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Flow Cytometric Analysis of Respiratory Burst Activity in Phagocytes With Hydroethidine and 2′,7′-Dichlorofluorescin

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Hydroethidine (HE) and 2′,7′-dichlorofluorescin (DCFH) were used for the flow cytometric measurement of reactive oxygen metabolites in leukocytes. Hydroethidine and DCFH were both rapidly oxidized in a cell-free cuvette assay to ethidium bromide (EB) and 2′,7′-dichlorofluorescein (DCF) by H_2O_2 and peroxidase, but not by H_2O_2 alone, while only HE was oxidized by KO_2, a source of O_2^-. Quiescent lymphocytes, monocytes, and neutrophils spontaneously oxidized HE to EB, while DCFH was only oxidized to a low degree. Neutrophils increased 6.9-fold in EB red fluorescence and 12.5-fold in DCF green fluorescence during the respiratory burst induced by phorbol 12-myristate 13-acetate or 6.1-fold and 4.7-fold, respectively, during the respiratory burst induced by *Escherichia coli* bacteria. The HE or DCFH oxidation during the respiratory burst, unlike the spontaneous HE oxidation, was not inhibitable by 10 mM NaN_e indicating a non-mitochondrial source of cellular oxidants during the respiratory burst such as NADPH oxidase, which produces O_2^-. The oxidation of DCFH, but not of HE, was decreased in stimulated neutrophils, which were simultaneously loaded with HE and DCFH. Intracellular DCFH oxidation induced by incubation of resting neutrophils with extracellular H_2O_2 was not influenced by the presence of HE. This indicates that HE is oxidized at an earlier step in the reactive oxygen metabolism of neutrophils than DCFH, i.e., by early oxygen metabolites like O_2^-, while DCFH is oxidized in part by H_2O_2 and phagosomal peroxidases. The differential oxidation of HE and DCFH during simultaneous cellular staining permits the analysis of up to three functionally different neutrophil populations in septic patients. This is of interest for the determination of disease-related alterations of oxygen metabolism in quiescent and stimulated leukocytes.

Key words: neutrophils, flow cytometry, free radical, reactive oxygen species (ROS)

INTRODUCTION

Phagocytosis or stimulation of neutrophils induces a metabolic burst. The non-mitochondrial oxygen consumption increases, and a variety of reactive oxygen metabolites are released into the phagosome and into the extracellular environment [6]. This respiratory burst is important for the killing of ingested microorganisms [24] but may also be detrimental to the organism through endothelial damage in rheumatic diseases [35], in sepsis [18], and in the adult respiratory distress syndrome [42].

Superoxide anion (O_2^-) is the primary metabolite generated through a one-electron reduction of molecular oxygen by a membrane-bound NADPH oxidase [1,6,36]. It dismutates either spontaneously or via superoxide dismutase (SOD) to H_2O_2, which is converted by myeloperoxidase to hypochlorous acid and chloramines inside the phagosome [37].

Specific biochemical methods for the quantitation of respiratory burst activity are based on the measurement of extracellular oxygen consumption or on the extracellular release of O_2^- and H_2O_2 [2]. A certain part of the O_2^- and H_2O_2 produced during the respiratory burst is reconverted intracellularly to molecular oxygen via SOD or catalase. This part of the O_2^- production is not detectable by extracellular biochemical assays because it leads neither to extracellular release of O_2^- or H_2O_2 nor to extracellular oxygen consumption. The magnitude of the respiratory burst is, therefore, underestimated by extracellular assays. Neutrophils must also be isolated from

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whole blood for biochemical bulk assays. This may alter their functional properties [29,34].

Flow cytometric methods for the rapid measurement of intracellular oxidative reaction in single vital cells have been developed as an alternative. The oxidative burst of neutrophils can be measured through the intracellular oxidation of 2′,7′-dichlorofluorescin (DCFH) [5,11] or dihydrorhodamine 123 [33]. Non-fluorescent DCFH is first loaded into vital cells by enzymatic cleavage of the membrane-permeable 2′,7′-dichlorofluorescin-diacetate (DCFH-DA). Intracellular DCFH is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) upon activation of the respiratory burst. The oxidation of DCFH is mainly a measure of the phagosomal formation of reactive oxygen metabolites since the fluorescent product DCF is predominantly found inside the peroxidase-containing granules [31]. Soluble stimuli, however, cause the release of O_2^- at the whole plasma membrane and at the phagosomal membrane [9]. Subpopulations of neutrophils with enhanced respiratory burst activity have been detected through DCFH oxidation in patients with bacterial infections [4,11].

The goal of the present study was to develop a flow cytometric method for the direct measurement of oxygen reduction during the respiratory burst of phagocytes. Hydroethidine (HE), the sodium borohydride-reduced derivative of ethidium bromide (EB) [10,38], either alone or in conjunction with DCFH was found useful for this purpose. HE, in contrast to EB, permeates the cell membrane easily and can be directly oxidized to red fluorescent EB, which is trapped in the nucleus by intercalation into DNA, leading to enhancement of EB fluorescence.

MATERIALS AND METHODS

Preparation of Human Leukocytes

Buffy coat leukocytes (200 μl) were prepared from 21 samples of heparinized (10 U/ml) venous blood (5 ml, 9 healthy donors, aged 24–45 years) by centrifugation at 200*g* and 4°C for 10 min. The samples were diluted with approximately 300 μl of the supernatant plasma to obtain a concentration of 3.5 * 10^7 leukocytes/ml. Six additional blood samples were drawn from 5 septic patients.

Polymorphonuclear and mononuclear cells were separated in some experiments by the one-step method of Ferrante et al. [8,14] using Mono-Poly Resolving Medium (Flow Laboratories, Meckenheim, FRG). The cell fractions were washed once (200*g*, 10 min) with 10 ml HEPES-buffered saline (HBS) (0.15 M NaCl, 5 mM HEPES, pH 7.35, Serva Feinbiochemica, Heidelberg, FRG) and resuspended in 50 to 100 μl of autologous plasma to obtain a concentration of 3.5 * 10^7 cells/ml. All cell preparations were stored on ice, and the flow cytometric measurements were terminated within 6 hr after removal of the blood from the donors.

Cell Staining

The cell suspension (10 μl) was diluted with 1 ml HBS and stained for 15 min at 37°C with 2.5 μl of a 63.5-mM HE (Polysciences, St. Goar, FRG) solution in N,N-dimethylformamide (DMF) (E. Merck, Darmstadt, FRG) and/or 20 μl of a 0.5-mM DCFH-DA solution (1/20 prediluted with HBS from 10 mM DCFH-DA in DMF, Serva). This corresponds to a final concentration of 160 μM HE or 10 μM DCFH-DA in the cell assay. Sodium azide (Merck) or sodium cyanide (Merck) (20 μl of 500-mM, 50-mM, or 5-mM solutions in HBS) were added together with the dyes in enzyme inhibition experiments. Cyanide, in contrast to azide, was only added after a 15-min prestaining of the cells with DCFH-DA at 37°C to avoid cyanide inhibition of the intracellular DCFH-DA cleaving esterases.

The samples with HE- or DCFH-loaded cells were split into aliquots of 250 μl and further incubated either for 30 min with 5 μl of a 7.5-μM solution of phorbol 12-myristate 13-acetate (PMA) (1/200 prediluted with HBS from 1.5 mM PMA in DMF, Sigma Chemie, Deisenhofen, FRG), or for 15 min with 5 μl of a 65-mM solution of H_2O_2 (Merck) in HBS. The EB fluorescence of dead cells, which was due to contamination of HE with EB, was quenched by addition of 10 μl of 5.2 mM trypan blue (Serva) in HBS immediately prior to flow cytometric measurement. Trypan blue at this concentration did not alter the EB red or DCF green fluorescence of vital cells. The DNA of dead cells was counterstained with 5 μl of 3-mM propidium iodide (PI) (Sigma) solution in HBS 3 min before the measurement in samples stained with DCFH-DA.

Phagocytosis

Escherichia coli K12 bacteria (Sigma) were grown overnight in RPMI-1640 (Gibco BRL, Eggenstein, FRG) at 37°C and 5% CO_2. The bacteria (5 ml) were washed twice with 10 ml HBS and resuspended in HBS to a final concentration of 7 * 10^9 bacteria/ml. The leukocyte suspension in autologous plasma (100 μl) was incubated with 10 μl of the *E. coli* suspension at 37°C to avoid potential interference of the staining solution with the phagocytosis process. Aliquots (10 μl) were taken every 5 min, diluted with 1 ml HBS, and stained for 15 min at 37°C with 2.5 μl 63.5-mM HE and/or 20 μl of a 0.5-mM DCFH-DA solution corresponding to final concentrations of 160 μM HE and/or 10 μM DCFH-DA.

Fluorescence Spectrometry

Fluorescence spectra were kinetically recorded with a Perkin Elmer LS-5 luminescence spectrometer (Bodenseewerk Perkin-Elmer, Überlingen, FRG) connected on-line to a VAX 8550 computer (Digital Equipment, Munich, FRG) using excitation and emission slits of 5 nm nominal band width and quartz cuvettes of 10 mm path length.

Stock solutions of HE (31.75 mM), EB (12.68 mM, Serva), DCFH-DA (50 mM), and DCF (50 mM, Sigma) were prepared in DMF. A 50-mM DCFH solution was prepared by 30-sec hydrolysis of 10 μl of the 50-mM DCFH-DA solution with 20 μl of a 0.1-N NaOH solution followed by addition of 10 ml MOPS-buffer (MBS) (0.2 M MOPS, pH 7.20, Sigma). Horseradish peroxidase (HRP) (Sigma) and SOD (Sigma) were dissolved in MBS at concentrations of 20 and 200 U/ml (HRP), or 20,000 U/ml (SOD). Potassium superoxide (KO_2) (Fluka, Buchs, CH) was directly weighed into the cuvettes at 97.5 μM per cuvette. After addition of 2 ml of the final dye solution in MBS this corresponded to a 48.8-mM KO_2 solution yielding approximately 1.3 mM O_2^- [23].

Spontaneous oxidation of a 10-μM HE solution or of a 0.5-μM DCFH solution in MBS, the oxidation of HE or DCFH in the presence of 48.8 mM KO_2 with or without 1,000 U/ml SOD, and the oxidation of HE and DCFH in the presence of 0, 0.1, 1, 10, and 100 U/ml HRP and of 1.3 mM H_2O_2 were recorded for 40 min at 22°C by measuring the fluorescence intensities at the wavelengths corresponding to the maximum emission and excitation of EB (excitation 473 nm/emission 593 nm) and DCF (excitation 501 nm/emission 521 nm). The residual HE concentration was determined through HE blue fluorescence (excitation 348 nm/emission 421 nm). The residual DCFH concentration was determined as the increase of DCF fluorescence after a second addition of 1.3 mM H_2O_2 and 100 U/ml HRP at the end of the incubation. The oxidation of a 10-μM DCFH solution in MBS by the same amounts of oxidants was measured at its end point after 40 min of incubation, followed by 20-fold dilution with MBS to keep the fluorescence signals within the photomultiplier range.

Flow Cytometry

The electrical cell volume and two fluorescences of more than 2,000 leukocytes per sample were simultaneously measured with a FLUVO-II flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG). The electrical cell volume was determined by hydrodynamic focusing of the cells through the center of a cylindrical orifice (80 μm diameter, 80 μm length) at an electrical current of 0.15 mA using HBS as the sheath fluid. The fluorescence was excited between 400 and 500 nm with a HBO 100 W/2 high pressure mercury arc lamp (Osram, Augsburg, FRG). The green fluorescence of DCF was collected between 500 and 530 nm, and EB or PI red

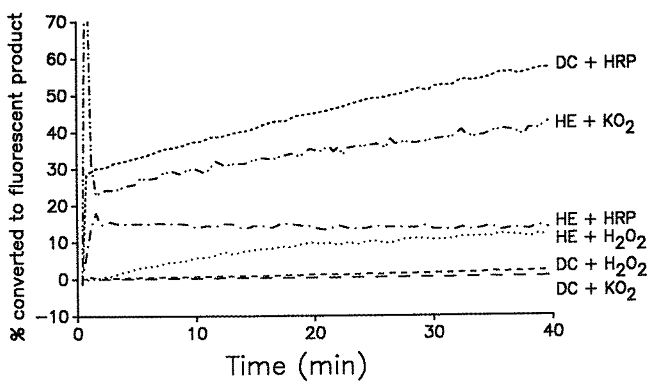

Fig. 1. Oxidation of HE to red fluorescent EB and of DCFH to green fluorescent DCF in a cell-free cuvette assay. Curves represent fluorescence generation from HE or DCFH in the presence of 1.3 mM O_2^- (48.8 mM KO_2) [23], 1.3 mM H_2O_2, or 1.3 mM H_2O_2 and 10 U/ml HRP in MBS at 22°C. Significant fluorescence is generated from DCFH and HE with H_2O_2 and HRP and from HE in the presence of O_2^-. The red fluorescence of EB was measured in intervals of 24 sec fluorimetrically at 593 nm with excitation at 473 nm. The green fluorescence of DCF was measured at 521 nm with excitation at 501 nm. The initial peak of fluorescence following contact of KO_2 with aqueous medium coincided with gas formation.

fluorescence was collected between 590 and 700 nm. The 365-nm line of the mercury arc lamp was used for excitation (350–380 nm) of HE blue fluorescence, which was collected between 418 and 500 nm. The flow cytometer was calibrated with porous, NH_2-group bearing particles (5 μm diameter, Paesel, Frankfurt, FRG) covalently stained with fluorescein isothiocyanate (Serva). The cell volume and fluorescence pulses were amplified by 2.5-decade logarithmic amplifiers. The maximum amplitude of each signal was digitized by 4096-step analog-to-digital converters. The list mode data were evaluated by the DIAGNOS1 program system for display, calculation, and databasing of flow cytometric data [40].

Statistical Analyses

Data are presented as mean ± standard deviation (SD). The significance ($P < .05$) of the difference of means was tested by the Student t-test.

RESULTS

Oxidation of HE and DCFH by KO_2 and H_2O_2 in a Cell-Free Cuvette Assay

Hydroethidine was only very slowly oxidized by H_2O_2 alone in a cell-free cuvette assay (Fig. 1). Less than 12% of 10 μM HE was oxidized after 40 min of incubation with 1.3 mM H_2O_2. Hydroethidine was, however, completely oxidized in less than 1 min with 1.3 mM H_2O_2 and 1 U/ml HRP as indicated by loss of HE blue fluo-

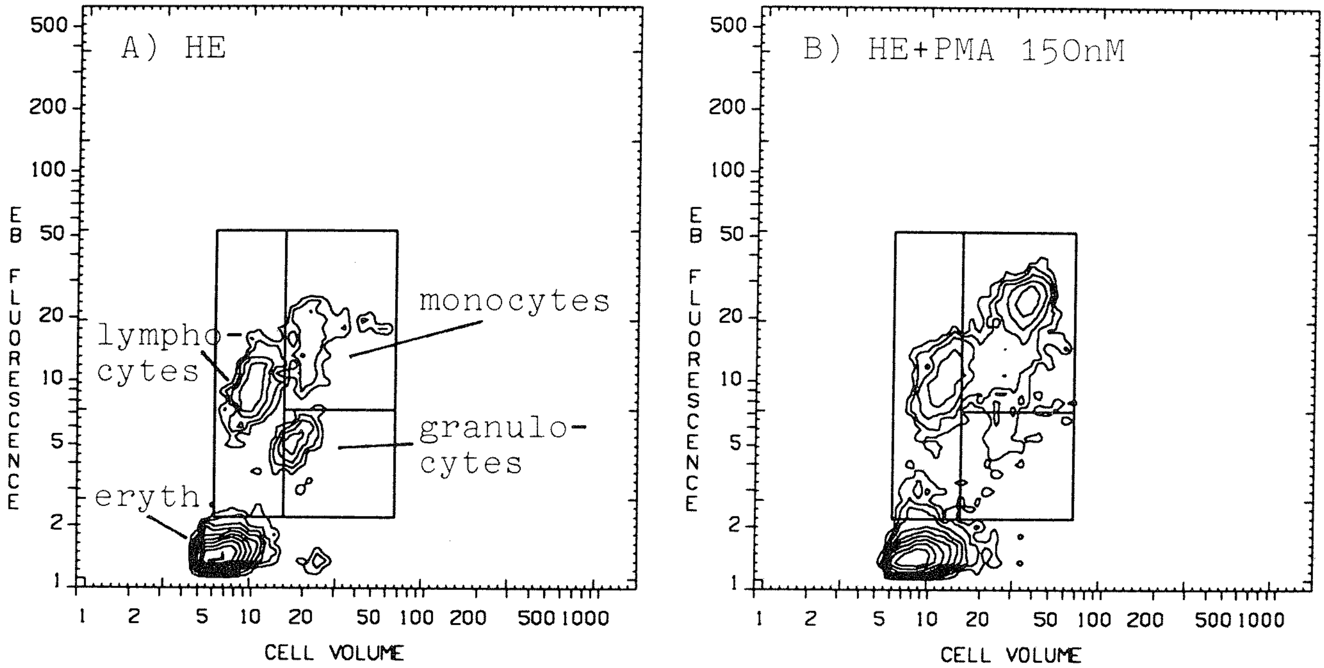

Fig. 2. Cell volume versus EB red fluorescence of buffy coat leukocytes incubated for 15 min with 160 μM HE at 37°C (A). The intracellular oxidation of HE to EB in resting cells is high in monocytes, intermediate in lymphocytes, and low in neutrophils. Following stimulation with 150 nM PMA for a further 30 min at 37°C, the EB fluorescence of neutrophils increased 6.9-fold (B). The graphs are standardized to the maximum logarithmic channel contents (100%). Contour lines were drawn in linear steps of 10% downward. The histograms contain 19,226 (A) and 32,971 (B) cells with 1,981 and 3,507 cells in the maximum channel.

rescence. Between 4.8% (1 U/ml HRP) and 13.6% (10 U/ml HRP) of the product was red fluorescent EB, the remainder being a non-fluorescent oxidation product. Less than 2% of 0.5 μM DCFH was converted after 40 min in the presence of 1.3 mM H_2O_2 alone. Addition of HRP induced the rapid conversion of DCFH to DCF (Fig. 1), and more than 56% of the DCFH was converted to DCF after 40 min of incubation with 10 U/ml HRP and 1.3 mM H_2O_2.

A substantial part (22.9%) of the 10μM HE was converted to EB after 1 min when incubated with 48.8 mM KO_2, a chemical source of O_2^-, but less than 1% of 0.5 μM DCFH was oxidized to DCF (Fig. 1). HE oxidation decreased to less than half of this value (10.5%) when 1,000 U/ml SOD was present together with KO_2 (48.8 mM) for 1 min. This indicates that the KO_2-induced oxidation of HE depended on the presence of O_2^-

The differences in the oxidation of HE and DCFH in the cell-free assay (Fig. 1) were not due to the different concentrations of the substrates (10 μM HE vs. 0.5 μM DCFH) since the degree of oxidation of 10 μM DCFH by 1.3 mM H_2O_2 and 10 U/ml HRP or by 48.8 mM KO_2 after 40 min of incubation followed by 20-fold dilution of the sample did not differ significantly from the results obtained with 0.5 μM DCFH. The dilution of the assay was necessary because the DCF fluorescence of the undiluted sample exceeded the range of the photomultiplier in the fluorimeter.

Intracellular Oxidation of HE and DCFH in Resting and Stimulated Cells

The membrane-permeable blue fluorescent HE rapidly accumulated in the cytoplasm and granules of neutrophils, monocytes, and lymphocytes. Saturation of cellular blue fluorescence was reached with 160 μM HE after a 15-min incubation at 37°C.

Spontaneous oxidation of HE resulted in red EB fluorescence of the cell nucleus. The spontaneous oxidation depended on the cell type (Fig. 2A; Table 1) and was high in monocytes, intermediate in lymphocytes, and low in neutrophils. The identity of the HE-stained cell populations was verified by staining the ficoll/hypaque-isolated mononuclear and the polymorphonuclear cell fraction separately with HE. The monocyte cell cluster was heterogeneous in fluorescence after incubation of unseparated cells for 30 min or more (Fig. 2A) suggesting the presence of two separate monocyte populations. The heterogeneity of monocyte fluorescence was not observed in the isolated mononuclear cell fraction.

Two stimuli, PMA and phagocytosis of *E. coli* bacte-

TABLE 1. Intracellular Oxidation of HE and DCFH in Resting Leukocytes[a]

Cells	EB red fluorescence (arbitrary units)	DCF green fluorescence (arbitrary units)
Neutrophils	0.0325 ± 0.0078	0.0221 ± 0.0042
Monocytes	0.0941 ± 0.0234	ND[b]
Lymphocytes	0.0472 ± 0.0130	0.0156 ± 0.0013

[a]Mean fluorescence ± SD (N = 12 for EB and 9 for DCF) of the different cell populations after 15 min incubation of buffy coat leukocytes of the same preparation with either 160 μM HE or 10 μM DCFH-DA.
[b]Not determined owing to overlap by the neutrophil cell cluster.

TABLE 2. Intracellular Oxidation of HE and DCFH in Stimulated Neutrophils[a]

Incubation	EB fluorescence (% of controls)	DCF fluorescence (% of controls)
PMA	685.3 ± 70.0	1,251.6 ± 135.5
H_2O_2	139.9 ± 7.9	336.1 ± 42.4
E. coli	608.2 ± 115.5	466.3 ± 22.6

[a]Buffy coat leukocytes were prestained for 15 min with 160 μM HE or 10 μM DCFH-DA and subsequently incubated for 30 min with 150 nM PMA or 1.3 mM H_2O_2. In the case of *E. coli,* leukocytes were preincubated for 10 min with bacteria before staining. Data represent means ± SEM (N = 3–15) of the peak fluorescence of neutrophils, expressed in % of the peak fluorescence of controls incubated without the stimulus.

ria, were used to determine whether respiratory burst activity led to an increase of the intracellular oxidation of HE. Stimulation with PMA (150 nM, 30 min, 37°C) induced a 6.9-fold increase of the EB fluorescence of neutrophils as compared to a 2.0-fold increase of the EB fluorescence of lymphocytes in HE-prestained cells (15 min, 37°C) (Fig. 2B; Table 2). Phagocytosis of bacteria similarly increased the EB red fluorescence of neutrophils. The highest EB fluorescence responses (6.1-fold) were obtained after a 10-min preincubation of leukocytes with *E. coli K12* in autologous plasma and subsequent staining of the cells with HE for 15 min (Table 2).

Spontaneous oxidation of DCFH to fluorescent DCF was higher in neutrophils than in lymphocytes (Table 1). The DCF fluorescence of neutrophils increased 12.5-fold upon stimulation with PMA, but only 4.7-fold upon phagocytosis of *E. coli* (Table 2).

The main difference between the intracellular oxidation of HE and DCFH was that HE was oxidized spontaneously to a significant degree not only in neutrophils but also in lymphocytes and monocytes. The spontaneous oxidation of HE was even higher in monocytes and lymphocytes than in neutrophils. This shows that HE was oxidized in resting cells by a mechanism different from respiratory burst activity.

Azide and cyanide as inhibitors of mitochondrial oxygen consumption were used to better characterize the oxidative mechanism. The spontaneous oxidation of HE to EB in resting neutrophils was inhibited through azide (10 mM) or cyanide (1 mM) by 72.4% ± 5.5% (Fig. 3) and 66.9% ± 4.3%, respectively, as compared to control assays (means ± SD). The EB fluorescence of PMA-stimulated neutrophils was reduced by 9.0% ± 0.4% by 10 mM azide (Fig. 3) and 48.6% ± 3.7% by 1 mM cyanide.

Azide (10 mM) inhibited the spontaneous oxidation of DCFH in unstimulated neutrophils. The response was, however, difficult to quantify owing to the low degree of DCFH oxidation in non-stimulated neutrophils. Azide (10 mM) or cyanide (1 mM) increased by 0.6% to 10.3%

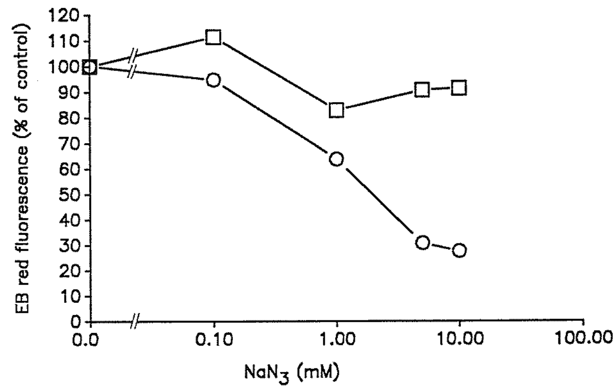

Fig. 3. Influence of sodium azide on the intracellular oxidation of HE in a typical experiment. The EB red fluorescence of neutrophils incubated for 15 min with 160 μM HE was measured with (squares) or without (circles) further stimulation with 150 nM PMA for 30 min. The fluorescence is expressed in percent of control assays incubated without azide.

the intracellular DCFH oxidation by PMA-stimulated neutrophils.

The mechanism of DCFH and HE oxidation was further compared by the addition of H_2O_2 or of KO_2, as a source of O_2^-, to a cellular assay. Addition of 1.3 mM H_2O_2 to dye-loaded cells increased neutrophil DCF fluorescence 3.4-fold, while the EB fluorescence of neutrophils was increased only 1.4-fold as compared to controls (Table 2). The experiments with KO_2 addition were not successful since cell lysis occurred at KO_2 concentrations above 200 μM (data not shown).

Intracellular oxidation of HE in resting cells, or in cells stimulated with PMA or in cells phagocytosing *E. coli,* was not altered by simultaneous presence of DCFH, when compared to cells loaded with HE alone (Fig. 4A). The intracellular oxidation of DCFH in PMA-stimulated or phagocytosing neutrophils was, however, significantly reduced by the presence of HE (Fig. 4B). The decrease of DCFH oxidation in the presence of HE was

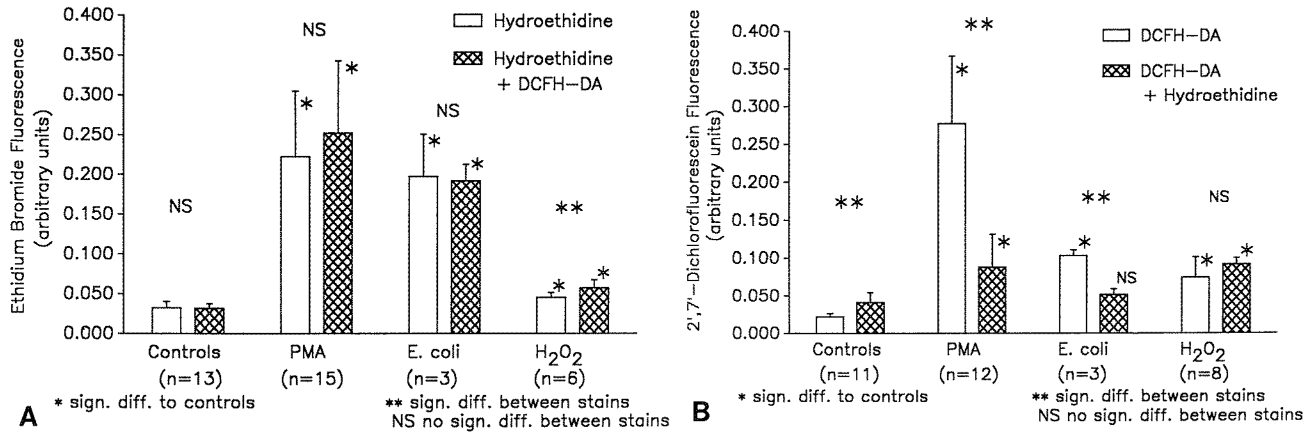

Fig. 4. Flow cytometrically determined EB red fluorescence (A) and DCF green fluorescence (B) of neutrophils in the presence of 150 nM PMA, following phagocytosis of *E. coli K12* or incubation with 1.3 mM H_2O_2. The significance of the difference between samples stained with HE alone (open bars) and samples simultaneously stained with HE and DCFH-DA (cross-hatched bars) (**) or of the results as compared to control assays (*) was evaluated by Student's t-test ($P < .05$).

not observed when 1.3 mM H_2O_2 was added extracellularly to resting neutrophils (Fig. 4B).

The simultaneous staining of different intracellular oxidative processes by HE and DCFH in stimulated neutrophils was used to analyze the functional heterogeneity of neutrophils from septic patients. Up to three subpopulations of neutrophils differing in intracellular oxidation of HE and DCFH were identified in PMA-stimulated blood of septic patients (Fig. 5). Subpopulations of neutrophils that increased only in EB fluorescence but not in DCF fluorescence upon PMA stimulation could be distinguished from neutrophils increasing in oxidation of both substrates and from unresponsive subpopulations, which although viable did not change their EB or DCF fluorescence upon stimulation. The difference between EB and DCF fluorescence in three different neutrophil populations in the blood of septic patients again demonstrated that HE and DCFH indicated different intracellular oxidative processes.

DISCUSSION

The oxidation of HE was significantly different from the oxidation of DCFH both in a cell-free cuvette assay and in cellular experiments with resting and stimulated leukocytes.

The cell-free cuvette assays show that HE and DCFH function as electron donors for peroxidases since they were oxidized by H_2O_2 and HRP but not by H_2O_2 alone (Fig. 1). Hydroethidine was furthermore oxidized by O_2^-, which was generated through the decay of KO_2 in aqueous solution. A substantial part of KO_2-induced HE oxidation was in fact due to O_2^- since addition of SOD, which dismutates O_2^- to H_2O_2, decreased HE oxidation in the cell-free assay. The additional involvement of a singlet oxygen intermediate in the oxidation of HE by O_2^- in aqueous solution cannot be excluded in our experiments [13].

Whether the different behavior of HE and DCFH toward oxidation in the cell-free assay was associated with different mechanisms of HE and DCFH oxidation in the more complex intracellular situation and whether HE and DCFH were useful for the discrimination of different intracellular oxidative pathways of neutrophils was analyzed in a series of cellular experiments.

The results of these experiments show that HE and DCFH are indeed metabolized by cells in different ways. The oxidation of HE in resting cells was substantial and highest in monocytes, followed by lymphocytes and neutrophils (Fig. 2). The oxidation of DCFH in resting cells was overall low by fluorescence. It was very low for lymphocytes and somewhat higher in neutrophils (Table 1) [11]. Furthermore, a separate monocyte cell cluster was not visible [11]. The oxidation of HE in resting leukocytes was sensitive to 10 mM azide (Fig. 3) or 1 mM cyanide, which inhibit the mitochondrial respiration at the cytochrome oxidase level [41]. Oxidation of HE by stimulated neutrophils was not inhibited by azide (Fig. 3) and was only partly inhibited by cyanide. The mechanism of HE oxidation by resting cells is, therefore, different from the HE oxidation during the respiratory burst. It is important to note that the azide and cyanide inhibition experiments are only useful to demonstrate differences in HE and DCFH oxidation between resting and stimulated cells. They do not permit us to obtain more detailed information on oxidative pathways in stimulated neutrophils because the inhibition of multiple heme-enzymes by azide or cyanide induces complex al-

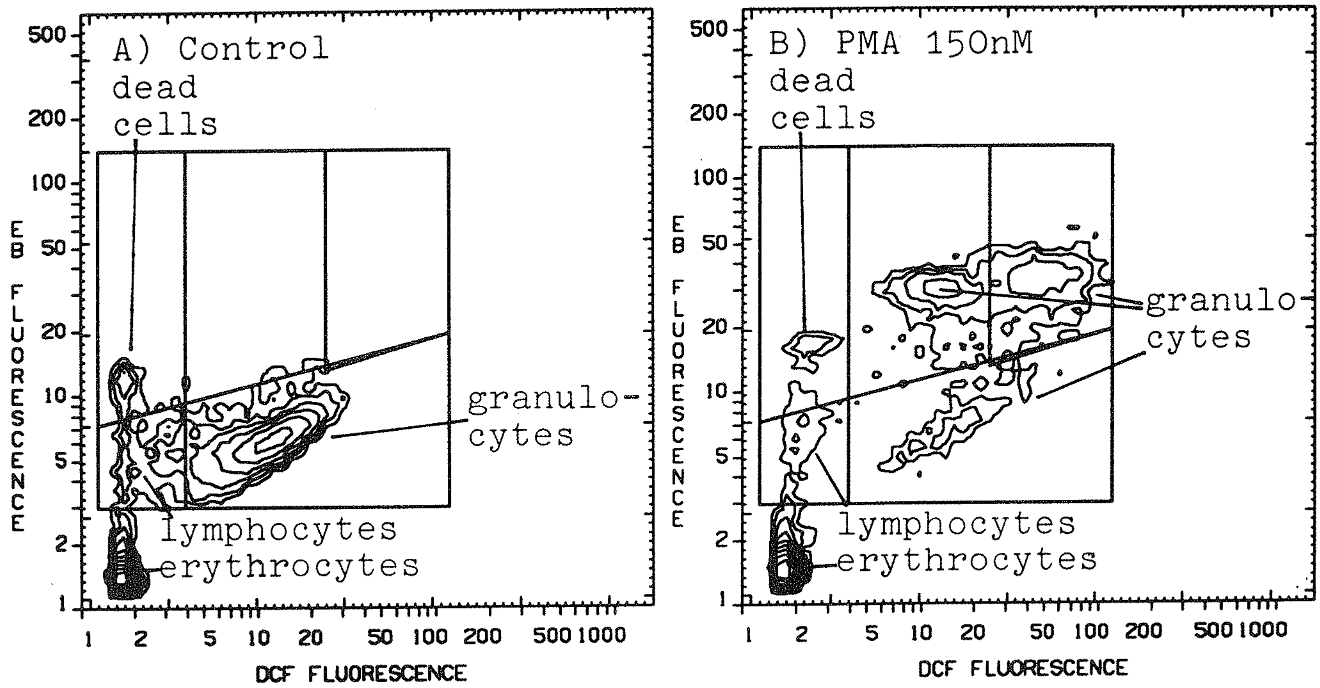

Fig. 5. DCF green and EB red fluorescence of buffy coat leukocytes, obtained from a septic patient, after simultaneous staining with 10 μM DCFH-DA and 160 μM HE for 15 min at 37°C and further incubation without (A) or with (B) 150 nm PMA for 30 min. The DNA of dead cells is counterstained with PI. More than 95% of the leukocytes were neutrophils as judged by morphological analysis of May-Grünwald-Giemsa-stained smears. The simultaneous measurement of peroxidase-dependent DCFH oxidation and NADPH oxidase-dependent HE oxidation revealed three subpopulations of vital neutrophils upon PMA stimulation.

terations of O_2^- production and H_2O_2 metabolism during the respiratory burst. Cyanide causes inhibition of the hexose monophosphate shunt [4], and the precise effect of cyanide on cellular O_2^- production cannot be easily determined in biochemical assays owing to interference of cyanide with SOD-inhibitable ferricytochrome-C reduction [27]. Azide increases oxidation of DCFH by H_2O_2 and myeloperoxidase already in a cell-free assay [4]. Results obtained in cellular experiments with azide or cyanide are, therefore, difficult to interpret.

The significant spontaneous oxidation of HE, but not DCFH, by resting leukocytes may be related to uncoupling of the mitochondrial oxidative phosphorylation by EB [26]. Mitochondria are the major source of O_2^- and H_2O_2 in resting cells. Most of the O_2^- is converted to H_2O_2 inside the mitochondria by a Mn-containing superoxide dismutase, but approximately 20% of the O_2^- escapes to the cytosol [28]. EB specifically binds to a mitochondrial hydrophobic protein and inhibits ATP synthesis but increases respiration [17]. This respiratory uncoupling of the mitochondria should lead to decreased mitochondrial membrane potential and increased intracellular oxidant levels. Incubation of leukocytes with HE in fact caused a 40% decrease of cellular rhodamine 123 fluorescence (data not shown), indicating decreased mitochondrial membrane potential [19], and an increase of the oxidation of DCFH to DCF in cells simultaneously stained with HE and DCFH-DA, indicating increased oxidant levels.

The incubation of resting neutrophils with extracellular H_2O_2, which diffuses into all cellular compartments [30], further confirmed the difference in intracellular HE and DCFH oxidation. Externally added H_2O_2 in neutrophils mainly induces oxidation inside the phagosome because cytoplasmic H_2O_2 is efficiently degraded by catalase and glutathione peroxidase [12]. Addition of H_2O_2 induced a 39.9% increase of EB fluorescence over controls (Table 2). This was less than 7% of the 585.3% increase of EB fluorescence over controls induced by PMA. The 236.1% increase of DCF fluorescence in the presence of H_2O_2, in contrast, corresponded to more than 20% of the 1,151.6% increase of DCF fluorescence in the presence of PMA. The low susceptibility of intracellular HE to oxidation by extracellular H_2O_2 could be due to the non-acceptance of HE as an electron donor by phagosomal or microsomal peroxidases, or to the trapping of non-membrane-permeable EB in the phagosomes where the 20-fold fluorescence increase [22] through binding of EB to nuclear DNA is not obtained.

The use of myeloperoxidase-deficient neutrophils seems useful to clarify which part of the DCFH or HE oxidation during the respiratory burst and upon addition of external H_2O_2 is due to myeloperoxidase activity. Such experiments are, however, difficult to interpret owing to complex alterations of O_2^- production and H_2O_2 metabolism in myeloperoxidase-deficient neutrophils. They differ in oxygen consumption and in the extracellular release of H_2O_2 and O_2^- from normal neutrophils [27]. The impaired balance of the H_2O_2-metabolizing enzymes in myeloperoxidase-deficient neutrophils also leads to uncharacterized alterations of the H_2O_2 metabolism by other peroxidizing enzymes such as microsomal cytochrome P-450-containing enzymes [25].

To further characterize differences in intracellular HE and DCFH oxidation and to better determine the sequence of oxidation of HE and DCFH, cells were simultaneously loaded with HE and DCFH. The separate reading of the simultaneously generated oxidation products EB and DCF was possible owing to their different fluorescence emission spectra. Neutrophils developed the same EB fluorescence in the presence of DCFH as cells stained with HE alone (Fig. 4A), indicating that HE oxidation was independent of the presence of DCFH. Cellular DCF fluorescence was increased in resting cells in the presence of HE, compatible with increased cytoplasmic oxidant levels caused by uncoupling of mitochondrial respiraton by EB (Fig. 4B). Cellular DCF fluorescence on the contrary was decreased in the presence of HE in PMA-stimulated cells, which produce O_2^- as the primary oxidant. The decrease of intracellular DCFH oxidation in the presence of HE did not occur when extracellular H_2O_2 was added to resting neutrophils. These results are compatible with the view that HE scavenges oxygen radicals, most likely O_2^-, the primary product of oxygen reduction during the respiratory burst, at an earlier step than DCFH. Dichlorofluorescin in this concept would be oxidized by follow-up products of O_2^- dismutation such as H_2O_2 in conjunction with phagosomal peroxidases or through reactive peroxidase metabolites.

Two other oxidants, hydroxyl radical, which can originate from an iron-catalysed reaction of O_2^- with H_2O_2 [3], and singlet oxygen, which is generated from a water-induced dismutation of O_2^- [13], have also been related to the respiratory burst. Recently published results suggest, however, that production of hydroxyl radical [10,21,32,43] or singlet oxygen [20] by neutrophils is unlikely to occur under physiological conditions. No products of free radical- or singlet oxygen-induced oxidation were found in phagocytosing neutrophils [39]. Thus, it is less likely that hydroxyl radical or singlet oxygen are responsible for the intracellular oxidation of DCFH or HE by neutrophils.

The different intracellular oxidation mechanisms of HE and DCFH in PMA-stimulated neutrophils of septic patients simultaneously stained with HE and DCFH permit the functional analysis of up to three subpopulations of viable neutrophils. These subpopulations cannot be discriminated with either HE or DCFH alone (Fig. 5). Our results are compatible with results from other flow cytometric assays, which revealed a functional heterogeneity of peripheral blood neutrophils. Approximately 30% of peripheral blood neutrophils depolarize to the chemotactic peptide N-for-met-leu-phe [15]. On the average only 40% of the neutrophils of patients with infections show enhanced intracellular oxidation of DCFH following stimulation with PMA [4]. These functionally different neutrophil populations do not correspond to morphologically distinguishable subpopulations such as the small amount of band form cells, a morphological subset of functionally immature neutrophils [7,16,44], in normal peripheral blood. The simultaneous HE and DCFH staining method seems of particular interest for the study of the biochemical and phenotypical characteristics and the disease-related significance of these functional subpopulations of neutrophils.

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