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Flow cytometric measurements of neutrophil functions: the dependence on the stimulus to cell ratio

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Abstract

Phagocytosis and antimicrobial killing of neutrophils has been quantitatively determined as a function of the stimulus (*Candida albicans*) to cell ratio *R* using two donor collectives containing a total of 115 blood samples. Analysis of the collectives in two different laboratories according to the same flow cytometric protocol for simultaneous measurement of neutrophil functions did not produce statistically significant differences. The number of phagocytosing leukocytes as well as that of killed fungi per leukocyte depends strongly on *R*. While each phagocytosing neutrophil kills one fungus at low values of *R*, each neutrophil kills on average 2.5 fungi for large *R*.

Keywords: Flow cytometry; Neutrophil function; Phagocytosis; Antimicrobial killing

1. Introduction

An increased susceptibility to bacterial and fungal infections is often related to an impaired antimicrobial neutrophil activity. Most of the methods used to analyze neutrophil functions, such as phagocytosis, oxidative burst and antimicrobial killing, are time consuming and do not allow one to measure different cell functions simultaneously [1]. However, the possibility to measure several parameters for individual neutrophils with flow cytometry in a short time by using multiple fluorochromes has improved the analysis of cell functions considerably [2–9]. A problem with the different flow cytometric assays so far has been major differences in preparative procedures, types of phagocytes used or the type of stimulating agent. The chosen conditions for the determination of phagocytosis or killing activity, i.e. the incubation time and the particle and cell concentrations, differ from one laboratory to another. This has in some cases led to contradicting results for certain medical conditions in different publications [10].

In most published assays only phagocytosis and oxidative burst have been reported although it is known that the killing function of neutrophils is a crucial property of neutrophil activity. To determine the killing capability of neutrophils it is not sufficient to measure the oxidative burst since it is not uniquely related to the killing of microorganisms [11].

In the present study we investigate the influence of the stimulus to cell ratio *R* on phagocytosis and antimicrobial killing of neutrophils and monocytes in a large number of healthy donors using a recently described test system [12]. To reduce systematic errors we analyzed data from two different laboratories.

The dependence of phagocytosis on the stimulus to neutrophil ratio *R* has been noted before [3,6,9]. However, the dependence on *R* has not been quantified except in one early experiment [13].

Our study comprises by far the largest number of blood samples (115) and allows us not only to quantify the influence of *R* on neutrophil functions, but also to characterize interassay variabilities. This was done with respect to personal constants and day to day variations on the one hand, and with respect to possible systematic differences

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between experiments conducted in different laboratories according to the same protocol on the other hand. Moreover, we present for the first time a direct quantitative measurement of the R dependence of antimicrobial killing.

2. Materials and methods

2.1. Assay for the evaluation of phagocytosis, oxidative burst and antimicrobial killing

We determined phagocytosis, oxidative burst and killing activity of neutrophils and monocytes as described recently [12]. The slightly modified protocol used for the present study is briefly described in the following.

2.1.1. Blood samples

One hundred and fifteen venous blood samples (57 in Freiburg, 58 in Dresden) were obtained from a total of 49 healthy volunteers ranging in age from 20 to 50 years. None of the volunteers had acute or chronic diseases or took drugs regularly. Heparin was used as anticoagulant (5 IE heparin ml^{-1} blood). Informed consent for blood sampling was obtained from all blood donors after the nature of the study had been fully explained. The present study is part of a study that was approved by the Medical Ethical Committees of the University Hospitals in Freiburg and Dresden.

2.1.2. Cell labeling

Culture of *Candida albicans* (DMS 1386) was performed in tryptic soy broth (Difco laboratories, Detroit, MI, USA).

For fluorescence labeling of *C. albicans* broth was centrifuged ($500\times g$, 15 min), the pellet was re-suspended in 1 ml of phosphate-buffered saline/glucose (100 ml PBS (Sigma, St. Louis, MO, USA) containing 0.5 g glucose, pH 7.5) with 5 μl calcein-AM (Molecular Probes, Eugene, OR, USA) and incubated for 50 min at 37°C in a thermomixer (Eppendorf-Nehler-Hinz, Germany) at 600 rpm. The stained yeast were washed in PBS, re-suspended in RPMI (RPMI 1640, Sigma) and stored in a refrigerator for a maximum of 7 days. The number of yeast per ml RPMI was determined with Neubauer's counting chamber, the percentage of stained yeast was controlled by flow cytometry ($>97\%$).

For detecting burst reaction, blood was incubated with dihydroethidium (DHE, Sigma, 20 μl of PBS containing 0.5 μg DHE per ml blood) for 10 min at 37°C in a shaking water bath. Reactive oxygen species released by neutrophils oxidize the dye, which turns from colorless to red fluorescent.

To identify neutrophils and monocytes, they were stained with orange fluorescence after the reaction had stopped (see below). The pellet of each tube was re-suspended with 100 μl PBS containing 1.8 μl CD13-RPE antibody (Coulter, Krefeld, Germany) and incubated at room temperature for 20 min.

For labeling dead yeast, after lysis of neutrophils and centrifugation (see below) the pellets were each mixed with 800 μl 2 μM ethidium homodimer-1 solution (EthD-1, Molecular Probes), incubated for 10 min at room temperature, mixed and stored on ice.

2.1.3. Preparation of blood samples

RPMI solution containing *C. albicans* was diluted to achieve the desired ratio R between neutrophils and *C. albicans*. According to the chosen kinetics for incubation (0, 6, 10, 30 min for phagocytosis per burst, 0, 15, 30, 60, 120 min for killing) polypropylene tubes were prepared with 100 μl RPMI and stored on ice.

Blood (after incubation with DHE, see above) and microorganisms were mixed, aliquots of 200 μl were quickly dispensed into the test tubes (separate tubes for phagocytosis and killing assay).

2.1.4. Assay for phagocytosis and oxidative burst

The tube for 0 min was immediately mixed with 400 μl of *N*-ethylmaleimide (NEM, Sigma) and stored on ice, the other tubes were incubated at 37°C in a thermomixer. At appropriate times the reaction was stopped by addition of 400 μl ice-cold NEM, tubes were stored on ice. The tubes were then centrifuged together at 4°C at $250\times g$ for 5 min (10 min for *C. albicans*/neutrophil ratios >2) to avoid attachment of microorganisms at the phagocyte surface. After staining neutrophils (see above) 1800 μl lysing buffer for erythrocytes (containing 8.27 mg ammonium chloride, 1 mg potassium hydrogen carbonate and 0.04 mg sodium EDTA) was added to each tube and incubated at room temperature for 10 min. Tubes were again centrifuged (4°C, $250\times g$, 5 min), 1000 μl of the supernatant was removed. After vortexing the tubes, cells were stored on ice and measured with a maximum delay of 2 h.

2.1.5. Assay for killing

After incubation of the cells (37°C, thermomixer) the reaction was stopped by placing the test tubes on ice and mixing immediately with 400 μl lysing buffer for human cells, consisting of 2 μl Triton X-100 (Sigma) and 2 μl Tween-20 (Sigma) in distilled water. After incubation at 37°C for 25 min in the thermomixer at 600 rpm the tubes were centrifuged at 4°C at $1000\times g$ for 5 min (10 min for *C. albicans*/neutrophil ratios >2). Dead yeast cells were then stained with EthD-1 solution as described above. Cells were measured within 2 h.

2.1.6. Flow cytometry

The samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) using Cellquest R software (BD, version 3.3). Green fluorescence (FL1) from calcein was collected through a bandpass filter of 530/30 nm, orange fluorescence (FL2) from CD13-RPE

was detected with a bandpass filter of 585/42 nm and red fluorescence (FL3) from ethidium and EthD-1 was analyzed with a longpass filter of 670 nm. Data were collected using linear amplifiers for the forward scatter (FSC) and logarithmic amplifiers for the sidescatter, FL1, FL2 and FL3. Data were displayed in single-parameter histograms or in two-parameter plots. Prior to each run 11 samples containing blood with or without yeast and all variations of red, orange and green fluorescence were measured and served as a control. In the assay for phagocytosis and burst 2500 neutrophils were counted, in the killing assay 5000 fungi. The results for phagocytosis, burst and killing were obtained through gating techniques and expressed in percentage of neutrophils which have phagocytosed (shown oxidative burst) and percentage of dead yeast as described in detail elsewhere [12]. The exact ratio R of yeast (H_0) to neutrophils (N_0) was determined in the FCS versus FL1 plot analysis and FSC versus FL2, respectively.

2.2. Parameters of neutrophil function

We explicitly focus on the measurement of all parameters as a function of the ratio of yeast (H_0) to neutrophils (N_0) which we call $R = H_0/N_0$. As usual we characterize phagocytosis $P = N_p/N_0$ and burst $B = N_b/N_0$ as the percentage of phagocytes N_p that are capable of ingesting targets (show oxidative burst N_b) at different incubation times. In contrast to the standard parameter k for the killing activity of neutrophils, namely the killed yeast H_k as the percentage of all yeast, $k = H_k/H_0$, we describe killing through killed yeast as a percentage of all neutrophils, $K = H_k/N_0$. This killing function can be easily obtained by multiplying the usual killing function k with the ratio R, i.e. $K = kR$. The advantage of this definition is that K behaves as a function of R similarly as phagocytosis P. We preferred yeast over other targets due to the relatively slow reaction of the neutrophils compared to bacteria, which facilitates the mapping of the time evolution of the neutrophil functions. Moreover, having in mind to apply an identical assay in patients who often receive antimicrobial therapy, yeast has the advantage of not being influenced by most of these drugs. We will not focus on the oxidative burst, which is only an indirect parameter for the antimicrobial neutrophil activity [14].

2.3. Model for the R dependence and statistical analysis

We have modeled the R dependence of the neutrophil functions with a simple exponential relation $X(R) = aR[1-\exp(-b/(aR))]$, which takes into account the asymptotic behavior for $X(R\to 0)\approx aR$ and $X(R\to\infty)\approx b = \text{const}$. Here, X stands for P or K. The constants a and b are fitting parameters where a gives phagocytosis P or killing k in the limit of low fungal concentration ($R\to 0$), while b represents the respective saturation value in the opposite limit of ($R\to\infty$). In the case of P only the fitting parameter a remains, while $b = 100\%$, since phagocytosis always saturates at 100% [15]. Note that apart from R all parameters, i.e. X, a and b, are given in percent. Fits were performed on the logarithm of the neutrophil functions, $\log(X)$, to ensure an equal weighting of all data. The correlation coefficient r for the fits was larger than 0.9 unless otherwise stated. The significance in the differences of $a \pm$ S.E.M. was analyzed with an unpaired t-test including a power analysis.

3. Results

We used two collectives with 57 samples in Freiburg and 58 samples in Dresden.

Our first goal was to see how robust measurements in different labs are using the same assay.

For clarity this analysis is here restricted to P_{30}, phagocytosis after 30 min incubation time, shown in Fig. 1. Fitting the data for Freiburg (F) and Dresden (D) separately yields $a_F = 53.2 \pm 3.3\%$ and $a_D = 58.6 \pm 2.0\%$. The difference $\Delta_{FD} = 5.4\%$ corresponds to $0.26 s_p$ (where s_p is the pooled standard deviation) and is not significant on the level of $P < 0.05$. Antimicrobial killing showed a similar insignificant difference. Given the number of patients and s_p of the means, the present test can detect a difference of the means of $0.75 s_p$ with more than 80% probability (power $1-\beta = 0.8$), or stated in a more abstract way: we could detect differences of $s_p/2$ with a probability of 76%. Hence, there is a high probability that the P_{30}s of the two collectives differ by less than one s_p. We conclude that the method is fairly robust and treat the data as one collective in the following. For P_{30} we obtained for all 115 samples $a = 55.8 \pm 2.0\%$.

The second issue investigated was the role of personal constants and day to day variations. To this end we made two separate analyses. First, we examined the effect of day to day variations by removing all multiple measurements

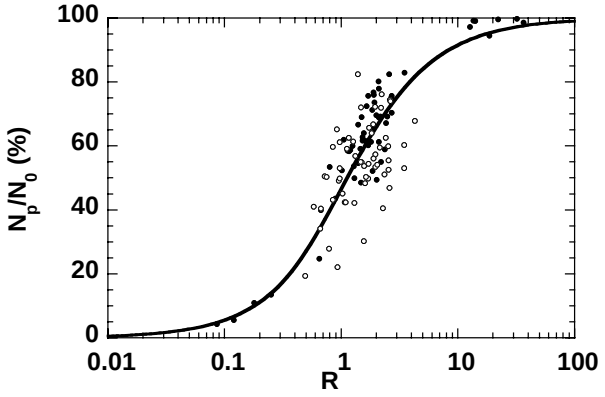

Fig. 1. Phagocytosis $P_{30}(R)$ for the Freiburg data (open circles) and the Dresden data (filled circles). The curve is a fit through the combined data, see text.

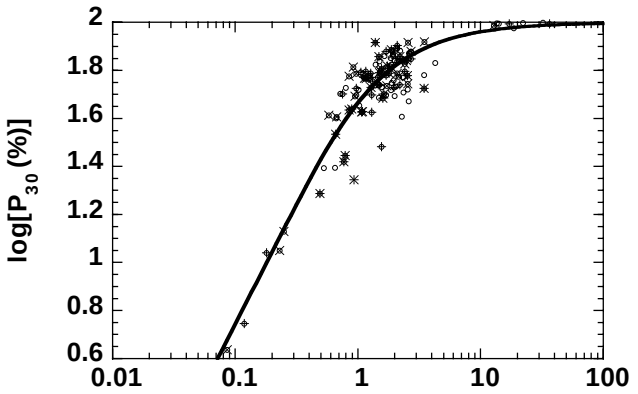

Fig. 2. Phagocytosis $P_{30}(R)$ for all 115 samples (open circles) and for two sets of data from 49 different probands only, (x, set A) and (+, set B). The fit produces the same line for all three data sets at the resolution of the figure.

which had been taken from 27 probands, leaving 49 data points from different probands (set A). Taking other (randomly chosen) data of the 27 multiply measured probands (set B) generated a second set of this kind. The result is shown in Fig. 2 and with $a = 54.6 \pm 2.9\%$ (set A) and $a = 52.2 \pm 2.8\%$ (set B) reveals no significant difference ($P < 0.05$), either between set A and B ($\Delta_{AB} = 2.4 \pm 4.0\%$) or between A or B and the full set including multiple measurements of probands (see above). Due to the smaller sample size this test can only detect differences $\Delta_{AB} = 16\%$ ($= 1.1\ s_p$) and more with a power of 0.8. We may conclude that within the accuracy of the data, there is no significant effect of day to day variations.

We further investigated the influence of personal constants by analyzing multiple measurements of eight probands, summarized in Table 1. Due to the small sample sizes we refrain from quantitative statistical analysis. As can be seen, personal constants influence measured values for phagocytosis considerably, which is also consistent with results reported in the literature [12,16]. However, the mean value of a for the eight individuals $\langle a \rangle = 54.2 \pm 6.7\%$ is basically the same as that for the full data set.

We proceed to the results of the killing function of neutrophils, expressed as killed yeast per neutrophil, $K = H_k/N_0$. While phagocytosis saturates approximately after 30 min incubation time, killing takes longer and saturation is reached after approximately 120 min, see Fig. 3. The killing function K grows linearly for small R and saturates for large R, like P. However, in contrast to $P(R \to \infty) = 100\%$ we found $K(R \to \infty) > 200\%$ (Fig. 3). This means that on average one phagocyte kills more than two fungi if enough fungi are available ($R \to \infty$) which is consistent with the known fact that phagocytes can take up two or three particles of the size of fungi [17]. The curves shown in Fig. 3 result from a fit with constants $a = 59.3 \pm 2.2\%$ and $b = 248 \pm 22\%$ for K_{120}.

Table 1
Phagocytosis P_{30} multiply measured (five to nine times) at different R in eight probands

Data points	$a \pm$ S.E.M.	$\langle R \rangle$	ΔR	r	χ^2
5	49.5 ± 6.2	1.76	0.4	0.93	0.02
5	35.2 ± 8.2	2.40	0.5	0.20	0.03
5	31.2 ± 6.1	2.08	0.3	0.6	0.05
5	79.6 ± 10	8.40	4.2	0.93	0.003
7	72.7 ± 3.9	1.45	0.3	0.95	0.005
8	50.4 ± 4.1	2.76	1.6	0.99	0.03
9	74.8 ± 6.3	5.00	3.4	0.88	0.01
9	40.6 ± 4.1	2.02	0.2	0.12	0.04

The fit result for a ($\pm$ S.E.M.), the correlation coefficient r and χ^2 are given, along with the mean $\langle R \rangle$ of the data sets and the corresponding fluctuation ΔR.

4. Discussion

The R dependence contains important information about neutrophil functions. There exists a maximum killing capability of approximately 2.5 fungi per neutrophil if enough fungi are supplied. This can be concluded from the value $b = 248\%$ of the fit to the data of K_{120}. From the same fit we obtained $a = 59.3\%$, very similar to $a = 55.8\%$ for phagocytosis. Since a characterizes the respective neutrophil function (killing or phagocytosis) at low fungal concentration for $R \to 0$, we can conclude that at appropriate incubation times of saturation $P \approx K$ at small R. This implies that each phagocytosing neutrophil kills one fungus in this situation and therefore there is a direct 1:1 correspondence of phagocytosis and killing.

From our results we conclude that the dependence of neutrophil functions on R should definitely be taken into account. This is even more important if $R \approx 1$ which is most commonly used in experiments with *C. albicans*. Exactly in this region the dependence of phagocytosis (and killing) on R is the strongest, as the large slope of $P(R)$ in Fig. 1 demonstrates.

To facilitate interassay comparison we propose for future work to determine and to state the ratio R for individual assays, as well as the incubation time which, of

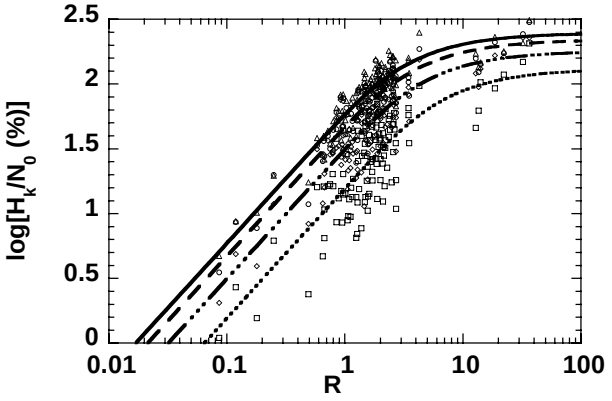

Fig. 3. The killing function K_t for incubation times t of 30 min (+, dotted), 60 min (x, dashed) and 120 min (triangles, solid), lines are fits, see text.

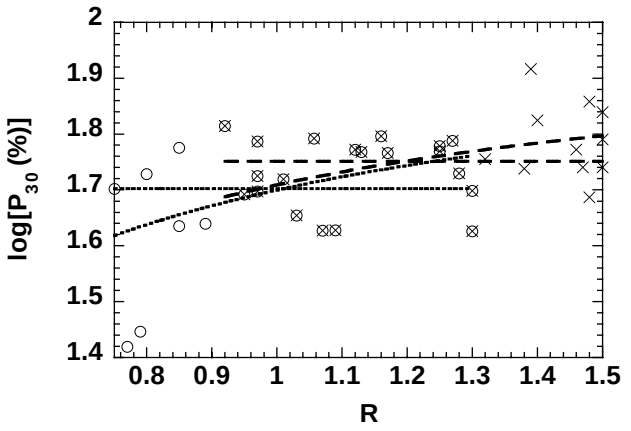

Fig. 4. Phagocytosis $P_{30}(R)$ for $0.75 < R < 1.3$ (set I: circles, fits dotted) and for $0.9 < R < 1.5$ (set II: crosses, fits dashed), see text.

course, also has a significant influence on the neutrophil parameters (see Fig. 3).

Often, the R dependence is ignored – this can even lead to a misinterpretation of results as we briefly demonstrate in the following. We take two subsets from our data of $P_{30}(R)$, set I in the range $0.75 < R < 1.3$, set II with $0.9 < R < 1.5$, i.e. shifted by $R = 0.2$, only. Set I contains 27 data points, set II 30 points, 20 points are common to both sets, see Fig. 4. This scenario simulates a situation where two experiments were performed with the same prepared ratio R. The actual values R, however, differ slightly as the exact flow cytometric analysis shows. Subjecting each data set to a simple statistical analysis ignoring any R dependence yields a mean of $50.4 \pm 2.2\%$ (set I) and $56.4 \pm 1.6\%$ (set II), indicated by straight lines in Fig. 4. From a t-test one would conclude a significant difference of the two sets ($P < 0.05$, $1-\beta = 0.61$). However, this (significant!) difference is only a consequence of the slightly different range of R in the two samples. It does not come from a different neutrophil activity of the probands from the two samples. Hence, one should measure the exact ratio R and take it into account when evaluating neutrophil functions as suggested.

If we fit set I and II with our R-dependent function we obtain $a = 63.0 \pm 4.1\%$ and $a = 65.4 \pm 3.5\%$, respectively, i.e. no significant difference ($P < 0.05$) between set I and II. (The correlation coefficients, $r = 0.45$ and $r = 0.21$, are small due to the small interval R covered.)

For simplicity we have described the tests on the R dependence for phagocytosis only. Clearly, the same reasoning also applies to the killing function.

To summarize, we have established the average neutrophil functions of healthy volunteers as a function of the stimulus to neutrophil ratio R. We found that at low fungal concentration there is a 1:1 correspondence between phagocytosis and killing, i.e. each phagocytosing neutrophil kills one fungus. At high fungal concentration the killing saturates on average at 2.5 dead fungi per neutrophil.

Since our results and those of other investigators show that interassay variations lead to large fluctuation in the data (in our case and in Salih et al. [12] about 20%, in Peltroche-Llacsahuanga et al. [16] even up to 50%) a comparison of neutrophil functions between *individuals* is not useful. However, the averaged neutrophil functions of healthy volunteers established in this investigation should make it possible in the future to arrive at statistically significant conclusions in comparison with similar data sets from patients with impaired neutrophil functions if the data are obtained under the same standardized conditions.

For this purpose the evaluation of data as a function of the ratio R, or at least the exact knowledge of R, is mandatory. Moreover, we have demonstrated that flow cytometric measurements represent a reliable and robust method to test neutrophil functions. This has been proved by the good agreement of average neutrophil functions determined from two different collectives whose blood samples were taken and analyzed in different laboratories according to the same protocol.

For the future it will be interesting and important to investigate possibilities for reducing the fluctuations in the data. This implies a closer look at the fluctuations induced by the rather complex laboratory treatment of the blood samples and the flow cytometric procedure as well as a better knowledge of determining factors for personal constants and day to day variations.

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