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Research Paper

Granulocyte activity in patients with cancer and healthy subjects

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In our study, we investigated the intracellular killing ability of granulocytes for healthy and ill subjects by measuring NADH oxidase activity and release of hydrogen peroxide. 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 counting granulocytes by flow cytometer and measurement of the kinetic curve of NADPH oxidase activity by fluorometer. Two parameters were used to describe the level of granulocyte activity: the initial rate of NADPH-oxidase enzyme activity and the level of hydrogen peroxide released after 20 min of granulocyte activation. The method was applied to measure granulocyte activity in 55 healthy subjects and 30 patients with cancer. It was demonstrated that applied procedure is sensitive for estimation of the disease activity. The granulocyte activity in patients with cancer was compared with the granulocyte activity of healthy subjects and demonstrated the downregulation of NADPH oxidase activity. We showed that granulocytes of cancer patients had inhibited oxidative burst and less NADPH oxidase activity.

Introduction

There is long-time interest in the human host mediated response to tumor growth and immunosurveillance against cancer. The concept of immunosurveillence against tumors was introduced in 1970 by Burnet,[1] where it was suggested that immunodeficient individuals or those being treated with immunosuppressive drugs would have an increased incidence of cancer.

Many researchers have carried in vitro and in vivo studies to support the significance of host defense against cancer or its metastasis (review see refs. 2 and 3). The studies demonstrated a decreased level of NK-activity in individuals with a high familial incidence of cancer and the clinical significance of NK activity.[4] The prospective cohort study among a Japanese general population showed a significant role of several naturally cytotoxic lymphocytes in preventing the development of cancer.[5]

In recent years, there is interest in a larger population of immune cells, granulocytes, which can kill microorganisms and tumor cells. In studies by Cui[6,7] it was shown that the granulocyte effectiveness to kill tumor cells varies from person to person. In addition, this study demonstrated that it is a possible to treat a range of different cancers in mice by injecting them with granulocytes from a strain of mice that are completely resistant to cancer.

The goal of our study was to find the intracellular killing ability of granulocytes in cancer patients and to compare this parameter with granulocytes of healthy volunteers. Decreased capacity of the host defense of granulocytes can be associated with some specific cellular abnormalities. The abnormalities can be found in the ability of migration of phagocytes, attachment to cell surface, phagocytosis and intracellular killing. We analyzed the ability of phagocytes to kill microorganisms or tumor cells by oxidative or peroxidative attack. 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.[8] 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.[9-14]

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 allowed us 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 the nitroblue tetrazolium dye reduction (NBT) test, which does not measure a distinct cell function and indicates the metabolic state of these cells, bacterial uptake by phagocytes and enzyme assay for selected enzymes of hexose monophosphate shunt. Other methods made use of the fluorescent dyes such as scopoletin with high background emission.

We used the fluorescent dye Amplex Red (AR), which has high sensitivity.[15] This method was developed and applied to measure the level of granulocyte activity in healthy subjects and patients with

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Table 1 **Examples of the parameters of granulocyte activity for cancer patients**

Diagnosis	Number of measured granulocytes	Intensity of maximum emission (cps)	Total amount of H_2O_2 released by cells (nM)	H_2O_2 released by 10000 cells (nM)	Initial rate of granulocyte activity (cps/min/cell)
Prostate cancer	22300	9.87E+05	72.37	32.45	3.3
Colon cancer	25000	2.00E+06	148.88	59.55	3.5
Liver cancer, hepatitis C	29000	2.40E+06	179.09	61.76	5.48
Liver cancer	34600	6.00E+06	151.02	43.65	4.15
Prostate cancer	41000	2.30E+06	171.54	41.84	3.88
Breast cancer with bone metastasis	43800	3.00E+06	224.42	51.24	3.77
Breast cancer	45000	4.20E+06	315.06	70.01	4.3
Breast cancer with bone and liver metastasis	45400	2.20E+06	163.99	36.12	2.36
Breast cancer	47800	4.50E+06	337.72	70.65	4.69
Ovarian cancer with invasion to the uterus and cervix	48000	3.30E+06	247.08	51.47	4.04
Colon cancer with liver metastasis	50000	2.70E+06	201.75	40.35	4.25
Non-Hodgkin's type lymphoma	50500	3.33E+06	249.34	49.37	3.9
Prostate cancer	50600	3.20E+06	239.52	47.34	4.08
Ovarian cancer with invasion to uterus and cervix	52500	3.50E+06	262.18	49.94	4.4
Prostate cancer	54000	4.00E+06	299.95	55.55	5.07
Breast cancer	54500	4.00E+06	299.95	55.04	4.5
Breast cancer	54800	4.25E+06	318.83	58.18	4.49
Ovarian cancer	55000	6.20E+06	466.12	84.75	6
Lung cancer with bone metastasis	57700	3.20E+06	239.52	41.51	3.5
Prostate cancer	60600	5.30E+06	398.14	65.70	5.07

cancer and demonstrated that the applied procedure is sensitive for estimation of the downregulation of NADPH oxidase activity.

Results

In this study, we analyzed the differences in phagocytic activity of cells for cancer patients and healthy volunteers. Fifty-five healthy volunteers (controls without known cancer, arthritis, diabetes or infection) and volunteer patients (30 patients with cancer) were employed in this study. The cancer patients were in various stages of the disease. Prostate cancer patients had a Gleason score of 8; breast cancer patients were mostly stage 3, while non-Hodgkin's lymphoma patients were in stage 3 and 4. Other types of cancers were in late stages with metastasis to various organs.

The distribution of the level of granulocyte activity for healthy volunteers is presented in Figure 5. The granulocytes' activity for healthy volunteers is approximately normally distributed with a coefficient of variation 12% (n = 55). Statistical Lilliefors's test for normality with the null hypothesis, that the cumulative probability function of observed variables is normally distributed, supported the null hypothesis.

Part of the data for the cancer patients is shown in Table 1. This includes the number of cells in the 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 the working solution, the intensity of emission, 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 granulocyte activity or the effect of the activator at the peroxidase-catalyzed reaction and NADPH oxidase activity of granulocytes was compared for healthy subjects and for patients with cancer. The parameters of granulocyte activity measured by two methods were compared by t-test for differences between means of the healthy and ill groups. For cancer patients the mean level of hydrogen peroxide released after 20 min of activation was 54.9 nM and average initial rate of NADPH activity was 4.18 cps/min/cell. For healthy volunteers the same values were 64.4 nM and 5.37 cps/min/cell.

The results of the statistical analysis of the level of granulocyte activity for groups of cancer patients and healthy volunteers demonstrated that the difference between mean values of H_2O_2 released by activated granulocytes and the levels of the initial rate of NADPH oxidase activity were statistically significant ($p < 0.05$ for mean released level of hydrogen peroxide and $p < 0.001$ for initial rate of NADPH activity).

Examples of the kinetic curves of emission after releasing hydrogen peroxide after activation of granulocytes for a healthy volunteer and two cancer patients are shown in Figure 6.

Distributions of the normal range of NADPH oxidase activity and measured levels of granulocyte activity in cancer patients are shown in Figures 7 and 8. In Figure 7 granulocyte activation for cancer patients and healthy volunteers is compared, which was calculated as the slope of fluorescence emission intensity at linear range normalized on the number of cells. Presented in Figure 8 the distributions of the level of granulocyte activity for healthy volunteers and patients with cancer are based on the level of hydrogen peroxide released by

10,000 granulocytes after activation. According to these data, the level of NADPH oxidase activity was suppressed in patients with cancer. Decreased levels of granulocyte activity were recorded for patients with cancers of the liver, lung, colon, prostate, ovaries, breast and with Non-Hodgkin's lymphoma. The reduced phagocytosis of granulocytes for cancer patients may be responsible for an increase in these patients being prone to microbial infection.

Our data demonstrated that activated granulocytes of cancer patients produced less H_2O_2, and this parameter may be useful for the detection of disease activity.

Discussion

The main purpose of this research was to demonstrate the impaired function of granulocytes in cancer patients. As the result of our study, we showed that granulocytes of cancer patients had inhibited oxidative burst and less NADPH oxidase activity.

The method described to measure the levels of granulocyte activity in the patients and healthy volunteers demonstrated the sensitivity of the measurements, and the possibility that differences in the level of granulocyte activity in cancer patients can be measured reproducibly.

According to our data, granulocytes from patients with cancer failed to produce the significant oxidative burst following stimulation. Average values for H_2O_2 released by 10000 granulocytes was 64 ± 11 nM for healthy subjects age 50 and older and 54 ± 9 nM for cancer patients in the same age range. The measured amount of hydrogen peroxide produced by granulocytes ranged from 32 nM to 80 nM for cancer patients and from 50 nM to 85 nM for healthy subjects.

We analyzed several possibilities the decreased efficiency of granulocytes in cancer patients. The reason of decreased granulocyte activity may be age, as aged phagocytes show decreased cytotoxicity against tumor cells, decreased ability to destroy pathogens and, possibly, impaired respiratory burst. As the immune system deteriorates with age, it may contribute to increased morbidity to infections, autoimmune disease and cancer. Our experiments with cell mediated cytotoxicity (data are not included), demonstrated the decreased efficiency of granulocytes against leukemia tumor cells HL-60, as the donor's age increased. Analysis was performed by Calcein-AM assay and ATP-based method for participants without cancer and other pathological conditions and showed decrease in cytotoxicity on 20%–30% for subjects older than 60 years in comparison with younger participants in 20–30 years of age.

However, analysis of the data of NADPH oxidase activity demonstrated that there was no significant changes with age of the hydrogen peroxide production by activated granulocytes for subjects without pathological conditions and the change in the granulocyte function cannot be explain by declining NADPH oxidase activity with age.

Another explanation may be that tumor growth may have influence on the NADPH oxidase or may change the activity of enzymes that regulate the granulocyte respiratory burst. The decreased granulocyte activity of patients with cancer may be the result of the treatment such as chemotherapy or radiation or the result of the cellular abnormalities of granulocytes.

In addition, we found that several patients with cancer of liver, prostate and breast had very abnormal levels of granulocyte NADPH oxidase activity (less than 40 nM) and had short overall survival

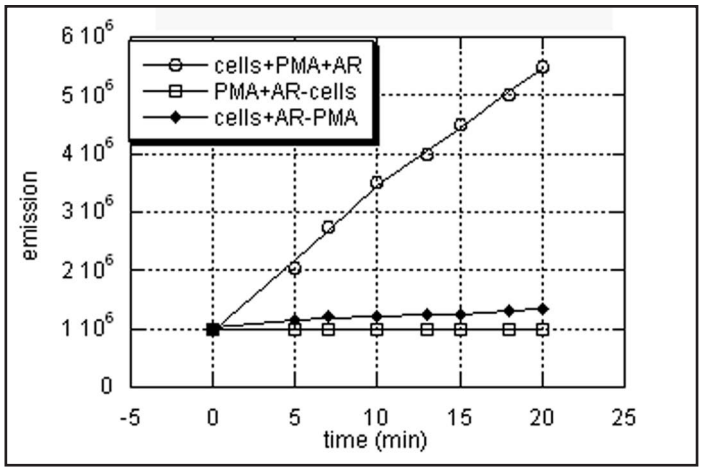

Figure 1. Fluorescence emission of activated granulocytes (cells with addition of PMA and Amplex Red), non-activated granulocytes (cells in reaction mixture with AR without addition of PMA) and background emission (reaction mixture with PMA and AR without cells).

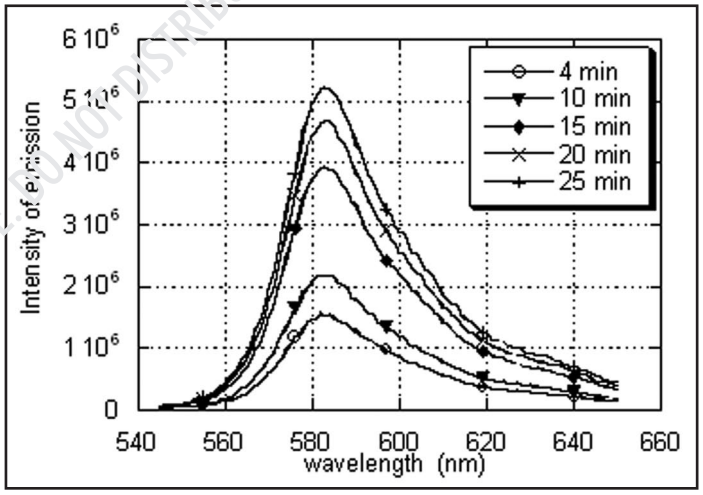

Figure 2. Time dependence of fluorescence emission after granulocyte activation.

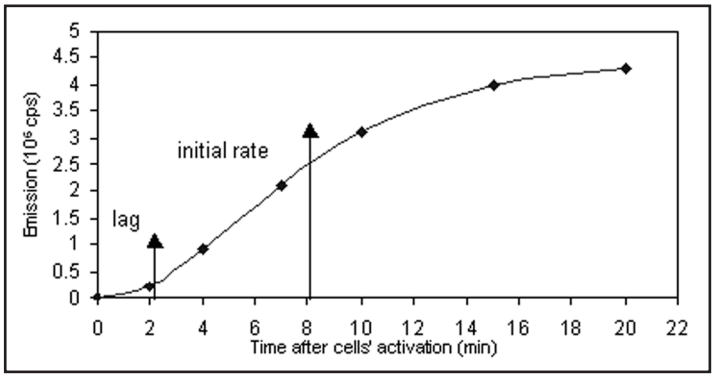

Figure 3. Time course of the change in fluorescence emission at 583 nm in response to cells' activation.

(less than year). This may indicate that the function of NADPH oxidase decreases as the disease progressed.

As the result of our research, we found that granulocyte activity is reduced in cancer patients compared with healthy subjects. Detection of the NADPH-depended production of active oxygen could be useful as a prognostic factor and as a factor of developing infection in cancer patients. For the patients who initially lie outside the normal range, phagocytic activity may be a useful parameter to monitor during and after a patient's medical treatment to ascertain improvement in health and return to normalcy.

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.[15,16] We modified the published method by using fluorometer measurements coupled with the counting of granulocytes 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 highly fluorescent product, resorufin, after enzyme-catalyzed oxidation of Amplex Red by H_2O_2 released from activated neutrophils. Granulocytes were stimulated with phorbol myristate acetate (PMA), a phorbol ester that directly activates protein kinase C. PMA is widely used in signal transduction studies. In granulocytes, it gives rise to a redistribution of protein kinase C, phosphorylation of the number of different proteins, and activation of phagocyte NADPH oxidase.[17,18] Emission of resorufin was measured by SPEX spectrofluorometer (double-grating spectrophotometer, sensitivity 4000:1). The excitation wavelength was 530 nm and the fluorescence emission 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 monitored during a 20-minute period with intervals of 2–3 min.

Separation Procedure

For the separation of granulocytes (polymorphonuclear leukocytes), 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) whole blood was treated with an anticoagulant (heparin, EDTA or citrate). Four mL of blood were layered over 4 mL of Polymorphoprep 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), layered on top of discontinuous percoll gradients with densities 1.1 and 1.077 g/mL and centrifuged at 600 g for 25 min.[19] Erythrocytes were removed by hypotonic red cell buffer (0.83% w/v NH_4Cl) that hemolysed the erythrocytes.

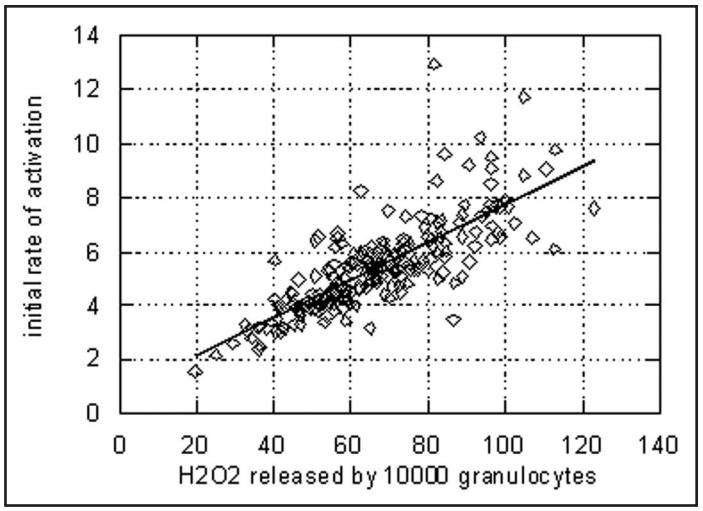

Figure 4. Correlation between two parameters of granulocyte activity: the initial rate of activation and the level of hydrogen peroxide released by 10000 granulocytes.

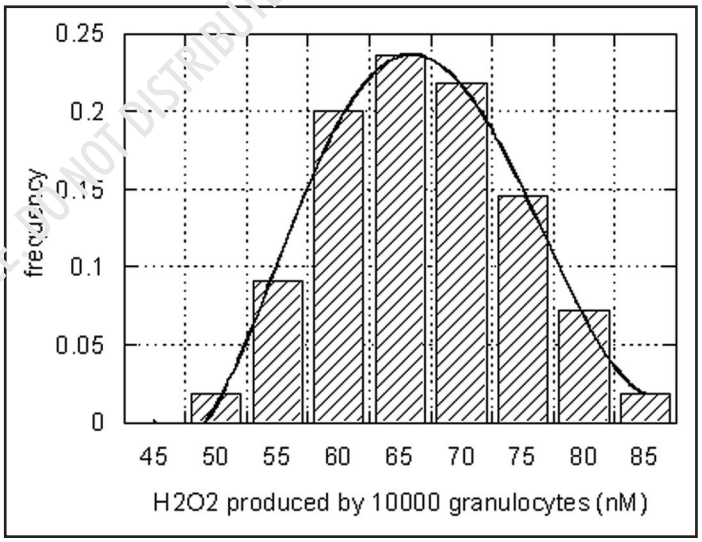

Figure 5. Distribution of the level of hydrogen peroxide production by 10000 granulocytes for healthy subjects.

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, which may be engulfed by granulocytes 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 cells 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 the range of (mean ± SD) 63.1 ± 10.3 nM for cells separated by Percoll and 65.2 ± 9.6 nM for cells separated by Polymorphoprep.

Count of Granulocytes 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. For immuno-fluorescence assay, 10^5 cells were resuspended in PBS with 1% bovine serum albumin and stained by antibody-CD15. Cells with a green tag were easily separated and counted.

Granulocyte Activity Assay

The stock solution of Amplex Red was prepared by dissolving 1 mg of Amplex Red in 200 µL of DMSO. For the reaction mixture, 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 on the level of fluorescent emission. The linear range of the reaction product, i.e., the linear range of the emission of resorufin and dependence on concentration, was established. 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 25 µL Amplex Red, 0.1 U/mL horseradish peroxidase (dissolved in PBS), 200 ng/mL phorbol 12-myristate 13-acetate (PMA), which was used as a stimulating agent, and 5.5 mM glucose in PBS. The properties of the reaction mixture were analyzed for spontaneous oxidation under the same conditions as for actual measurements, but without the addition of cells, and for the level of emission of non-stimulated cells. Results of the analysis are presented in Figure 1.

According to these data, the increase in intensities of the baseline emission after 20 min of measurements was in the range of 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. The emission curves for cells stimulated by PMA and for cells in the reaction mixture without the addition of PMA are also shown in Figure 1.

These experiments demonstrated that the effect of an increased level of fluorescence emission occurs only in the response to cells activation. The level of emission due to oxidant production by 0.05 million cells after 20 min of activation by PMA was 5.5 times higher than the initial emission level. For resting granulocytes in the reaction mixture without the addition of PMA the emission was increased only 1.3 times.

To determine the level of NADPH oxidase activity, 0.04–0.06 million granulocytes were added into 1 mL of working solution (25 µM Amplex Red, 0.1 U/mL of HRP, 200 ng/mL PMA) and the fluorescence curve was measured during 20–30 min with 3–5 min intervals between measurements. Figure 2 shows the change in the fluorescence of the reaction mixture due to the release of hydrogen peroxide by activated granulocytes at different times after the cells' activation.

For each measured curve, 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 in Figure 3. Figure 3 shows that there are several phases at the kinetic curve during the reaction. Immediately after the

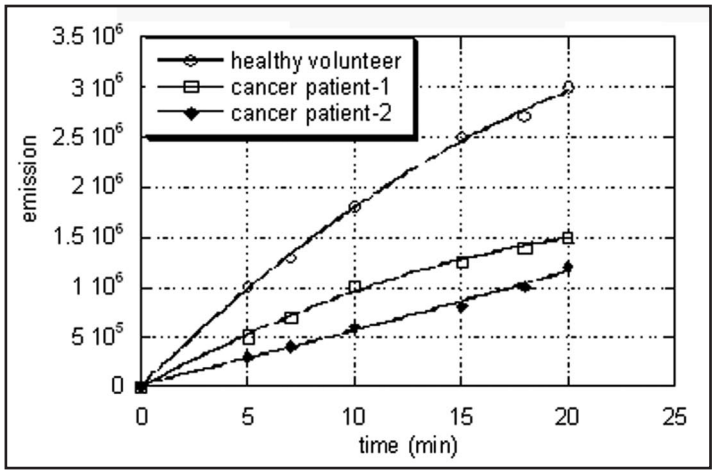

Figure 6. Comparison of the NADPH oxidase activity for healthy volunteer and patients with cancer.

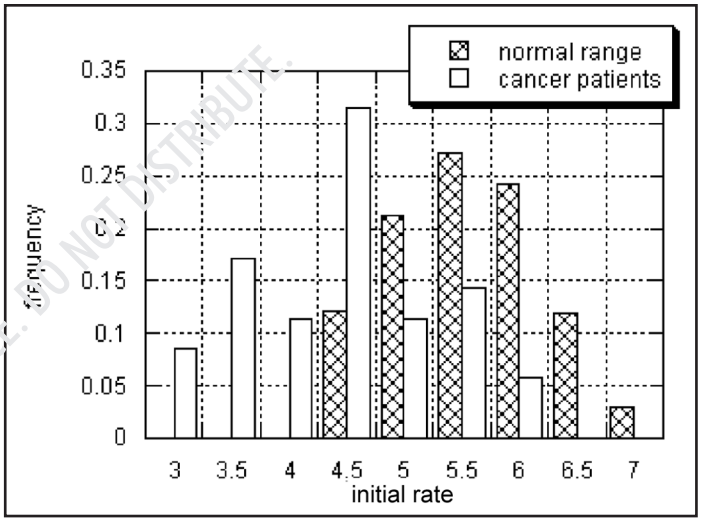

Figure 7. Comparison of the distributions of the initial rate of NADPH oxidase activity for cancer patients and healthy subjects.

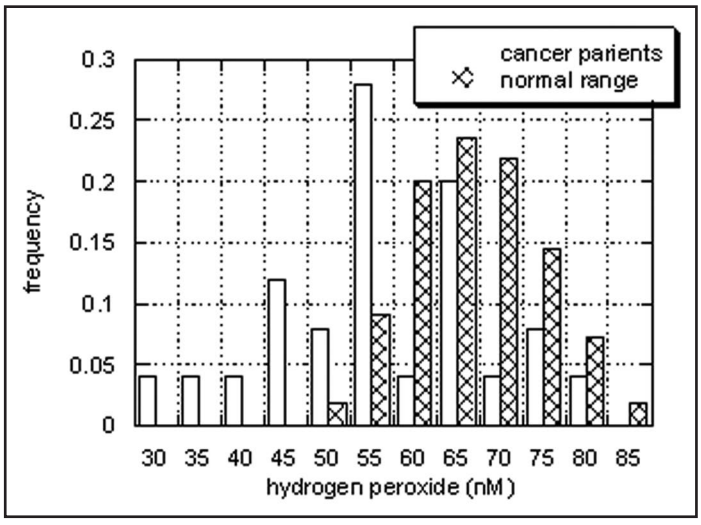

Figure 8. Comparison of the distributions of the level of hydrogen peroxide released by granulocytes for cancer patients and healthy subjects.

addition of cells to the working solution there is a period of equilibration during the 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 the cells after 20 min of activation. The initial rate of activation was calculated as the ratio of the difference of fluorescence emission in counts per seconds (cps) at the beginning and the end of linear range to the duration of linear range (scope of curve in linear range) to the number of cells and presented in units cps/min/cell.

In addition, two important quantitative relations were established for estimation of the NADPH oxidase activity from emission measurements: the linear range of the cells' response to the activator and the standard curve for the conversion of emission measurements to the concentration of H_2O_2 released by the 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 the 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 0.9.

For our experimental protocol, linear range of emission was found for concentration of cells less than 0.2 million per working solutions. All experiments were performed in the linear response in the range of 0.02–0.2 million granulocytes. Correlation between two parameters, which were used to describe the level of NADPH oxidase activity, is shown in Figure 4 (coefficient of correlation = 0.78).

To estimate the reproducibility of this method, each sample of blood was replicated two or three times using the same 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 granulocytes added in optimal range of concentrations was in the range of 2%–30%.

Acknowledgements

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