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Replication Rate of *Pseudomonas aeruginosa* in the Murine Lung

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Using a method recently developed at our laboratory, we determined the initial rate of *Pseudomonas aeruginosa* replication in the lung under different experimental conditions. Mice were exposed to aerosols containing mixtures of a temperature-sensitive (ts) mutant of *P. aeruginosa* and its parental wild type (wt). The changes in the ratio of ts:wt were determined by quantitatively culturing homogenates of lungs from animals sacrificed over different time periods. The doubling time (DT) was calculated as the reciprocal of the slope of the linear portion of the curve generated by plotting $n = (\log [r_0/r_t])/\log 2$ against time where r is the ratio of ts:wt at a given time. The DTs measured in both outbred ICR mice and F_1 hybrids (DBA/2J × B10.D2/nSnJ) were 32 and 30 min, respectively. These DTs were higher than that determined in the peritoneal cavities of ICR mice (20 min). The DT in the lungs of ICR mice rendered granulocytopenic by treatment with cyclophosphamide was 16 min. Experiments performed with inocula of different sizes showed that DTs tended to be higher in animals aerosolized with low doses of the ts-wt mixture.

Pseudomonas aeruginosa rarely causes infection in immunologically sufficient hosts, even though the microorganism may be present in the gastrointestinal and upper respiratory tracts or on the skin. *P. aeruginosa* lung infections, however, frequently occur in patients with specific underlying diseases (1). Once *P. aeruginosa* invades the lower respiratory tract, it replicates at a rate influenced by the particular conditions of the lung microenvironment; these are determined by, among other factors, the underlying disease. During the past two decades, many studies have been performed with animal models of bacterial lung infection. In most of these studies, the number of CFU in lung tissue was determined at different times after inoculation to evaluate lung clearance rates. With this procedure, researchers were only able to estimate the balance between bacterial replication in situ and clearance by pulmonary defense mechanisms. There is little information concerning the actual replication rates of bacteria in vivo (7, 9, 11, 12), one study on replication rates of mycobacteria in lungs (5), and, to our knowledge, no study evaluating the replication rate of *Pseudomonas* spp. in vivo. In a recent report, we described a procedure for determining in vivo replication rates of bacteria by using temperature-sensitive (ts) mutants (14). The present study was aimed at the evaluation of the replication rate of *P. aeruginosa* in the murine lung, using a new model in which we combined aerosol inoculation techniques and the unique properties of a ts mutant of *P. aeruginosa* that cannot replicate at mammalian body temperature.

MATERIALS AND METHODS

Bacteria and cultures. The ts mutant D/1/8 was isolated in our laboratory by a procedure described elsewhere (13). This mutant replicates well at 29°C and ceases growth immediately after transfer to body temperature. The parental wild-type (wt), Fisher-Devlin-Gnabasik immunotype 1, was obtained from C. Heifetz (Parke, Davis & Co., Detroit, Mich.) and used in all experiments. Both the ts mutant and the wt were propagated routinely on tryptic soy agar (England Laboratories, Beltsville, Md.); plates were incubated at 29 and 37°C, respectively. To prepare ts and wt suspensions, 50 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) was inoculated with a loopful from a fresh plate and incubated at 28°C and 300 rpm in a G-24 Environmental Incubator Shaker (New Brunswick Scientific, Co., Inc., Edison, N.J.). Cultures in mid-log phase were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. The pellet was suspended to the appropriate cell density in cold saline. Mixtures of ts and wt cells were prepared immediately before use.

Mice. Outbred, 3- to 4-week-old female ICR mice were purchased from Dominion Laboratories (Dublin, Va.). Hybrid F_1 (DBA/2J × B10.D2/nSnJ) mice were bred in our vivarium from parental inbred strains obtained from Jackson Laboratory (Bar Harbor, Maine). The animals were kept at our facilities under the conditions described by the *Guide for the Care and Use of Laboratory Animals*, Department of Health, Education and Welfare Publication no. 78-23 (NIH), until used in the experiments, when they were 4 to 6 weeks old. Several experiments were performed with ICR mice rendered granulocytopenic by a single injection of cyclophosphamide (Sigma Chemical Co., St. Louis, Mo.), 200 mg/kg intraperitoneally. Aerosol inoculation of the ts-wt mixture was performed 90 to 96 h after injection (10).

Aerosol inoculation. The animals were exposed to the bacterial aerosol for 30 min in a nebulization chamber built in our laboratory to the specifications of a previously described design (6), at 22 to 24°C and at −6-mm water pressure. Details of the procedure have been given in earlier publications from our laboratory (2, 15, 17). Animals were exposed to aerosols containing mixtures of ts and wt *P. aeruginosa* in the proportion 5 ts to 1 wt. Groups of four animals were sacrificed either immediately or at different times after aerosol exposure. Appropriate dilutions of lung homogenates in distilled water were plated quantitatively on tryptic soy agar in duplicate sets. One set was incubated at 37°C to determine the number of CFU of wt *P. aeruginosa* (D/1/8 does not replicate at this temperature); the other set was incubated at 29°C to determine the total number of CFU (wt + ts). The number of CFU of the ts mutant was determined by subtraction. The results were recorded as

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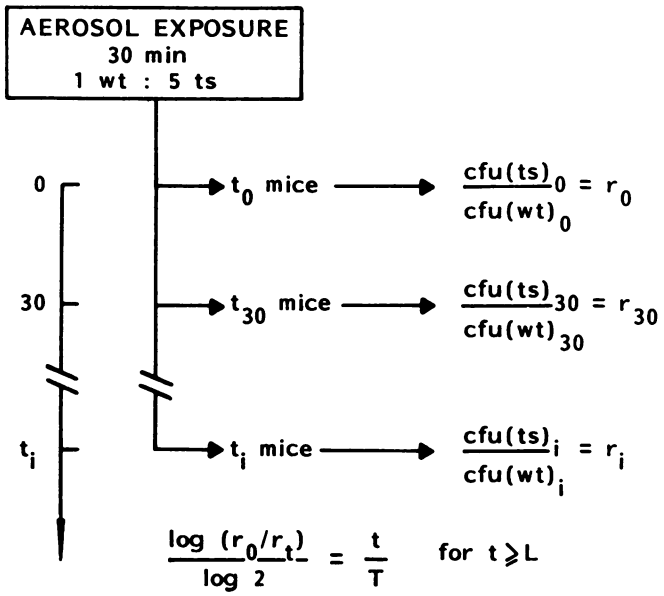

FIG. 1. Protocol for experimental determination of bacterial replication in vivo; n is the number of doublings of the wt over time t, r_0 is the ratio of the ts mutant to wt immediately after inoculation, and r_t is the ratio of the ts mutant to wt at time t after inoculation. Time L is indicated on the abscissa of the graph depicted in Fig. 3.

CFU of wt and ts *P. aeruginosa* per each mouse's lungs. The uncleared bacteria ratio (UBR) was determined as CFU_t/CFU_0 of either wt or ts *P. aeruginosa*.

Peritoneal inoculation. Mice of the ICR strain were injected intraperitoneally with mixtures of ts and wt *P. aeruginosa* in a 5:1 ratio and a total dose of 10^8 CFU per mouse. Lavage was performed by injecting 5 ml of saline into the peritoneal cavities of animals sacrificed at different times after inoculation; the abdomen was massaged gently for 1 min, and the lavage suspension was recovered. The number of CFU of the ts mutant and the wt *P. aeruginosa* was quantitated in the lavage suspensions, and the ratios of ts:wt was determined for each animal.

Estimation of the doubling rate. The number of CFU of ts and wt *P. aeruginosa* was determined for each animal at each time point. The number of doublings of the wt was estimated by the method depicted in Fig. 1. Derivation of the formula was described in detail in a previous report (14). The graph of n versus t has a linear portion after a short initial lag period. The reciprocal of the slope of the linear portion determined by regression analysis is an estimate of the in vivo mean generation time. Throughout the text, however, the term mean generation time will be used exclusively to define the time elapsed while bacteria replicate once in vitro; doubling time (DT) will be used in reference to in vivo replication of bacteria. Regression analysis was performed and the confidence intervals of the slopes were determined by standard methods (4).

RESULTS

Figure 2 depicts the changes in the number of CFU recovered at different times after aerosol exposure. The influence of bacterial replication was clearly seen when the UBR of ts and wt *P. aeruginosa* was plotted against time. The plot corresponding to wt *P. aeruginosa* followed a pattern similar to those described by others (18), but provided no indication of the rate at which the organisms are either being killed or replicating in the lungs. The plot of ts mutant UBR against time showed that the clearance mechanisms are acting very efficiently indeed, clearing ca. 90% of the initial inoculum of the ts mutant in the first 2 h and ca. 99% by 4 h. The difference between the two UBR plots was due to replication of wt *P. aeruginosa*. The plot of n (of the

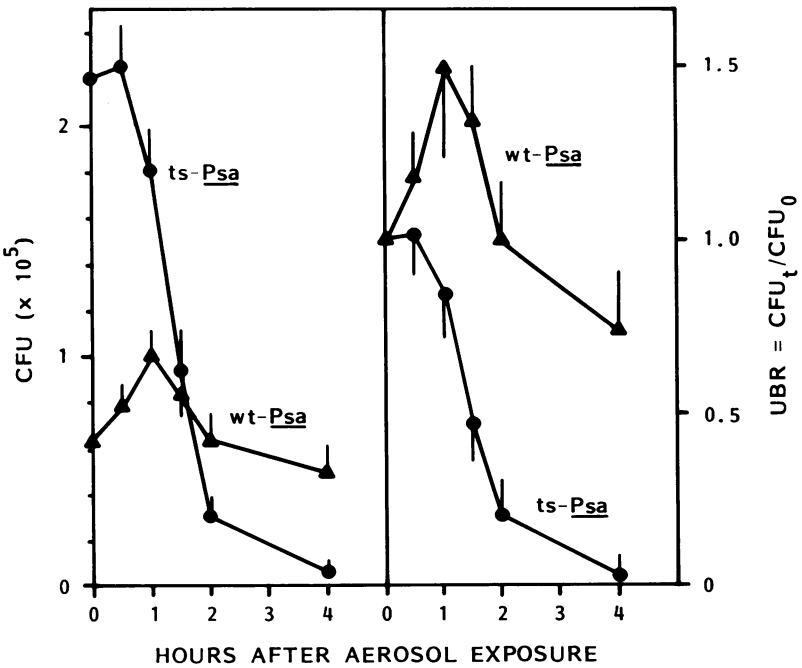

FIG. 2. Total number of CFU in lungs (left panel) and UBR (right panel) of both ts mutant D/1/8 and its parental wt *P. aeruginosa* (*Psa*) given to mice in a mixture by the aerosol route. Each point represents the arithmetic mean ± standard error of the mean from three mice.

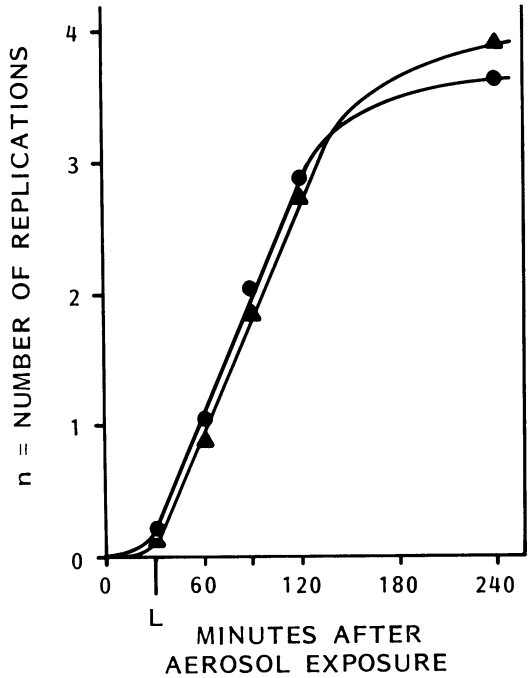

FIG. 3. Number of wt *P. aeruginosa* replications in vivo over time in the lungs of ICR mice (▲) and F_1 (DBA/2J × BB10.D2/nSnJ) hybrids (●). The DTs in these particular experiments were 32 and 30 min, respectively, after inoculation of 2×10^5 to 3×10^5 CFU of the ts-wt mixture. Each point represents the arithmetic mean from three mice, whereas 12 pairs of values were included in the regression analysis. Time *L* on the abscissa indicates the beginning of the linear portion of the plot of the number of generations against time.

wt) over time *t* had a linear portion (Fig. 3), and the DT, determined as the reciprocal of the slope, was 32 min (95% confidence interval, 28 to 36 min). The strain of mice used did not seem to influence the replication rate of *P. aeruginosa*. The DT determined in the F_1 hybrids (DBA/2J × B10.D2/nSnJ) under similar experimental conditions was 30 min (95% confidence interval, 23 to 39 min) (Fig. 3).

The DT was also determined by using lethal inocula of aerosolized mixtures of ts mutant and wt in the lungs of granulocytopenic animals which were kept at 37°C for the first 3 h after exposure to the aerosol. Under the conditions of these experiments, mice died within 48 h, and the DT measured in the first 3 h after aerosol exposure was 16 min (95% confidence interval, 15 to 17 min) (Fig. 4). The DT of *P. aeruginosa* in the peritoneal cavities of normal, untreated ICR mice, as shown previously, was 20 min (95% confidence interval, 18 to 22 min) (Fig. 4) (14).

The total intrapulmonary dose of the ts-wt mixture in the experiments described so far was kept at 2×10^5 to 3×10^5 CFU (ts plus wt) per each animal's lungs. A series of experiments was performed in which the total intrapulmonary dose of the ts-wt mixture received by the animals was adjusted to the lowest possible value within the limits of sensitivity of the method. At doses of 1×10^4 to 2×10^4 total CFU the estimation of the DT increased to 56 min (95% confidence interval, 44 to 78 min). The plots corresponding to this series of experiments are not presented because of their similarity to the ones shown in Fig. 3. The correlation coefficients were in all cases greater than 0.96, determined by regression analysis of the straight line generated from at least nine points.

DISCUSSION

It is generally accepted that the availability of nutritional factors modifies the mean generation time of bacteria. Estimation of the true mean generation time in vitro is facilitated by the fact that there is no substantial loss of bacterial viability during growth in log phase. The in vivo environment, however, is certainly more complex and contains multiple antibacterial mechanisms. Early studies showed that *P. aeruginosa* deposited in the lungs of mice by aerosol exposure is capable of proliferation before being cleared by the defense mechanism(s) of the host (8, 18). Although there are many studies addressing different aspects of all types of *P. aeruginosa* infections, to our knowledge the in vivo replication rate of the organism has never been measured before.

Before discussing the significance of in vivo replication rates of *P. aeruginosa* determined in this study, we should define first the characteristics of the microenvironment where the measurements were performed, i.e., the murine lung within the first hours after exposure of the animals to an aerosol containing the bacteria. Most (98%) aerosolized *P. aeruginosa* are retained in the lungs (15). In a previous report from our laboratory we have shown that intrapulmonary doses $>1 \times 10^5$ to 2×10^5 CFU of *P. aeruginosa* per mouse induce a certain degree of damage to the resident cell population of the respiratory tract (17). We have also shown

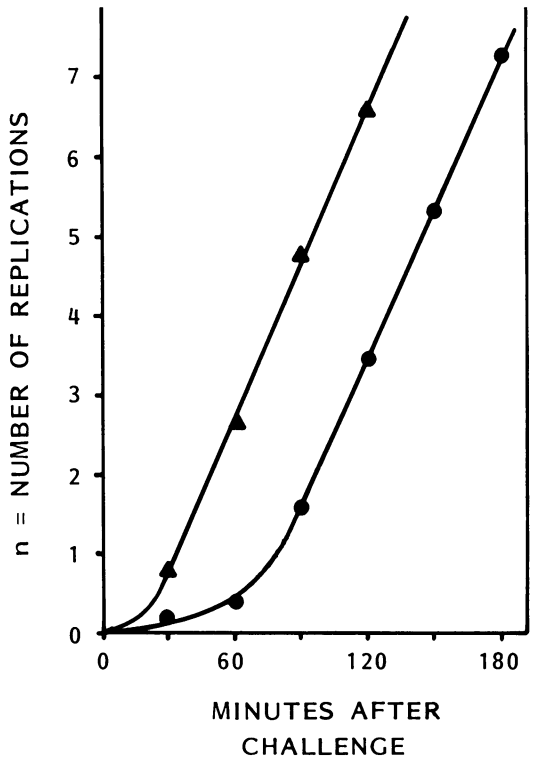

FIG. 4. Number of wt *P. aeruginosa* replications over time in the peritoneal cavities of normal ICR mice (●) and in the lungs of ICR mice rendered granulocytopenic by cyclophosphamide treatment (▲). The DTs in these experiments were 16 and 20 min, respectively. The intrapulmonary dose of the ts-wt mixture was 2×10^5 to 3×10^5 CFU per each animals's lungs, and the intraperitoneal, nonlethal dose of the wt-ts mixture was 5×10^8 CFU per mouse. Each point represents the arithmetic mean from three mice. Twelve pairs of values were included in the regression analysis.

that the extent of the damage may correlate with an impairment in the lung defense mechanisms against *P. aeruginosa* (D. O. Sordelli, M. C. Cerquetti, A. Morris Hooke, and J. A. Bellanti, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, E52, p. 84). An inflammatory cell response is triggered by *P. aeruginosa* in the lungs, and as early as 1 to 2 h after aerosol exposure polymorphonuclear leukocytes can be recovered by lung lavage (17). The development of lung edema is evident in the first 4 h after aerosol exposure, and histopathological analysis of lung sections reveals peribronchial and perivascular infiltrates accompanied by various degrees of congestion. The events described so far occur in the first 4 h after aerosol exposure. The present study shows that while these events are taking place, *P. aeruginosa* is replicating with a short DT and that the clearance mechanisms of the murine lung are nevertheless acting very efficiently, since *P. aeruginosa* is cleared totally within 48 h. Recent data from our laboratory have shown that either a ts mutant or the wt *P. aeruginosa* induced a recruitment of cells of similar type and magnitude in the murine lung (16). The changing ts:wt ratios during the first 4 h after aerosol exposure should not, therefore, have any impact on cell recruitment, and these lung defenses against both phenotypes should be the same.

P. aeruginosa can alter its own metabolism in subtle ways to adapt to the conditions of the environment (3). From the results of many experiments of in vivo replication performed in our laboratory, we have obtained different estimates of *P. aeruginosa* DT in murine lungs, according to the conditions in which the experiment was performed, e.g., dose of bacteria inoculated and temperature at which the animals were kept after aerosol challenge. The variations in the DT were not random but correlated with the conditions of each given experiment; in other words, we believe that there is no certain value for the DT of *P. aeruginosa* in vivo per se but, within the margin of experimental error, specific DTs for the bacteria can be reached under the conditions in which each particular experiment is performed, similar to that which occurs in vitro. Separate experiments performed under similar conditions yielded DTs that were not significantly different.

The DTs reported here probably represent the maximum rates of replication under given experimental conditions for the first hours in the murine lung (or the peritoneal cavity). Aerosolization of normal mice with *P. aeruginosa* is not a model of pneumonia as it occurs in patients with underlying diseases, but a model to study lung defenses against these organisms in a normal host. Therefore, the DTs obtained in the lungs of normal mice may have no clinical relevance. The DT determined in mice treated with cyclophosphamide, however, may represent a useful estimate of the replication rate of the bacteria in the lungs of granulocytopenic, terminally ill patients.

In conclusion, we obtained quantitative estimations of the replication rates of *P. aeruginosa* in the murine lung in the initial stages of bacterial invasion. The replication rate depended upon the conditions that the bacteria encountered in the lung microenvironment. We also present direct evidence that the defense mechanisms of the respiratory tract against *P. aeruginosa* are capable of operating at very high efficiencies. Our results demonstrated, in addition, that determination of the absolute number of CFU in lung tissue materials cannot reveal the dynamics of the interactions between *P. aeruginosa* and the murine respiratory tract.

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