

Determination of the Critical Concentration of Neutrophils Required to Block Bacterial Growth in Tissues

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Abstract

We showed previously that the competition between bacterial killing by neutrophils and bacterial growth in stirred serum-containing suspensions could be modeled as the competition between a first-order reaction (bacterial growth) and a second-order reaction (bacterial killing by neutrophils). The model provided a useful parameter, the critical neutrophil concentration (CNC), below which bacterial concentration increased and above which it decreased, independent of the initial bacterial concentration. We report here that this model applies to neutrophil killing of bacteria in three-dimensional fibrin matrices and in rabbit dermis. We measured killing of 10^3 – 10^8 colony forming units/ml *Staphylococcus epidermidis* by 10^5 – 10^8 human neutrophils/ml in fibrin gels. The CNC was $\sim 4 \times 10^6$ neutrophils/ml gel in the presence of normal serum and $\sim 1.6 \times 10^7$ neutrophils/ml gel in the presence of C5-deficient serum. Application of our model to published data of others on killing of $\sim 5 \times 10^7$ to 2×10^8 *E. coli*/ml rabbit dermis yielded CNCs from $\sim 4 \times 10^6$ to $\sim 8 \times 10^6$ neutrophils/ml dermis. Thus, in disparate tissues and tissuelike environments, our model fits the kinetics of bacterial killing and gives similar lower limits (CNCs) to the neutrophil concentration required to control bacterial growth.

Key words: rabbit dermis • bactericidal activity • *Staphylococcus epidermidis* • fibrin gels • critical neutrophil concentration

Introduction

Neutrophils play an essential role in defending vertebrates against bacterial infections. Leijh et al. (1) and Hammer et al. (2) reported that in stirred suspensions, which are thought to mimic the environment in blood, the neutrophil/bacteria ratio determines the efficiency of neutrophil bactericidal activity. However, the bacterial concentration in blood of bacteremic hosts is rarely >100 CFU/ml (3). Under this circumstance, the blood of a neutropenic ($\sim 10^5$ – 5×10^5 neutrophils/ml), bacteremic (~ 100 CFU bacteria/ml) host will have 1,000–5,000 neutrophils per bacterium. Because each neutrophil in suspension can kill >20 bacteria (4), there is a large apparent excess in neutrophil capacity to kill bacteria. Yet, in neutropenic hosts, bacteria survive and grow in blood.

Previously, we reported that neutrophil bactericidal activity in stirred serum-containing suspensions, a condition that mimics the blood, is dependent on the neutrophil concentration and is independent of the ratio of neutrophils to

bacteria (5). We showed that the time course of the bacterial concentration in such suspensions could be modeled as the competition between a first-order reaction (bacterial growth) and a second-order reaction (neutrophil killing of bacteria). The resulting kinetic equation fit the time-course of bacterial concentration over wide ranges of initial concentrations of bacteria and neutrophils. The equation is as follows: $b = b_0 e^{-(kp - g)t}$ (Eq. 1), in which b is the bacterial concentration at any time t , b_0 is the initial bacterial concentration, p is the neutrophil concentration, k is the second-order rate constant for neutrophil killing of bacteria, and g is the first-order rate constant for bacterial growth. Furthermore, the model provided a useful parameter, the critical neutrophil concentration (CNC), where $\text{CNC} = g/k$ (Eq. 2).

When $p = \text{CNC}$, the bacteria grow and are killed at the same rates, and the bacterial concentration, b , is in a steady state. We demonstrated that at neutrophil concentrations below the CNC, the bacterial concentration increased with time, whereas at neutrophil concentrations above the

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Abbreviations used in this paper: CNC, critical neutrophil concentration; NEE, neutrophil extraction efficiency; NS, normal human serum.

CNC, the bacterial concentration decreased with time. Moreover, the killing rate constant, k , the bacterial growth constant, g , and the CNC were independent of the initial bacterial concentration between 10^3 – 10^8 CFU/ml. For example, in stirred suspensions containing varying concentrations of human neutrophils, 10^3 – 10^7 CFU/ml *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa*, and 10–40% vol/vol normal human serum (NS), the CNC was $\sim 400,000$ neutrophils/ml, a value close to the empirically determined blood neutrophil concentration of $\sim 500,000$ /ml that is known to predispose neutropenic patients to potentially fatal bacteremia.

Treating neutrophil killing of bacteria as a consequence of the collision of two particles, the bacterium and the neutrophil, made sense under the conditions of stirred suspensions (5), but we asked whether this abstraction applied to more complex conditions in which neutrophils search for and kill bacteria in unstirred tissue-like environments and tissues. Comparatively little is known about neutrophil bactericidal mechanisms in tissues. Specifically, there are no analyses of the relationships between neutrophil concentration and neutrophil bactericidal activity in tissues, and the question of a CNC in tissues has not been considered. In the studies reported here, we used our kinetic model (5) to analyze neutrophil killing of *S. epidermidis* in fibrin gels in vitro and to reanalyze previously reported data on neutrophil killing of *E. coli* in rabbit dermis in vivo. We report that our model applies to the killing of bacteria by neutrophils in both situations and conclude that neutrophil concentration is one of the critical determinants of neutrophil bactericidal activity in tissue as well as in blood.

Materials and Methods

Bacteria, Sera, and Other Materials. *S. epidermidis* H753 was obtained, cultured, and assayed as described previously (6). Plasma-derived NS was prepared from AB plasma, and C5-deficient, plasma-derived serum was prepared from C5-deficient human plasma (a gift from J. Leddy, University of Rochester, Rochester, NY) was prepared as described previously (6). Sera, except for that used in experiments shown in Fig. 1, were dialyzed at 4°C versus Dulbecco's PBS with calcium, magnesium, and 0.1% glucose (wt/vol) before use. Purified human C5 and pertussis toxin were obtained from Calbiochem. A concentration of $\geq 40\%$ (vol/vol) NS was required for optimal opsonization of *S. epidermidis* (5). To conserve C5-deficient serum, we used this reagent at a concentration of 10% (vol/vol). Neutrophil killing efficiency in stirred suspensions and in fibrin gels containing 10% NS or C5-deficient serum was approximately 70% of that observed in suspensions and gels containing 40% NS (6).

Fibrin Gels. Fibrin gels were formed using 1 mg/ml fibrinogen and contained 10 or 40% NS or C5-deficient serum as indicated. Lymph draining normal human or rabbit skin contains complement, IgG, fibrinogen, and other plasma proteins at concentrations ~ 10 – 25% of those in normal plasma (7, 8), and clots when it is incubated with thrombin (8). Thus, the gels used in these experiments contain fibrin and other plasma proteins at concentrations similar to those in normal interstitial fluid.

Neutrophil Viability and Intercellular Distances. Fibrin gels, 60–80 μm in thickness, were formed by placing 10 μl buffer contain-

ing 1 mg/ml fibrinogen, 40% NS, 1 U/ml thrombin (Sigma-Aldrich), 6 μM Syto-13 (Molecular Probes), and the indicated number of neutrophils on a 12-well multispot microscope slide (Shandon Inc.). Z-series images of 20 μm -thick optical sections (optimal for resolving the relative locations of adjacent neutrophils in all directions) were captured at 20 μm intervals by confocal fluorescence microscopy using a $25\times$ oil-immersion objective. Distances between a randomly chosen neutrophil and the five or six nearest neutrophils in the same or adjacent optical section were determined using LSM 5 Image Browser (Carl Zeiss MicroImaging, Inc.).

Neutrophil Killing of *S. epidermidis* in Fibrin Gels. Fibrin gels (100 μl in volume) containing 1 mg/ml of purified human fibrinogen (American Diagnostica, Inc.), human neutrophils, *S. epidermidis*, C5-deficient serum, or NS (10–40% vol/vol) were incubated at 37°C and lysed, and their content of viable bacteria was assayed by pour-plate method as described previously (6). Control experiments showed that $>99\%$ of viable *S. epidermidis* were recovered from fibrin gels immediately after their formation, even when they contained $>10^8$ neutrophils (6), and that $>98\%$ of neutrophils were viable (determined by exclusion of propidium iodide [Molecular Probes]), after a 90-min incubation in fibrin gels containing 10^6 or 10^8 CFU/ml *S. epidermidis*.

Calculation of k , g , and CNC for Neutrophil Killing of *S. epidermidis* in Fibrin Gels. Fibrin gels containing 10^5 – 10^7 human neutrophils/ml (p) and the indicated concentrations of serum and *S. epidermidis* (b_0), were incubated for 90 min (t) and assayed for the number of viable bacteria remaining (b) as described before. k and g were obtained by least-squares fit of Eq. 1 to b at $t = 90$ min as a function of p . The values of k and g for the rate of killing of *S. epidermidis* over time also were determined at different neutrophil concentrations as described before. To fit these data, Eq. 1 was rewritten as follows: $b = b_0 e^{-k't}$ (Eq. 3), where $k' = kp - g$ (Eq. 4).

At each neutrophil concentration, k' was obtained by the fit of Eq. 3 to the time-dependent data, and k and g were obtained by a fit of Eq. 4 to k' versus p . The CNC was calculated from Eq. 2.

The quotient of b_{90}/b_0 is the factor relating the bacterial concentration after 90 min to the initial bacterial concentration. For $p \geq \text{CNC}$, $1 - b_{90}/b_0$ is the fractional decrease in bacterial concentration, and ranges from 0 to 1. However, for $p < \text{CNC}$, $b_{90}/b_0 > 1$ and $1 - b_{90}/b_0$ is negative. To have a convenient measure of the extent of bacterial killing at all neutrophil concentrations, independent of bacterial growth, we compared the bacterial concentration after a 90-min incubation in the presence of neutrophils, b_{90} , to the bacterial concentration after 90 min in the absence of neutrophils, $b_{90, p=0}$. We calculated a new parameter, κ_{90} , as $\kappa_{90} = 1 - (b_{90}/b_{90, p=0})$ (Eq. 5). From Eq. 1, κ_{90} also is given by $\kappa_{90} = 1 - e^{-90kp}$ (Eq. 6).

Thus, the meaning of κ_{90} is the fraction of bacteria initially present that would be killed by neutrophils at concentration p in the absence of bacterial growth (i.e., if $g = 0$). At all neutrophil concentrations (p), κ_{90} is a positive fraction.

Calculation of k , g , and CNC for *E. coli*-infected Rabbit Dermis. Movat et al. (9, 10) inoculated *E. coli* intradermally into normal and neutropenic rabbits and measured blood flow, neutrophil influx, and *E. coli* growth/killing in a 1.5 cm-diameter, full-thickness biopsy of skin centered on the inoculation site. They reported that all bacteria and inflammatory cells were contained in this 1.5 cm-diameter biopsy, and were restricted to its 0.2 cm-thick layer of dermal collagen. Thus, the volume of dermis in which the *E. coli* and neutrophils were contained was $\sim 0.35 \text{ cm}^3$ (i.e., $\pi \times 0.2 \text{ cm} \times 0.75 \text{ cm}^2$), and the concentrations of *E. coli* and neutrophils per ml of dermis were calculated as CFU of *E. coli* per biopsy/0.35 ml and number of neutrophils per biopsy/0.35 ml.

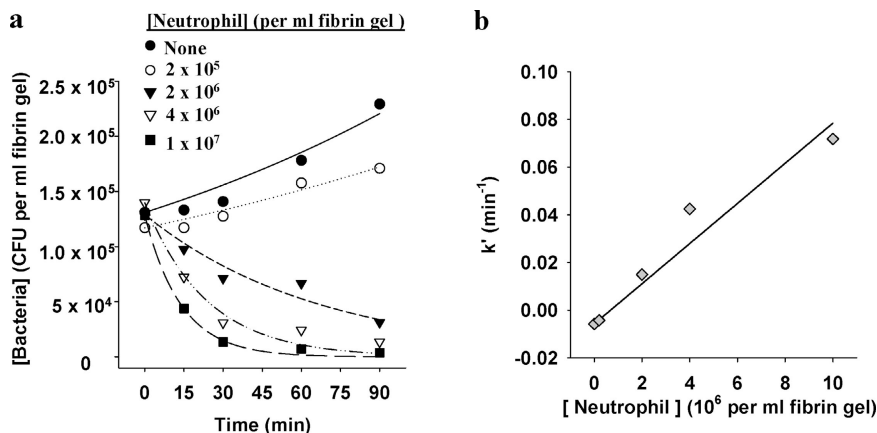


Figure 1. Effect of various concentrations of neutrophils on the time course of bacterial concentration in fibrin gels. (a) Fibrin gels containing 10^5 CFU/ml *S. epidermidis*, 40% NS (dialyzed vs. PBS without glucose), with or without human neutrophils were incubated at 37°C . Symbols represent the concentrations of viable bacteria (b_t) recovered from individual fibrin gels at the indicated times of incubation. The curves represent the fits of Eq. 3 to the data at each concentration of neutrophils ($R^2 = 0.93 - 0.99$). The fit yielded k' (Eq. 4). (b) Plot of k' versus p . A least-squares fit of Eq. 4 to these data yielded the values of k and g reported in the text. The data shown in a and b are from one experiment representative of two, each performed in duplicate. The mean \pm SEM of k and g from both experiments were as follows: $k = 1.03 \pm 0.19 \times 10^{-8}$ ml/neutrophils/min and $g = 5.38 \pm 0.4 \times 10^{-3}$ /min.

Movat et al. (9, 10) measured the number of neutrophils that entered each dermal site each hour after *E. coli* inoculation. We summed these hourly values to obtain the total neutrophil concentration (p_t) at the end of each hour after *E. coli* inoculation (see Figs. 6 a and b and Table III), and calculated the average neutrophil concentration/ml dermis for each 60-min interval after *E. coli* inoculation [e.g., $(\{p_t\} + \{p_{t+60\text{min}}\})/2$].

The rate constant for *E. coli* growth in rabbit dermis (g) of 0.017/min was calculated using Eq. 1 with $p = 0$, $b_0 = 5.7 \times 10^7$ CFU/ml dermis (9, 10), and $b_t = 1.2 \times 10^8$ CFU *E. coli*/ml dermis 1 h after *E. coli* inoculation of rabbits rendered neutropenic ($<5 \times 10^5$ neutrophils/ml blood) by cyclophosphamide treatment.

Because dermal neutrophil concentration varied over time, we calculated k for every 60-min interval using a modified form of Eq. 1, $b_{t+60} = b_t e^{(-kp + g)60}$ (Eq. 7), where b_t and b_{t+60} are the dermal concentrations of *E. coli* at time t and $t + 60$ min, p is the average dermal neutrophil concentration in a 60-min interval $[(p_t) + (p_{t+60})]/2$, and $g = 0.017$. CNCs were calculated using k , g , and Eq. 2. The values of k and CNC for rabbit dermis are summarized in Table III.

Neutrophil Extraction Efficiency (NEE). As described by Doerschuk et al. for *S. pneumoniae*-infected rabbit lung (11), NEE is the ratio of the number of neutrophils accumulated per milliliter tissue/unit time to the average number of neutrophils in the nonmargined neutrophil pool perfusing the same milliliter of tissue in that time. Blood neutrophil concentration and blood flow in 1 ml of uninfected rabbit dermis were 2.5×10^6 neutrophils/ml blood (10) and 3.6 ml of blood/h (12), respectively, and changed as reported (10 and 16) at various times after *E. coli* inoculation. The total number of neutrophils perfusing each *E. coli*-inoculated site per hour was estimated to be the product of the average blood neutrophil concentration during the preceding hour \times basal blood flow per ml dermis per hour (i.e., 3.6 ml/h) \times the average change in blood flow to the site during the preceding hour.

Results

Time Course of Neutrophil Killing of *S. epidermidis* in Fibrin Gels. Fibrin gels containing serum, 1.3×10^5 CFU *S. epidermidis*/ml gel, and neutrophils at concentrations ranging from 2×10^5 to 10^7 /ml gel, were incubated for 15, 30, 60, and 90 min at 37°C . The gels were lysed and their con-

tent of viable *S. epidermidis* was assayed. As shown in Fig. 1 a, the rate and extent of bacterial killing varied with neutrophil concentration.

In previous studies of neutrophil killing of bacteria in stirred suspensions, we modeled neutrophil bactericidal activity as a competition between neutrophil killing of bacteria and bacterial growth. Modeling the former as an analogue of a bimolecular reaction and the latter as an analogue of a unimolecular reaction yielded Eq. 1, which closely fitted findings for neutrophil killing of bacteria in stirred suspensions (5). In the present work, we have examined whether the same model also describes neutrophil bactericidal activity in fibrin gels. Because the kinetic model leading to Eq. 1 assumes a uniform distribution of a constant number of viable neutrophils throughout the course of an experiment, we confirmed experimentally that neutrophils were distributed uniformly in fibrin gels (Fig. 2), and were viable (not depicted) throughout the 90-min course of experiments. We also confirmed that $>99\%$ of bacteria embedded in fibrin gels with or without neutrophils were recovered from these gels at zero time (6).

To determine whether Eq. 1 describes neutrophil killing of *S. epidermidis* in fibrin gels at time periods varying from 15 to 90 min, we rewrote Eq. 1 as Eq. 3 (Materials and Methods), obtained k' , and fitted Eq. 4 to k' to obtain k and g . The data in Fig. 1 a are fit well by Eq. 3 ($R^2 > 0.98$),

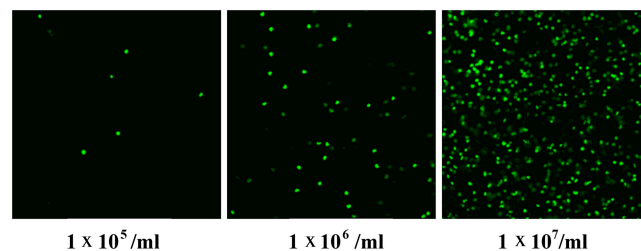


Figure 2. Distribution of neutrophils in fibrin gels. Confocal fluorescence micrographs of fibrin gels containing Syto-13-stained neutrophils at the indicated concentrations.

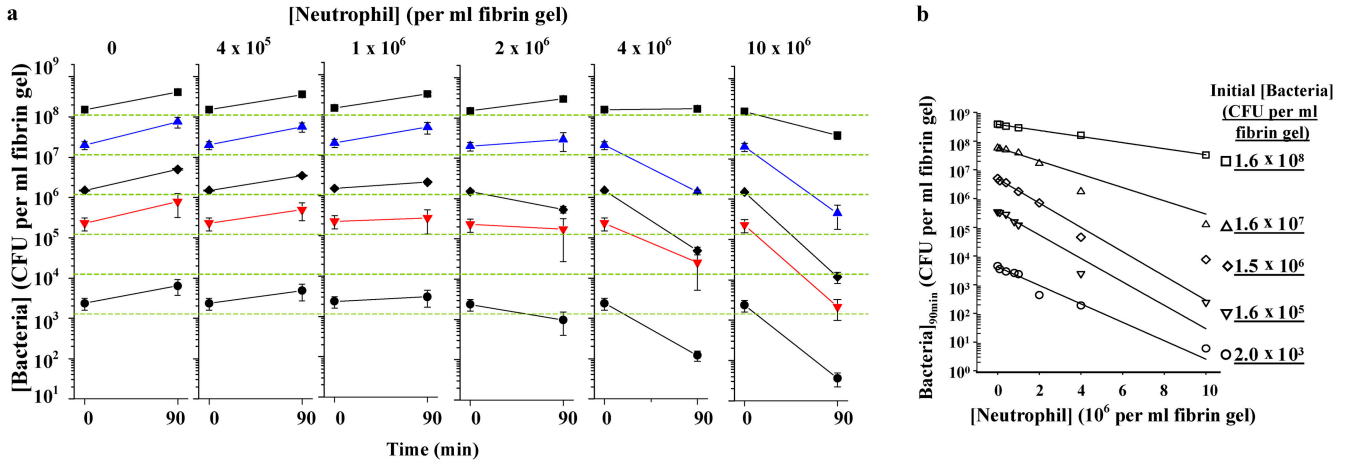


Figure 3. Neutrophil bactericidal efficiency in fibrin gels depends on neutrophil concentration and on the initial concentration of *S. epidermidis*. (a) CFU of *S. epidermidis* recovered from fibrin gels containing 40% NS and the indicated concentrations of these bacteria and human neutrophils at time zero (b_0) and after a 90-min incubation at 37°C. Each data point represents the mean \pm SEM of five independent experiments, each performed in duplicate. (b) b_{90} at each initial bacterial concentration from panel a plotted as a function of neutrophil concentration, p. Symbols are the mean values from one out of four independent experiments. The fit of Eq. 1 to these data with $t = 90$ yielded the values of k and g as reported in Table I.

yielding k' as a function of neutrophil concentration (p ; Fig. 1 b). The fit of Eq. 4 to these data yielded $k = 1.03 \pm 0.19 \times 10^{-8}$ ml/neutrophil/min and $g = 0.005 \text{ min}^{-1}$. We derive two conclusions from these experiments. First, that Eq. 1 describes neutrophil killing of 10^5 CFU/ml *S. epidermidis* in fibrin gels. Second, that the rate constant for neutrophil killing of *S. epidermidis* in fibrin gels (i.e., $\sim 10^{-8}$ neutrophils/ml) is 40% of that reported previously (i.e., $\sim 2.5 \times 10^{-8}$ neutrophils/ml) for stirred suspensions (5).

The experiments reported in Fig. 1 a show that the time course of killing of a single concentration of *S. epidermidis* in fibrin gels depended on neutrophil concentration. To determine whether neutrophil concentration determines the efficiency of killing of *S. epidermidis* in fibrin gels over a much broader range of bacterial concentrations, we incubated fibrin gels containing *S. epidermidis*, at concentrations ranging from 10^3 to 10^8 CFU/ml, and neutrophils, at concentrations ranging from 10^5 to 10^7 neutrophils/ml, for 90 min at 37°C. The gels were lysed, and their content of via-

ble *S. epidermidis* was assayed. As shown in Fig. 3 a, the number of bacteria remaining viable at 90 min compared with the initial bacterial inoculum depended primarily on the concentration of neutrophils. At 4×10^6 neutrophils/ml, fewer viable bacteria were recovered after 90 min than were present in the inoculum, even when there were 10^8 CFU *S. epidermidis*/ml gel, and the ratio of neutrophils/bacteria was 1:25 (Fig. 3 a). Conversely, at 4×10^5 neutrophils/ml, more viable bacteria were recovered after 90 min than were present in the inoculum, even when there were only 10^3 CFU *S. epidermidis*/ml gel, and the ratio of neutrophils/bacteria was 400:1 (Fig. 3 a). These experiments confirmed that it is the neutrophil concentration, not the ratio of neutrophils to bacteria, that determines whether the bacterial concentration increases or decreases.

Nonlinear regression analysis of the data in Fig. 3 a using Eq. 1 yielded lines closely fitting the experimentally determined results (Fig. 3 b). The slope of each line yielded k . k was $\sim 10^{-8}$ ml/neutrophils/min for *S. epidermidis* inocula

Table I. Rate Constants k , g , and CNC for *S. epidermidis* in Fibrin Gels

b_0	k	g	CNC = g/k
CFU/ml	ml/neutrophil/min	min^{-1}	neutrophils/ml
10^3	$9.6 \pm 0.7 \times 10^{-9}$	$1.0 \pm 0.1 \times 10^{-2}$	$1.1 \pm 0.1 \times 10^6$
10^5	$10.5 \pm 0.03 \times 10^{-9}$	$1.2 \pm 0.2 \times 10^{-2}$	$1.2 \pm 0.2 \times 10^6$
10^6	$9.9 \pm 0.5 \times 10^{-9}$	$1.3 \pm 0.04 \times 10^{-2}$	$1.3 \pm 0.07 \times 10^6$
10^7	$6.6 \pm 0.3 \times 10^{-9}$	$1.4 \pm 0.1 \times 10^{-2}$	$2.2 \pm 0.2 \times 10^6$
10^8	$2.7 \pm 0.1 \times 10^{-9}$	$1.1 \pm 0.1 \times 10^{-2}$	$4.2 \pm 0.4 \times 10^6$

Neutrophils and *S. epidermidis* were incubated in fibrin gels containing 40% normal serum as described in Materials and Methods. The means and SEM for k , g , and CNC are shown, which were determined from four independent experiments, each performed in duplicate with the indicated b_0 .

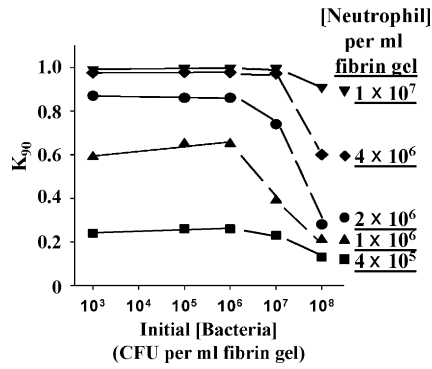


Figure 4. The fraction of bacteria that would be killed in 90 min in the absence of bacterial growth. κ_{90} , the fraction of *S. epidermidis* killed by the indicated concentration of neutrophils, as a function of initial bacterial concentration, was calculated using Eq. 5, as described in Materials and Methods. Data are from Fig. 3 a.

$\leq 10^6$ CFU/ml, approximately the same value determined in studies of the time course of neutrophil killing of *S. epidermidis* in fibrin gels (Fig. 1, a and b). However, for *S. epidermidis* inocula of 10^7 and 10^8 CFU/ml, k was 6.6×10^{-9} and 2.7×10^{-9} ml/neutrophils/min, respectively (Table I), values that are 1.5-fold and 3.7-fold smaller, respectively, than those observed for bacterial concentrations $\leq 10^6$ CFU/ml. Elaboration on the constancy of k at bacterial concentrations $> 10^6$ CFU/ml will continue in the next section (see Discussion). For the moment, it is noteworthy that we observed a similar departure from constancy of k at bacterial concentrations $> 10^7$ CFU/ml in our studies of neutrophil bactericidal activity in stirred suspensions (5).

The Fraction of Bacteria Killed by Neutrophils. To examine the effect of bacterial concentration on k uncomplicated by bacterial growth, we determined κ_{90} (Fig. 4). κ_{90} depends formally only on k and t (Eq. 6), and should not depend on b_0 , the initial bacterial concentration. κ_{90} is constant at b_0 up to 10^6 CFU/ml (Fig. 4). However, κ_{90} drops off at bacterial concentrations $> 10^6$ CFU/ml (Fig. 4), as noted before and as reflected in decreased values of k in Table I. This is discussed in the second paragraph of the Discussion.

Phagocytosis Is Required for Neutrophil Killing of *S. epidermidis* in Fibrin Gels. In stirred suspensions, neutrophils must phagocytose bacteria to kill them (13). Killing is not a result of secretion of bactericidal agents. Two lines of evidence indicate this also applies to fibrin gels. First, cytochalasin D blocks neutrophil movement and phagocytosis, and facilitates neutrophil secretion of bacteriostatic and bactericidal substances (14, 15). Indeed, we have found that cytochalasin D blocked neutrophil phagocytosis of *S. epidermidis* in fibrin gels by $> 95\%$ (as measured by transmission electron microscopy) and killing of *S. epidermidis* at all concentrations tested ($10^3 - 2 \times 10^8$ CFU/ml; unpublished data). Second, there was no change in the rate constant k for bacterial killing by neutrophils from 2×10^5 to 10^7 neutrophils/ml; i.e., the slope of the line in Fig. 1 b is constant. If bacteriostatic or bactericidal products secreted by neutrophils (e.g., peptides, proteases, and reactive oxygen species) killed significant

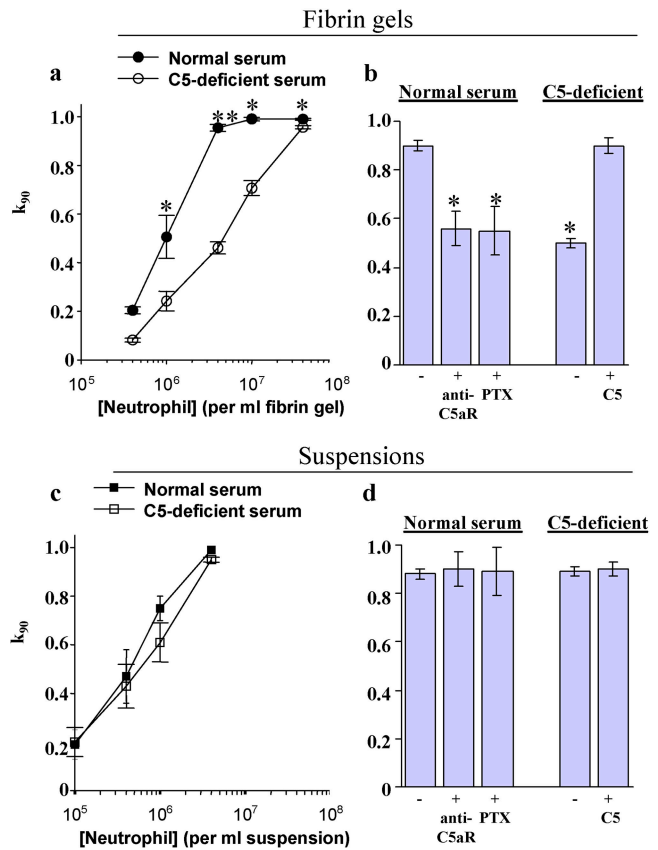


Figure 5. Effects of C5-deficient serum, anti-C5a receptor IgG, or pertussis toxin on killing of *S. epidermidis* by neutrophils in fibrin gels and in stirred suspensions. (a and c) Fraction of *S. epidermidis* killed (κ_{90} ; see Eq. 5), in 90 min at 37°C in fibrin gels (a) and in stirred suspensions (c) as a function of neutrophil concentration. The initial *S. epidermidis* concentration was 10^5 CFU/ml, and the medium contained 10% (vol/vol) normal or C5-deficient serum, as indicated. (b and d) Fraction of *S. epidermidis* killed (κ_{90}) in fibrin gels (b) or in suspensions (d) containing 4×10^6 neutrophils/ml and initially 10^5 CFU *S. epidermidis*/ml. The medium contained 10% (vol/vol) normal or C5-deficient serum and, where indicated, $7.5 \mu\text{g/ml}$ human C5, $10 \mu\text{g/ml}$ rabbit anti-C5a receptor IgG, or $1 \mu\text{g/ml}$ pertussis toxin. Data are means \pm SEM of three independent experiments, each performed in duplicate. In a, differences in means of κ_{90} in normal serum versus C5-deficient serum were significant by Student's t test at $P < 0.05$ (*) or at $P < 0.01$ (**). In b, differences in means of κ_{90} in normal serum versus normal serum plus anti-C5a receptor IgG or pertussis toxin, and in means of κ_{90} of C5-deficient serum versus C5-deficient serum plus C5 were significant at $P < 0.05$ (*).

numbers of extracellular bacteria, the rate constant for killing would have increased as the neutrophil concentration increased. It did not. Therefore, we conclude that bacteria had to be engulfed by the neutrophils to be killed by them.

Determination of the CNC Required to Control *S. epidermidis* Growth in Fibrin Gels. The CNC, the neutrophil concentration at which the rate of bacterial growth is matched by the rate of bacterial killing, is given by g/k . The CNC required to block growth of *S. epidermidis* inocula of $\leq 10^6$ CFU/ml in fibrin gels containing 40% NS was 10^6 neutrophils/ml (Table I). The CNCs required to block growth of *S. epidermidis* inocula of 10^7 and 10^8 CFU/ml gel were 2×10^6 and 4×10^6 neutrophils/ml, respectively (Table I).

Table II. Effect of C5-deficient Serum on k and CNC for Neutrophils and *S. epidermidis* in Fibrin Gels

Serum	k ml/neutrophil/min	g min ⁻¹	CNC = g/k neutrophils/ml
Normal	$0.63 \pm 0.02 \times 10^{-8a}$	$0.4 \pm 0.1 \times 10^{-2}$	$0.66 \pm 0.3 \times 10^6$
C5 deficient	$0.17 \pm 0.01 \times 10^{-8}$	$0.3 \pm 0.04 \times 10^{-2}$	$1.94 \pm 0.2 \times 10^6$

Fibrin gels containing *S. epidermidis* (10^5 CFU/ml), neutrophils (10^5 – 10^7 /ml), and 10% (vol/vol) normal or C5-deficient human serum were incubated for 90 min at 37°C. The number of viable bacteria at time zero and at 90 min, the rate constants k , g , and the CNC were determined as described in Materials and Methods. The means \pm SEM for k , g , and CNC from three independent experiments are shown, each carried out in duplicate. R^2 for each experiment was >0.98 . For normal serum, the k , g , and CNC differed from those reported for 10^3 – 10^6 CFU/ml *S. epidermidis* in Table I because of the use of 10 versus 40% serum. Statistical significance was determined by paired Student's t test. k was compared in NS versus C5-deficient human serum.

^a $P < 0.005$.

Substances That Affect Neutrophil Migration in Fibrin Gels Affect k and the CNC. Previous studies (6) showed that neutrophils must migrate to kill *S. epidermidis* in fibrin gels and that neutrophils migrate at a rate of ~ 4 $\mu\text{m}/\text{min}$ in gels containing C5-replete serum and at ~ 0.6 $\mu\text{m}/\text{min}$ in gels containing C5-deficient serum. C5 serves as a substrate for the convertases that generate C5a. C5a stimulates neutrophil migration by binding to $\text{G}\alpha_i$ -coupled heptahelical C5a receptors. This was confirmed in control experiments that showed that neutrophils treated with pertussis toxin or with anti-C5a receptor IgG migrated through fibrin gels containing C5-replete serum at rates comparable to those of untreated neutrophils through fibrin gels containing C5-deficient serum (i.e., ~ 0.6 $\mu\text{m}/\text{min}$; unpublished data). We made use of these conditions to determine whether they affect neutrophil bactericidal activity as reflected in the values of k and the CNC. Indeed, neutrophils killed *S. epidermidis* less efficiently in gels containing C5-deficient serum than in gels containing normal serum at every neutrophil concentration examined from 4×10^5 to 4×10^7 /ml (Fig. 5 a). k was $6.3 \pm 0.02 \times 10^7$ ml/neutrophils/min in 10% normal serum, and fourfold less, 1.7 ± 0.010^7 ml/neutrophils/min, in 10% C5-deficient serum ($P < 0.005$; Table II). An approximately fourfold decrease in k implies an approximately fourfold increase in CNC in C5-deficient versus normal serum at these neutrophil concentrations (Table II).

Control experiments showed that neutrophils incubated with *S. epidermidis* in fibrin gels containing C5-deficient serum supplemented with purified C5 killed these bacteria as efficiently as neutrophils in C5-replete serum (Fig. 5 b). Conversely, neutrophils treated with pertussis toxin or anti-C5a receptor IgG and incubated with *S. epidermidis* in fibrin gels containing normal serum killed these bacteria as inefficiently as untreated neutrophils incubated with *S. epidermidis* in gels containing C5-deficient serum (Fig. 5 b). Additional control experiments showed that in contrast with neutrophils in fibrin gels, neutrophils in stirred suspensions killed *S. epidermidis* with equal efficiency regardless of whether they had been treated with pertussis toxin or anti-C5a receptor IgG (Fig. 5, c and d) and/or incu-

bated in C5-replete, C5-deficient serum (Fig. 5 c), or in normal serum containing fMLP or LTB4 (6). These experiments show that C5-deficient serum contains sufficient IgG anti-*S. epidermidis* and complement to fully opsonize these bacteria for phagocytosis and killing by neutrophils, and that neither stimulation of neutrophils by chemoattractants (including C5a) nor $\text{G}\alpha_i$ activity is required for optimal killing of *S. epidermidis*. Together, they provide strong evidence that the reduction in killing efficiency observed in C5-deficient serum results from a decrease in the efficiency with which neutrophils contact bacteria in fibrin gels.

These experiments demonstrate that, as reported for neutrophils and bacteria in stirred suspensions (5), the rate of killing of bacteria by neutrophils in the unstirred tissue-like environment of fibrin gels is proportional to neutrophil concentration. However, in unstirred, tissue-like environments, the proportionality constant k depends on factors that promote the locomotion of the neutrophils to the bacteria.

*Determination of k and the CNC Required for Control of *E. coli* Growth in Rabbit Dermis In Vivo.* Movat and et al. (9) studied the number of neutrophils that migrated into the dermis of normal and neutropenic rabbits at hourly intervals after intradermal inoculation of *E. coli*, and the number of CFU of *E. coli* remaining viable at these sites. To determine whether Eq. 1 and the CNC concept are applicable in vivo, we converted data obtained by Movat et al. (9, 10, 16) to concentrations of neutrophils and *E. coli* per ml dermis (Fig. 6, a and b), fitted Eq. 1 to these data to obtain k , and used k to calculate the CNC required to control *E. coli* growth in vivo.

In normal rabbits, the *E. coli* concentration increased from 5.7×10^7 CFU/ml dermis at the time of inoculation to 1.1×10^8 CFU/ml dermis at 1 h, was $\sim 1.1 \times 10^8$ CFU/ml dermis at 2 h, and decreased to 5×10^6 CFU/ml dermis over the ensuing 6 h (Fig. 6 a). The neutrophil concentration at these sites increased from an undetectable level before *E. coli* inoculation to 2.3×10^6 and 12×10^6 CFU/ml dermis 1 and 2 h after *E. coli* inoculation, respectively, and continued to increase at a decreasing rate for 6 h thereafter (Fig. 6 b). In contrast, in neutropenic rabbits

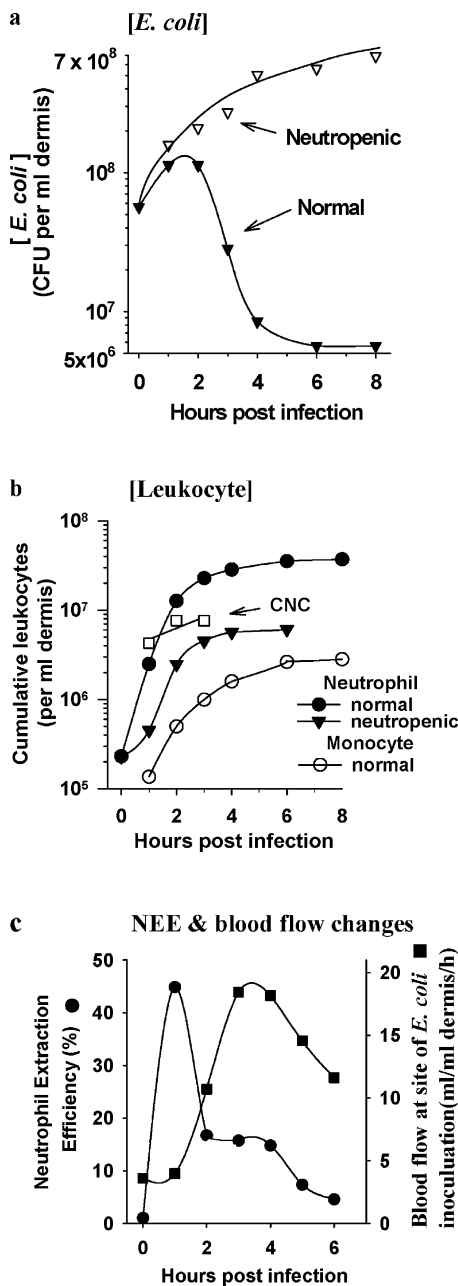


Figure 6. Neutrophil and *E. coli* concentrations, and blood flow per milliliter *E. coli*-inoculated rabbit dermis. Concentrations of *E. coli*/ml dermis (a) and of neutrophils/ml dermis (b) of normal and neutropenic rabbits were calculated using previously described data (reference 9). Concentrations of monocytes/ml *E. coli*-inoculated dermis of normal rabbits (b) were calculated previously (reference 20). (c) Effect of inoculation of *E. coli* into the dermis of normal rabbits on blood flow/ml dermis (replotted from reference 16) and on NEE were calculated as described in Materials and Methods.

($\leq 5 \times 10^5$ neutrophils/ml blood; reference 9), *E. coli* grew from 5.7×10^7 CFU/ml dermis at the time of inoculation to 1.2×10^8 CFU/ml dermis at 1 h (Fig. 6 a), and increased continuously over the ensuing 7 h, albeit at a slower rate than during the first hour (Fig. 6 a). Kopaniak et al. (16) reported that almost no neutrophils migrated into

the dermis of neutropenic rabbits in the first hour after *E. coli* inoculation. Therefore, we used *E. coli* growth in the first hour to calculate the bacterial growth rate constant (g) in rabbit dermis. g was 0.017/min, equivalent to an *E. coli* doubling time of 41 min.

Substituting the dermal concentrations of neutrophils (p) and of *E. coli* at the time of inoculation (b_0), at various times thereafter (b_t), and the value of g into Eq. 1, we determined that k was $2.2\text{--}2.3 \times 10^{-9}$ ml/neutrophils/min for neutrophil killing of $\sim 10^8$ CFU/ml *E. coli* in rabbit dermis (Table III). This is close to the value of k of 2.7×10^{-9} ml/neutrophils/min observed for killing of 10^8 CFU/ml *S. epidermidis* by human neutrophils in fibrin gels (Table I). Using $k = 2.25 \times 10^{-9}$ ml/neutrophil/min and $g = 0.017$ /min, we calculated CNCs of 7.7 and 7.6×10^6 neutrophils/ml rabbit dermis 1 and 2 h, respectively, after *E. coli* inoculation (Table III).

At the CNC, the bacterial concentration should remain constant over time as mentioned before. The *E. coli* concentration in rabbit dermis peaked between 1 and 2 h after *E. coli* inoculation (Fig. 6 a). By chance, it was 10^8 CFU/ml of dermis at both 1 and 2 h after *E. coli* inoculation, indicating that immigrating neutrophils had reached the critical concentration during this interval. The neutrophil concentration during this interval averaged $\sim 7.4 \times 10^6$ neutrophils/ml rabbit dermis (i.e., $[2.3 \times 10^6/\text{ml} + 12.5 \times 10^6/\text{ml}]/2$; Fig. 6 b). The very close correspondence between the empirically observed CNC (i.e., the average neutrophil concentration of 7.4×10^6 neutrophils/ml dermis 1–2 h after *E. coli* inoculation), and the CNC calculated using values of k obtained by substituting experimentally determined values for b_t , b_0 , p , g , and t into Eq. 1 (i.e., 7.7×10^6 neutrophils/ml dermis at 1 h after *E. coli* inoculation; Table III) supports the argument that Eq. 1 describes neutrophil bactericidal efficiency in rabbit dermis.

The applicability of the CNC concept to bacterial infections in vivo is further indicated the Movat et al. (9) finding that neutropenic rabbits inoculated intradermally with *E. coli* were unable to control the growth of these bacteria (Fig. 6 a). This suggests that neutrophils did not reach the CNC in the dermis of these rabbits. Indeed, the accuracy of this prediction is documented in Fig. 6 b.

The CNCs for human neutrophils to control growth of *S. epidermidis* and *E. coli* in stirred suspensions are nearly identical (5). Moreover, there is only a 1.8-fold difference between the CNC for rabbit neutrophils to control growth of $\sim 10^8$ CFU *E. coli*/ml rabbit dermis in vivo (i.e., $7.4\text{--}7.7 \times 10^6$ neutrophils/ml), and for human neutrophils to control growth of $\sim 10^8$ CFU *S. epidermidis*/ml fibrin gels (i.e., 4.2×10^6 neutrophils/ml). The 1.2-fold difference in k for neutrophil killing of *E. coli* in rabbit dermis versus for killing of *S. epidermidis* in fibrin gels (i.e., $k = 2.7 \times 10^{-9}$ vs. 2.2×10^{-9} ml/neutrophil/min, respectively), and the 1.5-fold difference in g (i.e., 0.017/min vs. 0.01/min, respectively). This suggests that values of k , g , and CNC obtained in fibrin gels will be useful for estimating the CNC in tissues and organs in vivo.

Table III. Rate Constants and CNCs for *E. coli* Killing in Rabbit Dermis In Vivo

t	p_t^a	$P_{[t, t + 60 \text{ min}]}^b$	b_t^a	k^b	CNC ^b
min	neutrophils/ml dermis	neutrophils/ml dermis	CFU <i>E. coli</i> /ml dermis	ml/neutrophil/min	neutrophils/ml dermis
0	2.3×10^5	1.4×10^6	5.7×10^7	4.0×10^{-9}	4.3×10^6
60	2.5×10^6	7.6×10^6	1.1×10^8	2.2×10^{-9}	7.7×10^6
120	1.3×10^7	1.8×10^7	1.1×10^8	2.3×10^{-9}	7.6×10^6
180	2.3×10^7		2.8×10^7		

^aTissue concentrations of neutrophils (p_t) and *E. coli* (b_t) at the times (t) specified were calculated as described in Materials and Methods using previously described data (references 9, 10).

^bValues for the average neutrophil concentration ($P_{[t, t + 60 \text{ min}]}$) between 0 and 60 min, 60 and 120 min, and 120 and 180 min were calculated and used to calculate k and the CNC for each of these time intervals as described in Materials and Methods.

Discussion

These experiments and those reported previously (5) show that in stirred suspensions (5), in fibrin gels in vitro (Figs. 1, 3, and 4), and in rabbit dermis in vivo (Fig. 6), the major determinant of neutrophil bactericidal efficiency is the neutrophil concentration, not the ratio of neutrophils to bacteria. A simple kinetic model treating killing and growth of bacteria as competing reactions fits the time course of neutrophil killing of bacteria in different environments and gives rise to the concept of the CNC.

Neutrophil Bactericidal Activity in Diverse Environments. Bacteria and neutrophils diffuse freely in stirred suspensions, but not in fibrin gels. Thus, it was not obvious that Eq. 1, which was derived to describe neutrophil bactericidal activity in stirred suspensions (5), would accurately model neutrophil bactericidal activity in fibrin gels and in tissues. The model leading to Eq. 1 assumes a rate constant, k , for bacterial killing that is independent of neutrophil and bacterial concentrations. This assumption proved true only when the initial bacterial concentration was $\leq 10^7$ CFU/ml in stirred suspensions or $\leq 10^6$ CFU/ml in fibrin gels. Our experiments show that k is affected in stirred suspensions (5) and in fibrin gels (Tables I and III) by serum concentration, by bacterial concentrations $> 10^7$ CFU/ml in stirred suspensions, and $> 10^6$ CFU/ml in fibrin gels, and by factors that affect neutrophil migration (Fig. 5, Table II, and reference 6).

The decrease in k at bacterial concentrations $> 10^6$ CFU/ml in fibrin gels (Tables I and III), and $> 10^7$ CFU/ml in stirred suspensions (5) could be due to saturation of neutrophil phagocytosis or to inhibitory or toxic factors released by bacteria. A more complicated model would be needed to include such effects. However, the premise of such a model would still be that neutrophil killing of bacteria is kinetically analogous to a bimolecular reaction.

Factors That Affect the Rate and Extent of Neutrophil Accumulation in E. coli-infected Rabbit Dermis. The finding that it takes < 2 h for neutrophils to reach the CNC of $\sim 8 \times 10^6$

neutrophils/ml in the dermis of rabbits inoculated with *E. coli* (Fig. 6 b) gave us the impetus to assess the relative contributions of the various physiological adaptations (e.g., endothelial cell activation, chemoattractant expression, and changes in blood flow and in blood neutrophil concentration) that facilitate accumulation of so large a number of neutrophils at these sites in so short a time span. Movat et al. (9, 10, 16) reported that in the first 4 h after intradermal inoculation of normal rabbits with *E. coli*, tissue neutrophil concentration increased at least 150-fold (i.e., from $\sim 2 \times 10^5$ /ml dermis to $\sim 3 \times 10^7$ /ml dermis; Fig. 6 b). During this period, blood neutrophil concentration varied from 0.5- to 1.5-fold of control, and blood flow at the site of *E. coli* inoculation increased approximately fivefold (Fig. 6 c). At most, these changes in blood neutrophil concentration and blood flow could have caused a 7.5-fold increase in neutrophil delivery to *E. coli*-infected dermis (i.e., 1.5-fold increase in blood neutrophil concentration \times fivefold increase in blood flow). However, the observed rate of neutrophil accumulation in *E. coli*-infected dermis was not closely linked to increased blood flow or to changes in blood neutrophil concentration (Fig. 6 c and reference 9). For example, the rate of neutrophil accumulation was most rapid during the first hour after *E. coli* inoculation, a period when blood neutrophil concentration averaged only $\sim 75\%$ of its control value (10) and blood flow to *E. coli*-inoculated dermis remained at control levels. These findings strongly suggest important roles for increased neutrophil adhesion to, and chemotaxis across, the endothelium of dermal capillaries perfusing *E. coli* inoculation sites. Indeed, up-regulation of endothelial selectins and other adhesion-promoting receptors, and formation of chemoattractants by endothelium, have been shown to occur within minutes of endothelial cell exposure to lipopolysaccharide.

To assess the relative magnitude of the many types of physiological adaptations that are likely to be responsible for recruitment of a critical concentration of neutrophils to

dermal sites of *E. coli* infection within the first 2 h after inoculation, we used a method introduced by Doerschuk et al. (11) to estimate the overall efficiency of neutrophil emigration into segments of *S. pneumoniae*-infected rabbit lung (Materials and Methods). We have termed this NEE. The NEE was ~ 0.45 in the first hour after *E. coli* inoculation (Fig. 6 c). That is, $\sim 45\%$ of neutrophils in the unmarginated blood pool perfusing 1 ml of *E. coli*-inoculated dermis accumulated in it during the first hour after *E. coli* inoculation. Because blood flow through *E. coli*-inoculated dermis was unchanged from preinoculation levels during the first hour (Fig. 6 c), and the concentration of neutrophils in the unmarginated pool decreased during this period (10), changes in expression of adhesion-promoting receptors and chemoattractants by endothelial cells in *E. coli*-infected dermis must have been the primary factors responsible for the increased rate of neutrophil emigration during this period.

By the end of the second hour after *E. coli* inoculation, blood flow through *E. coli*-inoculated dermis had increased approximately threefold (i.e., from ~ 3.6 ml/h per ml dermis to ~ 11 ml/h per ml dermis; Fig. 6 c), and blood neutrophil concentration had returned to its preinoculation level (i.e., 2.5×10^6 neutrophils/ml). Thus, in the second hour after *E. coli* inoculation, the total volume of blood perfusing each ml of *E. coli*-inoculated dermis contained $\sim 37 \times 10^6$ neutrophils. 8×10^6 of them migrated from the blood into each ml of *E. coli*-inoculated dermis during this second hour, approximately threefold more than had migrated into these sites during the first hour; and the NEE declined from 45% in the first hour to $\sim 15\%$ in the second (Fig. 6 c). Had the same fraction of neutrophils migrated from the blood into *E. coli*-inoculated sites in the second hour after *E. coli* inoculation as in the first hour (i.e., $\sim 45\%$), $\sim 1.6 \times 10^7$ neutrophils would have accumulated in each ml of *E. coli*-inoculated dermis during this second hour. The finding that only 8×10^6 neutrophils accumulated per ml *E. coli*-inoculated dermis during this second hour suggests that endothelial adhesion molecules and chemokines were less efficient in stimulating neutrophil immigration in the second hour than in the first.

What Regulates NEE? The findings described in Fig. 6 and discussed before suggest that of the several physiological adaptations thought to promote neutrophil accumulation at sites of infection and inflammation (e.g., increases in blood flow, changes in blood neutrophil concentration and vascular permeability, and expression of adhesion-promoting receptors and of chemokines by endothelium; references 15, 17), expression of chemokines and adhesion-promoting receptors by postcapillary venular endothelial cells exert the largest effects. The Movat et al. (16) finding that neutrophils accumulated at a similar rate and to a similar extent in *E. coli*-inoculated dermis of cobra venom factor-depleted rabbits as in normal rabbits suggests that C3a and C5a played minor roles, at best, in promoting neutrophil entry into *E. coli*-inoculated dermis. They are consistent with a dominant role for endothelial cell-derived factors (e.g., IL-8) in this process.

NEE at *E. coli*-inoculated skin sites in normal rabbits declined markedly in the second and subsequent hours after

E. coli inoculation (Fig. 6 c) despite continued increases in blood (16) and tissue neutrophil concentration (Fig. 6 b), and local blood flow (Fig. 6 c and references 9, 10, and 16). Although we had insufficient data to calculate the NEE in *E. coli*-inoculated neutropenic rabbits, it is evident that the rate of neutrophil accumulation also declined in the second hour after *E. coli* inoculation in these rabbits (Fig. 6 b).

Many of the mechanisms and molecules responsible for increases in NEE (e.g., expression of adhesion promoting receptors and chemokines by activated endothelial cells and up-regulation of neutrophil adhesion-promoting receptors by chemokines and chemoattractants), have been identified (for review see reference 17). Less is known about the mechanisms and molecules responsible for decreases in NEE.

The findings of Levy et al. (18) suggest an important role for lipoxins inhibiting inflammation. They are formed by endothelial cells and monocytes from 5-lipoxygenase products (e.g., leukotrienes A4 and B4) produced by chemokine/cytokine-stimulated neutrophils. They are formed at sites of inflammation after entry of the first wave of neutrophils, but before the rate of neutrophil entry declines (18). Lipoxins inhibit IL-8 release by endothelial cells, monocytes, and neutrophils; reduce endothelial cell adhesiveness for neutrophils; attenuate CD11/CD18 expression by neutrophils; and inhibit neutrophil chemotaxis and migration across endothelium (19). At the same time, they stimulate monocyte chemotaxis (19). These activities are consistent with ones that might be expected to produce the effects observed two or more hours after *E. coli* inoculation into rabbit dermis (i.e., a decrease in the rate of neutrophil accumulation, and continued monocyte entry (Fig. 6 b and reference 20)

Why Does Neutropenia Predispose to Bacteremia? The findings reported here (Figs. 1 a, 3 a, and 6, a-c, and Tables I and III) as well as those reported previously (5), provide three related insights into the relationships between neutropenia and bacteremia. First, they show that the CNC required to control bacterial growth is 10–19-fold higher in tissues than in blood. Second, they suggest that a decrease in NEE, which occurs between 2 and 3 h after commencement of neutrophil immigration, may prevent neutrophils from ever reaching the CNC in tissues when the blood neutrophil concentration is $\leq 5 \times 10^5$ neutrophils/ml. Accordingly, in neutropenic hosts, the small number of bacteria that penetrate skin and mucous membranes will grow to a higher concentration and persist longer in tissues than in hosts with a normal blood neutrophil concentration. By this means, neutropenia increases opportunities for bacterial entry into the vascular compartment. This scenario is consistent with Crosby's suggestion that tissue neutrophil concentration is the primary determinant of host defense against bacteremia (21) and with the Koene et al. (22) finding that bacteremia in neutropenic patients correlates more closely with total body mass of neutrophils than with blood neutrophil concentration. Third, they show that when the blood neutrophil concentration in a neutropenic host falls below the CNC (i.e., $< 4-5 \times 10^5$ neutrophils/ml), bacteria that enter the blood will continue to grow. Under these circumstances, the fixed phagocytes of the liver and spleen

will be the last line of host defense. Bacteria that escape them will inexorably increase in concentration in the blood at a rate described by Eq. 1.

Other Applications of Eq. 1 and of the CNC Concept. Information about the CNC for known or suspected bacterial pathogens may be helpful in determining whether and when to administer granulocytes and/or antibiotics to neutropenic patients. Such information will enable clinicians to estimate the minimum neutrophil concentration needed to control bacterial growth in specific organs and tissues, and may encourage them to consider whether administration of agents that block continued ingress of neutrophils into a site of infection, inflammation, or infarction may be therapeutically beneficial. For example, Wright et al. (23) reported that limiting neutrophil influx into the cerebrospinal fluid of rabbits with pneumococcal meningitis reduced mortality from this infection in these animals.

The concept of a critical leukocyte concentration may be applicable to other situations. For example, a critical concentration of antigen-presenting cells expressing a given antigen may be required to elicit a physiologically meaningful T or B cell response to that antigen, a critical concentration of platelets may be required to maintain vascular integrity, and a critical concentration of cytotoxic lymphocytes may be required to block growth of tumors. The concepts and systems described here should facilitate exploration of these questions.

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