Impact of Burden on Granulocyte Clearance of Bacteria in a Mouse Thigh Infection Model

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Abstract:

Background: We wished to delineate granulocytes’ impact on the clearance of different bacterial burdens of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in a granulocyte-replete mouse thigh infection model.

Methods: A mouse thigh model was employed. Bacterial challenges from $10^5\text{–}3\times10^7$ CFU (*S. aureus*) and from $3\times10^4\text{–}3\times10^8$ CFU (*P. aeruginosa*) were injected into murine posterior thighs. Organism quantitation was at baseline, 2 (Pseudomonas only) and 24 hours. A Michaelis-Menten population model was fit to the data for each organism.

Results: Breakpoints for microbial containment by granulocytes were identified. Bacterial burdens exceeding that breakpoint value, resulted in organism multiplication. The Michaelis-Menten model fit the data well. For *P. aeruginosa*, the observed-predicted plot had a regression equation that explained over 98% of the variance (p<< 0.001). For *S. aureus*, this relationship explained greater than 94% of the variance (p << 0.001). Maximal growth rate constants, maximal population burdens and the bacterial load at which granulocytes kill if half-saturated were not different. The kill rate constant for *P. aeruginosa* was almost 10 times that of *S. aureus*.

Conclusions: Bacterial kill by granulocytes is saturable. No difference in saturation point was seen between isolates. Higher bacterial burden means an increasing reliance on chemotherapy to drive bacterial clearance.
Introduction:

Humans are endowed with an immune system that protects them from a vast number of infectious assaults. When dealing with many (but not all) bacterial infections, the granulocyte plays a key role in the clearance of the infection.

With the advent of antibiotics, we have become somewhat complacent about our ability to deal with serious bacterial infections. A great multiplicity of antibiotics of different classes and types within classes have been discovered and, after appropriate review by the Food and Drug Administration, have made their way into the clinician’s armamentarium.

One issue not well addressed is the question of how much of the ability to cure infections is due to the antibiotics and how much is due to granulocytes. While there has been an extensive literature developed in many animal model systems in which the ability of an antibiotic to kill bacteria at the primary infection site has been linked to the drug exposure (2, 4, 11, 12), little has been done to examine this issue in vivo for the granulocytes. Indeed, the vast majority of this literature has been developed in a setting in which the animals were rendered severely neutropenic by cyclophosphamide.

In this investigation, we examined the impact of granulocytes on bacterial cell clearance in a mouse thigh infection model. We looked at two common and important bacterial pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

We also felt it important to examine the impact of bacterial burden upon the clearance of the bacterial pathogen. Clinicians have long known of the importance of bacterial load (3) in terms of its effect on the likelihood of a good clinical outcome. We are unaware, however, of a quantitative analysis relating bacterial burden to the clearance of the pathogens by granulocytes.
Methods:

Microorganisms:

A methicillin-susceptible *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* ATCC 27853 were used. Stocks of the microbes were stored in skim milk at -80°C. The isolate was sub-cultured on blood agar plates twice before each experiment.

Animals:

Female, 24-26 g outbred Swiss-Webster (Taconic Farms, NY) mice were used in all *in vivo* studies. They received food and water *ad libitum*. All animal experimentation procedures were approved by and conducted in accordance with the guidelines of our Institutional Animal Care and Use Committee (IACUC).

Murine thigh infection model:

A mouse thigh infection model, pioneered by Eagle (6) and greatly expanded upon by Craig (2, 4, 5) was adapted to examine the relationship between granulocyte presence and reduction in the density of bacteria in thigh muscles of mice. These experiments were performed in normal mice.

Growth/Death Determination Studies:

Inocula at six different levels of *Staphylococcus aureus* ATCC 29213 ranging from $10^5$ through $3 \times 10^7$ were injected into the posterior thighs of mice. For *Pseudomonas aeruginosa* ATCC 27853, inocula ranging from $3 \times 10^4$ through $3 \times 10^8$ were used. Bacterial challenges were verified by quantitative culture performed at the time of inoculation. At zero, 2 and 24 hours after bacterial inoculation (for *Pseudomonas aeruginosa*), 5 mice per were sacrificed by CO$_2$.
asphyxiation to establish the number of organisms. At zero hours and 24 hours (*Staphylococcus aureus*), cohorts (n = 5) of animals were sacrificed to determine the CFU/g of organisms present. Both posterior thighs were carefully dissected and homogenized in ice cold 0.9% saline (10:1 volume:wt). The homogenates of infected thigh muscles were quantitatively cultured.

**Modeling Methods:**

To mathematically determine the kill of micro-organisms by granulocytes, the inhomogeneous differential equation (shown below) was used to describe the time course of organisms at the mouse thigh primary infection site. Multiple inocula were simultaneously co-modeled by use of the Big Non-Parametric Adaptive Grid population modeling program of Leary, Jelliffe, Van Guilder and Schumitzky (BigNPAG - 9). Weights were the inverse of the observation variance for any cohort.

\[
\frac{dX_1}{dt} = K_{\text{max-growth}} \times (1-(X_1/\text{POPMAX})) \times X_1 - (260 \times K_{\text{max-kill}} \times X_1 / (K_m + X_1)) \times X_1 \quad (\text{Eqn 1})
\]

Where \( X_1 \) is the number of organisms present in the mouse thigh at time “t”; \( \text{POPMAX} \) is the theoretical stationary phase maximal number of organisms; \( K_{\text{max-kill}} \) and \( K_m \) are the Michaelis-Menten constants, where \( K_m \) is the number of organisms per g of tissue at which the granulocytes are half saturated. The number “260” is the average number of granulocytes/\( \mu l \) present at baseline (determined experimentally) that multiplies \( K_{\text{max-kill}} \). The baseline number of organisms were placed into the tissue compartment as an initial condition, which was a random variable and fit as part of the modeling process.

Goodness of fit was determined by examining observed-predicted plots, as well as determining 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 the Thigh muscles of Immunocompetent Mice:

In Figure 1A, we display the colony counts at times zero, 2 and 24 hours of *P. aeruginosa* in the mouse thigh. The initial burden injected into the posterior thigh ranged from $3 \times 10^4$ to $3 \times 10^8$ CFU. At the 2 hour evaluation, the 2 lowest burdens decreased from baseline, while the others showed net growth. At 24 hours, the initial challenges of $3 \times 10^4$ through $3 \times 10^5$, demonstrated net kill by the granulocytes of approximately $1.1 - 2.0 \log_{10}$ (CFU/g). The challenge of $1 \times 10^6$ achieved net stasis. All other challenges showed net growth of $2.2 \log_{10}$ (CFU/g), because of attaining stationary phase (POPMAX).

The changes in bacterial burden for all challenges are shown in Figure 1B.

Application of the Mathematical Model to the *Pseudomonas aeruginosa* Data:

The model fit the data well (Figure 2). After the Bayesian step, the predicted-observed plot demonstrated a regression line of:

$$\text{Observed} = 0.977 \times \text{Predicted} + 0.104; \ r^2 = 0.989; \ p << 0.001.$$  

As can be seen by inspection, the regression was adequately precise and was unbiased.

Table 1 shows the point estimates of the parameters and their dispersions. The organism grew well, as demonstrated by the estimate of maximal growth rate ($K_{\text{max-growth}}$), which was $4.299 \ h^{-1}$. The estimate of the maximal population count was physiologic at $1.07 \times 10^{10}$ CFU/g. The estimate of the maximal kill rate induced by granulocytes was ($V_{\text{max-kill}}$) was $1.295 \ h^{-1}$. It
should be noted that this number needs to be multiplied by the baseline granulocyte count, which for these experiments was 260/µl. This was determined separately in a pre-experiment in this laboratory. The number of organisms that half saturate the granulocytic kill ability was 4.30 x 10^6 CFU/g. This number is believable as the initial challenge of 1 x 10^6 CFU in Figure 1 (resulting in 6.9 x 10^5 CFU/g at time zero) shows near net stasis (actually 0.15 Log_{10} (CFU/g) kill) at 24 hours.

**Colony Counts over Time for Different Challenges of *Staphylococcus aureus* in the Mouse Thighs of Immunocompetent Mice:**

In Figure 3A, we display the colony counts at times zero and 24 hours of *S. aureus* in the mouse thigh. The initial burden injected into the posterior thigh ranged from 1 x 10^5 to 3 x 10^7 CFU. In this evaluation, the 2 hour time point was not obtained, as it was relatively non-informative. At 24 hours, the initial challenges of 1 x 10^5 and 5 x 10^5 demonstrated net kill by the granulocytes of approximately 1.0 Log_{10} (CFU/g). The challenges of 1 x 10^6 and 3 x 10^6 achieved net stasis. All other challenges showed net growth, of 1.55 and 1.45 Log_{10} (CFU/g) increase from baseline to 24 hours.

The changes in bacterial burden for all challenges are shown in Figure 3B.

**Application of the Model to the *Staphylococcus aureus* Data:**

The model fit the data well (Figure 4). After the Bayesian step, the predicted-observed plot demonstrated a regression line of:

\[
\text{Observed} = 0.917 \times \text{Predicted} + 0.679; r^2 = 0.947; p << 0.001.
\]

As can be seen by inspection the regression was adequately precise and was unbiased.
Table 2, shows the point estimates of the parameters and their dispersions. The organism grew well, as demonstrated by the estimate of maximal growth rate ($K_{\text{max-growth}}$) of 5.678 h$^{-1}$, which is slightly, but not significantly faster than observed with $P. \text{aeruginosa}$. The estimate of the maximal population count was physiologic at $4.24 \times 10^{10}$ CFU/g, again, slightly but not significantly different from that seen with the strain of Pseudomonas studied. The estimate (Table 2) of the maximal kill rate induced by granulocytes ($V_{\text{max-kill}}$) was 0.115 h$^{-1}$ which is considerably less (circa $1/10^6$) than that observed for $P. \text{aeruginosa}$. Because of the variability identified, this number is not significantly different from the value identified for Pseudomonas. The number of organisms that half saturate the granulocytic kill ability was $5.55 \times 10^6$ CFU/g. This number is believable as the initial challenge of $1 \times 10^6$ and $3 \times 10^6$ CFU in Figure 3 (resulting in $3.55 \times 10^6$ and $1.35 \times 10^7$ CFU/g at time zero) shows approximate net stasis at 24 hours.

Discussion:

In the therapy of infections, antibiotics are a crucial tool to optimize therapeutic outcome. It is also clear that many people survived serious infections in the pre-antibiotic era because of their immune function. For most typical bacterial infections, granulocytes play a central role in control of the infection.

Here we have examined a strain of $P. \text{aeruginosa}$ and a strain of methicillin-susceptible $Staphylococcus aureus$ in a non-neutropenic mouse thigh infection model. The first finding from the studies was the impact of increasing bacterial burden on the adequacy of response of the granulocytes. For $P. \text{aeruginosa}$ (see Figure 1 A and B), bacterial challenges of $1 \times 10^6$ CFU (resulting in $6.92 \times 10^5$ CFU/g at baseline) resulted in stasis, whereas
3 x 10⁶ CFU as a challenge (resulting in 2.19 x 10⁶ CFU/g at baseline) lost control and grew to 3.16 x 10⁸ CFU/g at 24 hours. For *Staphylococcus aureus* (Figure 3 A and B), a challenge of 3 x 10⁶ (1.35 x 10⁷ CFU/g at baseline) achieved stasis (net granulocyte kill balanced *S. aureus* growth) whereas with a 1 x 10⁷ CFU challenge (4.52 x 10⁷ CFU/g) net microbial containment was lost and colony counts exceeded 9 Log₁₀ (CFU/g) at 24 hours. For both isolates, then, baseline challenges of 3 x 10⁶ CFU (resulting in 2.19 x 10⁶ CFU/g) - 1 x 10⁷ (resulting in 4.52 x 10⁷ CFU/g) overwhelmed the system, resulting in net growth to near maximal values of organisms over 24 hours.

Examination of Figures 1 and 3 resulted in a preliminary conclusion that the kill of bacterial pathogens by granulocytes followed Michaelis-Menten kinetics and, therefore, was saturable. It should be noted that Leijh et al (10) demonstrated granulocyte saturability for *Staphylococcus aureus* and *E. coli* in a set of *in vitro* experiments previously. We wrote a mathematical model in which bacterial growth was opposed by granulocyte killing, but where the system was explicitly saturable. For both the *P. aeruginosa* data and the *S. aureus* data (see Figures 2 and 4), the model fit the data quite well and, in both instances, explained greater than 94% of the variance.

The model parameters (see Tables 1 and 2) showed very similar rates of maximal growth \( (K_{\text{max-growth}}) \) for both organisms and similar maximal population values \( (\text{POPMAX}) \). For the Michaelis-Menten parameters, the colony counts that half saturate granulocyte killing are strikingly similar at 4.3 x 10⁶ CFU/g for Pseudomonas and 5.55 x 10⁶ for Staphylococcus. The only major difference is seen with the maximal kill rate term \( (V_{\text{max-kill}}) \), where this term is about 10-fold higher for Pseudomonas relative to Staphylococcus. We speculate that this may be because of the well known ability of *Staphylococcus aureus* to survive within granulocytes (7).
and is likely to explain the observed difference in maximal 24 hour kill of $1 \log_{10}(CFU/g)$ for *S. aureus* versus $2 \log_{10}(CFU/g)$ for *P. aeruginosa*.

These findings may also help explain the observation by clinicians that there are some antimicrobial agents that, at approved dose and schedule, are not particularly potent, yet patients with less severe community-acquired infections frequently recover from their infection. It is important to state that the following is speculation and that these findings on saturability and, more importantly, the degree of cell kill per day need to be repeated in the more appropriate murine pneumonia model. The Food and Drug Administration (1, 14) has started to question the validity of clinical trials of Community-Acquired Pneumonia (CAP) in which the bulk of the patients enrolled were of mild severity (PORT I and PORT II – [Pneumonia-patient Outcomes Research Team, also referred to as Pneumonia Severity Index or PSI]). Their hypothesis is that the actual recovery is due more to the patients’ immune system (especially the granulocytes) and less to the effect of the antimicrobial.

Generally, less severe community-acquired infections (using PORT I and PORT II CAP patients as an example) have lower bacterial burdens and the pulmonary infiltrates seen of chest X-rays are very infrequently multi-lobar. If the bacterial burden is around the value at which granulocyte kill is half-saturated, then relatively small amounts of drug effect are sufficient to move the total burden to below this value and the granulocyte kill can start the process of clearing the infection.

Alternatively, patients with greater bacterial burdens and with multi-lobar involvement will rapidly saturate their granulocytes and, in the absence of a major antimicrobial effect, net growth of the bacteria will occur with resultant failure of therapy. Treatment of PORT IV and
PORT V CAP patients with borderline antimicrobial agents is likely to lead to an unacceptable rate of therapeutic failure.

In the case of *Pseudomonas aeruginosa*, it is important to note that patients with Ventilator-Associated Pneumonia (VAP) often have their diagnosis made by quantitative bronchoalveolar lavage, where the smallest burden meeting the VAP definition is $10^4$ CFU/ml. The dilution associated with bronchalveloar lavage is on the order of 30-100 fold (seen explicitly when modeling drug penetration into ELF using a urea correction – 13). Consequently, the true concentration is $3 \times 10^5$ to $1 \times 10^6$ per ml. At a very small volume of 100 ml (many VAP patients would have close to 1 liter or more of organisms), the total bacterial burden would exceed $3 \times 10^7$ per ml, and would saturate the granulocytes. This is a case where only the most potent antimicrobial therapy will drive a good clinical outcome.

The next step is to delineate how antimicrobial agents and the immune system can work together to attain optimal clinical responses. Previous work has shown that even sub-inhibitory drug concentrations can help with intracellular killing (7). Our laboratory (8) has previously modeled the direct interaction of granulocytes and Amphotericin B for clearance of Candidal infection. This should be done with a range of bacteria and different antimicrobial agents. Finally, we focused on granulocytes in this investigation, but it will be increasingly important to understand what other parts of the immune system add to clearance of infection.
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References:


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Figure 1. A. Time Course of *P. aeruginosa* ATCC 27853 in Mouse Thigh (Granulocyte Replete) as a Function of Baseline Bacterial Burden

B. Change in Bacterial Burden from zero to 24 hours
Figure 2. Predicted-Observed Plot after the Bayesian Step for *P. aeruginosa* ATCC 27853 and Point Estimates of the Parameter Values
Figure 3. A. Time Course of *S. aureus* ATCC 29213 in Mouse Thigh (Granulocyte Replete) as a Function of Baseline Bacterial Burden

B. Change in Bacterial Burden from zero to 24 hours
Figure 4: Predicted-Observed Plot after the Bayesian Step for *S. aureus* ATCC 29223 and 301
Point Estimates of the Parameter Values

![Predicted-Observed Plot](image)

- Observed = 0.917 × Predicted + 0.679
- $r^2 = 0.947; p << 0.001$
Table 1: Point Estimates of the Parameter Values and Their Dispersions for the Growth of Pseudomonas aeruginosa ATCC 27853 in the Mouse Thigh and the Kill of the Organism by Granulocytes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$K_{\text{max-growth}}$</th>
<th>POPMAX</th>
<th>$K_{\text{max-kill}}$*</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units</td>
<td>h$^{-1}$</td>
<td>CFU/g</td>
<td>h$^{-1}$</td>
<td>CFU/g</td>
</tr>
<tr>
<td>Mean</td>
<td>4.299</td>
<td>0.107x10$^{11}$</td>
<td>1.295*</td>
<td>0.430x10$^{7}$</td>
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<td>SD</td>
<td>3.115</td>
<td>0.102x10$^{11}$</td>
<td>2.070</td>
<td>0.375x10$^{7}$</td>
</tr>
</tbody>
</table>

* Multiplied by the granulocyte count

$K_{\text{max-growth}}$ is the maximal growth rate of the organism in the mouse thigh; POPMAX is the maximal number of organisms per g of tissue at stationary phase; $V_{\text{max-kill}}$ is the maximal kill rate induced by granulocytes; $K_m$ is the number of organisms per g of tissue at which granulocyte kill is half saturated.
Table 2: Point Estimates of the Parameter Values and Their Dispersions for the Growth of *Staphylococcus aureus* ATCC 29223 in the Mouse Thigh and the Kill of the Organism by Granulocytes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$K_{max}$-growth</th>
<th>POPMAX</th>
<th>$K_{max}$-kill*</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units</td>
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<td>CFU/g</td>
<td>h$^{-1}$</td>
<td>CFU/g</td>
</tr>
<tr>
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<td>SD</td>
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<td>0.1241</td>
<td>0.2563x10$^7$</td>
</tr>
</tbody>
</table>

* Rate constant is multiplied by the granulocyte count

Definitions are the same as above in Table 1.