Assessment of Granulocyte Activity with Application to Healthy and Ill Subjects

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Abstract

A protocol was developed to measure granulocyte activity. The protocol methodology measured the hydrogen peroxide released after granulocytes activation by PMA (phorbol 12-myristate 13-acetate) by using the Amplex Red assay, which included count cells by flow cytometer and measurement kinetic curve of NADPH oxidase activity by fluorometer. Two parameters were used to describe the level of granulocyte activity: (1) the initial rate of NADPH-oxidase enzyme and (2) level of hydrogen peroxide released after 20 min of granulocyte activation. The method was applied to measure granulocyte activity in healthy and ill subjects and demonstrated that applied procedure is sensitive for estimation of the disease activity. There was a decrease in granulocyte activity in patients with cancer or diabetes mellitus, and an increase in activity in patients with osteo- and rheumatoid arthritis or with viral and fungal infection.

Introduction

Phagocytes play a crucial role in host protection by combating infection. For this purpose they have a molecular mechanism that is able to generate toxic oxygen derivatives. The signaling pathways involved in the stimulus-response of the plasma membrane oxidase system and the properties of the NADPH oxidase activity of granulocytes have been described previously.[1-6]

The initial product of NADPH oxidase-mediated oxygen reduction is primarily superoxide anion (O_2^-), a very potent free radical. The superoxide is converted to H_2O_2 either spontaneously or by superoxide dismutase. The electron donor for NADPH oxidase is cytosolic NADPH. The O_2^- generated by the respiratory burst is released at the outer surface of the granulocyte membrane, or into the phagocytic vacuole (phagolysosome) when bacteria are engulfed. The movement of an electron from the inside to the outside of the membrane is electrogenic, leading to the depolarization of the plasma membrane. This is followed by charge compensation through the opening of a H^+ sensitive channel. The relative stability and membrane permeability of H_2O_2 allows one to use its production and to quantify the extracellular release of reactive reduction products in phagocytes, which reflects the phagocytic index of the phagocyte.

Many attempts have been made to measure the phagocytic activity of neutrophils. This was done with nitroblue tetrazolium dye reduction (NBT) test,[7] bacterial uptake by phagocytes, enzyme assay for selected enzymes of hexose monophosphate shunt,[8] and other methods, which made use of the fluorescent dyes such as scopoletin with high background emission.[9] We used the fluorescent dye Amplex Red, which has superior sensitivity.[10]

Method of measurements

In our experimental detection of the level of phagocytes' activity we chose a very sensitive method of detection of H_2O_2 from activated cells by using Amplex Red.[10,11] We modified the published method by using a fluorometer coupled with the counting of cells by flow-cytometer.

In this method, the Amplex Red reagent was used in combination with horseradish peroxidase to detect H_2O_2 released from the biological samples or generated in enzyme-coupled reactions. In the presence of peroxidase, the Amplex Red reagent reacts with hydrogen peroxide to produce a red–fluorescent product. The principle of the determination of H_2O_2 is that the working solution is colorless and produces a

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highly fluorescent product, resorufin, after enzyme-catalyzed oxidation of Amplex Red by H_2O_2 released from activated neutrophils. Instead of a fluorescence microplate reader, we used a more sensitive SPEX spectrofluorometer (double-grating spectrophotometer, sensitivity 4000:1). The excitation was at 530 nm and the fluorescence was scanned from 540 to 650 nm. The maximum of the emission spectra occurred at 583 nm.

For estimation of the NADPH oxidase activity the product formation was monitor during a 20-minute period with intervals of 2-3 minutes.

Separation Procedure

For the separation of granulocytes (polymorphonuclear leukocytes or neutrophils) we used two different methods. The principle of separation utilized the fact that the granulocyte fractions of white blood cells have a density predominantly above 1.08 g/mL, while mononuclear cells (lymphocytes and monocytes) have a density below this value. For the separation by Polymorphoprep (Greiner Bio, Oslo, Norway) (sodium diatrizoate, dextran 500) whole blood was treated with an anticoagulant (heparin, EDTA or citrate). 4 mL of blood are layered over 4 mL of Polymorpho-prep and centrifuged at 450-500 g for 30-35 min. The resulting two layers contain mainly mononuclear cells on top and the desired polymorphonuclear cells below. For separation by Percoll (Amershampharmacia Biotech, Piscataway, New Jersey), the blood was diluted by Phosphate Buffered Saline (PBS) (1:1) and layered on top of discontinuous percoll gradients with densities 1.1 and 1.077 g/mL and centrifuged at 600 g for 25 min.[12] Erythrocytes were removed by hypotonic red cell buffer (0.83% (w/v) NH_4Cl) that hemolysed the erythrocytes. We compared the two different separation procedures to see if the separation procedure influenced the metabolic parameters of the cells. For example, the Percoll solution is composed of particles 15-30 nm in size and may be engulfed by neutrophils and cause activation during the separation procedure. A comparison of the level of granulocyte activity using the same volunteer's samples and statistical analysis of the distribution of the level of cells' activation for cell separated by two different methods, demonstrated that the level of metabolic activity was not influenced by the separation procedures. For blood samples of 30 volunteers the values of hydrogen peroxide released by 10^4 granulocytes were in range (mean±SD) 63.1±10.3 nM for cells separated by Percoll and 65.2±9.6 nM for cells separated by Polymorphoprep.

Cell counts by Flow Cytometer

The number of granulocytes was obtained by the specific binding of the FITC (fluorescein isothiocyanate) -conjugated antibody (CD15) to the 220 kD carbohydrate structure at the cell surface, expressed at 95% of granulocytes (including neutrophils and eosinophils). For immuno-fluorescence assay, 10^5 cells were resuspended in PBS with 1% bovine serum albumin and stained by antibody-CD15. After the washing procedure, cells were counted by flow-cytometer. The result of analysis was expressed as green fluorescence intensity versus the number of cells.

Analysis of the forward scattering (FS) and side scattering (SS) diagram on a flow-cytometer identified the neutrophils, lymphocytes and monocytes. Cells with a green tag were easily separated and counted. Figure 1 (p.153) shows the result of FS/SS gating and determination of the number of granulocytes by using a staining procedure (the number of granulocytes was determined by counting cells in rectangular region D).

The data were also collected using the logarithmic scale and fluorescein amplification (Figure 2, p.153). This diagram shows the number of cells from region D, which are bounded to the anti-human monoclonal antibody CD15.

Figure 1. The gating and determination of the number of granulocytes using the Flow-cytometer.

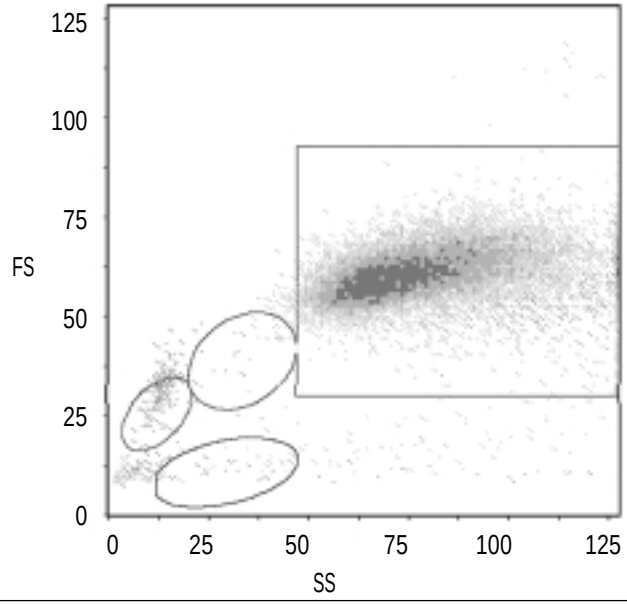
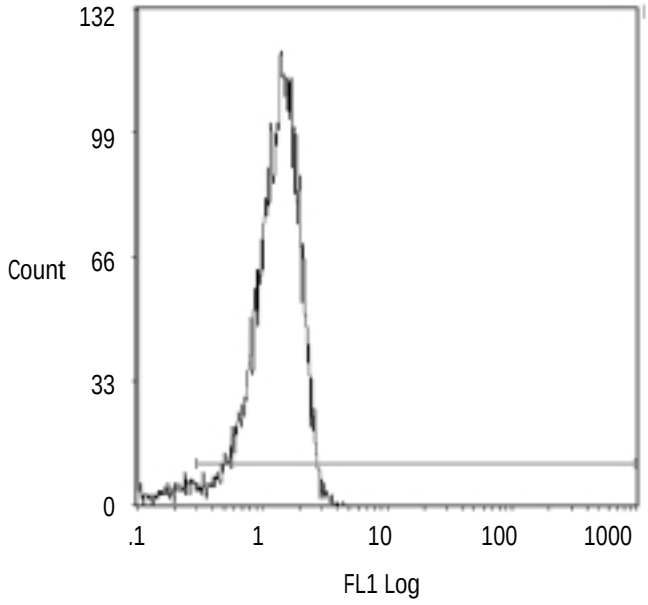


Figure 2. The fluorescence histogram for granulocytes stained with FITC-conjugated antibody CD15.



Granulocyte Activity Assay

The stock solution of Amplex Red was prepared by dissolving 1 mg of Amplex Red in 200 mL of DMSO. We determined the optimal range for the concentration of the dye, the effect of different horseradish peroxidase enzyme concentrations on kinetic curves and the effect of different scanning protocols at the level of fluorescent emission, with the purpose of finding the linear range of reaction product, i.e., the linear range of the emission of resorufin in dependence on concentration. For the control the linear range of the emission of resorufin also was determined by using different concentrations of the stock solution of resorufin.

To measure the level of the granulocytes' activation, the reaction mixture contained 10-50 μM Amplex red, 0.1 U/mL horseradish peroxidase (dissolved in PBS), 200 ng/mL phorbol 12-myristate 13-acetate (PMA), which was used as stimulating agent, and 5.5 mM glucose in PBS. The properties of reaction mixture were analyzed for spontaneous oxidation under the same conditions as for actual measurements, but without addition of cells. The increase in intensities in counts per second (cps) of baseline emission after 20 min of measurements was in range 2%-4%.

The level of emission for activated cells and resting cells (reaction mixture with the addition of activator and without activator PMA) was also compared. These experiments demonstrated that the effect of increased level of fluorescence emission occurs only in the response to cells activation, as the level of emission due to oxidant production by $6*10^4$ cells activated and not activated by PMA after 20min of activation was 80% and 12%.

To determine the level of granulocyte activity, $4*10^4$-$6*10^4$ cells were added into 1 mL of working solution (50 μM Amplex Red, 0.1 U/mL of HRP, 200 ng/mL PMA) and the fluorescence curve was measured during 30 min with 3-5 min intervals between measurements. Figure 3 (p155) shows the change in fluorescence of the reaction mixture due to release of hydrogen peroxide by activated granulocytes at different times after of cells activation.

For each time, the maximum intensity at 583 nm was determined and the results were plotted versus time since activation. An example of the curve of the granulocytes' response to PMA is presented at Figure 4. (p. 155)

Figure 4 shows that there are several phases at the kinetic curve during the reaction. Immediately after the addition of cells to the working solution there is a period of equilibration during first several minutes (lag phase). After the lag phase the initial rate of the reaction occurs. In this reaction phase the rate of product formation is increasing linearly with time. For the calculation of the level of enzyme activity two parameters were estimated, first the initial rate of the reaction normalized to the number of cells, and secondly, the level of hydrogen peroxide released from cells after 20 min of activation.

Also, two important quantitative relations are needed to estimate the NADPH oxidase activity from emission measurements. The first is the linear range of the cells' response to activator and the second is a standard curve for the conversion of emission measurements to the concentration of H_2O_2released by cells. For the preparation of H_2O_2 standard curve, the stock solution of H_2O_2 was diluted to produce concentrations from 50 nM to 400 nM in working solution. The level of the intensity of the fluorescence emission at 583 nm for different concentrations of hydrogen peroxide demonstrated a linear dependence and the correlation coefficient for the linear least-squares fit was 0.99.

Results

In this study we analyzed the sensitivity and reproducibility of a method for the detection of differences in phagocytic ac-

Figure 3. Time dependence of fluorescence emission after granulocyte activation (times in minutes).

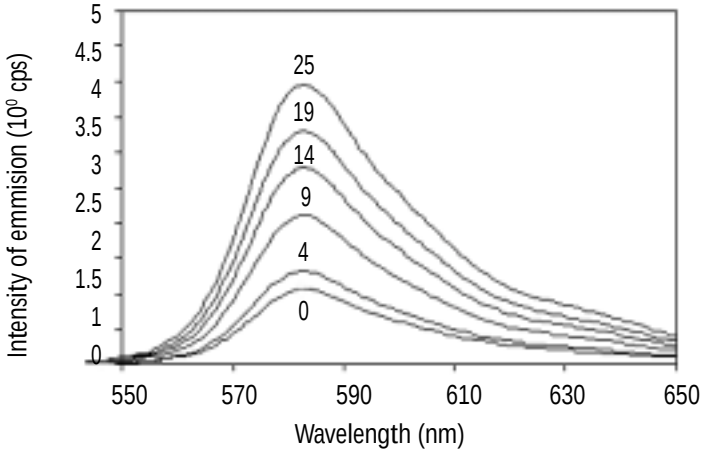
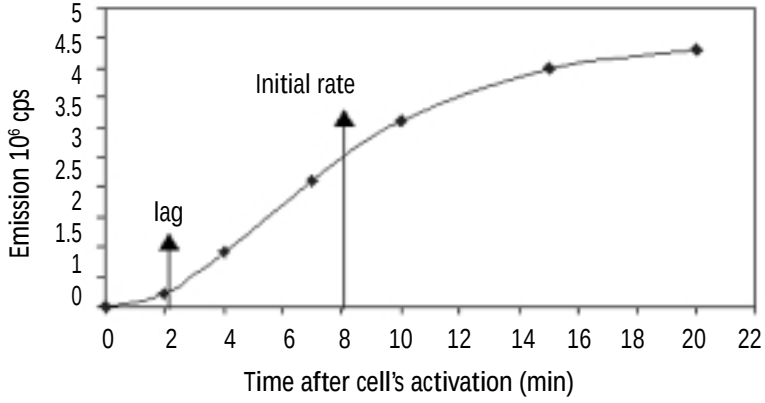


Figure 4. Time course of the change in fluorescence at 583nm in response to cells' activation. The range of initial rate is delineated with arrows.



tivity of cells for ill and healthy volunteers. Staff employees (controls without known cancer, arthritis, diabetes or infection) and volunteer patients of the Center were employed in this study.

Twelve healthy subjects were sampled to determine the dependence of emission on the number of granulocytes in working solution.

An example of the cell dose response to the level of fluorescent emission is presented on Figure 5. (below) We found a linear response in range of $2*10^4$–$1*10^5$ cells.

To estimate the reproducibility of this method, each sample of blood was replicated two or three times using the experimental conditions. The comparison of granulocyte activity for the same sample of blood but for different experiments demonstrated that the variation in the level of H_2O_2 released by 10000 granulocytes was in range of 2%-30%.

Granulocyte activities in normal subjects

Test values for the healthy volunteers are presented in Table 1 (p.157) and Figure 6. (p.158) Table 1 includes the method of cell separation, the number of cells in working solution, the initial rate of granulocyte activity, calculated as the ratio of kinetic curve slope at the linear range to the number of granulocytes in working solution, the intensity of emission per activated cell, the concentration of H_2O_2 released by all cells after 20 min of activation and the concentration of H_2O_2 released by 10,000 activated cells.

The distribution of the level of granulocyte activity with estimated values of descriptive statistics is presented at Figure 6.

The granulocytes' activity for healthy volunteers is approximately normally distributed with a coefficient of variation of about 12% (n=55). Statistical Lilliefors's test for normality with the null hypothesis, that the cumulative probability function of observed variables is normal distribution, gave probability $p>0.53$ to support the null hypothesis.

Granulocyte activity in ill subjects

We analyzed granulocyte activity, i.e., the effect of the activator at the peroxidase-catalyzed reaction and NADPH oxidase activity of granulocytes, for several groups of patients: patients with cancer, inflammatory disorder (osteo- and rheumatoid arthritis), infection (EBV, fungal infection, candidiasis, cytomegalic virus) and diabetes mellitus.

Presented in Figure 7 (p.159) is the distribution of the level of granulocyte activity, based on the initial rate in cps/min/#cells, for 55 healthy volunteers and patients with cancer, arthritis and infection.

Figure 5. Dependence of emission on cell concentration.

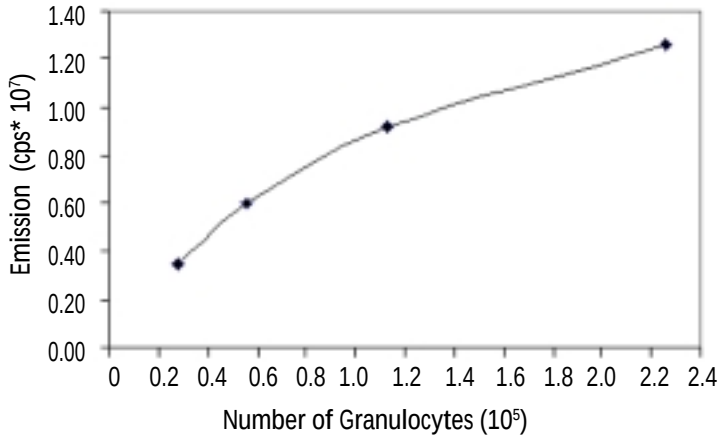


Table1. Results of granulocyte activity measurements for healthy subjects.

Age, sex	Method of separation	Number of granulocytes	Initial rate of granulocyte activity	Intensity of emission (intensity/#cells)	Concentration of H_2O_2 released by cells	Concentration of H_2O_2 per 1000 cells
28,f	pm*	53000	6.6	245.2	327.7	61.8
30,m	per**	54800	5.8	273.7	378.0	68.9
40,f	per	55000	5.3	254.5	352.9	64.1
40,f	pm	58000	5.6	241.3	352.9	60.8
41,f	pm	64000	5.1	203.1	327.7	51.2
65,f	per	46700	6.9	336.1	395.6	84.7
68,f	per	52000	5.5	211.5	277.4	53.3
45,m	per	30000	6.4	283.3	214.6	71.5
40,m	pm	52000	5.3	192.3	252.3	68.5
46,m	pm	53700	4.3	182.5	247.3	56.0
34,m	pm	49000	5.6	357.1	440.9	64.9
36,f	per	41800	5.8	263.1	277.4	66.3
41,f	per	47600	4.4	210.1	252.3	53.0
31,m	pm	47000	4.3	234.0	277.4	59.0
31,m	per	44000	5.3	245.4	272.4	61.9
34,m	pm	43500	5.9	275.8	302.6	69.5
59,m	pm	53000	4.9	264.1	352.9	66.5
32,f	per	46500	5.8	322.5	378.0	81.3
24,f	per	49500	4.9	303.0	378.0	76.4
68,m	pm	50700	5.9	286.2	365.5	72.1
62,f	pm	53700	4.9	366.4	403.8	63.8
68,m	per	52000	4.6	269.2	352.9	67.8
48,f	per	50000	5.7	320.5	403.2	68.6
63,f	pm	54000	4.7	222.2	302.6	56.0
51,f	pm	55000	5.8	254.5	352.9	64.1
57,f	per	63000	6.7	328.5	521.4	82.7
52,f	pr	58800	5.5	289.1	428.3	72.8
43,f	per	49400	5.1	222.6	277.4	56.2
52,f	pm	46700	5.4	256.9	302.6	64.8
55,f	per	59200	5.7	236.4	352.9	59.6
65,f	pm	55000	6.2	290.9	403.2	73.3
62,f	per	48000	5.6	229.1	277.4	57.8
62,f	per	36400	6.5	247.2	227.2	62.4
57,f	pm	39000	5.3	217.95	214.6	65.0
47,m	per	49800	5.7	196.7	247.3	59.6
49,f	per	61000	5.4	221.3	340.3	65.7
59,m	per	42000	5.3	211.9	224.6	63.4

*separation by Polymorphoprep

** separation by Percoll

Table 2 (p.158) shows the mean granulocyte activity for each group by two methods, the number of subjects analyzed, and the results of t-tests for differences between means of the healthy and ill groups.

The results of the statistical analysis of the level of granulocyte activity for different groups of patients demonstrated that the difference between mean values of H_2O_2 released by activated granulocytes was statistically significant for cancer patients ($p<0.05$), for patients with arthritis ($p<0.001$), patients with infection (EBV in-

Figure 6. Distribution of hydrogen peroxide released by 10000 granulocytes for healthy volunteers.

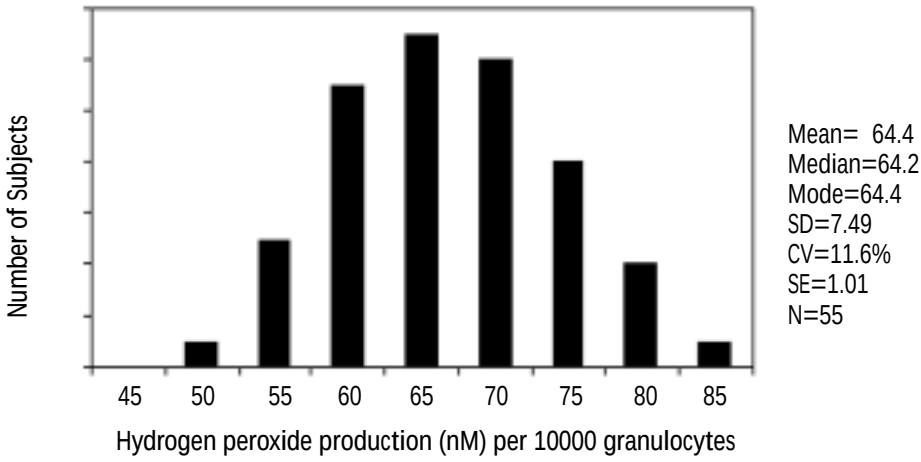
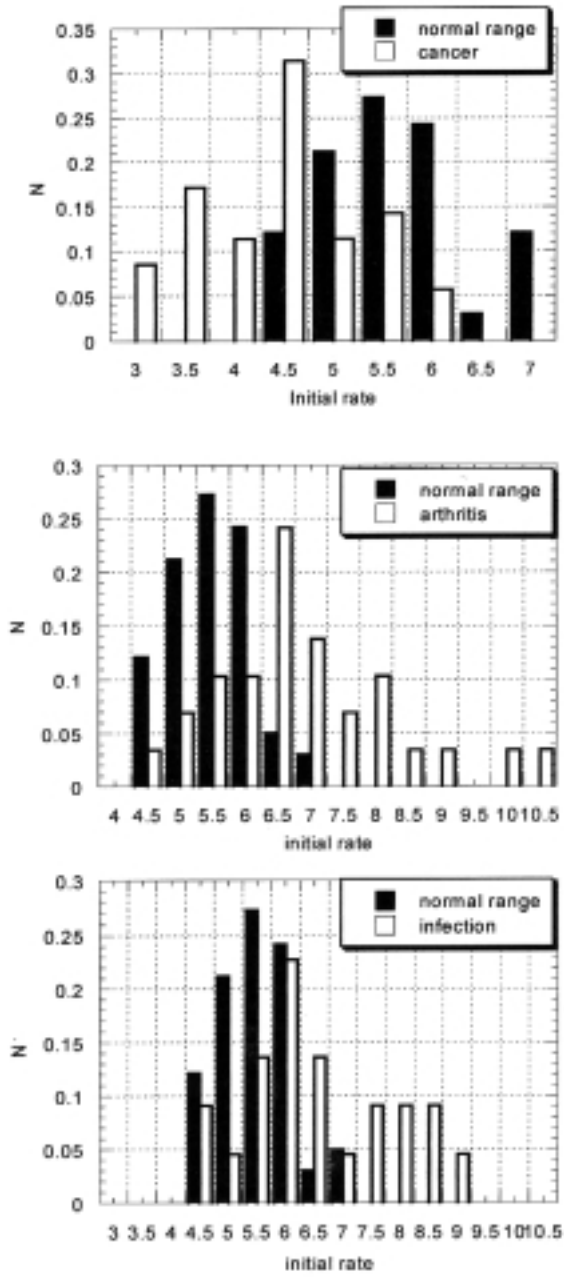


Table 2. Comparison granulocyte activity for different groups of patients.

	Healthy volunteers	Cancer	Arthritis	Infection	Diabetes
Level of H_2O_2 released after 20min of granulocyte activation					
Mean	64.4	54.9	76.1	81.4	44.4
Number of observations	55	30	33	22	5
p value		<0.05	<0.001	<0.01	>0.05
Initial rate of NADPH activity					
Mean	5.37	4.18	6.8	6.45	3.52
Number of observations	34	30	30	22	5
p value		<0.001	<0.001	<0.01	<0.01

Figure 7. Distribution of the level of granulocyte activity (initial rate of the NADPH-oxidase versus normalized number of cases) for patients with cancer, arthritis and infection.



fection, yeast, candida) ($p<0.01$). The second parameter (level of initial rate of activity of NADPH oxidase enzyme) was significant different from normal subjects for patients with cancer ($p<0.001$), patients with arthritis ($p<0.001$), patients with infection ($p<0.01$) and patient with diabetes ($p<0.01$).

The level of granulocyte activity was suppressed for patients with cancer (possibly an effect of chemotherapy or decreased energy metabolism). The data demonstrated that activated granulocytes produced less H_2O_2 and this parameter may be useful for the detection of disease activity in those patients who lie from the normal range. Decreased levels of granulocyte activity were recorded for some patients with cancer (Non-Hodgkin lymphoma, liver, lung, colon, prostate, ovarian and breast).

We also noticed that the level of metabolic activity and granulocyte activity was very low for patients with hepatitis (more than 40% lower than average normal value). Granulocyte showed the clear defect in natural immunity.

For some patients with arthritis and rheumatoid arthritis granulocyte activity was higher than in healthy subjects. The increased level of granulocyte activity may well relate to processes involved in the cause of disease and the inflammation due to the reactive oxygen intermediate molecules and other toxic molecules, which are over-produced by overly productive macrophages and neutrophils.

For some patients with viruses and fungal infection the level of NADPH activity was higher than normal range. A level of H_2O_2 production greater than 75nM was recorded for 62% of cases indicating an increased level of the immune defense mechanisms during infection.

For four patients with diabetes mellitus the level of granulocytes' activity was on 20-34% lower than average normal value for healthy volunteers.

Conclusions

The main purpose of this research was to improve the sensitivity of methods to estimate phagocytic activity. For the patients who initially lie outside the normal range, phagocytic activity may be useful parameter to monitor during and after a patient's medical treatment to ascertain improvement in health and return to normalcy.

The method described to measure the levels of granulocyte activity among different groups of patients demonstrated the sensitivity of the measurements, and demonstrated the possibility that differences in the level of granulocyte activity between different subsets of patients can be measured reproducibly.

The procedure developed showing the amount of H_2O_2 produced during phagocytosis may be used to replace the manual method for doing phagocytic index of neutrophils using *E-coli*, bacteria or yeast and NBT.

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