

Measurement of Intracellular Fluorescence of Human Monocytes Relative to Oxidative Metabolism

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Human monocytes (MN) produce O_2^- and H_2O_2 when stimulated by agonists. Dichlorofluorescein diacetate (DCFH-DA) has been used as a substrate for measuring intracellular oxidant production in neutrophils. DCFH-DA is hydrolyzed by esterases to dichlorofluorescein (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by action of cellular oxidants. DCFH-DA can not be appreciably oxidized to a fluorescent state without prior hydrolysis. We have examined the utility of DCFH-DA for the assessment of monocyte oxidative responses. The levels of intracellular fluorescence measured by flow cytometry were considerably less than expected from reported levels of O_2^- -production or chemiluminescence assays. Compared with neutrophils, monocytes produced minimal increases in DCF fluorescence after stimulation with phorbol myristate acetate as measured by flow cytometry, but both cell types showed increases in fluorescence when bulk cell suspensions were measured by spectrofluorometry. To determine the intracellular location of the DCFH, bulk fluorescence measurements were made on both whole and sonicated cell preparations. When intact mononuclear cells were preloaded with DCFH-DA, then sonicated and oxidized with added excess H_2O_2 , the increase in fluorescence was only 30% of the fluorescence of mononuclear cell sonicates to which DCFH-DA was added and oxidized in a similar manner. These results suggest that a portion of the DCFH-DA incorporated by intact cells, is not susceptible to oxidation by the added H_2O_2 . Addition of NaOH to induce hydrolysis of any residual DCFH-DA in the sonicates of DCFH-DA-loaded intact mononuclear cells resulted in a further increase in fluorescence upon addition of H_2O_2 , suggesting that a significant portion of the DCFH-DA was not hydrolyzed despite ample uptake of this dye by these cells. In contrast, no further increase in fluorescence was observed in sonicates of DCFH-DA-loaded intact neutrophils, suggesting complete hydrolysis of all incorporated DCFH-DA to DCFH. When monocytes were allowed to phagocytose DCFH-DA-loaded *Staphylococcus aureus*, intracellular fluorescence was measurable by flow cytometry, indicating intracellular oxidation of the fluorochromes. We therefore propose that in monocytes the mechanism of intracellular processing of these fluorochromes differs from that in neutrophils owing to differences in intracellular localization of fluorochromes, site of oxidant production, and/or accessibility of the DCFH-DA to esterolysis.

Key words: neutrophil, dichlorofluorescein, H_2O_2

INTRODUCTION

The use of intracellular fluorochromes, particularly dichlorofluorescein diacetate (DCFH-DA), to monitor the production of oxidants by neutrophils has become a valuable research tool [2,3,6]. An intracellular measure of oxidant production would enable further understanding of the differences in oxidative metabolism between monocytes and neutrophils. We have used DCFH-DA in an attempt to measure the generation of intracellular oxidants in human monocytes but have been unable to measure significant quantities of fluorescence by flow cytometry. Previous workers, using bulk measurement techniques, have demonstrated that appropriately stimu-

lated human monocytes produce O_2^- [7], chemiluminescence (CL) [12,14] and H_2O_2 [13] in almost the same quantities as neutrophils, albeit with different kinetics.

Current methods measure primarily extracellular production of H_2O_2 or O_2^- in phagocytes [1]. It is not clear

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whether CL measurements are related to intra- or extracellular events or what oxidant is being measured. On the other hand, the measurement of DCF fluorescence by flow cytometry is mostly intracellular.

Recently, Bender and colleagues [4] indicated that they could measure intracellular oxidant production in human monocytes, although in that study it is unclear how much oxidant is actually produced by these cells. This report suggests that DCF-associated fluorescence can only be detected in human monocytes after stimulation with particulate but not soluble activators.

MATERIALS AND METHODS

Buffers and Lysing Solution

Phosphate-buffered saline (PBS) contained 0.147 M NaCl, 4.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2.3 mM NaH_2PO_4 , 15 mM NaNO_3 , pH 7.4 PBS-gel is PBS which also contained 2 mM EDTA, 10 mM glucose, and 1% gelatin (Difco Laboratories, Detroit, MI) and was used for all cell incubations. Lysing solution consisted of 0.15 M NH_4Cl , 10 mM NaHCO_3 , 10 mM ethylenediamine tetraacetic acid (EDTA) at pH 7.4.

Reagents

2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Eastman Kodak Co, Rochester, NY) was dissolved in absolute ethanol at a concentration of 5 mM and kept at 4°C for up to 4 wk. 5',6'-Carboxy-2',7'-dichlorofluorescein diacetate (CF-DA) (Molecular Probes, Junction City, OR) was dissolved under the same conditions. Phorbol myristate acetate (PMA) (Consolidated Midland, Brewster, NJ) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 1 μM (2 mg/ml) and stored in 20- μl aliquots at -20°C.

Preparation of Cells

Human blood was drawn into a syringe containing preservative-free heparin (5 IU/ml blood) (Elkins-Sinn Inc, Cherry Hill, NJ). Blood was diluted 1:2 with PBS and layered onto Lymphocyte Separation Medium (LSM) (Bionetics Laboratory Products, Kensington, MD) at a ratio of 2 ml diluted blood per 1 ml LSM. Blood was centrifuged at 600g for 25 min at 25°C in a Beckman bench centrifuge (model TJ-6) according to the methods of Boyum [5]. The interface containing the mononuclear cells was removed and diluted 1:2 with PBS in a 50-ml sterile polyethylene tube (Corning Glass Works, Corning, NY) followed by three washes to remove excess LSM and platelets. Cells were resuspended at a 1×10^7 /ml in PBS-gel.

Fluorescence Assay

Flow cytometry. Cell populations (1×10^6 /ml) were incubated with fluorochromes (5 μM) for 15 min at

37°C. These loaded cells were then placed into a temperature-controlled chamber in the flow cytometer (EPICS 753, EPICS Division, Coulter Electronics, Hialeah, FL). All measurements were performed using an argon ion laser emitting 300 mw at 488 nm with emission measured using 525-nm band-pass filters. At zero time, PMA (100 ng/ml, final concentration) was added, and the samples were constantly mixed while running through the cytometer. Temperature was maintained at 37°C throughout. Signals were collected as ungated list mode data. Parameters collected included forward angle light scatter (FLS), 90° light scatter (90LS), green fluorescence (GFL), and time. A typical kinetics experiment included the analysis of 250,000–750,000 individual cells over 15 min.

Spectrofluorometry. All bulk fluorescence measurements were performed on a Varian SF-330 spectrofluorometer (Varian Associates Inc, Palo Alto, CA) using 3-ml quartz cuvettes. Temperature was maintained at 37°C with a heated sample compartment. All measurements were made with the instrument excitation wavelength set at 488 nm and emission wavelength at 530 nm, and both with 10-nm slit widths.

Sonication of cells was performed with a sonicator model W255R (Heat Systems-Ultrasonics, Inc., Plainview, NY). Cells (1×10^6 /ml in 5-ml volumes) were sonicated on ice three times each with 12-sec bursts at 30 W. Microscopic analysis showed complete cell disintegration. Additions of H_2O_2 and NaOH, where indicated in the text, refer to final concentrations unless otherwise stated.

RESULTS

Flow Cytometric Measurement of DCFH-DA Fluorescence in Mononuclear Cells

To assess the intracellular fluorescence of DCFH-DA-loaded cells, mononuclear cells (1×10^6 /ml) were loaded with DCFH-DA (5 μM) for 15 min at 37°C and run on the flow cytometer. Using light-scatter gating techniques the fluorescence of each cell population was monitored for 15 min. Two populations of cells were identified by light scatter. Lymphocytes (low FLS and 90LS) exhibited a basal fluorescence with no increase in the mean fluorescence channel during stimulation with PMA over 15 min. The second population consisted of monocytes and showed variable fluorescence. The majority of cells (>90%) showed almost no increase in fluorescence (mean channel No. from 25.5 to 51.85) after stimulation with PMA (0.1–100 ng/ml). A very small percentage of cells (<5%) became slightly fluorescent during the 15-min measurement (mean channel No. 102.5). This responsive population of cells may represent neutrophils which have gated within the gates set for the monocytes. These cells may be less dense than normal neutrophils and were collected in the interface with mononuclear cells.

Measurements were made with both density-gradient-separated cells (LSM) and whole blood samples in which erythrocytes had been lysed, to determine whether separation procedures altered the ability of mononuclear cells to utilize the DCFH-DA. In both cases mononuclear cells were gated by forward and 90° light scatter. Cell preparations were measured kinetically for up to 20 min while maintaining the cell suspension at 37°C. All samples were mixed continuously using the standard sample mixer on the EPIC 753 flow cytometer. No, or very low, fluorescence was measured in either monocyte or lymphocyte populations despite consistently observing dose-dependent increases in fluorescence by neutrophils (mean channel No. from 17.0 to 221) in the same samples (Fig. 1). Thus, accurate and reliable measurement of intracellular monocyte oxidative metabolism using the DCFH-DA fluorochrome under these conditions was not possible by

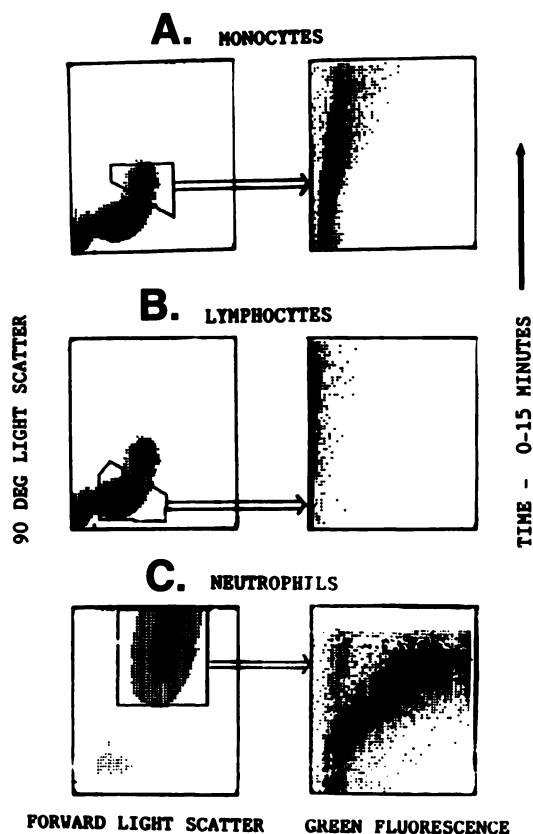


Fig. 1. Flow cytometric histograms showing forward-angle light scatter versus 90° light scatter on the left of the figure and, on the right, the resultant green fluorescence (GFL) from the gated population plotted as GFL versus time (0–15 min). All cell preparations were stimulated at zero time with PMA (100 ng/ml). A shows the gated monocytes from Hypaque-separated mononuclear cells. Almost no increase in fluorescence was observed over 15 minutes. B shows the same for the gated lymphocyte population, and C shows the increase in GFL observed with PMA stimulation of neutrophils. In the neutrophils, a small nonresponsive population was observed and consisted of less than 10% of the neutrophils.

flow cytometry. The following studies were undertaken to investigate the apparent discrepancy between flow cytometric and bulk suspension assays.

Spectrofluorometric Measurement of DCFH-DA and CF-DA Fluorochromes

The use of DCFH-DA as an intracellular probe for intracellular oxidation products is based upon the intracellular hydrolysis of DCFH-DA to form DCFH by esterase(s), which is then susceptible to oxidation to form the fluorescent product DCF. Thus, the lack of fluorescence in the flow cytometric assay could be the result of poor hydrolysis of DCFH-DA and/or oxidation of DCFH. First, to examine this esterolytic step in monocytes and neutrophils, two fluorochromes which are hydrolyzed by similar esterases were used. Hydrolysis of DCFH-DA forms a nonfluorescent molecule, DCFH. However, hydrolysis of nonfluorescent CF-DA results in the direct formation of a fluorescent product, carboxyfluorescein (CF). CF-DA is therefore a good substrate for the direct measurement of the intracellular esterolytic capacity. DCFH-DA was added to mononuclear cells (containing 1×10^6 monocytes) or neutrophils (1×10^6) for 15 min at 37°C. Spectrofluorometric tracing indicated no or minimal increase in fluorescence with mononuclear cells or neutrophils in the absence of added stimulus. CF-DA addition to the same number of mononuclear cells or neutrophils caused a rapid increase in the fluorescence within seconds of addition which continued for at least 30 min, suggesting that the CF-DA was being hydrolyzed rapidly as soon as it entered the cells (fluorescence after 15 min for mononuclear cells $70\% \pm 5\%$ of final fluorescence, and for neutrophils fluorescence was $63\% \pm 7\%$ of final fluorescence, $N=2$). Addition of base (0.1 N NaOH) to the cell suspensions followed by neutralization back to pH 7.4 failed to increase the fluorescence further, suggesting total hydrolysis of intracellular CF-DA in both cell types. When DCFH-DA-loaded mononuclear cells or neutrophils were stimulated with PMA (100 ng/ml), an increase in fluorescence of the cell suspension was observed for both cell types (Fig. 2), suggesting the hydrolysis of the DCFH-DA to DCFH and subsequent oxidation to DCF. In this type of bulk suspension assay, however, the location of the fluorescent product is uncertain, but subsequent data showed that the bulk of the fluorescence was in the cell supernates. This suggests that the flow cytometric assay failed to show significant fluorescence because of a lack of intracellular hydrolysis of DCFH-DA and/or intracellular oxidation of DCFH. The CF-DA data, however, seems to indicate adequate hydrolytic mechanisms for that probe, although DCFH-DA may either partition differently inside the cell, or be less susceptible to hydrolysis by the same esterase, or both. These possibilities were then explored further in the following experiments.

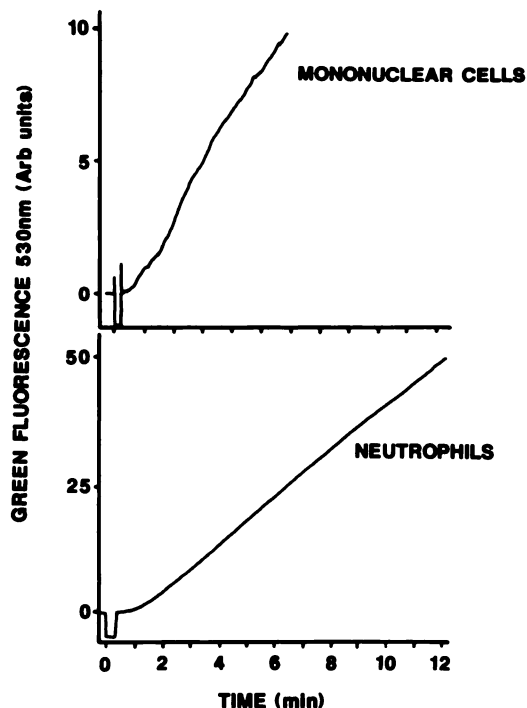


Fig. 2. Spectrofluorometric tracings showing the increasing fluorescence of DCFH-DA loaded (but unwashed) monocytes (top) and neutrophils (bottom) after stimulation with PMA (100 ng/ml). Green fluorescence was measured with excitation at 488 nm and emission at 530 nm at 37°C. Both mononuclear cells and neutrophils demonstrated increasing fluorescence after stimulation.

Efficiency of Mononuclear Cells' Hydrolytic Mechanisms

To establish whether there was residual intracellular DCFH-DA which had not been converted to DCFH, intact mononuclear cells and neutrophils were loaded with 5 μ M DCFH-DA for 15 min at 37°C, washed, and sonicated. NaOH (0.1 N, final concentration) was added to the sonicates for 3 min (pH 10.4) (to hydrolyze any DCFH-DA available), and the fluorescence was measured spectrophotometrically after readjusting to pH 7.4, before and after addition of H₂O₂ (1 μ M final concentration). H₂O₂ added to DCFH-DA alone did not result in any fluorescent products. In the absence of added H₂O₂ there was no significant difference in the fluorescence of cell sonicates before or after the treatment with NaOH. Upon addition of H₂O₂, the fluorescence of mononuclear cell sonicates increased significantly, with the increase being approximately 100% greater after base hydrolysis than that without base hydrolysis. A smaller difference (approximately 25%) was seen in neutrophil sonicates between base-hydrolyzed versus nonhydrolyzed preparations. The final fluorescence after both base hydrolysis and H₂O₂ addition was greater in mononuclear cells than

in neutrophils, suggesting equal or greater DCFH-DA uptake in mononuclear cells. These data suggest less efficient intracellular hydrolysis of DCFH-DA in mononuclear cells relative to neutrophils despite equal if not greater uptake of DCFH-DA in mononuclear cells.

This difference between the two cell types is more apparent when the effect of base hydrolysis was analyzed in cell sonicates to which DCFH-DA was added (Table 1). In this experiment, cells were sonicated first, and DCFH-DA (5 μ M) was added to the sonicates and incubated at 37°C for 15 min. Aliquots were then taken which were hydrolyzed with 0.1 N NaOH and reneutralized as described above. Upon addition of 1 μ M H₂O₂ the increase in fluorescence in mononuclear cells was approximately 300% greater in base-hydrolyzed versus nonhydrolyzed sonicates. In contrast, neutrophil sonicates showed no significant difference in H₂O₂-induced fluorescence increase as a result of base hydrolysis (Table 1). The same experiments were performed with CF-DA, using mononuclear cells, and these experiments confirmed that approximately 70% of the fluorochrome was hydrolyzed in both sonicates and whole cells (Table 2). These results demonstrate a markedly less efficient system for DCFH-DA hydrolysis in mononuclear cells than neutrophils, which was not apparent with CF-DA.

Susceptibility of Cellular Esterases to Heat Treatment

To determine if the fluorescence generated by cells was due to enzymatic hydrolysis of DCFH-DA, mononuclear cells were sonicated. An aliquot was heated at 100°C for

TABLE 1. Percentage of Baseline Fluorescence After Addition of NaOH^a

	Mononuclear cells	Neutrophils
Whole cells	362 \pm 134	75.5 \pm 15.4
Sonicates	586 \pm 235	106.7 \pm 44.9

^aData (mean \pm SD, N=4) showing the percentage increase over baseline fluorescence for both mononuclear cells and neutrophils after addition of 0.1 N NaOH and neutralizing the pH before measurement of fluorescence as described in Materials and Methods.

TABLE 2. Percentage of Probe Not Hydrolyzed by Mononuclear Cells^a

	Sonicates	Whole cells
DCFH-DA	85.2 \pm 20.3	58.8 \pm 12.7
CF-DA	10.35 \pm 4.8	28.6 \pm 10.5

^aTable 2 shows data (mean \pm SD, N=4) indicating the amount of probe (DCFH-DA or CF-DA) not hydrolyzed by either the sonicates or whole cells in the absence of added NaOH hydrolysis. Whole cell fluorescence was corrected for background fluorescence in the absence of added H₂O₂. Measurements were made spectrofluorometrically with excitation at 488 nm and emission at 525 nm.

5 min to destroy enzymatic activity and 5 μM DCFH-DA was added to heated and unheated sonicates, and incubated for 15 min at 37°C. The fluorescence in the heated sonicates, owing mainly to basal oxidant activity, was less than half that of the untreated samples. Addition of H_2O_2 increased the fluorescence of the heated sonicates by 2.7-fold, while the unheated sonicate's fluorescence increased 6.3-fold. Similar values were observed for neutrophils, demonstrating that DCFH-DA hydrolysis in both these cell types is primarily enzymatic.

Comparison Between PMA Stimulation and H_2O_2 Oxidation

To determine the degree of hydrolysis of the DCFH-DA in mononuclear cells and neutrophils, 1.5×10^6 monocytes (approximately 4.5×10^6 mononuclear cells) or neutrophils were loaded with DCFH-DA (5 μM) for 15 min at 37°C. After washing, each cell suspension was divided into two equal portions, and one was stimulated with PMA (100 ng/ml) for 15 min at 37°C, while PBS was added to the other. Bulk fluorescence was measured spectrofluorometrically as previously described. The PMA-stimulated samples of both cell types increased in fluorescence intensity, indicating oxidation of fluorochromes as a result of cell activation. However, the increase was markedly less in mononuclear cells than in neutrophils (Fig. 2).

To determine whether further oxidation could occur if excess oxidant (H_2O_2) was added to these PMA-stimulated samples, 1 mM H_2O_2 was added to each sample and the spectrofluorometric measurements repeated. The addition of H_2O_2 to DCFH-DA-loaded cells should oxidize intracellular DCFH to the fluorescent product DCF. In resting cell preparations (which are loaded with DCFH-DA), the addition of H_2O_2 should produce a response similar to that of PMA stimulation. Fluorescence of both cell types increased after addition of H_2O_2 . Each measurement was made 7 min after addition of H_2O_2 . The results indicated that both PMA and H_2O_2 were capable of causing cellular fluorescence, but that the PMA-stimulated cells were consistently more fluorescent than in cells which the fluorescence was produced by H_2O_2 oxidation of the intracellular DCFH (Table 3). Mononuclear cells again produced markedly less fluorescence than did neutrophils treated identically with H_2O_2 . These results again confirmed deficient hydrolysis of DCFH-DA in the mononuclear cells. We then examined whether the introduction of DCFH-DA in a different manner would affect hydrolysis and oxidation.

Measurement of DCFH-DA in Phagocytosing Mononuclear Cells

To determine whether intracellular fluorescence could be measured if DCFH-DA were introduced inside bacte-

TABLE 3. Percentage Increase in Fluorescence After Addition of 1 mM H_2O_2 ^a

	Monocytes	Neutrophils
Unstimulated	287.3 \pm 45.5	830.7 \pm 70.9
Stimulated	176 \pm 23.3	154.2 \pm 21.6

^aWhole cells (neutrophils or mononuclear cells) were loaded with DCFH-DA (5 μM). One portion of each cell type was stimulated with PMA (100 ng/ml) and the other was left unstimulated. Spectrofluorometric measurements were made on each sample. Immediately, H_2O_2 (1 mM) was added to each tube and spectrofluorometric measurements repeated after 7 min. Results indicate that both mononuclear cells and neutrophil intracellular DCFH can be oxidized by either cell activation (PMA) or exogenous oxidant (H_2O_2). Monocyte whole cell absolute fluorescence per cell was considerably less than neutrophil whole cell fluorescence. Data represent means \pm SD, N=4.

ria, the following experiment was undertaken. *Staphylococcus aureus* (1×10^9 /ml) were preloaded by incubation with DCFH-DA (5 μM) for 15 min at 37°C in PBS-gel. Bacteria were extensively washed in PBS-gel (4 times at 10,000g for 3 min) and opsonized with pooled human serum for 30 min at 37°C. After washing, *S. aureus* were added to mononuclear cells which had not been loaded with DCFH-DA. DCFH-DA-loaded *S. aureus* alone were not fluorescent by flow cytometry and neither were mononuclear cells fluorescent in the absence of *S. aureus*. Flow cytometric fluorescence measurements were then made on the light-scatter-gated mononuclear cells population over 15 min, during which the bacteria were being phagocytosed. A steady increase in mononuclear-cells-associated fluorescence was observed during this time (Fig. 3). The cells were also observed by fluorescence microscopy. Mononuclear cells exposed to DCFH-DA-loaded bacteria contained many diffusely fluorescent granules, while no free bacteria were fluorescent. This suggests that monocytes were able to oxidize intrabacterial DCFH, despite their inability to oxidize intracellular (monocyte) DCFH.

DISCUSSION

A preliminary report by Bender et al. [4] has indicated that monocytes produce fluorescent products from the intracellular fluorochrome DCFH-DA, in a similar manner to neutrophils [3]. We have extended these studies by examining the oxidative metabolism of the human mononuclear cells using this fluorochrome by both spectrofluorometric and flow cytometric studies.

We have used the fluorochrome DCFH-DA, which is hydrolyzed intracellularly to nonfluorescent DCFH in neutrophils. During the oxidative burst, DCFH is converted into fluorescent DCF which can be quantified by flow cytometry. When mononuclear cells were isolated,

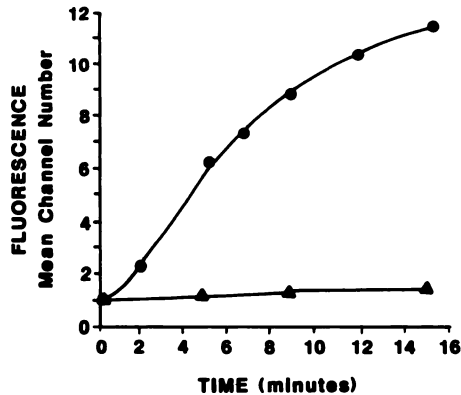


Fig. 3. Plot of mean fluorescence channel number standardized so that background fluorescence is channel 1. This figure shows the increase in mean fluorescence channel No. in monocytes (which did not contain DCFH-DA) after stimulation with preopsonized DCFH-DA-loaded *S. aureus*. A rapid increase in fluorescence was observed as monocytes phagocytosed the *S. aureus*. Almost no increase in fluorescence could be detected in DCFH-DA-loaded monocytes stimulated with PMA. ●—● *S. aureus* loaded with DCFH-DA plus unloaded monocytes; ▲—▲ monocytes alone (unloaded).

loaded with DCFH-DA and stimulated with PMA, no or minimal fluorescence was observed within the monocytes using flow cytometric methods.

To test the possibility that the lack of fluorescence may have been related to the isolation procedure, unseparated leukocytes from whole blood were also evaluated in this assay. DCF fluorescence of monocytes, lymphocytes, and neutrophils was measured by simultaneously gating each population based on light-scatter properties. The neutrophils always responded with a rapid increase in DCF fluorescence, whereas the lymphocytes and monocytes remained nonfluorescent. The lack of fluorescence, therefore, appeared to be unrelated to the technical procedures of cell isolation and preparation for assay. Thus, mononuclear cells appear to be incapable of forming intracellular DCF fluorescence in significant amounts after stimulation with PMA.

Ample evidence exists that stimulated monocytes produce oxygen radicals [8–11,13]. Although we were unable to measure intracellular fluorescence by flow cytometry, we were able to measure spectrophotometrically an increase in whole cell bulk fluorescence (Fig. 2) and O_2^- production by measuring superoxide dismutase (SOD) inhibitable cytochrome C reduction (data not presented) after PMA stimulation.

The source of this extracellular DCF is unknown, but several possibilities exist. The extracellular DCF could have been formed either intracellularly, or in the monocyte plasma membrane and subsequently released from the cell, although this is less likely due to the polar nature

of DCF. Alternatively, secreted oxygen metabolites may have converted any extracellular DCFH to DCF. To discriminate between these hypotheses, bacteria loaded with DCFH-DA were phagocytosed by monocytes and the resultant intracellular fluorescence measured by flow cytometry. After phagocytosis had occurred, the fluorescence inside the monocytes had increased in a similar manner (although not to the same extent) as that observed with PMA stimulation of DCFH-DA-loaded neutrophils. Free, extracellular bacteria remained nonfluorescent, suggesting that the bacteria-related DCF was formed by H_2O_2 generated intracellularly by the monocytes. The possibility exists that the H_2O_2 could have been formed by lysosomes or in the plasma membrane of the monocytes. The former seems likely in view of the lack of fluorescence generated in DCFH-DA-loaded PMA stimulated monocytes. Also, under fluorescence microscopy, the fluorescence was observed as a diffuse cytoplasmic glow within the monocytes. This is further strengthened by the fact that DCFH-DA-related fluorescence is not measurable in alveolar and peritoneal macrophages after stimulation with PMA or IgG or IgA immune complexes (Robinson et al., manuscript in preparation). Our findings support the concept of H_2O_2 release into the phagosome after phagocytosis, leading to the possibility that DCFH may be either leaking from DCFH-DA-loaded monocytes or formed on the plasma membrane after stimulation with nonparticulate substances.

There are a number of other explanations as to why little DCF-related fluorescence was observed with nonparticulate stimuli. One might be that esterases necessary for the hydrolysis of DCFH-DA were either lacking or partitioned in such a way as to be unavailable. A similar fluorochrome, CF-DA, which fluoresces immediately upon hydrolysis, was used to test for esterase availability. No difference was observed in the final fluorescence of either mononuclear cells or neutrophils with this dye, suggesting that the esterases were indeed available. Our studies with addition of NaOH to hydrolyze DCFH-DA did indicate that with mononuclear cells, further hydrolysis was possible, suggesting that sufficient DCFH-DA was taken up by the mononuclear cells. This was particularly evident with the monocyte sonicates where significantly more hydrolysis was possible compared with the neutrophil sonicates. In this case, where DCFH-DA was added to the sonicates of mononuclear cells, a greater percentage increase in fluorescent product could be achieved than from the neutrophil treated in a similar manner. Despite this finding, it would not be sufficient to account for the lack of fluorescence observed within whole cells.

Our studies utilizing CF-DA demonstrated that both mononuclear cells and neutrophils were capable of hydrolyzing the CF-DA directly to the fluorescent carboxy-

fluorescein. There does appear to be some difference between the way the mononuclear cells hydrolyze DCFH-DA and CF-DA. Both cell types rapidly form the fluorescent product carboxyfluorescein, indicating the presence of esterases for hydrolysis; however, the reasons for the different hydrolytic activity with DCFH-DA are not clear. Despite this, there are sufficient esterases present in the mononuclear cells for the formation of some DCFH. In DCFH-DA-loaded monocytes, DCFH equilibrates within the hydrophobic-lipid-containing areas of the plasma membrane. During PMA stimulation a considerable proportion of the H_2O_2 or O_2^- is generated extracellularly, where the DCF so formed would be unavailable for flow cytometric determination.

We conclude that there are significant differences in the oxidative mechanisms of neutrophils and monocytes not previously observed. It appears that in response to soluble stimuli which are not internalized by phagocytic mechanisms, monocytes mainly secrete their oxygen metabolites, whereas such metabolites are also formed within neutrophils. These data also suggest that there may be incomplete intracellular hydrolysis of DCFH-DA, probably as a result of esterase compartmentalization and thus unavailability. Further, since stimulated cells release esterases and peroxidases owing to degranulation, it is likely that extracellular hydrolysis of DCFH-DA could occur. The DCFH so formed could then be converted by H_2O_2 in the presence of peroxidase to the fluorescent DCF, extracellularly. During phagocytosis cytotoxic oxygen species are released into bacterial-containing phagosomes of both neutrophils and monocytes, allowing the measurement of the intracellularly formed fluorescence. The exact mechanisms of these interactions is yet to be determined; however, it appears likely that the lysosomal enzymes and peroxidase inside the phagolysosome penetrate the bacteria, converting the DCFH to DCF. The extracellular bacteria remain nonfluorescent because of the inability of extracellular peroxidase to penetrate the bacteria.

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