Investigations of Phagosomes, Mitochondria, and Acidic Granules in Human Neutrophils Using Fluorescent Probes

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The oxidative burst is frequently evaluated by the conversion of dihydrorhodamine 123 (DHR) to rhodamine 123 (R123) and hydroethidium (HE) to ethidium with the use of flow cytometry (FCM). Added R123 accumulates in mitochondria, but during phagocytosis R123 originating from DHR has been observed in neutrophil granules. The present study was designed to identify the site of reactive oxygen species (ROS) formation and the intracellular traffic of R123 in neutrophils by using mitochondrial membrane potential probes and the lysosomotropic probe LysoTracker Red, which have not previously been applied to neutrophils. Quiescent and phagocytosing human peripheral blood neutrophils were incubated with DHR, HE, R123, MitoTracker Green (MTG), MitoTracker Red (CMX-Ros), and LysoTracker Red alone and in all combinations of red and green probes, and studied by FCM and confocal laser scanning microscopy (CLSM). Phagosomes were filled with R123 originating from DHR. Phagocytosis also triggered the oxidative burst in oxidative response granules that differed from acidic granules. All the neutrophils stained with mitochondrial and lysosomotropic dyes. Added R123 and MTG selectively accumulated in mitochondria. Added R123, MTG, and DHR increased the fluorescence of CMX-Ros and LysoTracker Red. This is the first FCM and CLSM demonstration of ROS formation in phagosomes. A distinct subpopulation of neutrophil granules, termed oxidative response granules, also was identified. Neutrophil mitochondrial membrane potential may be evaluated by incubating the cells with R123 and MTG, but results with CMX-Ros should be interpreted with caution. HE and DHR seem to measure a common pathway in the oxidative burst. The simultaneous application of several probes for investigations of organelles carries the risk of probe interference. Cytometry Part B (Clin. Cytometry) 51B:21–29, 2003. © 2002 Wiley-Liss, Inc.

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The oxidative burst of neutrophils plays a crucial role in the defense against life-threatening bacterial infections (reviewed in 1–5). The oxidative burst is routinely measured by the conversion of non-fluorescent dyes to fluorescent counterparts. Dihydrorhodamine123 (DHR) is converted to rhodamine123 (R123) and reflects hydrogen peroxide (H_2O_2) production (3,6,7). The conversion of dihydroethidium (HE) to ethidium is an indicator of superoxide (O_2^-) production (3,8). DHR is the most sensitive and stable indicator of the oxidative burst and is used in routine clinical evaluations of the oxidative burst (7,9).

Added R123 accumulates in mitochondria and originally was not observed in other organelles such as lysosomes and endosomes (10). For this reason R123 was used as a supravital mitochondrial probe (11,12). However, Rothe and coworkers suggested that the R123 that is generated from DHR may accumulate in neutrophil compartments other than mitochondria (7). Jankowsky and Grinstein recently found that, after stimulation with phorbol myristate acetate, DHR accumulates in neutrophil granules and is converted to R123, whereas added R123 is taken up into mitochondria (13).

To be effective in combating infections, reactive oxygen species (ROS) have to be produced near or on their target microorganisms in phagosomes. An intracellular

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production of ROS is demonstrated by autoradiography using iodine nuclides (14). There is abundant evidence for the production of H_2O_2 and O_2^- inside phagosomes (reviewed in 1,15,16). However, results from experiments using DHR seem to be inconsistent with ROS formation restricted to phagosomes. By using a bead phagocytosis assay, Lehmann and coworkers observed that R123 originating from DHR accumulates in neutrophil granules during phagocytosis (8,9). These fluorescent granules are round and evenly distributed throughout the cytoplasm. However, surprisingly little R123 is associated with phagosomes, and no mitochondria-related fluorescence is observed. These results accord with the findings of Jankowski and Grinstein (13). The nature of these R123-fluorescent granules and their relation to acidic organelles remain to be established.

One way to track the site of metabolic processes is by using fluorescent dyes that localize to defined intracellular compartments. Several dyes are available for this purpose. When added to cells R123 accumulates in mitochondria (17,18). MitoTracker Green (MTG) and MitoTracker Red (CMX-Ros) also may allow the visualization of mitochondria, and LysoTracker Red is useful for staining acidic intracellular compartments, in particular lysosomes (19). The distribution of these potent organelle probes has not been investigated in neutrophils.

The present study aimed to localize R123 after the addition of R123 to cells and R123 formation from DHR during phagocytosis. To achieve this goal we incubated human neutrophils with HE to identify superoxide production. R123, MTG, and CMX-Ros were selected for visualizing mitochondria, and LysoTracker Red was chosen for staining acidic granules. In addition, DHR was used to track ROS-producing neutrophil granules and phagosomes. To detect the phagosomes we selected porous zymosan particles prepared from *Saccharomyces cerevisiae*. The uptake and distribution of these organelle probes were quantified with flow cytometry (FCM), and probe localization was achieved by confocal laser scanning microscopy (CLSM).

The results showed that R123 added to the cells stained only mitochondria of quiescent neutrophils. This R123 also was taken up into phagosomes of phagocytosing neutrophils but was bound to the remaining nucleus of *S. cerevisiae* and did not appear elsewhere in the phagosome. In contrast, R123 originating from DHR during the phagocytosis of zymosan particles stained a subpopulation of granules that was different from acidic granules. In addition, neutrophil phagosomes were filled with green fluorescent R123 emerging from DHR. The lack of passive diffusion of R123 between organelles and the different staining pattern of phagosomes exposed to R123 and DHR confirmed that R123 is compartmentalized in neutrophils. Because the production of R123 from DHR is mediated by ROS and R123 originating from DHR was found in phagosomes, the results strongly suggested that ROS are produced locally in phagosomes. The findings demonstrated selective uptake into mitochondria of R123 and MTG that were added to the cells, whereas even low concentrations of CMX-Ros stained neutrophil granules. Association of selected dyes with specific subcellular organelles should be interpreted with care. In particular, the cell type must be taken into account.

MATERIALS AND METHODS

Instrumentation

Two flow cytometers, an EPICS Elite and an EPICS XL (Coulter Corp., Hialeah, FL), were used throughout, with excitation at 488 nm. Emissions were collected with a 525-nm bandpass filter for R123 and MTG and a 610-nm longpass filter for CMX-Ros, ethidium, and LysoTracker Red. Fluorescence compensation was performed electronically. Lymphocytes, monocytes, and neutrophils were discriminated by forward and side scatter. For this study, log red and green fluorescences were collected from the neutrophils. CLSM was performed with a Bio-Rad MRC 1024-UV (Bio-Rad, Hercules, CA) instrument equipped with a krypton-argon laser with 488-nm excitation and interfaced to a Nikon 300 inverted microscope (Nikon, Tokyo, Japan). Emission filters were a 525- to 535-nm bandpass for R123 and MTG and a 585-nm longpass for CMX-Ros, ethidium, and LysoTracker Red.

Human Subjects

Leukocytes were obtained from healthy female and male volunteers aged 30–54 years. Peripheral blood was drawn into 10-ml Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing 100 U of preservative-free heparin according to an approved human-use protocol. Lysis of red blood cells (see below) was performed immediately after blood withdrawal.

Reagents

The following probes were used, at the final concentrations given in parentheses DHR (500 nM), R123 (500 nM), MTG (100 nM), CMX-Ros (100 nM), JC-1 (100 nM), or LysoTracker Red (100 nM; all from Molecular Probes, Eugene, OR), and HE (100 nM; Polysciences, Warrington, PA). The dyes were also tried at 10× these concentrations, and CMX-Ros was also used at an intermediate concentration (see details in Results).

Dulbecco's phosphate buffered saline (DPBS) consisted of 8 g of NaCl, 1.15 g of Na_2HPO_4 and 0.2 g each of KCl and KH_2PO_4 dissolved in 1 liter of distilled water. DPBS supplemented with glucose and albumin (DPBS-GA) contained 1 g of glucose and 5 g of bovine serum albumin per liter of DPBS. DPBS-GA supplemented with Ca^{2+} and Mg^{2+} (Sørnes' buffer) contained 132 mg of $CaCl_2 \cdot 2H_2O$ and 121 mg of $MgSO_4 \cdot 7H_2O$ per liter of DPBS-GA. A standard ammonium chloride lysing solution was used throughout (20).

Incubation Procedure

Erythrocytes were lysed according to a standard procedure (20). Cells were washed once in DPBS-GA and maintained in 2–5 ml of DPBS-GA at room temperature until use. The suspensions were adjusted to 1.25×10^7 leukocytes/ml. Zymosan A particles (50 μg; Sigma, St. Louis,

MO) were opsonized in a mixture of 1 ml of DPBS-GA and 1 ml of human serum and incubated for 1 h under gentle mixing at 37°C. The particles were washed once in DPBS-GA and adjusted to 1.25×10^8/ml in DPBS-GA using FCM. The zymosan particles were frozen in 1-ml vials and kept at −20°C until use. In phagocytosis experiments, 20 μl of leukocytes and 20 μl of zymosan particles were added to each microwell and adjusted to a total incubation volume of 100 μl with Sørnes' buffer.

Incubations were performed in flat-bottom, low-evaporation lid, tissue-culture–treated Costar microwell plates (Costar Corporation, Cambridge, MA). A total incubation volume of 100 μl was used in all experiments. The total volume was obtained by first adding the appropriate amount of Sørnes' buffer to each microwell. All probes were dissolved in Sørnes' buffer from the appropriate stock concentrations. A total of 20 μl of each probe was added to the appropriate microwells (see Results). Finally, 20 μl of leukocytes or 40 μl of the leukocyte–zymosan mixture was added to appropriate microwells (see Results) to start the reaction. If not otherwise stated, incubations were done for 30 min at 37°C in the dark with mixing supplied by a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ). For FCM the reaction was stopped by adding 200 μl of ice-cold (4°C) halting solution (0.02% ethylene-diaminetetra-acetic acid in PBS) and placing the microtiter plates on ice. A small proportion (3–5 μl) of each cell suspension was transferred to a Lab-Tek chambered coverglass (Nalge Nunc International, Naperville, IL) for CLSM investigations. The incubation mixtures were immediately subject to FCM and CLSM. Thus, data from FCM and CLSM were obtained from corresponding experiments run on the same day.

Statistics

All experiments were performed in triplicate at least three times. Values in bar diagrams are expressed as the mean of the triplicates. Differences between samples were evaluated with Students' *t*-test.

RESULTS

Autofluorescence and Staining of Zymosan Particles

Zymosan A particles originate from *Saccharomyces cerevisiae*. When particles are stained with fluorescein isothiocyanate the "nucleus" stains brightly, and a broader rim of the remaining "cytoplasm" stains more faintly (not shown). Native zymosan particles dissolved in Sørnes' buffer displayed a faint green autofluorescence that was seen only in the core of the particle, corresponding to the site of the remaining "nucleus" of the *Saccharomyces*. Zymosan particles exposed to DHR and HE displayed only this faint autofluorescence. In contrast, the nucleus of the zymosan particles stained green with R123 and MTG added to the cells, as did those stained red with CMX-Ros and LysoTracker Red.

Staining of Phagosomes by Fluorescent Probes

When DHR was present during phagocytosis, zymosan nuclei inside phagosomes were clearly green fluorescent, and in many neutrophils the entire phagosome was filled with green-fluorescent dye (Fig. 1A,F). When similar experiments were performed with R123 and CMX-Ros, zymosan nuclei inside phagosomes were clearly yellow-green fluorescent, whereas the cytoplasm of the particles exhibited no fluorescence, and a wide unstained rim between the zymosan nucleus and the phagosome wall was observed (Fig. 1B,C). MTG gave rise to a similar staining pattern (not shown). The observed phagosomal staining patterns for CMX-Ros and HE was similar, except that the fluorescence was red. HE also stained the zymosan nuclei (not shown). LysoTracker Red, like DHR, stained the entire phagosome in many neutrophils. The combination of R123, MTG, or DHR with CMX-Ros, LysoTracker Red, or HE rendered the nuclei of the zymosan particles red-yellow (Fig. 1A–C,F). When DHR was added after phagocytosis and incubated for 1 min with the cells, the zymosan nuclei inside phagosomes became fluorescent (not shown).

Association of Organelle Probes With Granules

A granular staining pattern was found in quiescent neutrophils stained with CMX-Ros or LysoTracker Red (Fig. 1D–E). The granules were round and localized all over the neutrophil except in the area of the cell nucleus. CMX-Ros$^+$ granules (Fig. 1D) were smaller and more numerous than the large LysoTracker Red$^+$ granules (Fig. 1E). When CMX-Ros or LysoTracker Red was combined with R123 or MTG, two distinct sets of organelles were observed: tubular structures were colored green only, and granules were red fluorescent (Fig. 1D,E). Tubular and granular structures with a mixture of the two colors were not observed. HE and DHR did not cause visible cellular fluorescence in resting peripheral mononuclear cells as leukocytes.

In neutrophils phagocytosing zymosan particles in the presence of DHR, the granules were brightly green fluorescent (Fig. 1A,F). However, when DHR was added after phagocytosis and incubated for 1 min with the cells under the same incubation conditions, none of the granules were fluorescent (not shown). When HE was present during phagocytosis, the neutrophil nuclei invariably stained red, but the granules were not colored. In phagocytosing neutrophils incubated with CMX-Ros or LysoTracker alone, the granular staining pattern was similar to that of quiescent neutrophils. In some of the neutrophils coincubated with CMX-Ros and DHR, the granules displayed a yellow fluorescence.

Mitochondrial Staining by Fluorescent Probes

When quiescent neutrophils were stained by adding R123 or MTG alone or in combination with any of the red dyes, many green tubular structures were clearly observed (Fig. 1D,E). Tubular structures were seen most often at the periphery of the cells and in areas of adhesion of the neutrophils to the object glass surface. By light micros-

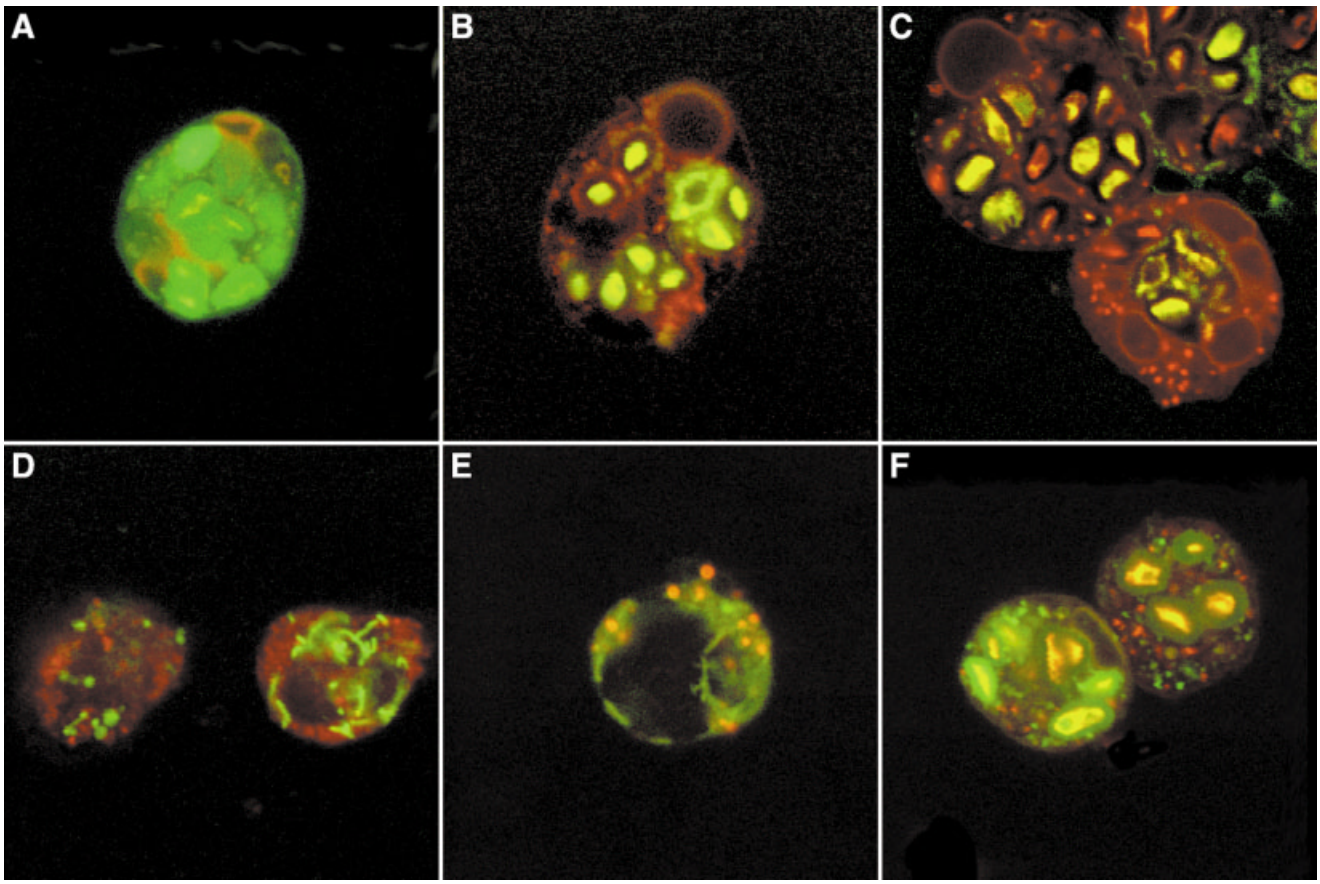

FIG. 1. Confocal scanning laser microscopy of resting neutrophils and neutrophils phagocytosing zymosan particles in the presence of various fluorescent indicator dye combinations. The functional status and the dye combinations are given for each panel. **A:** Phagocytosis, dihydrorhodamine (DHR) and hydroethidine (HE). **B:** Phagocytosis, rhodamine123 (R123) and a low concentration of MitoTracker Red (CMX-Ros). **C:** Phagocytosis, R123 and a high concentration of CMX-Ros. **D:** Resting neutrophils, R123 and CMX-Ros. **E:** Resting neutrophils, MitoTracker Green and LysoTracker Red. **F:** Phagocytosing neutrophils, DHR and CMX-Ros. Three-dimensional rotations of neutrophils stained as above are available at: www.cyto.purdue.edu/flowcyt/research/pub1.htm.

copy these tubular structures were seen to extend into lamellipodia. CMX-Ros stained only a few such tubular structures. No fluorescence was observed in tubular structures with DHR, HE, or LysoTracker Red.

In neutrophils phagocytosing zymosan particles in the presence of added R123 or MTG, the green tubular fluorescence from these probes was more dim than in the quiescent cells, and in most of the neutrophils examined, a green fluorescence was not observed at all. However, when R123 was added after phagocytosis and incubated for 1 min with the cells, tubular structures were as clearly stained as when the neutrophils had not phagocytosed. In the presence of HE, cell nuclei stained red, but the color of the mitochondria did not change. CMX-Ros, DHR, and LysoTracker Red did not affect the staining pattern of the tubular structures (not shown).

Fluorescence Patterns of Neutrophil Nuclei

Exposure of quiescent and phagocytosing neutrophils to a high concentration of CMX-Ros resulted in a clear red fluorescence corresponding to the double-layered nuclear envelope of the segmented nuclei (Fig. 1C). The inside of the nuclei were not stained. HE did not stain quiescent cell nuclei, but the densely packed chromatin bordering the nuclear membrane displayed a variable but clear red fluorescence (Fig. 1A). The added R123, MTG, LysoTracker Red, and DHR did not stain any part of the neutrophil nuclei.

FCM Investigations of Organelle Probes in Quiescent Neutrophils

Leukocytes were incubated with single fluorescent dyes. The reactions were stopped by adding 200 μl of halting solution and placing the microtiter plates on ice. The percentage of fluorescent neutrophils and their mean fluorescence intensity were recorded. With the probes R123 and MTG, all neutrophils were fluorescent in the green part of the spectrum, whereas neutrophils in the red part were not. With equimolar concentrations the fluorescence intensity of added R123 was greater than that of MTG (cf. Fig. 2C and 2F). Neutrophils exposed to DHR alone displayed only a faint fluorescence. CMX-Ros and LysoTracker Red displayed a unimodal fluorescence in the red part of the spectrum; but

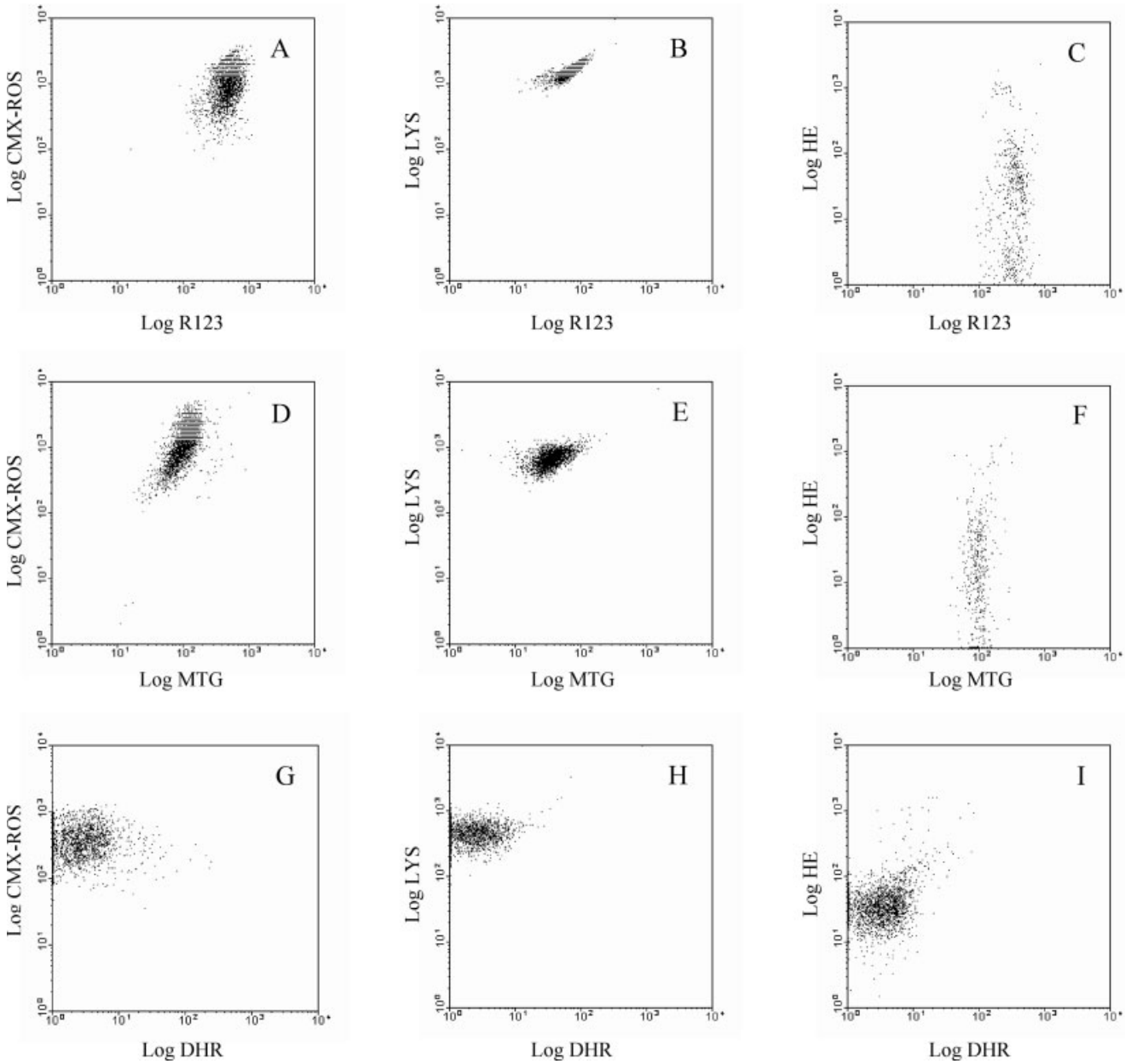

FIG. 2. Flow cytometric investigations on fluorescent mitochondrial and granule stains and oxidative burst in non-phagocytosing neutrophils. **A–I:** The cytograms represent dual measurements with all possible combinations of yellow-green fluorescent dyes rhodamine 123 (R123), dihydrorhodamine (DHR), and MitoTracker Green (MTG) and the red fluorescent dyes MitoTracker Red (CMX-ROS), hydroethidine (HE), and LysoTracker Red (LYS).

with due compensation, fluorescence from these probes was undetectable in the green. Neutrophils exposed to HE displayed a significant red fluorescence.

The interaction of organelle probes was studied by checkerboard additions of R123, MTG, and DHR with CMX-Ros, LysoTracker Red, and HE. The same volume (20 μl) of each probe was added to each microwell. This experimental approach may allow the detection of neutrophil subpopulations differing in membrane potentials and ROS formation. The neutrophils stained positive with the added R123 and CMX-Ros and with R123 and LysoTracker Red (Fig. 2A,B), whereas with the combination of R123 and HE, a significant but weaker ethidium fluorescence was observed (Fig. 2C). Only one population of neutrophils was observed. MTG behaved like the added R123 in combination with CMX-Ros and with HE (Fig. 2A,C,D,F). LysoTracker Red noticeably diminished the fluorescence of R123 and slightly diminished that of MTG (Fig. 2B,E). DHR gave rise to only a faint fluorescence with CMX-Ros, LysoTracker Red, or HE (Fig. 2G–I). There was a profound and highly statistically significant ($P < 0.01$) enhancing effect of added R123 and MTG on the red

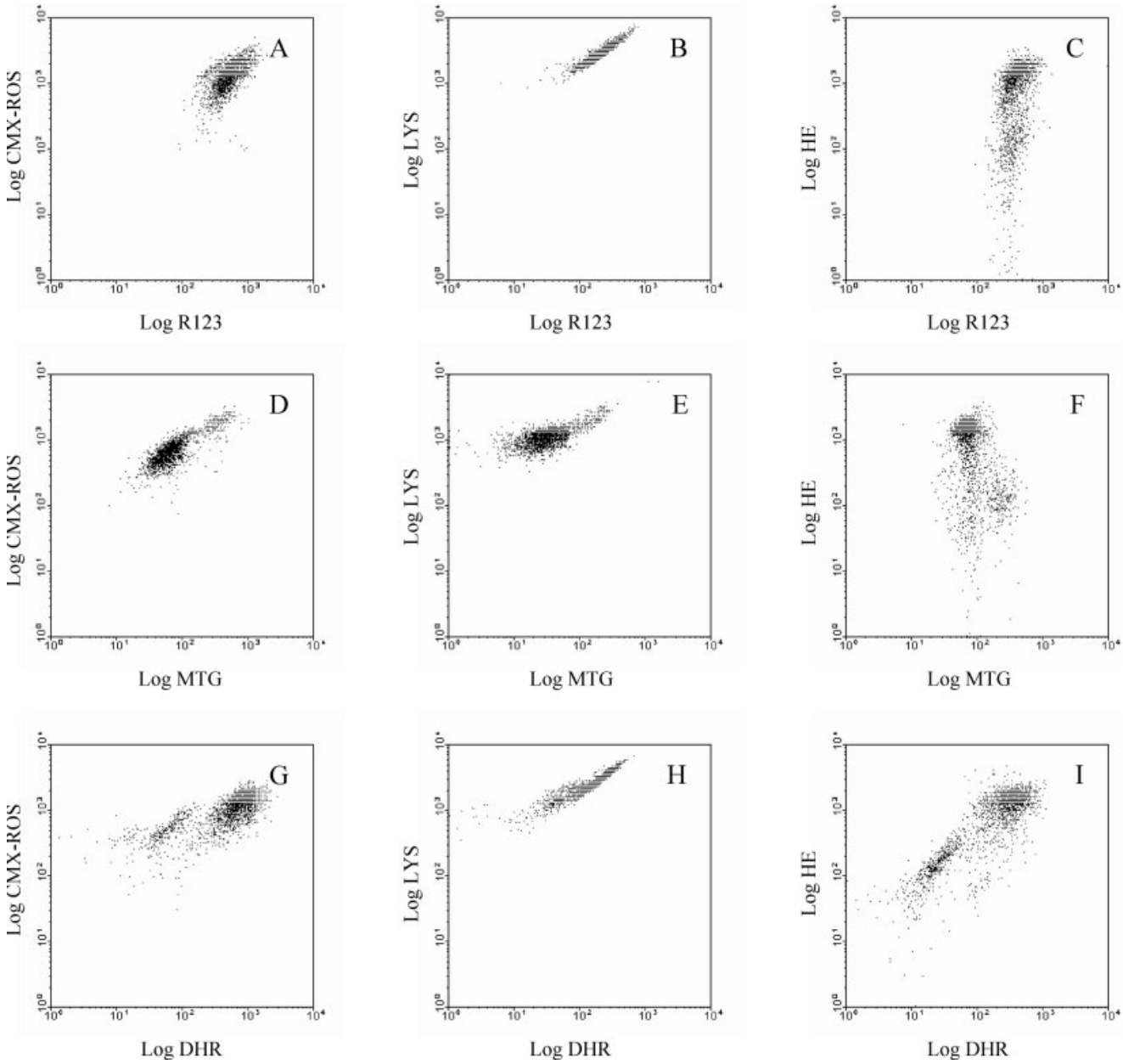

FIG. 3. Flow cytometric investigations on fluorescent mitochondrial and granule stains and oxidative burst in neutrophils phagocytosing zymosan particles. **A–I:** The cytograms represent dual measurements with all possible combinations of yellow-green fluorescent dyes rhodamine 123 (R123), dihydrorhodamine (DHR), and MitoTracker Green (MTG) and the red fluorescent dyes MitoTracker Red (CMX-ROS), hydroethidine (HE), and LysoTracker Red (LYS).

fluorescence of CMX-Ros and LysoTracker Red, but the converse effect of CMX-Ros and LysoTracker Red on the green fluorescence of added R123 and MTG was not observed (results not shown).

Dual-Parameter Assay of the Oxidative Burst

To induce complement-mediated phagocytosis, pre-opsonized non-fluorescent zymosan particles were used as targets, and all combinations of green and red fluorescent dyes were investigated (Figs. 3–5). Several neutrophil subpopulations were observed.

There was a profound and statistically significant increase ($P < 0.001$) in mean DHR green fluorescence compared with quiescent neutrophils (cf. Fig. 2G,H and Fig. 3G,H), whereas lymphocytes remained unstained (not shown). Similar results were obtained with HE (cf. Fig. 2C,F,I and Fig. 3C,F,I). With the instrument settings used during these experiments, a green fluorescence from free zymosan particles was not detected, and resting neutrophils did not convert DHR into R123.

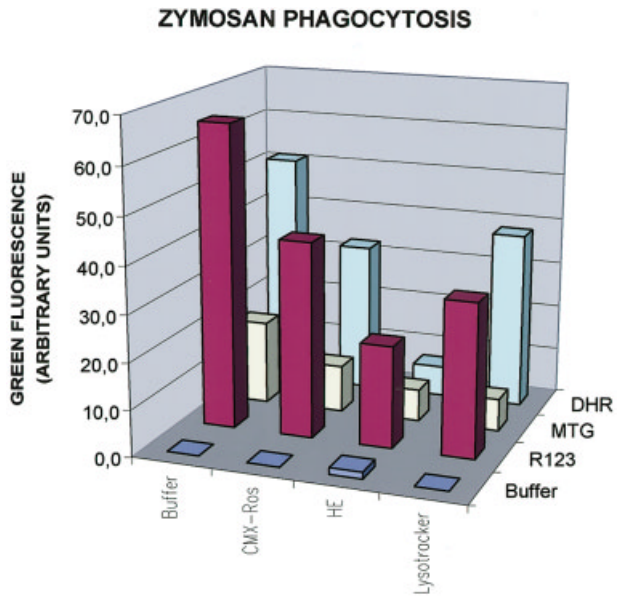

FIG. 4. Flow cytometric investigations on fluorescent mitochondrial and granule stains and oxidative burst in neutrophils phagocytosing zymosan particles. Measurements were performed with all possible combinations of yellow-green fluorescent dyes rhodamine 123 (R123), dihydrorhodamine (DHR), and MitoTracker Green (MTG) and the red fluorescent dyes MitoTracker Red (CMX-ROS), hydroethidine (HE), and LysoTracker Red (LYS). The mean green fluorescence values were recorded. Results are presented as typical mean values of duplicate investigations performed three times.

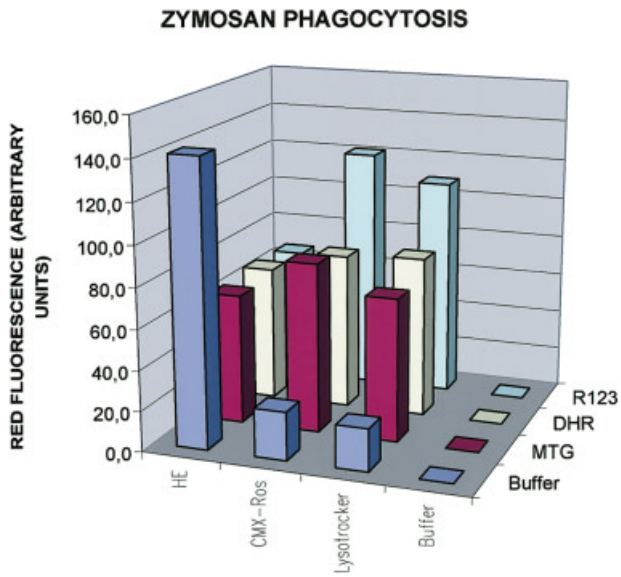

FIG. 5. Flow cytometric investigations on fluorescent mitochondrial and granule stains and oxidative burst in neutrophils phagocytosing zymosan particles. Measurements were performed with all possible combinations of yellow-green fluorescent dyes rhodamine 123 (R123), dihydrorhodamine (DHR), and MitoTracker Green (MTG) and the red fluorescent dyes MitoTracker Red (CMX-ROS), hydroethidine (HE), and LysoTracker Red (LYS). The mean red fluorescence values were recorded. Results are presented as typical mean values of duplicate investigations performed three times.

Phagocytosis was allowed to proceed in the presence of HE or DHR alone or in combination, and the mean fluorescence emitted by ethidium and R123 from DHR was measured. The presence of HE significantly diminished the fluorescence emission of R123 emerging from DHR (Fig. 4).

The level of baseline neutrophil fluorescence is suitably taken as the fluorescence of resting neutrophils in the presence of DHR. When this baseline was used, we observed a 400-fold increase in neutrophil fluorescence during phagocytosis after incubation with added DHR or R123. Complement-mediated phagocytosis also was measured by using opsonized, unstained zymosan particles as targets in the presence of 5% (v/v) hypogammaglobulinemic serum. Phagocytosis increased the uptake of added R123 into neutrophils by 219% with a 51-min preincubation time.

Uptake of a Lysosomotropic Probe During Phagocytosis

Leukocytes were incubated with LysoTracker Red in all combinations of green fluorescent organelle probes (Fig. 3B,E,H). Phagocytosis was arrested by diluting the cells in ice-cold halting solution and placing the microtiter plates on ice in the dark. The suspensions were immediately subjected to FCM. Phagocytosis significantly increased the uptake of LysoTracker Red into neutrophils. MTG influenced the red fluorescence from LysoTracker Red only slightly (cf. Fig. 2E and Fig. 3E). The presence of DHR and added R123 profoundly increased LysoTracker Red fluorescence emission of individual neutrophils (cf. Fig. 2B,H and Fig. 3B,H).

Uptake of Mitochondrial Probes During Phagocytosis

Leukocytes were incubated with all combinations of green and red fluorescent organelle probes during phagocytosis of pre-opsonized zymosan particles. After phagocytosis the suspensions were immediately subjected to FCM (Fig. 3). Phagocytosis of zymosan particles profoundly increased the uptake of added R123 into neutrophils and slightly diminished that of MTG (cf. Fig. 2B,E and Fig. 3B,E). In addition, CMX-Ros and HE diminished the fluorescence of added R123 and MTG (cf. Fig. 2A,C,D,F and Fig. 3A,C,D,F) and that of R123 emerging from DHR (Fig. 4).

Complement-mediated phagocytosis more than doubled neutrophil red fluorescence emission from CMX-Ros (cf. Fig. 2A,D,G and Fig. 3A,D,G). Mitochondrial membrane potential dyes R123 and MTG profoundly increased the fluorescence intensity from CMX-Ros (Fig. 5). In addition, the oxidative burst probe DHR increased CMX-Ros fluorescence about as much as MTG did (Fig. 5).

DISCUSSION

To be effective in combating microbial infections, ROS should be produced near their target in phagosomes. Although ROS formation in phagosomes has been demonstrated with electron microscopy (1), such a reaction has not been demonstrated with fluorescent probes. Rather, reaction products used to measure ROS formation have

been localized to neutrophil granules or neutrophil nuclei (8,9,13). This is the first combined FCM and CLSM study showing ROS formation inside phagosomes with the use of fluorescent dyes.

In the present investigation, FCM and CLSM demonstrated the conversion of DHR to R123 and of HE to ethidium during complement-mediated phagocytosis, strongly suggesting ROS formation. By CLSM, neutrophil phagosomes were filled with green-fluorescent R123 originating from DHR, suggesting that the conversion of DHR to R123 took place inside the phagosomes themselves. An alternative explanation is that R123 emerging from DHR might have originated from the passive uptake of R123 generated from DHR in mitochondria or extracellularly from ROS generated by the secretory activity of neutrophils. In accordance with such a hypothesis, added R123, MTG, CMX-Ros, and LysoTracker Red were taken up into phagosomes. However, all these probes were bound to the remaining nucleus of *S. cerevisiae* and did not appear elsewhere in the phagosome. In particular, the phagosomes were not filled with any of these fluorescent probes. In addition, when DHR was incubated for 1 min with leukocytes immediately after phagocytosis had occurred, only phagosomes became fluorescent, indicating that diffusion of DHR into phagosomes was followed by its local conversion to R123. These studies demonstrated local production of R123 from DHR and excluded other possible sources of R123. Because ROS mediate the production of R123 from DHR and R123 was found in phagosomes, the results strongly suggested that ROS are produced inside phagosomes.

CMX-Ros diffuses across the plasma membrane, accumulates in the negatively charged mitochondrial matrix, and is therefore used to evaluate mitochondrial membrane potentials (21,22). Such a distinct staining pattern requires CMX-Ros concentrations in the range of 100–200 nM. However, at these dye concentrations, staining of mitochondria was not apparent with CLSM, but neutrophil granules were clearly red fluorescent. Even with a CMX-Ros concentration of 1 μM, the probe was observed in association with neutrophil granules, but staining of mitochondria could not be ascertained with CLSM. Although these findings are in accordance with the known ability of CMX-Ros to associate with other organelles at higher dye concentrations (22), it remains unclear why neutrophil mitochondria were not discernible. The mechanism(s) responsible for the uptake and retention of CMX-Ros in neutrophil granules remains to be established.

DHR is converted into R123 and accumulates in neutrophil granules in neutrophils phagocytosing antigen-coated, opsonized fluorescent beads (8,9), but the nature of these granules is unknown. CLSM confirmed that this R123 emerging from DHR is found in granules and extended the observation to complement-mediated phagocytosis.

When neutrophils were co-stained for R123 emerging from DHR and LysoTracker Red, green and red but no yellow granules were observed by CSLM, demonstrating that R123 emerging from DHR and LysoTracker Red were associated with different granules. This seems to be at variance with the "good correspondence between CD63 and the R123 fluorescence" reported by Jankowski and Grinstein (13). However, their Figure 4A,B clearly showed that some $R123^+$ granules are $CD63^-$. In addition, the staining intensities of R123 and CD63 in the $R123^+CD63^+$ granules did not correlate. The investigation by Jankowski and Grinstein was performed on whole cells, and superposition of different granules cannot be excluded. In the present investigation we were able to obtain more precise information on the localization of fluorescence because confocal microscopy allowed us to make thin optical sections through individual cells, thereby providing a fully three-dimensional spatial structure (images available at: www.cyto.purdue.edu/flowcyt/research/pub1.htm). The results of the study by Jankowski and Grinstein also were at variance with the granular distribution of cytochrome b_{558}; see review by Borregaard and Cowland (23). The present results and those of the existing literature indicated that R123 is produced from DHR in a compartment that is different from azurophilic granules.

CSLM revealed green and red but no yellow granules in neutrophils that were co-stained for R123 emerging from DHR and for CMX-Ros. FCM confirmed that the neutrophils were doubly green and red fluorescent. The granular distribution of CD66b differs from that of R123 emerging from DHR (13). CD66 stains secondary but not gelatinase-positive and secretory granules (23). When combined, these results indicated that DHR is converted to R123 in gelatinase-positive and secretory granules or that a specific type of neutrophil granules, hereafter referred to as oxidative response granules, is responsible for the conversion of DHR into R123.

Oxidative response granules differ from acidic granules and granules stained by CMX-Ros. Because the uptake of CMX-Ros was limited to a subpopulation of the granules, it is quite selective. The relation of oxidative-response and $CMX\text{-}Ros^+$ granules to the established granule types remains to be determined.

Neutrophils were shown by FCM and CLSM to accumulate added R123, MTG, and CMX-Ros. All these organelle probes are known to accumulate in mitochondria (22). CLSM associated added R123 and MTG with tubular structures that were often localized in lamellipodia and near the plasma membrane. The morphology, localization, staining pattern, and number of these tubular structures closely corresponded to the mitochondria known to reside in neutrophils. There are few, if any, reports demonstrating neutrophil mitochondria by FCM or CLSM. The present results strongly suggested that added R123 and MTG can be used as mitochondrial probes in neutrophils, whereas the use of CMX-Ros for the same purpose is questionable.

The present study confirmed that HE is converted to ethidium during complement-mediated phagocytosis and that ethidium stains cell nuclei. Likewise, DHR is converted to R123. When neutrophils were co-incubated with HE and DHR, FCM results showed a severely reduced conversion of DHR to R123. Because observations with

CLSM clearly localized the dyes to different intracellular compartments, the blunting of the DHR response cannot be explained by quenching of R123 by a direct interaction with ethidium. Rather, the inhibition of the DHR response suggested interference with an underlying complex metabolic process. HE is considered a measure mainly of superoxide production (3). The conversion of DHR to R123 depends on ROS and is independent of catalysis (3). Our results suggested that inhibition of R123 formation induced by HE may be due to depletion of superoxide by HE by reducing the amount of H_2O_2 available for the conversion of DHR to R123.

In neutrophils, CMX-Ros, HE, and LysoTracker Red displayed specific fluorescences in the red part of the spectrum, but fluorescence was undetectable in the green. In contrast, with DHR, added R123, and MTG all neutrophils were fluorescent in the green part of the spectrum but did not fluoresce in the red. With JC-1, neutrophils were only faintly fluorescent, and the percentage of positive cells was variable. For that reason, we did not use JC-1 to monitor mitochondrial membrane potentials of neutrophils. Rather, we made use of checkerboard combinations of CMX-Ros, HE, and LysoTracker Red with the addition of R123, MTG, and DHR. This experimental approach allowed the detection by FCM and CLSM of neutrophil subpopulations differing in membrane potentials, ROS formation, and acidic granules.

In conclusion, FCM and CLSM investigations confirmed that ROS are formed in phagosomes. The combined use of several probes allowed for the detection of a subpopulation of neutrophil granules that we have termed oxidative response granules. Further, neutrophil mitochondrial membrane potential could be evaluated from cellular uptake of added R123 and MTG, but results with CMX-Ros should be interpreted with caution. In addition, we confirmed that DHR and HE seem to measure the same pathway, but at different points in the oxidative burst. The simultaneous application of several probes for the measurement of organelle function and number carries the risk of probe interference and may make the interpretation of the results difficult. The current increase in the use of functional probes by FCM and CLSM raises a number of concerns, particularly when these probes are used in combination. Clearly, results from one cell type cannot be carried over to other cell types.

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