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Flow-cytometric characterization of stimulation, free radical formation, peroxidase activity and phagocytosis of human granulocytes with 2,7-dichlorofluorescein (DCF)

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A standardized four-step assay for the flow cytometric determination of the oxidative activity of human polymorphonuclear leukocytes (PMNL) from normal human individuals and from septic patients was developed, using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator for the intracellular formation of H_2O_2 and free radicals. Spontaneous H_2O_2 and free radical formation was measured by preincubation of buffy coat PMNLs from fresh peripheral venous blood at 37 °C and *p*H 7.4 with 10 μM DCFH-DA. Intracellular peroxidase activity was determined by addition of 1 mM external H_2O_2 to this assay. A maximum of granulocyte oxidative burst activity was elicited by the addition of 150 nM phorbol-myristate-acetate (PMA). A physiological burst was generated by incubating buffy coat PMNLs together with E. coli bacteria. The DNA of dead cells was in all instances simultaneously counterstained with propidium iodide (PI).

Quiescent or H_2O_2 or bacteria treated granulocytes moved as a single cell cluster to higher fluorescences. Stimulation with PMA, in contrast, generated always a bimodal distribution of granulocyte fluorescence with the high activity cell cluster being approximately sevenfold more active than the low activity cell cluster. Roughly half of the granulocytes in normal individuals had high fluorescence. An increase of the high activity granulocytes was observed in septic patients.

Model experiments with the nonfluorescent DCFH-DA cleavage product DCFH (2,7-dichlorofluorescin) showed that DCFH was quickly photo-oxidized to fluorescent DCF (2,7-dichlorofluorescein) by UV-light and to a lower degree by daylight. DCFH even slowly autooxidized in the dark. As a consequence, DCFH-DA was dissolved and stored in organic solvents in the dark to prevent spontaneous hydrolysis and autooxidation. All cellular assays prior to the flow-cytometric measurements were stored and incubated strictly in the dark.

Introduction

Phagocytosis of polymorphonuclear leukocytes (PMNL) induces a burst of CN^--insensitive oxidative metabolism [18]. The burst is believed to be due to a membrane-bound pyridine-nucleotide-dependent oxidase which reduces oxygen to O_2^- [6, 10]. Subsequently, the oxygen products: peroxide, hydroxyl radicals, singlet oxygen and hypochloric acid which is formed from chloride through myeloperoxidase activity are associated with the bactericidal activity of polymorphonuclear leukocytes (PMNL) [15].

Several methods have been described to monitor the metabolic burst activity in PMNL using luminol-dependent chemiluminescence assays [1] or the reduction of nitroblue tetrazolium [5] and cytochrome C by superoxide [17]. The main disadvantage of these methods is that the mean value of all granulocytes is measured and possible heterogeneities in the granulocyte population remain undetected.

2,7-Dichlorofluorescin-diacetate (DCFH-DA) has been used for the fluorimetric determination of peroxide concentration in solution [7, 14]. It has more recently been introduced into flow-cytometry as a metabolic burst indicator for single H_2O_2-producing vital PMNLs [3]. DCFH-DA diffuses through the cell membrane, is hydrolyzed by cellular esterases to nonfluorescent, polar 2,7-dichlorofluorescin (DCFH) which remains trapped in vital cells. Intracellular oxidation of DCFH during a metabolic burst results in the formation of highly fluorescent dichlorofluorescein (DCF) [3].

Although DCFH-DA is a very useful reagent, difficulties were encountered in obtaining stable results with vital cell preparations. The purpose of our study was to investigate the conditions of stability of the cellular reaction and to develop a standardized assay protocol which would extract a maximum of information on the functional activity of PMNLs.

Materials and methods

Reagents

Phorbol-myristate-acetate (PMA, MW: 662), propidium-iodide (PI, MW: 668), bovine catalase, bovine superoxide-dismutase (SOD) and bovine carboxylic-ester hydrolase were purchased from Sigma-Chemicals (Taufkirchen/FRG). 2,7-Dichlorofluorescin-diacetate (DCFH-DA, MW: 485) and horseradish peroxidase (PO) were obtained from Serva (Heidelberg/FRG). 5 mM HEPES-buffered saline, *p*H 7.4 (HBS) was used for incubations and cell washings. DCFH-DA and PMA were dissolved in dimethyl-formamide (DMF) at concentrations of 4.85 mg/ml and 1 mg/ml, respectively. PI was dissolved at 2 mg/ml in HBS-buffer. H_2O_2 Solutions (100 mM were freshly made by diluting a 30% stock solution (Merck, Darmstadt/FRG) 100-fold with HBS.

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Leukocytes, bacteria and cell staining

Ten ml heparinized blood (10 IU/ml) were obtained from healthy donors by venipuncture and adjusted to *p*H 7.35 by addition of 100 µl 1N HCl. After 10 min centrifugation at 120*g*, 2 ml of supernatant plasma followed by 300 µl of leukocyte-rich buffy coat were carefully removed in separate tubes. The buffy coat sample was diluted with autologous plasma until a cell concentration of leukocytes of 1×10^7 cells/ml was reached. Fifty µl buffy coat, 5 ml HBS and 50 µl of a 1 mM DCFH-DA solution (fresh 1/10 dilution with HBS of the DCFH-stock solution in DMF) were preincubated for 15 min at 37 °C in a waterbath. The assay was then split into 4 aliquots of 1 ml. Ten µl of a 100 mM H_2O_2 solution, a 15 µM PMA solution, a 3.2×10^9 E. coli bacterial/ml suspension in HBS or of HBS alone were added and each assay was further incubated at 37 °C. Portions (250 µl) from the assays were taken at various time intervals and 5 µl of a 2 mg/ml propidium iodide solution in HBS were added to stain the DNA of dead cells during 5 min at room temperature. K12 strain E. coli bacteria were grown in 10 mM HEPES buffered RPMI medium at 37 °C in air with 5% CO_2. Five ml bacteria suspension of a 72-h old culture were washed twice in HBS by centrifugation. The pellet was resuspended in 250 µl HBS and diluted to an $OD_{350nm} = 0.1$ extinction units which corresponded to 3.21×10^9 bacteria/ml.

DCFH-DA was hydrolyzed for some experiments in the following way. Thirty-five µl of a 0.1N NaOH solution were added to 15 µl of 10 µM DCFH-DA solution in HBS and incubated for 2 min at 22 °C to obtain complete hydrolysis of DCFH-DA. The solution was neutralized by addition of 2950 µl HBS, *p*H 7.4 containing 10 mM HEPES. Alternatively, 15 µl of a 1 mM DCFH-DA solution in HBS were incubated for 20 min at 22 °C with 10 µl of a 1600 U/ml solution of carboxylic esterase in HBS. The enzymatic hydrolysis was stopped by adding 2975 µl HBS and by decreasing the temperature to 0 °C. This solution was 100-fold diluted with HBS for fluorimetric measurements.

Flow-cytometry

The electrical cell volume and the green and red fluorescence of each cell were simultaneously measured in a Fluvo-Metricell flow-cytometer [13] (HEKA, Lambrecht/Pfalz/FRG) using a cylindrical orifice of 80 µm diameter and 85 µm length with hydrodynamic focusing of the cells through the center of the orifice. HBS-buffer was used as sheath fluid. The electrical current through the orifice was 0.15mA. Fluorescence was excited by a HBO-100 high pressure mercury arc lamp between 400 and 500 nm. The green DCF-fluorescence was collected between 500 and 530 nm and the red PI-fluorescence between 550 and 700 nm. The maximum amplitude of the logarithmically amplified volume and fluorescence pulses of each cell were digitized with a resolution of 128 steps and stored on magnetic tape. Data evaluation was performed by Fortran computer programs developed earlier [23].

All measurements were standardized with 5 µm monosized and porous latex particles [22], substituted with free NH_2-groups (Paesel, Frankfurt/FRG). The particles were rendered fluorescent by incubation for 2 h at *p*H 9.5 in a 50 mM $NaHCO_3$-buffer with 0.25 mg/ml of fluorescein-isothiocyanate (FITC) followed by three washing steps with HBS.

Fluorescence spectroscopy

Fluorimetric measurements were made with a fluorescence-spectrometer LS-5 (Perkin-Elmer, Norwalk/USA) mounted on-line to a VAX 11/782 computer. Spectra were measured in HBS-buffer, *p*H 7.4. Slit setting was 5 nm or 10 nm for excitation and 5 nm for emission. DCF-fluorescence was excited at 501 nm and the emission was measured at 521 nm in quartz cuvettes with 10 mm light-path.

Results

The examination of DCFH-DA stained human leukocytes under the fluorescence microscope revealed a rapid and uniform increase in cellular fluorescence on exposure to ultraviolet light. It was investigated whether the fluorescence increase was due to photo-oxidation. Nonfluorescent DCFH was prepared for this purpose from a DCFH-DA solution either by alkaline hydrolysis or through cleavage by carboxylic esterase. The DCFH-solutions (50 nM thus obtained were exposed to UV-light, to daylight or kept in the dark. UV-irradiation lead quickly to a significant increase of fluorescence (Fig. 1). The fluorescence was determined by exciting the solution at 501 nm in the fluorimeter and by measuring the fluorescence emission at 520 nm. The fluorescence increase was independent of the presence of oxygen since oxygen removal from the solution by continuous insufflation of helium gas did not significantly influence the development of fluorescence. The spectrum of the newly generated fluorescence was superimposable to the DCF-fluorescence spectrum. The results favor the conclusion that fluorescence is generated by photo-oxidation of DCFH to DCF.

Besides photo-oxidation, DCFH is also converted to DCF by oxidation with H_2O_2. It was investigated whether the oxidation was influenced by the presence of enzymes such as catalase, superoxide dismutase or peroxidase. The enzymes at a concentration of 5 U/ml were incubated in the dark at 37 °C in HBS with 50 nM DCFH and 1 mM H_2O_2. The fluorescence of aliquots of each assay was determined at various times in the fluorimeter. Peroxidase generated substantially higher fluorescences than catalase, superoxide dismutase or H_2O_2 alone (Fig. 2). A 400-fold

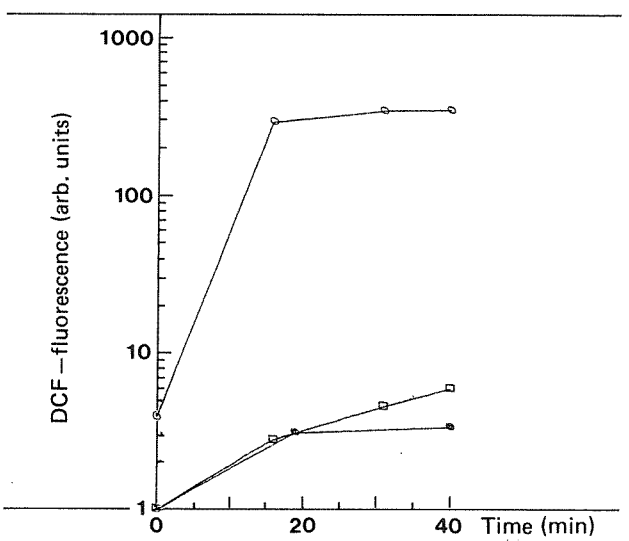

Fig. 1. Oxidation of non-fluorescent DCFH to fluorescent DCF caused by irradiation with 260 nm UV-light (○) or daylight (□) and by storage in the dark (●). DCFH at a concentration of 50 nM in HBS-buffer, *p*H 7.4 was filled into quartz cuvettes for the irradiation with light. Fluorescence was measured at 521 nm in the fluorimeter following excitation at 501 nm and using slit settings of 5 nm for excitation and emission.

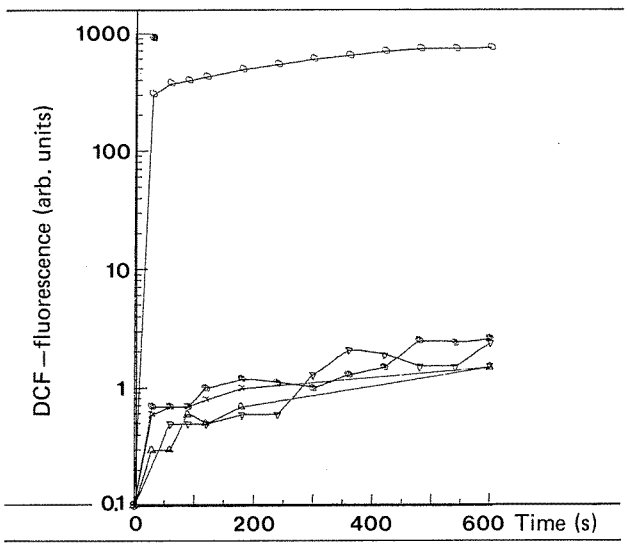

Fig. 2. Oxidation of non-fluorescent DCFH to fluorescent DCF by 0.1 mM H_2O_2 in HBS-buffer, *p*H 7.4 (•) or together with peroxidase (○), catalase (×) and superoxide-dismutase (△). DCFH-concentration was 50 nM and 5 U/ml of the respective enzyme was present in the assay. DCFH was quantitatively converted to DCF by peroxidases since the same levels of fluorescence were reached as in a 50 nM DCF control solution (*black dot, top left*). The other enzymes did not accelerate the conversion above the DCFH control assay without enzyme (▽). The experiments were performed in HBS-buffer, *p*H 7.4 at 22 °C. The slit setting of the fluorimeter was 10 nm for excitation and 5 nm for emission.

higher level of fluorescence was reached after 10 min of incubation in the peroxidase assay as compared to incubation with H_2O_2 alone. The comparison with the fluorescence of an equimolar DCF solution showed that all available DCFH was transformed to fluorescent DCF.

In several experiments with cells, the incubation time, temperature, and DCFH-DA concentration were optimized. Incubation with 5 μM DCFH-DA at 0, 20 and 37 °C showed that 15 min at 37 °C were sufficient to develop stable levels of fluorescence. Cells were also incubated at 37 °C in the presence of 1, 5 or 10 μM DCFH-DA. At 5 μM DCFH-DA concentration, saturation of the cellular fluorescence was reached within 15 min of incubation. Cellular vitality as determined from the number of PI-stained cells of the assays (Figs. 3a, b) was >95% at all three DCFH-DA concentrations over a period of 45 min at 37 °C. Based on these experiments a 15-min preincubation of cells with 10 μM DCFH-DA at 37 °C, followed by the addition of H_2O_2, PMA or bacteria to the assay and by 15 or 30 min further incubation at 37 °C were chosen for further experiments. These assays permitted to measure the spontaneous generation for H_2O_2 or free radicals by cells (Fig. 4a), the peroxidase activity in the presence of a high concentration of diffusible external H_2O_2 (Fig. 4b), the stimulation of metabolic burst activity by PMA (Fig. 4c) and the response of cells to externally offered E. coli bacteria (Fig. 4d). Granulocytes were easily distinguishable from lymphocytes, erythrocytes, cell debris and dead cells by their larger cell volume (Figs. 4a–d). More than 95% of the lymphocytes and granulocytes were vital in all experiments

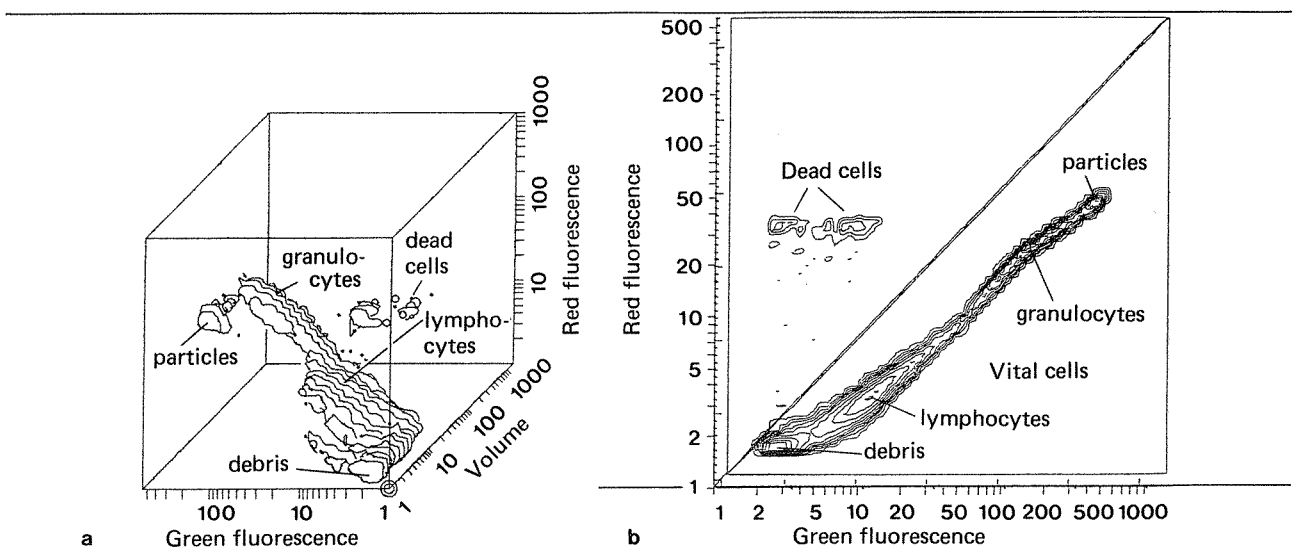

Fig. 3. Cell volume, green and red fluorescence of peripheral human leukocytes (**a**) which had been preincubated for 15 min at 37 °C with 10 μM DCFH-DA followed by addition of 1 mM H_2O_2 and further 15 min incubation at 37 °C to obtain the intracellular peroxidase activity of vital cells. The cells were simultaneously incubated with 60 μM PI to measure the DNA-content of dead cells. The data of the cube display of (**a**) were projected onto the red and green fluorescence plane and are displayed in (**b**) to show the discrimination between vital and dead cells. Granulocytes, lymphocytes, dead cells, calibration particles and cell debris (debris) are distinguishable in both graphs. A total of 11710 cells and particles were measured. The cube graph (**a**) is standardized to the maximum logarithmic channel content as 100% value (450 cells). Contour lines are plotted which connect cube channels at 10% of this value. The contour lines display the position of more than 95% of all cells or particles (n = 43750). The matrix plot (**b**) is also standardized to the maximum logarithmic channel content as 100%. Contour lines are plotted in linear decrements of 10%.

(Fig. 3b). Granulocytes showed a high degree of peroxide and free radical formation, when stimulated by 150 nM PMA (Fig. 4c) and to a lower degree by 3.2×10^7/ml E. coli bacteria (Fig. 4d) and exhibited a small spontaneous H_2O_2 and free radical formation when being unstimulated (Fig. 4a). Lymphocytes had peroxidase activity (Fig. 4b) but could not be stimulated by PMA or bacteria. No fluorescence was associated with erythrocytes or cell debris. It was unexpected that stimulation with PMA yielded two distinct granulocyte clusters. The low reactivity granulocyte cluster remained at the level of unstimulated control cells (Figs. 4a, c) while the high activity cluster had approximately a seven-fold greater activity (Fig. 4c). In 5 normal adult male and 5 female persons, between 45 to 55% of the granulocytes could be highly stimulated. There were no apparent sex differences. More than 95% of the granulocytes were neutrophils on morphological examination of May-Grünwald-Giemsa stained samples. No correlation between morphological features of May-Grünwald-Giemsa stained smears of the leukocyte preparations and the biochemically revealed heterogeneity of normal granulocytes was found. Values between 60 and 85% of high activity granulocytes were obtained in the blood of 5 septic patients.

Non-fluorescent DCFH was slowly converted into fluorescent DCF by H_2O_2 alone but at a much accelerated rate was observed in the presence of peroxidase (Fig. 2). It was the question whether DCFH oxidation under intracellular conditions was mainly due to the slow direct action of H_2O_2 on DCFH or to the indirect reaction via intracellular

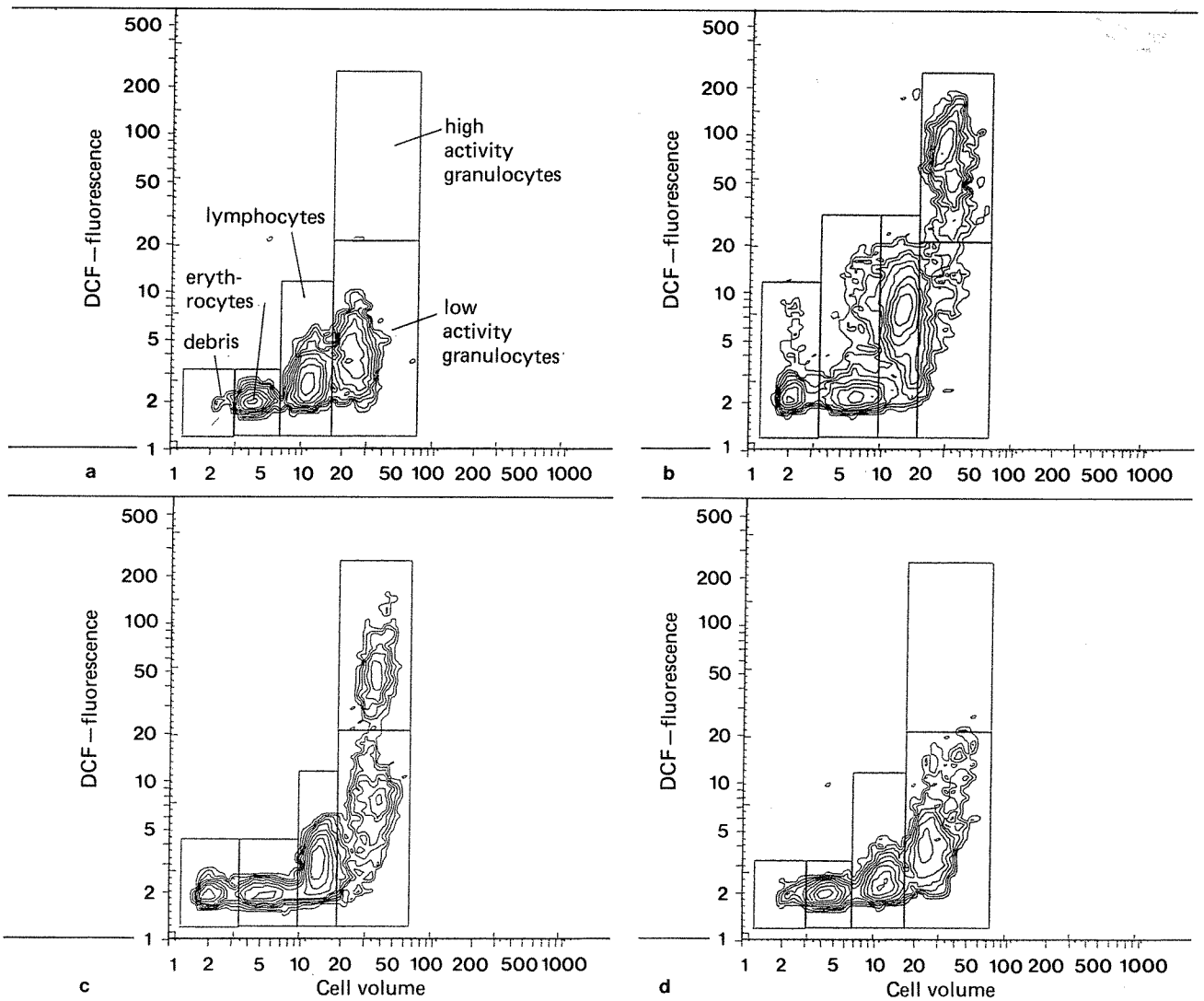

Fig. 4. Cell volume versus DCF-fluorescence of human buffy coat cells incubated during 15 min at 37 °C with 10 μM DCFH-DA, followed by a second incubation of 30 min at 37 °C in HBS alone (**a**), or with addition of 1 mM H_2O_2 (**b**), 150 nM PMA (**c**) or 3.2×10^7 E. coli bacteria/ml (**d**). Only the position of the vital cells (Fig. 3b) of each assay (>95%) is indicated in the above graphs. 4860, 11350, 7690 and 4340 cells were measured per graph. The standardization and display was as indicated in the legend of Figure 3. One logarithmic cell volume class corresponds to a cell volume of 20 μm^3.

peroxidases. If the second mechanism is operative, one would expect a quick conversion of most of the intracellular DCFH. It was investigated how much of the intracellular DCFH was indeed converted into fluorescent DCF. Granulocytes from 10 ml heparinized fresh blood were separated from lymphocytes by centrifugation through 1.077 g/ml ficoll/hypaque. The cell sediment was resuspended in 1 ml HBS and erythrocytes were lysed by addition of 6 ml of a 0.84% NH_4Cl-solution during 20 min at 22 °C. The granulocytes were washed twice by centrifugation with HBS and resuspended at a concentration of 2.4×10^6 cells/ml in HBS. One ml cell suspension (2.4×10^5 cells/ml) was incubated for 15 min at 37 °C with 10 µl of a 1 mM DCFH-DA solution in HBS containing 10% DMF. Ten µl of a 100 mM H_2O_2 solution were then added and the incubation was continued for another 20 min at 37 °C. Cells were then washed twice with 5 ml HBS by 10 min centrifugation at 200*g* and finally resuspended in 250 µl HBS. The cells were destroyed by sonication (100 W Branson sonifier with microtip, Lorch/FRG) and the cell debris was sedimented at 40 °C by 10 min centrifugation at 12000*g*. The fluorescence of the supernatant was measured in the fluorimeter (230 arbitrary units). The remaining, non-oxidized DCFH was determined by addition of 10 µl of 100 mM H_2O_2 and 5 µl peroxidase solution (150 U/ml), followed by 15 min incubation of the assay at 22 °C and remeasuring the fluorescence (260 arbitrary units). The results showed that 88% of total fluorescence was generated during the incubation of intact cells indicating that most of the intracellular DCFH had been converted to fluorescent DCF in the presence of 1 mM H_2O_2.

Discussion

DCFH-DA [3] permits the rapid functional estimation of the oxidative capacities in single vital polymorphonuclear leukocytes by flow cytometry. The single cell measurement is advantageous since it reveals the existence of granulocyte subpopulations (Fig. 4c) which are undetectable by normal biochemical assay with the same dye.

Surprisingly, polymorphonuclear leukocytes of normal, adult human individuals exhibited a bimodal distribution of DCF-fluorescence after 30 min stimulation with PMA (Fig. 4c). The highly reactive granulocyte subpopulation amounted to between 45 and 55% of all granulocytes. Bimodal distributions have been described after PMA stimulation in heterozygotes for the classical X-linked recessive form of chronic granulomatous disease (CGD) [20]. Recently, the existence of a highly active "primed" subpopulation of polymorphonuclear leukocytes in patients with acute bacterial infections [4] was described after PMA stimulation. Forty % of PMNLs of infected patients were primed on average, but the rate of primed PMNLs was widely scattered (0–80%). In contrast, the stimulation of granulocytes from normal individuals was comparatively low [4]. Our own results do not support the concept that the existence of highly activated granulocytes is coupled to abnormal conditions in patients [4, 20]. They rather suggest that both granulocyte populations exist in normal adult human individuals and that the balance between both populations is impaired in disease. In the limited experience with the blood of five septic patients, the highly active granulocyte fraction regularly was increased to between 60 and 85% of all granulocytes.

Care was taken in all experiments to interfere as little as possible with granulocyte function. The preparation of granulocytes was started less than 2 h after the collection of the heparinized blood. Only one centrifugation step of 10 min 120*g* was performed to obtain the buffy coat cells, the cells were never washed and no chelators were added to the incubation medium.

Further work has to clarify to what extent granulocyte subpopulations which have been detected by other methods [2, 8, 9, 11, 12, 16, 19] are identical to the subpopulations described here. It is of interest whether they are due to a different maturation process of precursors in the bone marrow or whether they are the consequence of granulocyte aging or of functional stress while circulating through the body.

In contrast to the bimodal response on stimulation with PMA (Fig. 4c), the incubation of cells with exogenous H_2O_2 (Fig. 4b) always generated a unimodal cluster of highly active granulocytes. Lymphocytes were not significantly stimulated by PMA but exhibited consistently a low peroxidase activity on incubation with H_2O_2 (Fig. 4b).

Besides the cell biochemical results, the study has revealed several useful technical details. It was important to protect the assays from irradiation with light because DCFH is easily photo-oxidized to fluorescent DCF (Fig. 1). Prolonged storage of incubated assays is not recommended, since DCF-oxidation proceeds slowly in the dark. It was essential to keep the amount of the organic solvent DMF in the cellular assay at or below 0.1% to prevent depression of granulocyte functions which occurred above DMF-concentrations of 1% or 2%. With regard to the mechanism of intracellular fluorescence generation, it is believed that both H_2O_2 and intracellular peroxidases are required to generate fluorescent DCF from DCFH. It seems unlikely that 88% of the intracellular DCFH can be converted to DCF within 20 min by H_2O_2 alone when only 0.1% are converted within 10 min by 100 nM H_2O_2 in a cuvette assay (Fig. 2).

The measurement of granulocyte function is of interest for the understanding of the biochemical regulation mechanisms in phagocytic cells. Besides the DCF-method several other techniques for multifunctional probing of granulocytes by flow cytometry have been developed recently, like the determination of intracellular glutathione [21], of calcium levels [25] and of *p*H-values [24] in vital cells. It seems possible that these functional measurements will permit to recognize the deterioration of the defense mechanisms in patients substantially earlier than by measuring leukocyte counts or differentiating leukocyte morphology.

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