Comparative Use of 2,7-Dichlorofluorescin Diacetate, Dihydrorhodamine 123, and Hydroethidine to Study Oxidative Metabolism in Phagocytic Cells

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Tests carried out with model systems to assess the sensitivity of 2,7-dichlorofluorescin diacetate, dihydrorhodamine 123, and hydroethidine to the reactive oxygen species generated in these systems showed that all three fluorochromes are sensitive to hydroxyl and superoxide radicals and to hypochlorite ions and less sensitive to hydrogen peroxide. They do not react with singlet oxygen. Hydroethidine reacts with superoxide radicals in the 1:1 molar ratio. The relative proportions of reactive oxygen species generated extra- and intracellularly were estimated for phorbol myristate acetate-stimulated peritoneal and alveolar rat macrophages and for polymorphonuclear leukocytes from the blood of healthy donors. In activated leukocytes of different types, intracellular generation accounted for approximately 20% of the total reactive oxygen species produced. Intracellular production of these species was found to show considerable cyanide sensitivity, which indicates that cyanide-resistant NADPH oxidase located in the plasma membrane is not implicated in the generation of reactive oxygen species.

Key Words: *2,7-dichlorofluorescin diacetate; dihydrorhodamine 123; hydroethidine; oxygen radicals*

Altered activity of free-radical processes is associated with a variety of pathological conditions including, among others, inflammation, pneumoconiosis, fibrosis, autoimmune states, and carcinogenesis [4,5].

So far, most of the studies concerned with oxidative metabolism in phagocytes have considered only the extracellular generation of reactive oxygen species. However, some radicals are known to be formed extracellularly, for example by enzymes of the mitochondrial respiratory chain or endoplasmic reticulum enzymes [1]. Unfortunately, the intracellularly generated products of incomplete oxygen reduction are usually undetectable with conventional techniques (e.g., spectrophotometrically from cytochrome C reduction or using chemiluminescence). Promising substances in this regard are fluorescent dyes, for they are capable of penetrating inside phagocytes to be oxidized there by reactive oxygen species (ROS) with the formation of fluorescent products [11,14].

In this investigation we explored the feasibility of using the fluorochromes 2,7-dichlorofluorescin diacetate (2,7-DCFH-DA), dihydrorhodamine 123 (DHR-123), and hydroethidine in comparative studies of the role played by extracellular ROS production in the oxidative metabolism of phagocytic cells.

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MATERIALS AND METHODS

The proposed method of measuring the intracellular production of ROS relies on the ability of the vital fluorochromes hydroethidine, 2,7-DCFH-DA,

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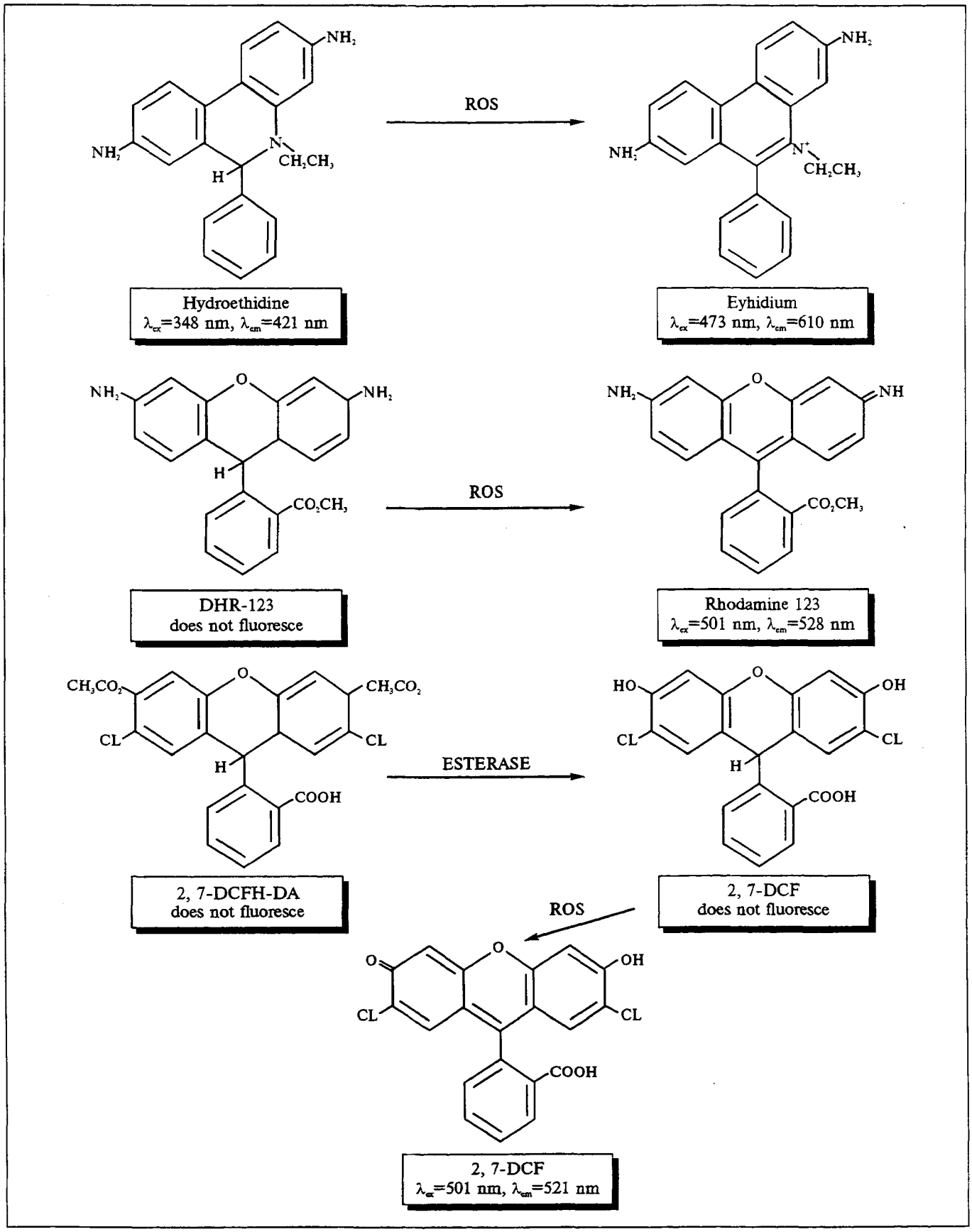

Fig. 1. Schemes showing conversions of 2,7-DCFH-DA, DHR-123, and hydroethidine into 2,7-DCF, rhodamine 123, and ethidium after interaction with ROS. λ_{ex} = excitation wavelength; λ_{em} = emission (fluorescence) wavelength.

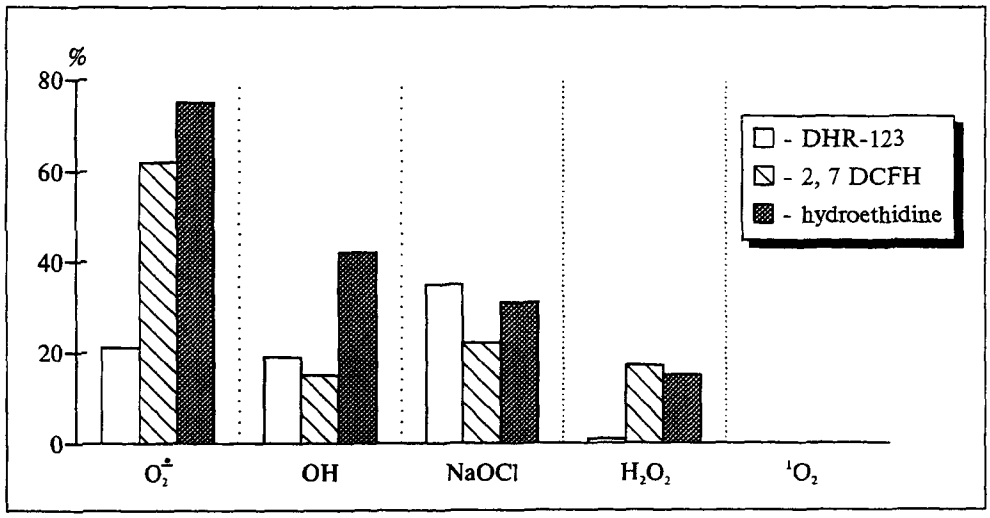

Fig. 2. Comparative sensitivities of 2,7-DCFH, DHR-123, and hydroethidine to ROS.

and DHR-123 to accumulate inside cells and to be converted into ethidium, 2,7-dichlorofluorescein (2,7-DCF), and rhodamine 123, respectively, after interacting with ROS (Fig. 1). All three fluorochromes were synthesized at and kindly donated by the Research Institute of Organic Intermediates and Dyes. Their stock solutions were prepared in dimethylformamide and stored at -18°C.

Conversions of the fluorochromes under the action of free oxygen radicals were studied using model systems, namely a modified Fenton's reagent to study the action of hydroxyl radicals ($OH^{\bullet}$) and the xanthine oxidase reaction to study that of superoxide radicals ($O_2^{\bullet-}$). Xanthine oxidase activity was estimated by uric acid formation [13]. To each control sample, 20 μg/ml superoxide dismutase were added. The amount of $O_2^{\bullet-}$ was estimated by cytochrome C reduction knowing that one $O_2^{\bullet-}$ molecule reduces one cytochrome C molecule [12]. The source of singlet oxygen was $NaOCl$-H_2O_2 solution [3].

Liposomes from the total fraction of egg phospholipids (0.1 mg/ml) were prepared as previously described [8]. Peritoneal and alveolar macrophages and polymorphonuclear leukocytes were isolated from donor blood by the conventional technique [9,10].

For measurement of intracellular ROS production, each cell suspension in Hanks' balanced salt solution without phenol red (pH 7.4) was incubated for 15 min with 10^{-4} M hydroethidine, carefully washed by centrifugation (5 min at 800 g and 4°C) in a 20-fold excess of Hanks' solution, finally resuspended to a concentration of 10^6 cells/ml, and stimulated with phorbol myristate acetate (PMA) in a concentration of 2×10^{-7} M. Ethidium formation from hydroxyethidine was estimated by measuring the intensity of ethidium fluorescence at an excitation wavelength of 610 nm and an emission (fluorescence) wavelength of 473 nm in an MPF-44 spectrofluorometer (Perkin Elmer) in centimeter quartz cuvettes thermostatically controlled at 37°C, with constant stirring [2].

Two groups of cell samples were prepared to compare extra- and intracellular ROS production. One group comprised hydroethidine-loaded phagocytes carefully washed free of excess fluorescent dye and then stimulated with PMA; in these samples, only intracellular ROS production was measured. The other group contained hydroethidine-loaded phagocytes that still contained the excess dye and were also stimulated with PMA in the same concentration; in such samples both intra- and extracellular (i.e., total) production of ROS was measured, after which their extracellular production was estimated by subtracting the intracellular production from the total production [2].

TABLE 1. Relative Proportions of Extra- and Intracellularly Produced ROS in Different PMA-Activated Phagocytic Cells ($M\pm m$)

Phagocytes	Percentage of ROS generated	
	intracellularly	extracellularly
Peritoneal macrophages, rats	28±5	72±7
Alveolar macrophages, rats	11±2	89±5
Blood neutrophils, healthy donors	19±7	81±7

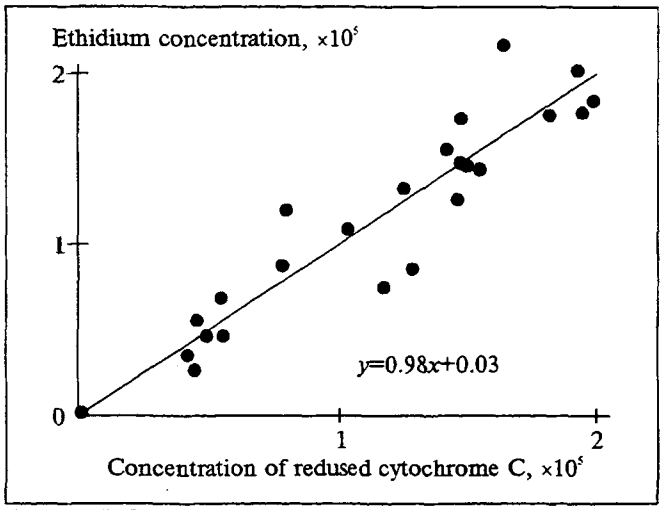

Fig. 3. Relationship between cytochrome C sensitivity to superoxide radicals and hydroethidine conversion into ethidium in the xanthine oxidase reaction. Xanthine oxidase: 1 activity unit/ml; oxidized cytochrome: 5×10^{-5} M; catalase: 0.8 mg/ml; hydroethidine: 3×10^{-5} M. Four different xanthine concentrations were used: 0.8, 1.7, 2.7, and 3.4 μM in 50 mM potassium–phosphate buffer (pH 7.4).

RESULTS

In the first part of the study we sought to ascertain whether the fluorescent dyes exhibit specific sensitivity to a particular type of oxygen radicals and whether they can be utilized for quantifying ROS production in cells. To these ends, the fluorochromes hydroethidine, 2,7-DCFH, and DHR-123 were tested for sensitivity to ROS in model systems that each produced radicals of only one type and also for NaOCl. In these tests the percentage of dye molecules whose fluorescent properties were altered after their reaction with ROS was determined.

The initial concentration of the fluorochromes was 10^{-6} M. The concentration of their oxidized forms was determined by comparing the fluorescence intensity of the test sample with a calibration curve prepared using standard fluorochrome solutions. In the concentrations used, none of the fluorochromes had an inhibitory effect on xanthine oxidase activity (Fig. 2).

We also explored how many $O_2^{\bullet}$ molecules would convert one dye molecule into the fluorescent form and found that one DHR-123 molecule was converted into rhodamine by 370 $O_2^{\bullet}$ molecules and one 2,7-DCFH molecule into 2,7-DCF by 15 $O_2^{\bullet}$ molecules, and that hydroethidine interacted with $O_2^{\bullet}$ in a 1:1 molar ratio (Fig. 3).

The specificity of each fluorescent dye for ROS was tested in order to estimate the likelihood of artefactual results. The tests showed that DHR-123 was converted into rhodamine 123 not only after exposure to ROS but also after interacting with the polyanion bovine serum albumin (0.5 mg/ml) as well as with the polycation protamine sulfate (0.5 mg/ml), and that 2,7-DCFH was converted into 2,7-DCF in the presence of 0.1 mg/ml bovine serum albumin. Protamine (0.5 mg/ml) and DNA (0.05 mg/ml) did not affect 2,7-DCFH conversion into 2,7-DCF, nor did liposomes. Proteins, DNA, phospholipids, or a change in pH within the range of 4 to 8 had no effect on hydroethidine conversion into ethidium. The use of 2,7-DCFH-DA or DHR-123, but not of hydroethidine, may therefore produce artefactual results, and for this reason hydroethidine was used in all further tests.

Table 1 shows the relative proportions of extra- and intracellularly produced ROS as estimated using hydroethidine for alveolar and peritoneal macrophages from rats and blood neutrophils from healthy donors. The mean proportion of ROS generated intracellularly by different types of activated neutrophilic leukocytes was approximately 20% (range, 11-28%).

Intracellular ROS production in phagocytic cells was sensitive to cyanide (NaCN). Thus, it was decreased by 85±6% and 98±4%, respectively, in PMA-stimulated peritoneal and alveolar rat macrophages preincubated with 1 mM NaCN for 3 to 5 min. At this concentration NaCN also reduced intracellular ROS production in PMA-stimulated neutrophils, though only by 47±5%. Nonactivated polymorphonuclear leukocytes responded to the presence of 1 mM NaCN by a slight increase in spontaneous ROS generation. For peritoneal rat macrophages and human blood neutrophils extracellular ROS production was found to be less sensitive to NaCN than intracellular: preincubation with 1 mM NaCN reduced extracellular ROS generation only by 20-40% in the former cells and by 22±9% in the latter. Alveolar macrophages proved to be more sensitive to NaCN, which reduced extracellular ROS generation by as much as 82-86%.

The most convenient of the fluorescent dyes used to determine intracellular ROS generation proved to be hydroethidine.

Alveolar macrophages are strikingly aerobic cells with intensively working mitochondria, whereas peritoneal macrophages are anaerobes in which glycolysis predominates [7]. The observed difference between these cells in the ratio of extracellular to intracellular ROS production is possibly due to differences in oxidative metabolism. The ability of NaCN to depress intracellular ROS generation confirms that radicals such as ROS are

indeed produced in cyanide-sensitive systems, most likely in mitochondria [1,5]. The reaction to cyanide is also a measure of the quality of the method we used. The finding that intracellular ROS generation is more sensitive to NaCN than extracellular demonstrates that hydroethidine penetrates into the cell and is converted there into ethidium.

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