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How many neutrophils are enough (redux, redux)?

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Many chemotherapeutic regimens produce neutropenia, which predisposes to microbial infection. However, not all neutropenic individuals develop infections, so the ability to predict this outcome would be a powerful clinical tool. In this issue of the JCI, Malka et al. describe a dynamic system model of neutrophil bactericidal activity that confirms and extends the concept of critical neutrophil concentration. The authors demonstrate that when the neutrophil concentration approaches the critical concentration, bacterial populations in contact with them exhibit bistability. Their experimental findings raise the intriguing possibility of greater variability in bactericidal activity of neutrophils from healthy adults than heretofore recognized; their model predicts that this could have life-and-death consequences.

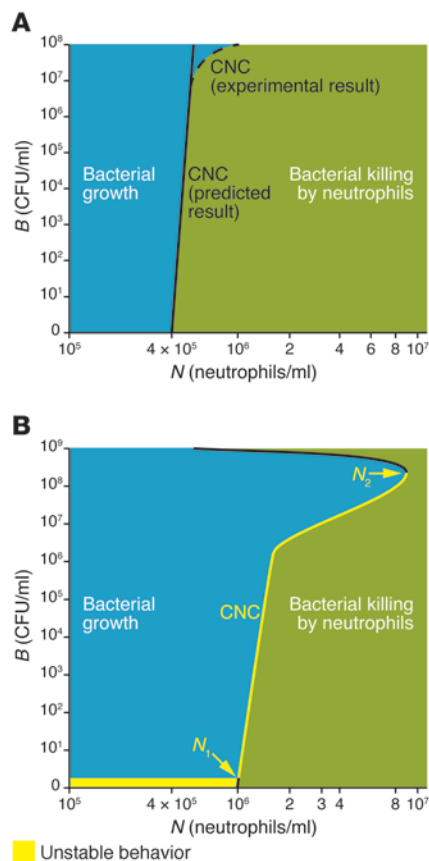
Although the link between neutropenia and infection risk is clear, the precise number of neutrophils required to maintain health remains a topic of intense research interest. Neutrophils continuously patrol the luminal surfaces of endothelial cells, searching for signs of infection or inflammation. Such signs stimulate them to emigrate from blood into extravascular compartments (1). There, their armamen-

tarium of chemoattractant and phagocytosis-promoting plasma membrane opsonin and pattern-recognition receptors enable them to phagocytose approximately 40–50 bacteria/neutrophil (2), and their preformed granule proteins and capacity to produce high intravacuolar concentrations of reactive oxygen species (3) enable them to kill their bacterial prey. The extracellular release of DNA-histone antimicrobial protein-containing nets (4) that entrap bacteria and kill yeast/fungi (5) extends their bactericidal and fungicidal activities into their afterlives and further enhances their efficacy as guardians of tissue sterility.

Chemotherapeutic agents that inhibit neutrophil biogenesis (6–8) and/or reduce their bactericidal activity (7) produce neutropenia (i.e., <500,000 neutrophils/ml blood). Although neutropenia predisposes to infection, it has little direct effect on the sterility of blood because under most (9) — but not all (10) — conditions, hepatic and splenic macrophages are the cells primarily responsible for clearing bacteria from the circulation. Indeed, as suggested by Crosby (11) and supported by the studies of Wright et al. (12) and Koene et al. (13), it is the tissue neutrophil concentration (N) that determines whether the small number of bacteria that breach mucosal surfaces each day will find fertile soil for growth, or be engulfed and killed. Thus, while a blood N value of 5×10^5 neutrophils/ml is a call for vigilance, it is an imprecise measure of the likelihood of infection. This is so because blood is primarily the conduit by which neutrophils travel from bone marrow to tissues, and the blood N reflects the sum of the rates at which neutrophils are produced and released from bone marrow into the circulation, and the rates at which they are consumed in tissues (14) and/or recycled to spleen and bone marrow for destruction (15).

Conflict of interest: Raul Rabadan is a consultant for Novartis and the Geneva Foundation. Samuel C. Silverstein owns stock in Roche Pharmaceuticals and Teva Pharmaceuticals.

Citation for this article: *J Clin Invest*. 2012; 122(8):2776–2779. doi:10.1172/JCI63939.

**Figure 1**

Comparative behaviors of bacterial populations at or near the CNC, as predicted by the equations of Li et al. (20, 21) and Malka et al. (25). **(A)** Li et al. derived an equation that includes an experimentally determined bacterial killing constant and the bacterial growth rate (20, 21). It predicts the CNC is constant at all B values. Their experiments show, however, that the bacterial growth rate and killing constant decrease, and the CNC increases (curved dashed line), at B values greater than 10^7 CFU/ml in stirred suspension and greater than 10^6 CFU/ml in fibrin gel (not shown). Most importantly, this equation predicts B will decrease or increase at all N values below or above the CNC, respectively, and remain constant at the CNC. **(B)** In contrast, as reported here by Malka et al., the bacterial population is bistable at N at or very near the CNC, creating favorable conditions for uncontrolled bacterial growth (25). N_1 and N_2 and the dashed line indicate the N and B values that define the zone of bistability in the bacterial population. For N below the CNC (solid yellow line), the bacterial population grows exponentially. Note also that in fibrin gels and rabbit dermis, the CNC at all N values is at least 2-fold larger than in stirred suspensions, a reflection of the effect of the former environment on the bacterial killing constant.

They discovered that neutrophil-bactericidal activity in dermis of living rabbits and in fibrin gels is controlled by N and is independent of bacterial concentration (B), up to approximately 10^6 CFU/ml (20). They derived an equation that describes bacterial killing as a function of N and bacterial growth rate (Figure 1A) and showed that it accurately described neutrophil-bactericidal activity in suspension (21), fibrin gels, and dermis of living rabbits (20). Additionally, they found the ratio of the experimentally determined bacterial growth rate (g) and neutrophil-bacterial killing rate constant (k) defined a new and extremely useful parameter: the critical neutrophil concentration (CNC), namely, the N required to hold B constant (Figure 1A and refs. 20, 21). At N values below the CNC, B increases; at N above the CNC, B decreases. The CNC in stirred suspensions is approximately 4×10^5 neutrophils/ml (21), very close to the clinically determined value of 5×10^5 neutrophils/ml that defines neutropenia and characterizes neutropenic patients in danger of infection (6–8). The CNC in fibrin gels and rabbit dermis is approximately 1×10^6 and 8×10^6 neutrophils/ml, respectively (20), 2- to 20-fold larger than in stirred suspensions. Apparently, evolution has taken note of the CNC, as the blood neutrophil concentration is no less than approximately 5×10^5 neutrophils/ml in all adult vertebrates (22–24).

What is the physical meaning of the CNC? The answer is evident from the value and units of k (ml/neutrophil/min), which for these purposes can be viewed as the volume of medium or tissue a neutrophil can explore per minute. For neutrophils in stirred suspension and in dermis of living rabbits, k is equivalent to 2×10^{-8} and 4×10^{-9} ml/neutrophil/min, respectively. In stirred suspension, 1 neutrophil

can explore approximately 20 pl medium/min, whereas in rabbit dermis, it can only explore approximately 4 pl dermis/min. Accordingly, 5×10^6 neutrophils/ml – the average concentration in human blood – is required for these cells to explore 1 ml of a stirred bacterial suspension in 10 minutes (i.e., 2×10^{-8} ml/neutrophil/min $\times 5 \times 10^6$ neutrophils $\times 10$ min = 1 ml). Likewise, N of 2.5×10^7 neutrophils/ml or neutrophils/g rabbit dermis is required for neutrophils to explore 1 milliliter or gram of dermis in 10 minutes. Higher concentrations of neutrophils or longer exploration times are required when B exceeds 10^7 per milliliter stirred suspension (21) or 10^6 per milliliter or gram of tissue (20).

Dynamic system model of neutrophil bactericidal activity: effects of bistability

The equation Malka et al. report here (25) and previously (26), namely $dB(t)/dt = \rho B(t)/[1 + \beta B(t)] + s - \delta B(t) - (\alpha NB(t)/[1 + \gamma B(t) + \eta N])$, more accurately describes the rate of bacterial clearance than does the one previously reported by Li et al. (20, 21). This is because Malka et al. included the saturation rate for bacterial growth (β) and the spontaneous rate of bacterial death [$\delta B(t)$]. The bacterial growth rate (ρ) behaves like a carrying capacity and saturates (βB) at high B values. The authors also included in their equation the rates of bacterial influx into the circulation or site of infection(s) and of spontaneous bacterial death. If these rates could be measured, their inclusion would add significantly to the predictive power of the equation; however, there are no established methods at present for measuring them. Hence, Malka et al. assign them a value of 0, thereby eliminating this difference between the two groups' equations.

A critical concentration of neutrophils is required to produce a net reduction in bacterial concentration

Since Robertson and Sia's 1924 report (16), most in vitro studies of neutrophil-bactericidal activity have used neutrophils stirred continuously for 15–120 minutes at 37°C in plasma- or serum-containing medium with known concentrations of bacteria. The neutrophils then are lysed, and the concentration of viable bacteria remaining is measured by bacteriological colony assay. This system mimics neutrophil-bactericidal activity in blood. Comparatively few investigators have studied neutrophil-bactericidal activity in tissues of living animals or in three-dimensional gels composed of extracellular matrix proteins. The findings that extracellular matrix proteins exert potent physiological effects on phagocytes (17, 18), and that the chemoattractant N -formyl-methionine-leucine-phenylalanine (fMLP) (19) inhibits neutrophil migration in fibrin-containing matrices, but not in collagen matrices, prompted Li et al. to examine neutrophil-bactericidal activity in tissues and tissue-like environments (20).



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The equation's quadratic term represents the killing rate ($-\alpha NB(t)/[1 + \gamma B(t) + \eta N]$). It involves the law of mass action: the neutrophil-bactericidal rate is proportional (α) to the product of N and B multiplied by the amount of time (t ; in minutes) the neutrophils interact with bacteria. It saturates at high concentrations of both neutrophils (ηN) and bacteria (γB). The system yields two very different outcomes depending on the magnitude of the initial parameters: it is monostable when N is well above or below the critical concentration, but becomes bistable when N fluctuates close to the CNC. Bistability is a characteristic of dynamic systems in which two stable coexisting fixed points exist for a range of parameters (Figure 1B), and the system's outcome depends on its initial conditions.

Unresolved questions

Although the equation derived by Malka et al. (25, 26) provides a more complete description of neutrophil-bactericidal activity than that of Li et al. (21), additional experiments are needed to assess its clinical utility. First, the conclusion that there is substantial patient-to-patient variability in neutrophil-bactericidal activity relies on studies of the staphylococidal activity of neutrophils from only four healthy adults (25). Li et al. observed an approximately 2-fold variation in values of k and CNC for neutrophils from more than 20 healthy adult donors killing *S. epidermidis* in stirred solutions (21), and variations in these parameters of similar magnitude for neutrophils killing *S. aureus*, *E. coli*, and *P. aeruginosa* in stirred suspensions in studies are reported by others. Variations in neutrophil-bactericidal activity of this magnitude near or at the CNC are certainly sufficient to produce bistability in the bacterial population. Nonetheless, additional measurements of the efficiency of killing of both Gram-positive and -negative bacteria by neutrophils, from a larger number of uninfected and infected eupenic and neutropenic donors than was tested by Malka et al., are needed to define precisely the frequency and magnitude of variation in bactericidal activity of neutrophils. Second, the authors measured neutrophil-bactericidal activity in stirred suspensions (25), a condition that mimics neutrophil-bactericidal activity in blood. However, neutrophil-bactericidal activity in tissues is of greatest relevance for neutropenic patients (8,

11–13). Thus, the method Malka et al. use might better predict the likelihood of infection in neutropenic patients if it employed fibrin gels to determine their neutrophils' bactericidal activity. Li et al. showed that the kinetics of neutrophil-bactericidal activity in these gels closely mimics that for neutrophils in tissues in vivo (20). Third, Malka et al. ascribed the variability they observed in killing of *S. aureus* to differences in bactericidal activity of neutrophils from different healthy adult donors (25). However, their studies used 10% autologous serum, close to the concentration at which opsonin concentration becomes limiting (21, 27); 20%–40% serum would have been a better choice (21). Moreover, Malka et al. did not control for variations in opsonin concentration in each leukocyte donor's serum. Thus, the variations in killing efficiencies observed could reflect variations in serum opsonin concentrations (27, 28), not in neutrophil bactericidal activity. From both mechanistic and practical standpoints, this is an important matter to resolve.

Clinical experience now suggests that the use of G-CSF to prevent bone marrow-depressive effects of chemotherapy in patients at high risk for neutropenia is far more efficacious than using it to treat neutropenia once it has occurred (8, 29). Accordingly, it may be that the most useful clinical application of this new equation will be to use it prospectively in conjunction with other predictive tools (29), including low-tech methods such as the one devised by Wright et al. (12), to assess the likelihood that chemotherapy will cause the tissue bactericidal activity of a patient's neutrophils to fall below the CNC and/or into regions of bacterial bistability.

These concerns notwithstanding, we believe the findings that neutrophil-bactericidal activity can be described by equations very similar to those describing enzyme-substrate interactions provide an entirely new and quantitative way of viewing cellular defense against microbial pathogens. Budhu et al. reported that the equation described by Li et al. (21) also describes antigen-specific CD8⁺ T cell killing of cognate antigen-expressing melanoma cells in vitro and in living mice (30), which suggests that the same physical principles that govern neutrophil-bacterial interactions also govern the interactions of cytolytic lymphocytes with eukaryotic target cells. Indeed, it seems likely that these principles govern

the effector activities of all immune cells. Consistent with this idea, Babbs (31) and Lejune et al. (32) report bistability of tumor cell growth under conditions in which the intratumoral concentration of tumor antigen-specific CD8⁺ T cells fluctuates around the critical CD8⁺ T cell concentration.

Conclusions

Malka et al. have derived a dynamic system model (25, 26) that describes neutrophil-bactericidal activity in stirred solutions more accurately than did its linear predecessor (20, 21). Although further work is needed to determine whether the variability in bactericidal activity described for neutrophils from normal adults applies to neutrophils from neutropenic and/or cytotoxic drug-treated patients, this model may enable clinicians to better distinguish patients who are at risk of infection from those who are not, thereby reducing the morbidity, mortality, and expense associated with unnecessary hospitalizations and/or administration of antibiotics and/or G-CSF.

Acknowledgments

This work was supported by NIH grant AI-20516-026 to S.C. Silverstein and by grants U54 CA121852-05 from the NIH and 1R01LM010140-01 from the National Library of Medicine to R. Rabadan. The authors note that two previous reviews of neutropenia have used the approximate title, "How many neutrophils are enough?" (11, 33); hence their use of "redux, redux" in this title.

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