was maintained by intraperitoneal passage in rats prior to use.

Animals were lightly anesthetized with ether, and a small tracheotomy was performed. A beaded-tip needle was advanced into the left lower lobe, and 10⁴ pneumococci in 0.05 ml of PBS were inoculated. Preliminary studies showed that 10⁴ pneumococci produced a pneumonia with a suitable time course for our study. Lesser inocula (10⁶ organisms) were cleared spontaneously, and greater inocula (10⁶ organisms) caused an infection that was rapidly fatal.

At intervals after inoculation, animals were sacrificed by cross-clamping the neck to sublux the cervical spine and to prevent agonal aspiration of oropharyngeal contents. The lungs were removed aseptically and washed in buffer. The lobes were dissected, suspended together in 5 ml of buffer, and homogenized. Initially, the right and left lungs were handled separately, but it became apparent that within 4 h after inoculation equal counts were obtained from both lungs, so this practice was abandoned. Samples of the lung homogenate were serially diluted in buffer, inoculated onto Trypticase soy agar plates containing 5% sheep blood, and incubated for 24 h at 37°C prior to quantitation of pneumococcal colonies. Blood was obtained for culture from either a neck or tail vein. Blood samples were serially diluted and quantitated similarly. Total circulating leukocytes and differential counts were performed on peripheral blood by standard techniques, and the counts were expressed as total polymorphonuclear leukocytes per mm³.

Specimens obtained at intervals after inoculation from some animals were used only for histological examination. The lungs and heart were removed en bloc and fixed inflated with Formalin under 20 cm of hydrostatic pressure. Sections 6 μm thick were cut, stained with hematoxylin and eosin, and examined by light microscopy.

Tetracycline determinations. Tetracycline is a bacteriostatic antibiotic with no known direct effect on lung defense mechanisms (22). Its action against S. pneumoniae was investigated by inoculating 10⁶ of Todd-Hewitt broth containing various concentrations of tetracycline with 10⁶ log-phase organisms. Minimal inhibitory and bactericidal concentrations were determined by culture after 6 h of incubation at 37°C.

Animals treated with tetracycline received a single injection of tetracycline, 50 mg/kg, intraperitoneally. Subsequent serum levels were measured by the method of Simon and Yin (29).

The concentration of tetracycline in pneumonic lungs was measured in an additional group of animals with pneumococcal pneumonia. Tetracycline was administered to four animals 12 h after onset of infection and to three animals 24 h after onset of infection. Six hours after tetracycline administration, the lungs were removed. Sections of lung, including separate portions of atelectatic and consolidated lung, were dissected aseptically, weighed, and homogenized in 5 ml of buffer. The concentration of tetracycline in lung homogenates and simultaneous serum samples was measured with a bioassay technique (29). Lungs from two infected but untreated animals were assayed as controls.

Studies with PCP. Purified pneumococcal capsular polysaccharide (PCP) obtained from S. pneumoniae, type III, kindly supplied by Francis R. Cano, American Cyanamid Co., Lederle Laboratories
Div., Pearl River, N.Y., containing 3.1% protein and 0.6% nucleic acid was dissolved in distilled water. To test the effects of PCP on antipneumococcal defenses, groups of animals were inoculated intrabronchially with 0.25 ml of PBS containing 10⁴ S. pneumoniae cells and 0.036, 0.075, 0.15, 0.3, 0.6, or 1.2 mg of PCP. Controls were inoculated with 10⁴ S. pneumoniae cells in 0.25 ml of PBS without PCP. Tetracycline was administered 12 h after inoculation to one-half of the controls and to all animals receiving PCP. The lungs of all animals were removed and cultured 24 h after pneumococcal inoculation.

Studies with S. epidermidis. Pneumococcal pulmonary infections were produced as above in a group of animals. Twenty-four hours later, 10⁵ S. epidermidis (ATCC 12228) cells, grown to log phase in Trypticase soy broth and resuspended in PBS, were instilled through a tracheotomy into the lungs of the pneumococcus-infected animals and simultaneously into the lungs of a group of uninfected controls. Subsequently, the animals were sacrificed and cultured as previously described.

We studied the effects of PCP on the lung clearance of S. epidermidis in two additional groups of uninfected animals. One group was inoculated with 10⁵ S. epidermidis cells in 0.25 ml of PBS, and the second group received 10⁵ S. epidermidis cells mixed with 0.3 mg of PCP in 0.25 ml of PBS. The lungs of all animals were removed and cultured 6 h after inoculation.

Differences between control and experimental groups in each experiment were evaluated with Student's t test for unpaired data. Probabilities equal to or less than 0.05 were considered significant.

RESULTS

Characteristics of the model of pneumococcal pneumonia. Animals inoculated intrabronchially with 10⁴ pneumococci consistently developed a progressive pulmonary infection. The number of pneumococci cultured from the lungs of these animals at various intervals after inoculation is shown in Fig. 1. Pneumococcal counts increased from 10⁴ organisms inoculated at zero time to 6 × 10⁷ organisms at 48 h.

Blood cultures demonstrated bacteremia with quantitative counts increasing from 10² organisms/ml at 12 h to 10⁴ organisms/ml at 48 h (Fig. 1). At these levels, the number of organisms in the blood could not have contributed significantly to the number of pneumococci cultured from the lungs. Although the mean total polynuclear leucocyte counts were increased at 24 and 36 h, individual animals varied widely, and there was no significant difference in counts between 0 and 24 h (P > 0.05) or between 0 and 36 h (P > 0.1) (Fig. 1). Sporadic deaths began occurring at 24 h, and most untreated animals died within 72 h.

The gross and microscopic findings in the lungs 12 h after inoculation were usually normal, although occasional mild atelectatic and congestive changes were noted. At 24 h, the lungs had extensive atelectasis, often with grossly visible consolidation and pleuropericarditis. Atelectasis was manifested by erythematous, small lungs which returned to a normal gross appearance when fully inflated with either air or fixative. Bacterial counts were similar in lungs demonstrating atelectasis, consolidation, or both. Representative microscopic sections of an area of consolidation in a 24-h infection are shown in Fig. 2 and 3.

The lungs of animals sacrificed 48 h after

FIG. 1. Course of pneumococcal pneumonia in the rat. Bacterial counts per lung (--; ) progressively increased from the initial inoculum of 10⁴ to 6 × 10⁷ by 48 h. Bacteremia was present at 12 h and increased to 10⁴/ml by 48 h (-----). Polymorphonuclear leucocyte (PMN) counts (---) showed no significant change. Vertical bars show the mean ± the standard error of the mean.
inoculation had changes similar to those found at 24 h, although consolidation was usually more extensive.

**Effect of tetracycline on pneumococcal clearance in pneumococcal pneumonia.** The increasing pulmonary bacterial population in untreated animals (Fig. 1) clearly indicates that pneumococci multiplied faster than they could be cleared by pulmonary antibacterial defenses. The capacity of lung defenses to kill pneumococci may be directly assessed if bacterial multiplication can be inhibited. To ensure that bacteriostatic levels of tetracycline were achieved, serum and lung concentrations were measured after the intraperitoneal injection of 50 mg/kg of body weight. The mean serum tetracycline concentration was 9.8 μg/ml at 4 h, 1.8 μm/ml at 6 h, and not detectable at 12 h. The concentration of tetracycline in pneumonic lungs 6 h after injection averaged 10.3 μg/g (8, 10, 10, and 13 μg/g) in animals with 12-h infections and 12.7 μg/g (12, 12, and 14 μg/g) in animals with 24-h infections. Concentrations of tetracycline between 1 and 20 μg/ml were bacteriostatic over a 6-h period of incubation in vitro; higher concentrations were bactericidal.

The effects of tetracycline on the course of infection with *S. pneumoniae*, type III, are shown in Fig. 4. When administered 1 h prior to pneumococcal inoculation, or 4 to 12 h after inoculation, tetracycline was associated with rapid disappearance of viable pneumococci (Fig. 4). Since the drug is only bacteriostatic, this finding indicates the preservation of effective antipneumococcal mechanisms in the lungs of these animals as late as 12 h after onset of infection. In contrast to the rapid killing of the pneumococci which occurred when tetracycline was given within 12 h of infection, bacterial killing did not occur if tetracycline administration was delayed until 24 h after pneumococcal inoculation (Fig. 5). A transient decrease in lung bacterial counts occurred 4 h after tetracycline, but by 48 h after the onset of infection the numbers of pneumococci were the same in tetracycline-treated animals and controls. This observation suggests that the bactericidal capacity of the lung became markedly impaired during the course of the pneumococcal infection.

An alternative explanation for our data could be that the bacterial population of the lungs at
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Fig. 3. Pneumococcal pneumonia 24 h after inoculation. At greater magnification the outlines of alveolar walls can be distinguished although the air spaces are filled with a polymorphonuclear leukocytic exudate. Hematoxylin and eosin. Original magnification, ×438.

Fig. 4. Effects of tetracycline on experimental pneumococcal pneumonia. All animals received $10^4$ S. pneumoniae, type III, cells at time zero. Pneumococcal counts per lung progressively increased in untreated animals (-), whereas counts decreased in animals which received tetracycline, 50 mg/kg, 1 h prior to pneumococcal inoculation (△--△), 4 h after inoculation (■--■), or 12 h after inoculation (○--○). Each group consisted of six to eight animals. Vertical bars show the mean ± the standard error of the mean.
24 h, $10^7$ organisms, is too large for even intact defense mechanisms to handle. This possibility was studied by inoculating additional animals with $10^7$ *S. pneumoniae* cells; such animals pretreated with tetracycline rapidly cleared an inoculum of $10^7$ log-phase pneumococci (Fig. 6).

Effect of PCP on the clearance of pneumococci. PCP is known to accumulate in pneumonic lungs and is produced by the pneumococcus in excess during logarithmic growth (33). Studies were done to determine whether the addition of PCP to the initial inoculum of $10^4$ *S. pneumoniae* cells would impair bacterial killing in the presence of tetracycline. The results of this experiment (Fig. 7) show that PCP caused a dose-related inhibition of antipneumococcal defenses. The addition of 0.038 mg of PCP produced no impairment of pneumococcal defense mechanisms.

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**Fig. 5.** Effects of tetracycline when administered 24 h after pneumococcal inoculation. Although a transient decrease in lung bacterial counts occurred 4 h after administration of the antibiotic in the tetracycline group (---), counts were subsequently similar to those in untreated controls (-). Each group consisted of six to eight animals. Vertical bars show the mean ± the standard error of the mean.

**Fig. 6.** Lung clearance of an initial inoculum of $10^7$ *S. pneumoniae*, type III, cells. Bacterial numbers increased in control animals (-), whereas clearance occurred in animals pretreated with tetracycline, 50 mg/kg (-----). Each group consisted of six to eight animals. Vertical bars show the mean ± the standard error of the mean.
killing, whereas with amounts of 0.3 mg or greater lung bacterial killing was markedly reduced and lung bacterial counts were similar to those in animals not receiving tetracycline.

**S. epidermidis** clearance. The specificity of the impairment in host defenses associated with pneumococcal pneumonia and the administration of PCP was studied with *S. epidermidis*. *S. epidermidis* was cleared at a similarly rapid rate from the lungs of animals with a 24-h pneumococcal pneumonia and from the lungs of controls (Fig. 8). The addition of 0.3 mg of PCP to the inoculum did not alter the clearance of *S. epidermidis* in uninfected animals, compared with controls (Fig. 9).

**DISCUSSION**

There are four important features in the data presented. First, the ability of the lung to eliminate viable pneumococci after intrabronchial instillation is inversely related to inoculum size. Second, lung antipneumococcal defenses are capable of inactivating enormous numbers of pneumococci in the early hours after infection, although they cannot keep pace with bacterial multiplication. Third, the capacity for pneumococcal inactivation ceases 24 h after infection. Fourth, this acquired defect in host defenses does not extend to other bacterial species and appears to be due to the accumulation of pneumococcal capsular material in the lung.

The rate of inactivation of inhaled bacteria appears to be largely independent of inoculum size (13), in contrast to our findings with the intrabronchial bolus model. This difference may not seem surprising at first. However, the evenly distributed deposition of 10⁸ bacteria in the rat lung by aerosol and our deposition of 10⁴ bacteria in 0.05 ml of fluid would result in similar numbers of bacteria per unit of lung volume since the functional residual capacity of the rat lung is about 5.0 ml (17). Further, we demonstrated that as many as 10⁹ bacteria in 0.05 ml were rapidly killed, if bacterial multiplication was prevented. This latter finding also suggests that the fluid bolus itself did not significantly impair phagocytosis and killing. On the basis of available data, it is not clear why viable pneumococci deposited in the lung by aerosol are rapidly eliminated (22), whereas those deposited by intrabronchial bolus produce a progressive infection.

Our data do emphasize the urgent requirement for swift phagocytosis of organisms capable of rapid multiplication in the lungs, such as the pneumococcus. About 50% of staphylococci deposited in the lung by an aerosol challenge are phagocytosed by the end of a 30-min exposure period, and 80% are phagocytosed within 1 h (14). This response involves only alveolar macrophages. Phagocytosis of bacteria in the alveolar spaces of a normal lung does not require opsonization by specific antibody. The role of nonspecific opsonins is less clear. Intra-alveolar killing of staphylococci by alveolar macrophages is enhanced by prior contact of the organism with the lipid fraction of the alveolar lining fluid, although the rate of bacterial ingestion is not affected (20, 24). In contrast to the response of the lung to aerosolized staphylococci, the intrabronchial deposition of type III *S. pneumoniae* causes a rapid recruitment of circulating polymorphonuclear leukocytes into the lung (34). Since alveolar edema precedes infiltration of the lung by polymorphonuclear leukocytes in the development of pneumococcal pneumonia (12), it is reasonable to believe that plasma proteins are present in greater concentrations in such areas of the lung than in normal alveolar spaces. Effective opsonization of many pneumococcal types is mediated nonspecifically by the alternate complement pathway,
allowing rapid phagocytosis and killing by polymorphonuclear leukocytes (2, 7). However, type III pneumococci, as well as types IV and VIII, do not activate the alternate pathway unless specific antibody is present, and type I pneumococci do not appear to activate complement in either the presence or absence of antibody (7). Although the role of these interactions in defense of the lung against pneumococcal infection has not been clearly demonstrated, it is intriguing to speculate that the lack of nonspecific opsonization of these capsular types may underlie their propensity to cause pneumonia in humans.

The administration of an antimicrobial agent in our study, as in those of Wood and co-workers (30–32, 35), had no effect on the course of the pneumococcal infection once the latter had been established for as long as 24 h. Wood et al. attributed the lack of antimicrobial effectiveness to the metabolic inactivity of microorganisms in the central portions of the pneumonia (31). Our findings suggest instead that the persistence of viable bacteria in the presence of tetracycline is due to a failure of lung antibacterial mechanisms. These two factors may well coexist. To support our conclusion, it was necessary to show that tetracycline was not bactericidal in our model system and that effective concentrations of tetracycline were achieved in the areas of lung consolidation. After the intraperitoneal injection of tetracycline, 50 mg/kg, peak serum concentrations of 9.8 μg/ml occurred after 4 h. The concentrations of tetracycline in pneumatic lungs, determined 2 h after achievement of peak serum levels, were 10 μg/g in animals treated 12 h after onset of infection and 13 μg/g in animals treated 24 h after infection. Tetracycline was bacteriostatic in vitro at concentrations as high as 20 μg/ml. Thus, tetracycline was present in similar, bacteriostatic concentrations in the pneumatic lung at 12 and 24 h, and the observed differences in bacterial elimination at these times can be attributed to the development of an impairment in host antipneumococcal defenses.

Despite the marked depression of antipneumococcal defenses associated with the development of pneumonia, the capacity of the lung to inactivate staphylococci was not impaired. Other types of lung injury, such as hypoxia, are known to preferentially affect the clearance of
only some bacterial species (16). These findings suggest that the inability of the pneumonic lung to kill pneumococci is due to the development of a selective defect and is not due only to edema, atelectasis, or other nonspecific mechanisms. The addition of PCP to the infecting inoculum mimicked pneumonia in that it markedly impaired pneumococcal clearance but did not affect the inactivation on staphylococci. The inhibitory effect of PCP on pneumococcal clearance was dose related; maximal inhibition occurred with the addition of 0.3 mg of PCP. This amount is small in comparison to the quantity of PCP found in the lungs of humans dying of pneumococcal pneumonia (9). The presence of detectable concentrations of PCP in the blood or urine of patients with pneumococcal pneumonia has been associated with azotemia (4), empyema (4), bacteremia (6, 23), and death (3, 6). The presence of large amounts of PCP in sputum of patients with type III pneumococcal pneumonia also has been associated with severe infection and increased mortality (9). However, in human infections it is not clear whether demonstrable increases in PCP cause more severe disease or are the result of it (5). Our results suggest that the accumulation of PCP in the lungs during the early hours of type III pneumococcal pneumonia inhibits bacterial phagocytosis and killing and is an important contributing factor in the pathogenesis of this infection.

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