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# Saturability of Granulocyte Kill of *Pseudomonas aeruginosa* in a Murine Model of Pneumonia▽

G. L. Drusano,* B. VanScoy, W. Liu, S. Fikes, D. Brown, and A. Louie

*Ordway Research Institute, Albany, New York 12208*

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**Outcomes for patients with dense bacterial burdens, such as ventilator-associated pneumonia (VAP) patients, are often critically influenced by the adequacy of antimicrobial chemotherapy and by the response of the immune system, particularly the granulocytes. Little information is available about the quantitation of kill of organisms over time by granulocytes. In this investigation, we examined the impact of the baseline bacterial burden on the ability of granulocytes alone (without chemotherapy) to keep the number of organisms in check or to kill them over a 24-h period. *Pseudomonas aeruginosa* ATCC 27853 was the study organism, and we employed a murine pneumonia model (granulocyte replete) for the study. We found that the ability of the immune system to kill *P. aeruginosa* was saturable. The burden at which the system was half saturated was $2.15 \times 10^6 \pm 2.66 \times 10^6$ CFU/g. Burdens greater than $10^7$ CFU/g demonstrated net growth over 24 h. These findings suggest the need for aggressive chemotherapy early in the treatment of VAP to keep the burden from saturating the granulocytes. This should optimize the outcome for these seriously infected patients.**

We have recently demonstrated that the kill of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* by granulocytes is saturable in a mouse thigh infection model in which the animals are granulocyte replete (1). As part of that evaluation, we speculated that the same would be true in the setting of pneumonia and that this phenomenon may explain why some antimicrobial agents that are predicted to be suboptimal based on the exposure achieved at the primary infection site still appear to work, particularly in trials where patients are drawn from the community and where the bacterial burden at the initiation of chemotherapy is modest.

While we believed it was likely that the same finding would be demonstrable in the setting of pneumonia, the considerable pathological differences between a murine model reflecting mostly a skin or skin structure type of infection and a murine pneumonia model might be large enough that the findings in the pneumonia model might be substantially different.

In this evaluation, we examined the impact of the initial bacterial burden on the ability of granulocytes to limit the expansion of the bacterial population over 24 h in a murine model of *Pseudomonas* pneumonia.

## MATERIALS AND METHODS

**Microorganisms.** *Pseudomonas aeruginosa* ATCC 27853 was employed. Stocks of the organism were stored in 10% glycerol at −80°C. The isolate was subcultured on blood agar plates twice before each experiment.

**Animals.** Female 24- to 25-g outbred Swiss Webster mice (Taconic Farms, NY) were used in all *in vivo* studies. They received food and water *ad libitum*. All animal experimentation procedures were approved by our Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with its guidelines.

**Animal model.** Animals were anesthetized with ketamine-xylazine (100 mg/kg ketamine and 6 mg/kg xylazine) via intraperitoneal (i.p.) injection. *Pseudomonas aeruginosa* challenge was performed by placing 20 μl of the proper bacterial suspension into each naris of an anesthetized animal to generate the desired initial challenge. Mice were allowed to recover from anesthesia and were then observed serially for 24 h. All mice were euthanized humanely at 24 h for the determination of lung colony counts.

**Growth determination studies.** *Pseudomonas aeruginosa* ATCC 27853 inocula at five different levels, ranging from $1 \times 10^6$ CFU through $1 \times 10^8$ CFU, were employed. Challenges with bacteria were verified by quantitative culture performed at the time of inoculation. At 0 and 24 h after bacterial challenge, five mice per bacterial challenge group were humanely sacrificed in order to estimate the number of organisms at the infection site. The lungs were carefully dissected, weighed (allowing weight-normalized bacterial numbers to be calculated), and homogenized in ice-cold 0.9% saline (10:1, vol/wt). The homogenates of infected murine lungs were cultured quantitatively.

**Modeling methods.** Given our previous results (1), we employed the same approach. The inhomogeneous differential equation shown below was employed to describe the time course of organisms in the primary infection site of the murine lung. All challenges were simultaneously comodeled with the big nonparametric adaptive grid population modeling program (BigNPAG, version 4) of Leary, Jelliffe, Schumitzky, and Van Guilder (4). To approximate the homoscedastic assumption, we employed the inverse of the observation variance weighting for any cohort: $dX_1/dt = K_{\text{max-growth}} \times [1 - (X_1/\text{POPMAX})] \times X_1 - [K_{\text{max-kill}} \times X_1/(K_m + X_1)] \times X_1$, where $X_1$ is the organism load in the mouse lung at time $t$, POPMAX is the estimated maximal number of organisms at stationary phase, $K_{\text{max-growth}}$ is the maximum growth rate constant, and $K_{\text{max-kill}}$ and $K_m$ are the Michaelis-Menten constants ($K_{\text{max-kill}}$ is the maximal granulocyte-induced kill rate, and $K_m$ is the number of bacteria per gram of tissue that half-saturates granulocyte kill). The baseline number of organisms was placed in the lung compartment by rapid (0.001-h) infusion.

Goodness of fit was determined by examination of observed-predicted plots and by determination of the estimates of bias and precision for the regression. Mean weighted error was the measure of bias, and bias-adjusted precision was calculated.

## RESULTS

**Colony counts over time for different challenges of *Pseudomonas aeruginosa* in a model of pneumonia in immunocompetent mice.** In Fig. 1A, we display *P. aeruginosa* colony counts in the mouse lung at 0 and 24 h. The bacterial burden of the initial challenge for the lung ranged from $1 \times 10^6$ to $1 \times 10^8$ CFU. At 24 h, the initial challenges of $1 \times 10^6$ through $1 \times 10^7$ CFU demonstrated net kill by the granulocytes of approximately 0.74 to 2.5 $\log_{10}$ CFU/g over 24 h. The challenges of 3 ×

* Corresponding author. Mailing address: Ordway Research Institute, 150 New Scotland Ave., Albany, NY 12208. Phone: (518) 641-6410. Fax: (518) 641-6304. E-mail: gdrusano@ordwayresearch.org.
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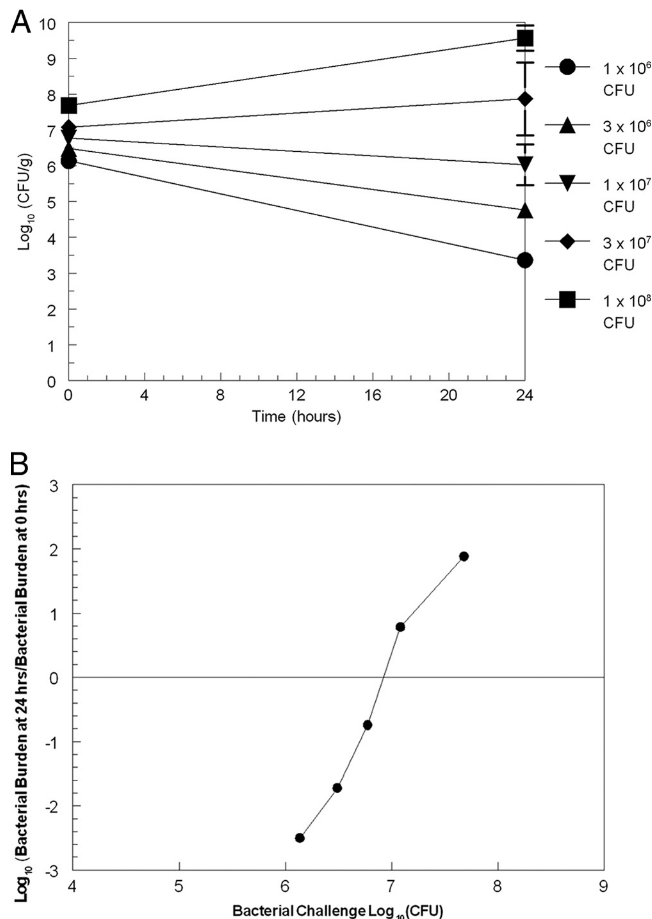


FIG. 1. (A) Numbers of *Pseudomonas aeruginosa* organisms in the lungs of granulocyte-replete mice infected with different challenges. (B) Net change in the number of *Pseudomonas aeruginosa* colonies over 24 h, as influenced by granulocyte killing.

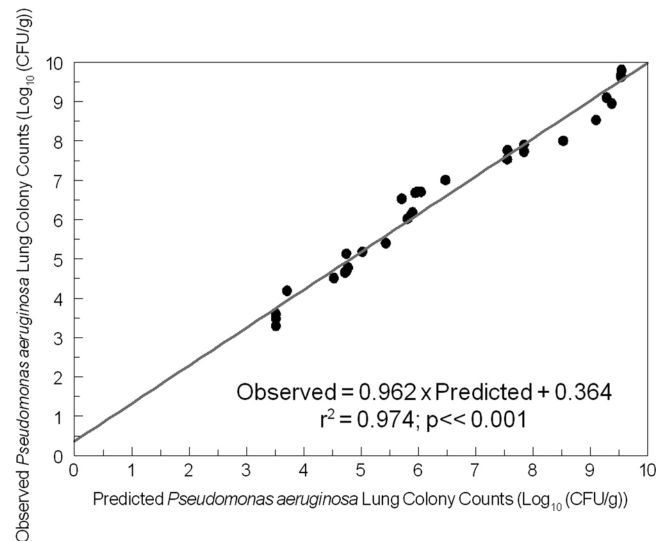


FIG. 2. Predicted-observed plot after the Bayesian step.

$10^7$ CFU and $1 \times 10^8$ CFU showed net growth of 0.78 and 1.88 $\log_{10}$ CFU/g, respectively, from the baseline to 24 h. The changes in bacterial burden for all challenges are shown in Fig. 1B.

We also examined cohorts of animals challenged with *Pseudomonas aeruginosa* that were rendered neutropenic with cyclophosphamide. Challenges as low as $3 \times 10^4$ CFU still produced growth up to $4.7 \times 10^7$ CFU/g at 24 h (data not shown), indicating the central role of the granulocyte in the infection-clearing process in the lung.

**Application of the mathematical model to the *Pseudomonas aeruginosa* data.** The fit of the model to the data was quite acceptable (Fig. 2). The predicted-observed plot after the Bayesian step demonstrated a regression line with the following equation: observed = 0.962 × predicted + 0.364 ($r^2$ = 0.974; $P \ll 0.001$). The regression was adequately precise and was unbiased. Table 1 shows the estimates of the system parameters and their dispersions. The organism growth rate constant ($K_{\text{max-growth}}$) was 0.745 ± 1.01 $h^{-1}$, smaller than that seen in the mouse thigh model (1).

The estimated maximal population count was $8.48 \times 10^{11} \pm 1.65 \times 10^{11}$ CFU/g. The estimate of the maximal kill rate induced by granulocytes ($K_{\text{max-kill}}$) was 1.97 ± 3.03 $h^{-1}$. The number of organisms that half-saturated the granulocyte killing system was $2.15 \times 10^6 \pm 2.66 \times 10^6$ CFU/g, a value quite concordant with that seen previously in the mouse thigh system (1).

## DISCUSSION

Antimicrobials are amazing drugs and have effected nothing less than a revolution in medicine. They allow patients to be treated with intensive cancer chemotherapy, undergo invasive procedures that cross anatomical barriers, and endure modulation of the immune system under circumstances such as severe rheumatoid arthritis. Still, antimicrobial chemotherapy is not the only factor influencing the outcome of infections; the immune system also plays an important role. While there are many facets to the immune system, when it comes to overwhelming infections such as ventilator-associated pneumonia (VAP), granulocytes clearly make an important contribution to the outcome.

We had shown previously that bacterial cell kill by granulocytes was saturable in the setting of a mouse thigh model (1). From this, we speculated that the outcomes for community-acquired pneumonia treated with antimicrobial agents of marginal efficacy could be explained by the fact that for many patients with PORT (patient outcome research team) I and PORT II community-acquired pneumonia, the bacterial burden was relatively low at the initiation of therapy, so that the drug needed only to have a marginal effect, resulting in a burden that remained below the point at which granulocyte

TABLE 1. Parameter values for growth and Michaelis-Menten death model

| Parameter | Mean | SD |
|---|---|---|
| $K_{\text{max-growth}}$ ($h^{-1}$) | 0.745 | 1.01 |
| POPMAX (CFU/g) | $8.48 \times 10^{11}$ | $1.65 \times 10^{11}$ |
| $K_{\text{max-kill}}$ ($h^{-1}$) | 1.97 | 3.03 |
| $K_m$ (CFU/g) | $2.15 \times 10^6$ | $2.66 \times 10^6$ |

killing was half-maximal. This would allow the addition of an extra 0.5- to 1.5-$\log_{10}$ CFU/g bacterial cell kill attributable to granulocytes over the first 24 h.

Clearly, the pathological process in pneumonia is quite different from that in a skin and skin structure infection, and the murine models for these infections (murine thigh model and murine pneumonia model) would also be expected to be quite different, so there would be no guarantee that granulocyte killing would be saturable in the murine pneumonia model. In this investigation, we show that, as before, granulocyte killing is saturable. Further, the amount of bacterial kill seen here is virtually identical to that seen in the mouse thigh model, with maximal kill at approximately 2.0 $\log_{10}$ CFU/g over the first 24 h. Also, the bacterial burdens at which granulocyte killing is half-saturated were strikingly similar in the two models ($4.3 \times 10^6 \pm 3.75 \times 10^6$ CFU/g for *P. aeruginosa* in the mouse thigh model versus $2.15 \times 10^6 \pm 2.66 \times 10^6$ CFU/g in the murine pneumonia model).

These findings have considerable implications for VAP therapy. When one diagnoses VAP, the gold standard is to obtain a quantitative culture via bronchoalveolar lavage (BAL). The diagnostic criterion is to have a minimal burden of $10^4$ CFU/ml. It should be recognized that the dilution obtained by the lavage is on the order of 30- to 100-fold when one corrects for the dilution by the ratio of urea in the BAL fluid to that in blood (2, 6). This means that the minimal burden acceptable for the definition of VAP is ca. 5.5 to 6.0 $\log_{10}$ CFU/ml. It is clear that many patients' burdens will be greater, and some will be in the range of 8.0 to 9.0 $\log_{10}$ CFU/ml (11).

For this patient group, then, chemotherapy must be considerably better than in patients with lower bacterial burdens in order to obtain a good outcome. The baseline number of organisms in a majority of VAP patients will exceed the threshold where the kill is half saturated. In order to maintain the maximal amount of granulocyte kill, the chemotherapy should generate a bacterial cell kill of 2 to 3 $\log_{10}$ CFU/ml early on. This is quite concordant with clinical observations, where early, appropriate chemotherapy has a significant impact on the outcome for VAP patients (5, 9, 10). It also implies that for serious *Pseudomonas* VAP infections, combination chemotherapy may be the optimal approach. Given the high exposures in the epithelial lining fluid (ELF) needed to mediate multilog kill and the difficulty of penetration into the ELF for many drugs, it may be difficult to attain a cell kill of 2 to 3 $\log_{10}$ CFU/ml with monotherapy (2, 7). Consequently, obtaining greater cell kill with combination therapy is important for two reasons. First, the greater cell kill will allow optimal granulocyte kill. Second, the combination therapy is likely to suppress the amplification of resistant mutant subpopulations (3, 8). Finally, it will be important to sort out the impact of prevention of the saturation of granulocyte killing, particularly in combination with optimal chemotherapy, on therapy duration.

There is one cautionary note. This work was performed with a single strain of *Pseudomonas aeruginosa*. For a fuller understanding of the impact of granulocytes, many more isolates will need to be studied.

In summary, granulocyte cell kill is saturable in a murine pneumonia model. This finding indicates that pneumonia patients with high bacterial burdens require optimal chemotherapy. Such optimal therapy will have two desirable consequences: suppression of resistance and optimal granulocyte kill. In the next series of investigations, we will attempt to examine the interaction of granulocyte kill and antimicrobially mediated cell kill in a murine pneumonia model.

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We have no conflicts to disclose.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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