

Reactive oxygen species in phagocytic leukocytes

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Abstract Phagocytic leukocytes consume oxygen and generate reactive oxygen species in response to appropriate stimuli. The phagocyte NADPH oxidase, a multiprotein complex, existing in the dissociated state in resting cells becomes assembled into the functional oxidase complex upon stimulation and then generates superoxide anions. Biochemical aspects of the NADPH oxidase are briefly discussed in this review; however, the major focus relates to the contributions of various modes of microscopy to our understanding of the NADPH oxidase and the cell biology of phagocytic leukocytes.

Keywords NADPH oxidase · Respiratory burst · Phagocytosis · Neutrophils · Cytochemistry

Introduction

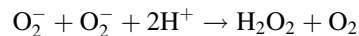
Phagocytic leukocytes, when appropriately stimulated, consume oxygen and produce superoxide (O_2^-) in a process often referred to as the respiratory burst. Much of the work that has led to our current understanding of the regulatory events and the molecular organization underlying the respiratory burst is derived from studies of neutrophils, the

most abundant of the phagocytic cells in blood. Therefore, this review will likewise focus on the neutrophil.

The respiratory burst is mediated by the NADPH-oxidase complex, a multicomponent system that is rapidly assembled following activation of neutrophils. As we have discussed previously, the neutrophil NADPH oxidase produces O_2^- and hydrogen peroxide (H_2O_2) following activation (Robinson et al. 2004). The NADPH oxidase catalyzes the production of O_2^- :

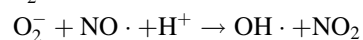
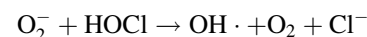


A dismutation reaction occurs with O_2^- to produce peroxide:



Both H_2O_2 and O_2^- are components of the oxygen-dependent, antimicrobial system of phagocytic leukocytes (e.g., Robinson and Badwey 1995).

In part, the cytotoxic effects of O_2^- and H_2O_2 relate to their ability to react with products of other microbicidal systems in these cells to generate additional reactive oxygen species (ROS) [e.g., hydroxyl radical ($OH\cdot$), singlet oxygen (1O_2), ozone (O_3)]. Superoxide can react with either hypochlorous acid ($HOCl$) or nitric oxide ($NO\cdot$) to produce $OH\cdot$ (Beckman et al. 1990; Ramos et al. 1992).

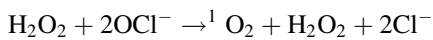


Hypochlorous acid and nitric oxide are produced in phagocytic leukocytes by myeloperoxidase and nitric oxide synthase, respectively (Harrison and Schultz 1976; Xie et al. 1992). Singlet oxygen can form by nonenzymatic dismutation of superoxide (Corey et al. 1987). Additionally, hydrogen peroxide and the

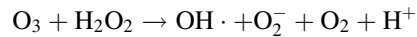
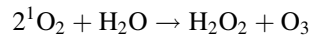
This paper is dedicated to the memory of late John A. Badwey, an insightful and creative scientist, with whom the author enjoyed a fruitful collaboration studying neutrophil biology that spanned over 20 years.

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deprotonated form of hypochlorous acid (OCl^-) can react to form $^1\text{O}_2$ (Steinbeck et al. 1992).



More recently it was shown that $^1\text{O}_2$ and H_2O_2 react in an antibody catalyzed reaction to form ozone and that ozone and H_2O_2 can react to form hydroxyl radical and superoxide (Wentworth et al. 2002).



The NADPH oxidase

Physiological and biochemical changes associated with leukocyte phagocytosis were noted some time ago (Baldridge and Gerard 1933; Sbarra and Karnovsky 1959; Iyer et al. 1961). It was subsequently shown that superoxide is a product of the respiratory burst and that superoxide production is defective in neutrophils from patients with the genetic disease chronic granulomatous disease (CGD) (Babior et al. 1973; Curnutte et al. 1974). However, a fuller understanding of the underlying mechanisms associated with the respiratory burst had to await a number of methodological and technological advances. Neutrophils from CGD patients are unable to generate normal amounts of O_2^- owing to the loss of components of the NADPH-oxidase complex (reviewed in Segal et al. 2000; Heyworth et al. 2003; Malech and Hickstein 2007).

Regulation of the activation of the NADPH oxidase in neutrophils is exquisite since the oxidase is dormant in resting cells but is rapidly activated following appropriate stimulation of these cells. Due to the toxicity of its products, it is not surprising that the NADPH oxidase is highly regulated and limited in its sites of action. Physiological stimuli capable of activating the neutrophil oxidase include phagocytizable particles such as bacteria and yeast, certain molecules that induce chemotaxis in these cells, certain bioactive lipids, and antibodies. The chemotactic peptides, such as *f*-Met-Leu-Phe, have served as models for the study of receptor-ligand mediated activation of the NADPH oxidase. Other “non-physiological” agents can also activate the oxidase and have been important in understanding the regulation of the phagocyte NADPH oxidase. This latter group of compounds includes certain phorbol esters, fatty acids, and retinoids as well as sodium fluoride. The time course of activation of the oxidase and the amounts of reactive oxygen species produced varies depending on the agent used to stimulate cells and activate the oxidase. The rapid activation of the oxidase and the response of neutrophils to agonists can be appreciated by comparing the kinetics of the production

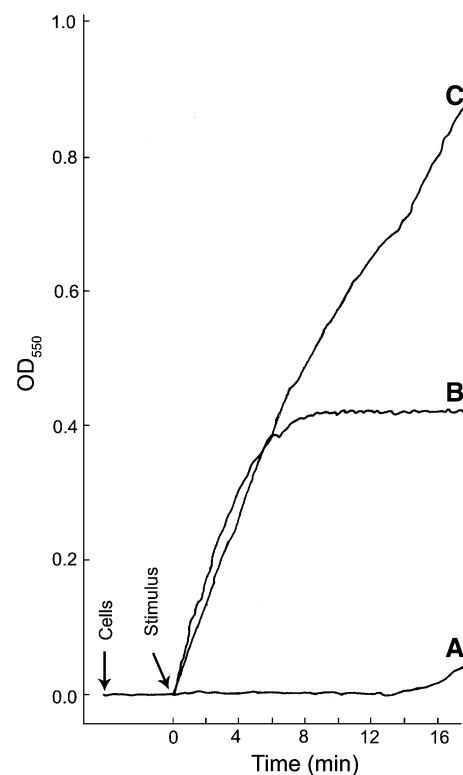


Fig. 1 The levels of O_2^- produced and the rate at which it is produced can vary depending upon the stimulus. Real-time kinetics of O_2^- production by thioglycolate-elicited guinea pig neutrophils stimulated in three different ways is shown; the cytochrome c reduction spectrophotometric assay was employed. Representative curves of cells stimulated with either (A) NaF (12.5 mM) plus AlCl_3 (100 μM), (B) the chemotactic peptide *f*-Met-Leu-Phe (10^{-7} M), or (C) a combination of *f*-Met-Leu-Phe and NaF plus AlCl_3 at the same concentrations used in curves A and B. Cells (1×10^6) were added and allowed to equilibrate at 37°C for 3 min before addition of the stimulus; during that time the resting cells did not generate O_2^- . Following the equilibration period, a stimulus was added (arrow). With NaF plus AlCl_3 there was a long lag period prior to the release of modest amounts of O_2^- (curve A). This lag period is likely due to the slow entry of the stimulus into the cells. The lag period was eliminated when cells were permeabilized by electroporation in the presence of NaF plus AlCl_3 (Hartfield and Robinson 1990). When cells were stimulated with the chemotactic peptide, production of O_2^- was essentially instantaneous and was largely shut down by 5 min post stimulation (curve B). When cells were stimulated by a combination of *f*-Met-Leu-Phe and NaF plus AlCl_3 , at the same concentrations used in curves A and B, the initial rate of O_2^- production was essentially the same as that for the chemotactic peptide alone; however, the shut off of the *f*-Met-Leu-Phe stimulated O_2^- was abrogated and the cells continued to produce O_2^- at a high rate throughout the course of the incubation period. This experiment shows that: (1) resting neutrophils in suspension do not generate O_2^- ; (2) activation of the NADPH oxidase and the production of O_2^- can be very rapid in intact neutrophils; and (3) different stimulatory conditions can lead to different levels of O_2^- production and the kinetics of O_2^- production vary with different stimulatory conditions. The continuous spectrophotometric assay for O_2^- production was as we have described previously (Robinson et al. 1987)

and release of superoxide from neutrophils in response to different stimulatory agents (Fig. 1) (Badwey et al. 1980). The rate of O_2^- released from neutrophils in response to a variety of different agonists has been summarized (Robinson and Badwey 1995). An overview of the various methods used to measure reactive oxygen species and limitations associated with these methods has been presented (Tarpey et al. 2004).

There are several excellent reviews describing the components of the NADPH oxidase and the assembly of these components into the multiprotein complex capable of producing superoxide (Lambeth 2000; Babior et al. 2002; Vignais, 2002; Bokoch and Knaus 2003; Roos et al. 2003; Babior 2004; Cross and Segal 2004; Nauseef 2004; Groemping and Rittinger 2005; Matute et al. 2005; Sheppard et al. 2005; Dagher and Pick 2007; Nauseef 2007). Therefore a detailed analysis of this system will not be presented herein; instead, a distillation of the major features of the oxidase will be given. As is the case for most aspects of cell function, location is a key factor. This is certainly the case for the NADPH oxidase. Indeed, separation of different components of the oxidase in specific locations within the cell account for its dormancy in unstimulated cells. This is important since products of the oxidase are highly reactive and can lead to cell and tissue damage. Thus activation of the oxidase should occur only when necessary. It is only after appropriate stimulation that all of the components of the oxidase come together in a coordinated manner that this complex multicomponent molecular system is functional in intact cells (see below).

The so-called cell-free system in which homogenized neutrophils were subjected to ultracentrifugation to generate cytosol and membrane fractions have been important in sorting out the various components of the NADPH oxidase (Bromberg and Pick 1984; Heyneman and Vercauteren 1984; Curnutte 1985; McPhail et al. 1985). With this approach in hand, investigators could take membranes from one source and cytosol from another source and mix them together and assay for O_2^- production [reviewed in Dagher and Pick 2007]. For example, membranes from normal neutrophils and cytosol from neutrophils from patients with chronic granulomatous disease (CGD) could be mixed, and vice versa. In addition to this genetic approach, recombinant forms of components of the oxidase complex, mutated forms of these proteins, chimeric proteins, and synthetic peptides have also been used in the cell-free system to analyze the NADPH oxidase (Dagher and Pick 2007). A large number of experiments have been carried out by several groups using a variety of approaches to study the NADPH oxidase in cell-free preparations. Taken together, these data have been crucial in leading to our current understanding of the components, their

assembly, and regulation of the phagocyte NADPH oxidase.

The current model for the NADPH oxidase has the catalytic component, flavocytochrome b_{588} , located in the membrane. This heterodimeric flavocytochrome is composed of the proteins gp91^{phox} and p22^{phox} (Segal and Jones 1978; Dinauer et al. 1987; Parkos et al. 1988). The other components of the oxidase complex are in the cytoplasm. It is only after cell stimulation that the cytoplasmic components migrate to the membrane and associate with the gp91^{phox} and p22^{phox} complex. The cytosolic components of the oxidase consist of the proteins p67^{phox}, p47^{phox}, and p40^{phox}, which appear to exist as a complex awaiting mobilization to join the cytochrome b_{588} upon cell stimulation (Wientjes et al. 1993, 1996; Zhan et al. 1998; Lapouge et al. 2002). An additional component of the NADPH oxidase is the GTP-bound form of Rac (Quinn et al. 1993; Abo et al. 1994; Bokoch and Diebold 2002). Until cell activation, Rac exists in association with RhoGDI in the GDP-bound form; upon cell stimulation, it dissociates from RhoGDI, exchanges GDP for GTP, and binds the NADPH oxidase (Bokoch et al. 1994; Kreck et al. 1996). As noted earlier, there are several excellent reviews covering the composition and assembly of phagocyte NADPH oxidase that have appeared since the turn of the century and the interested reader is referred to those publications. The present review will focus on the role microscopy has played in our understanding of the phagocyte NADPH oxidase.

Microscopy in the study of the respiratory burst

The tetrazolium method: detection of superoxide

Early microscopy studies of the respiratory burst in neutrophils relied on the dye nitroblue tetrazolium (NBT). Indeed, NBT was used to diagnose the absence of the respiratory burst in neutrophils from CGD patients (Nathan et al. 1969). The so-called NBT test predated the discovery that CGD neutrophils were unable to generate O_2^- (Curnutte et al. 1974). The chemical basis of the NBT test has been presented elsewhere and will not be discussed herein (Auclair and Voisin 1985). The NBT reaction was adapted for electron microscopy but it was noted that the diformazan reaction product lacked sufficient electron density to be useful in this application (Briggs et al. 1975a). The tetrazolium 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium has also been used to monitor the respiratory burst (Pruett and Loftis 1990; Oez et al. 1993).

A quantitative video-microscopy assay using NBT was used to show heterogeneity in O_2^- production among

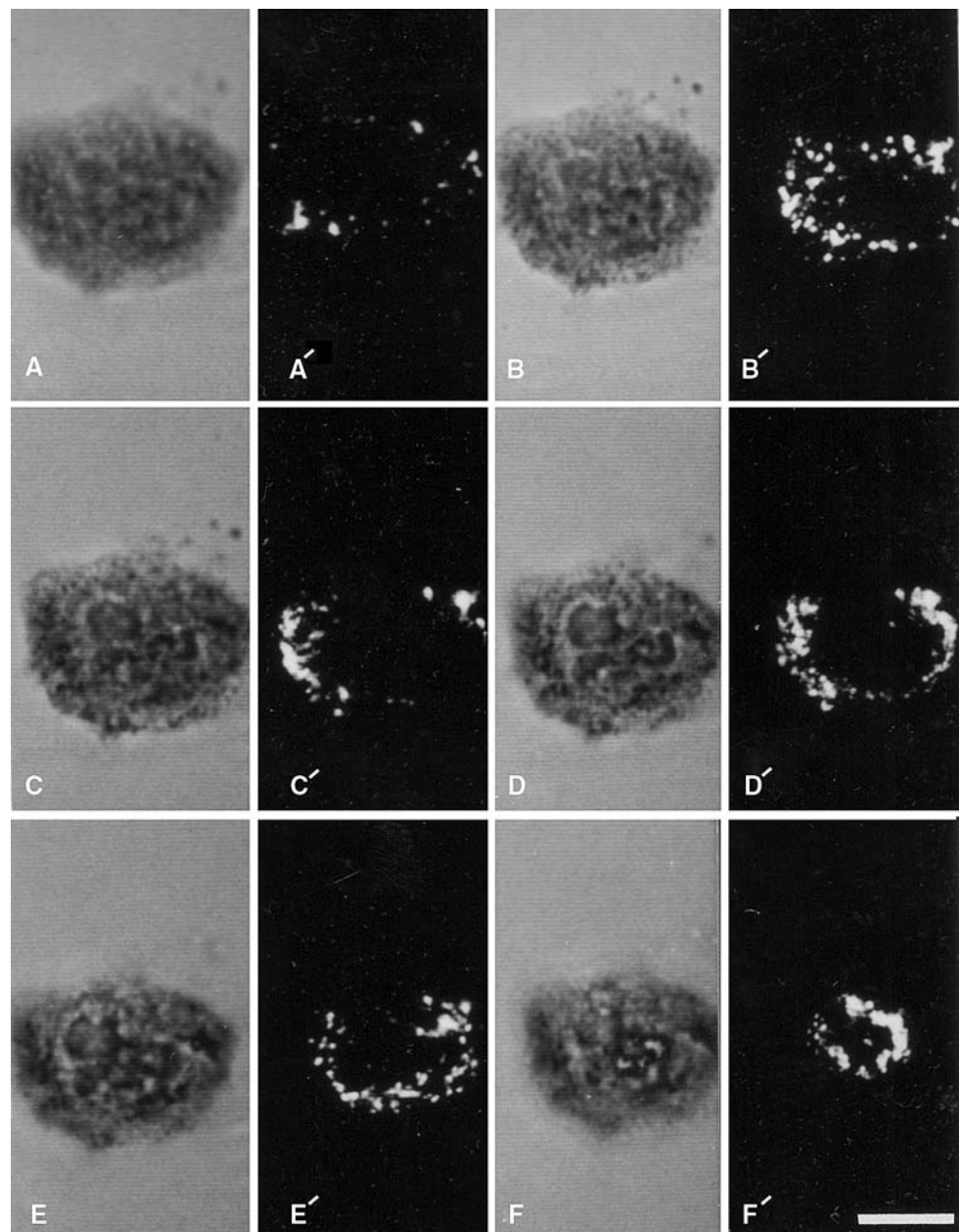
individual cells (DiGregorio et al. 1991). Other studies, using an end-point assay have also provided quantitative data for NBT reduction by activated leukocytes (e.g., Champelovier et al. 1993). A live cell imaging assay in which cytosolic Ca^{2+} was measured with the fluorescence probe Fura-2 and O_2^- production was detected with NBT, was developed. When neutrophils were stimulated with a chemotactic peptide, localized production of O_2^- correlated with the location of a cytosolic “calcium cloud” (Davies et al. 1991). We have shown that the diformazan deposits in activated neutrophils can be monitored by laser scanning confocal microscopy using the reflectance

mode (Robinson and Batten 1989; Robinson and Badwey 1995) (see Figs. 2, 3).

H_2O_2 detection in phagosomes with diaminobenzidine

The presence of H_2O_2 within neutrophil phagosomes was initially inferred from the cytochemical experiments of Briggs et al. (1975a). Myeloperoxidase (MPO), present in azurophil granules of neutrophils, is delivered to the phagosome by fusion of azurophil granules with phagosomes. Myeloperoxidase utilizes H_2O_2 in the generation of hypochlorous acid, a potent microbicidal agent, in the

Fig. 2 Cytochemical detection of sites of stimulated O_2^- generation with the NBT reaction and laser scanning confocal microscopy. A single neutrophil was allowed to adhere to a glass cover slip and was subsequently stimulated with 4 β -phorbol 12-myristate acetate in the presence of NBT is shown. The cell was then optically sectioned and viewed by transmitted light (a–f) and by confocal laser scanning microscopy in the reflectance mode (a'–f'). The non-confocal transmitted light images go from the substratum region (a) toward the apical portion of the cell (f). The reflectance images represent confocal sections corresponding to these different focal planes in the transmitted light images. Reaction product is present over the entire cell; however, there is heterogeneity in its distribution. Bar: 10 μm . Reprinted from Robinson and Badwey 1995



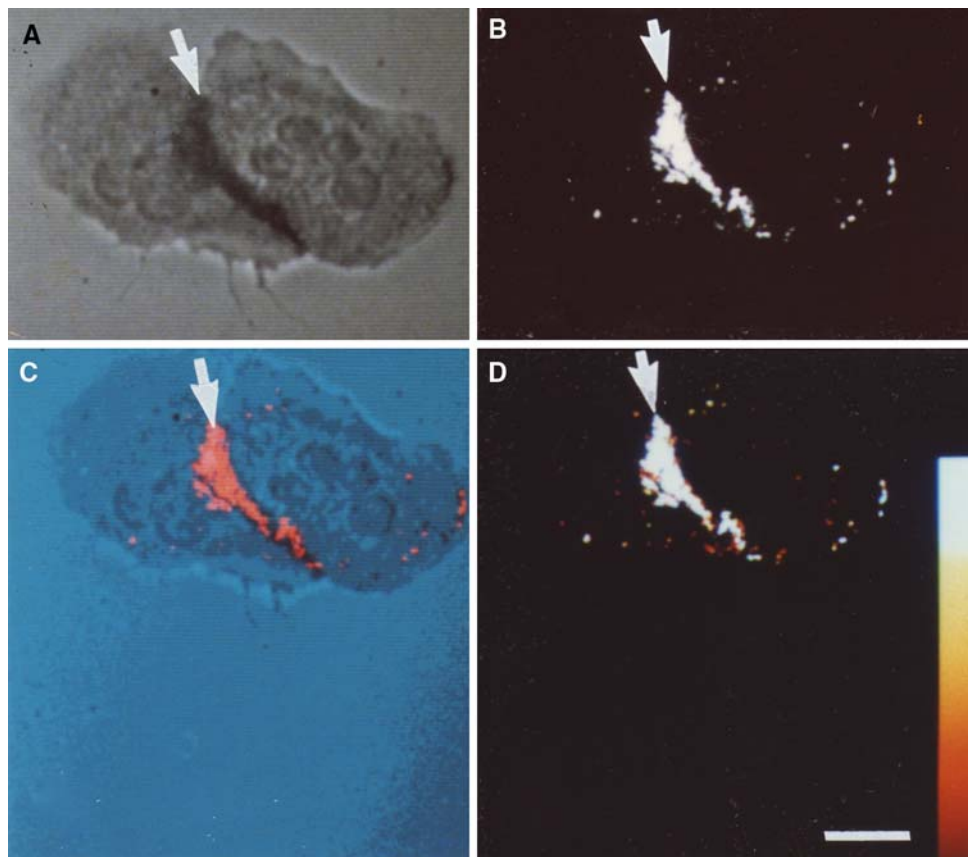


Fig. 3 Cytochemical detection of NBT-derived diformazan reaction product by reflected-light laser scanning confocal microscopy. Two neutrophils that were incubated with NBT in the absence of exogenous stimulating agents are shown. **a** Cells were viewed by non-confocal transmitted light. **b** The same cells were imaged by laser scanning confocal microscopy in the reflectance mode. The adherent neutrophils have a region of cell–cell contact. The reaction product, indicative of O_2^- production, is detected in both the transmitted light image and the confocal reflectance image (arrows). **c** The co-

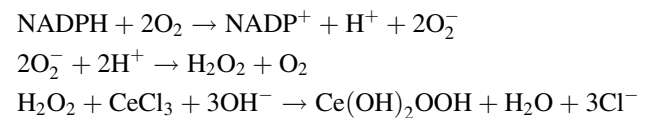
localization of the transmitted light image and the confocal reflectance image is the pseudocolor merge of the two images; the reaction product is indicated by the arrow. **d** A color wedge has been inserted to estimate the intensity of the reflectance signal. Note that the diformazan reaction product is almost entirely restricted to the area of cell–cell contact, indicating the lack of a global signal transduction cascade leading to the generation of O_2^- over the entirety of the cells. Bar: 10 μ m. Reprinted from Robinson and Badwey 1995

phagosome (Klebanoff 1970). The activity of MPO in the phagosome of intact neutrophils was detected with 3,3'-diaminobenzidine (DAB). Deposits of reduced DAB in the phagosome strongly suggested that endogenous H_2O_2 was produced within the phagosome since exogenous H_2O_2 was not added to the cytochemical medium (Briggs et al. 1975a). Other DAB-based cytochemical methods for the localization of ROS have been developed (see below). The presence of MPO in the phagosome is important for the production of the full complement of microbicidal agents. The demonstration of MPO delivery to neutrophil phagosomes at the electron microscope level is shown (Fig. 4).

Detection of hydrogen peroxide with cerium

Hydrogen peroxide, produced by the rapid dismutation of superoxide, was initially detected at the ultrastructural

level using a cerium-based cytochemical reaction (Briggs et al. 1975b). The cerium-based reaction proceeds as follows:



In this reaction, sequence endogenous phagocyte NADPH is utilized by the NADPH oxidase to generate O_2^- . The O_2^- subsequently undergoes dismutation to form H_2O_2 which, in turn, reacts with cerium to form cerium perhydroxide, an electron dense product detectable in the electron microscope. Cerium perhydroxide is a fine, non-crystalline, electron-dense reaction product that generally forms with low background. Thus the cerium reaction is ideal for electron microscopy. However, the cerium perhydroxide reaction product was not initially considered useful for

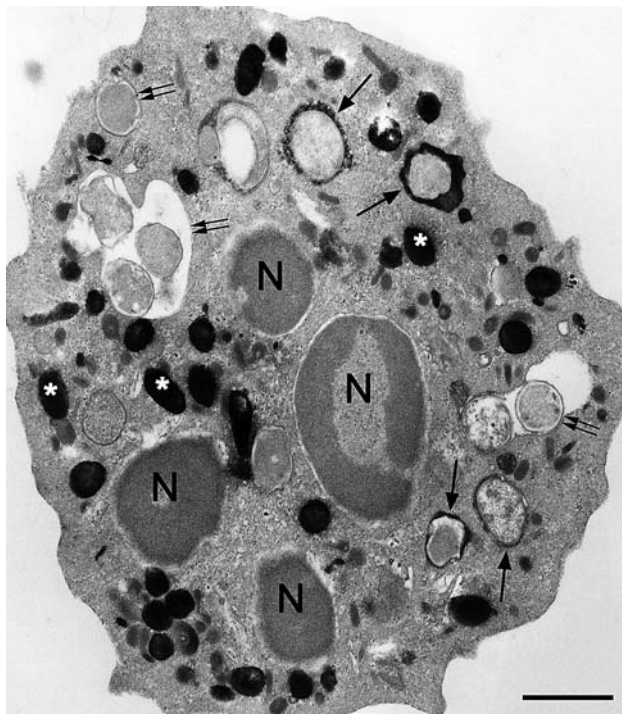


Fig. 4 Electron micrograph of a human neutrophil that has ingested *E. coli* minicells; the neutrophil was subsequently reacted for the cytochemical detection of MPO activity using the DAB method with exogenous H_2O_2 . Black electron dense deposits are present in intracellular granules, the azurophil granules (asterisks), and within a population of phagosomes (arrows). Under these incubation conditions, there were some phagosomes that had not yet acquired MPO (double arrows). The cytochemical reaction for the detection of MPO activity following ingestion of minicells was carried out as we have described (Fox et al. 1987). Bar: 1 μm

bright-field optical microscopy. This problem was overcome as cerium perhydroxide could be converted to other products visible by bright-field microscopy (Zimmerman and Halbhuber 1985; Okada et al. 1987; Angermüller and Fahimi 1988; Halbhuber et al. 1988). It was subsequently found that the cerium perhydroxide reaction product can be detected directly, without conversion to another product, using the reflectance mode of the laser scanning confocal microscope (Robinson and Batten 1990). This latter method allows for direct visualization of the cerium reaction product and permits precise localization by means of confocal optical sectioning and collection of three-dimensional data sets. Cerium has proven to be a versatile tool for enzyme cytochemistry since it can also be used in the detection of several phosphatases and has several advantages over older methods for detection of these enzymes (Robinson and Karnovsky 1983a, b). The use of cerium in oxidase and phosphatase enzyme cytochemistry has been reviewed (Van Noorden and Fredericks 1993; Halbhuber et al. 1994; Karnovsky 1994).

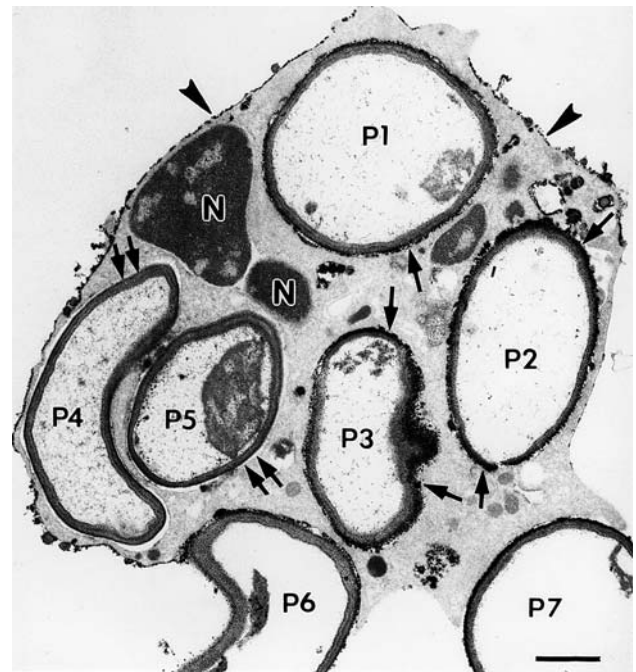


Fig. 5 Electron micrograph illustrating the cytochemical detection of H_2O_2 with the cerium method in a neutrophil that was stimulated by phagocytosis. This thin-section electron micrograph is of a cell that had phagocytized zymozan particles (yeast cell walls) and was then incubated for cytochemistry as described previously (Briggs et al. 1975b). Electron-dense reaction product is present on portions of the cell surface (arrowheads) and in some phagosomes. In this cell profile, five zymozan particles appear to be within phagosomes (P1–5), while two additional particles are bound to the cell surface, but are incompletely internalized (P6–7). Note that some particles (P1–3) have reaction product surrounding the luminal aspect of the phagosomes (arrows), while other particles (P4–5) lack reaction product in the phagosomes (double arrows). Portions of the nucleus (N) are evident. These results indicate an asymmetry in the spatial and/or temporal delivery of H_2O_2 to phagosomes. Bar: 1 μm . Reprinted from Robinson and Badwey 1995

When neutrophils are incubated with phagocytizable particles, such as yeast cell walls, in the cerium-based cytochemical medium developed by Briggs et al. (1975b), the reaction product is evident within phagosomes and at the cell surface. These results were confirmed in other studies (Badwey et al. 1980; Ohno et al. 1982). There are also deposits of reaction product in vesicle-like structures in the cytoplasm (see more below). We have noted that typically phagosomes can vary with regard to the apparent amount of reaction product per phagosome with some phagosomes lacking reaction product altogether suggesting an asymmetric delivery of H_2O_2 to phagosomes (Fig. 5). Similar asymmetry in the delivery of ROS to phagosomes was obtained in living cells by Soret band transmitted light microscopy of neutrophils that phagocytized antibody-coated red blood cells (Petty et al. 1992). Griffiths (2004) proposes that heterogeneity in phagosomes within the same

cell is the norm. The asymmetric delivery of ROS was also noted in neutrophils attached to aggregated IgG associated with glomerular basement membranes; in this case the reaction product, indicative of H_2O_2 production, was on the side of the neutrophil attached to the non-phagocytizable basement membrane (Vissers et al. 1985). The asymmetric generation of ROS has been observed by fluorescence microscopy (see more below) in polarized neutrophils (i.e., crawling over a substratum) (Kindzelskii and Petty 2002). We also observed polarized distribution of cerium perhydroxide, indicative of H_2O_2 production, in crawling cells (Heyworth et al. 1997). This appears to be similar to the situation reported by Kindzelskii and Petty (2002) who used fluorescence microscopy. Another example of the asymmetric generation of ROS was previously shown herein using NBT as the detection reagent; in this case, O_2^- was detected where two polarized cells made contact (see Fig. 3). These findings, enabled by microscopy, indicate the complexity of the regulation of production of ROS by phagocytic leukocytes and raise a number of intriguing questions related to the cell biology and biochemistry of the respiratory burst. Such asymmetric and/or polarized generation of ROS may have important implications in pathophysiology.

It is generally considered that the activated NADPH oxidase is associated with the plasma membrane or its derivative the phagosome (e.g., Nauseef 2004). However, there are several findings that suggest that there may be a separate intracellular pool of NADPH oxidase activity (Lundqvist et al. 1996; Kobayashi et al. 1998; Lundqvist-Gustafsson and Bengtsson 1999; Vaissiere et al. 1999; Karlsson et al. 2000; Karlsson and Dahlgren 2002; Brown et al. 2003; Ambruso et al. 2004). There are often small vesicle-like structures containing cerium perhydroxide indicative of the production of H_2O_2 in cells that have phagocytized particles (Fig. 6). These structures can vary in number from one thin section to another in conventional electron microscopy such that some sections have numerous cerium perhydroxide-containing vesicles and other sections have few or even none of the positive vesicles. A detailed analysis of serially sectioned neutrophils that had internalized particles and in which the localization of H_2O_2 had also been carried out, would be required to address this question adequately and to understand further this intracellular pool of NADPH oxidase in neutrophils following phagocytosis. Insofar as the author knows, such an analysis has not been conducted. The exact nature of these vesicles containing reaction product needs further investigation to determine if they are intracellular in origin or whether they are derived from the plasma membrane since it has been reported that a burst of pinocytosis accompanies phagocytosis (Botelho et al. 2002). However, even if these

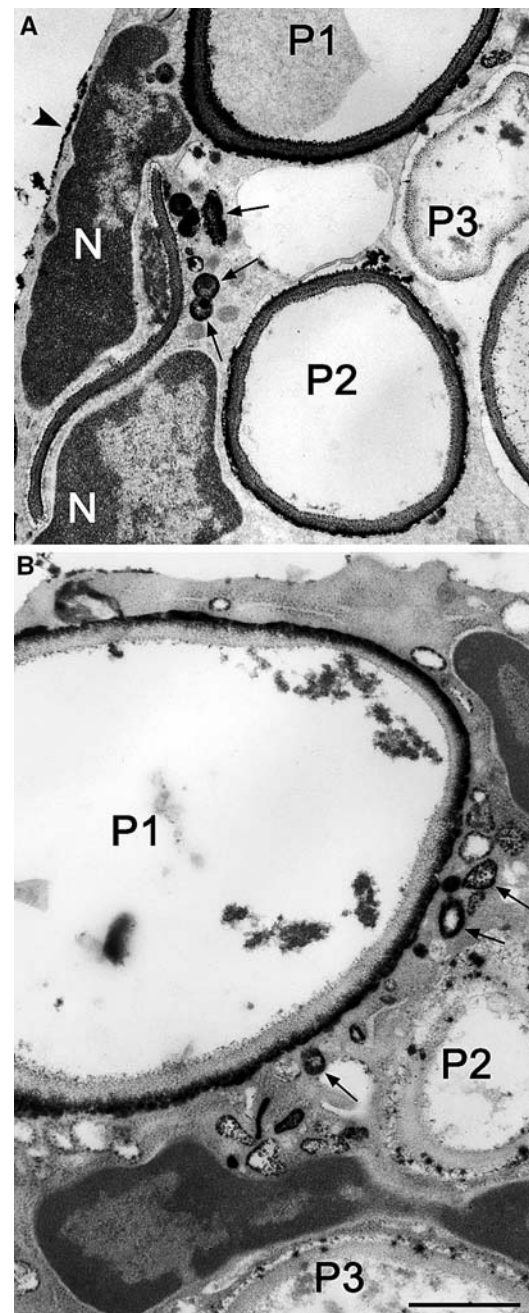


Fig. 6 Electron micrographs of portions of two different neutrophils that ingested zymosan particles; sites of H_2O_2 generation were detected with the cerium-based cytochemical reaction. **a** A portion of a cell is shown in which phagosomes show a spectrum of responses: *P1*, heavy reaction product; *P2*, moderate reaction product; *P3*, little reaction product. There are several small vesicular structures containing reaction product in the periphagosomal region (arrows) suggesting intracellular sites of H_2O_2 . Patches of reaction product are at the cell surface (arrowhead). Portions of the nucleus (*N*) are visible. **b** A portion of a cell is shown in which phagosomes show different responses: *P1*, heavy reaction product; *P2* and *P3*, little reaction product. There are several small vesicular structures containing reaction product in the periphagosomal region (arrows) suggesting intracellular sites of H_2O_2 . Patches of reaction product are at the cell surface (arrowhead). Portions of the nucleus (*N*) are visible. Bars: 1 μ m

vesicles containing reaction product showing H_2O_2 production are pinocytotic in origin, there remains the question of how (and when) are the cytosolic components of the oxidase directed to these sites.

O_2^- detection with DAB- Mn^{2+}

A cytochemical method for the ultrastructural localization of O_2^- was developed; the cytochemical medium contains DAB- Mn^{2+} as the key components (Briggs et al. 1986). Subsequent study showed that this method was specific for O_2^- and was not responsive to $^1\text{O}_2$ (Steinbeck et al. 1993). A modification of this methodology was used to localize sites of superoxide production in rat endothelium in situ (Babbs et al. 1991a, b). The DAB- Mn^{2+} procedure along with modification of this DAB method has been reviewed (Babbs 1994). The DAB- Mn^{2+} method was used to analyze intracellular sites of O_2^- in phorbol ester-activated neutrophils (Kobayashi et al. 1998). This latter study generated interest since it identified intracellular sites of O_2^- production as being distinct from plasma membrane derived vesicles. Two alternative explanations could be posited for this observation. One explanation is that bona fide sites of NADPH oxidase assembly and activation occur on certain intracellular compartments; the second explanation is that the cytochemical reaction occurs in vesicles that were internalized and are in reality derived from the plasma membrane and do not represent an intracellular pool of NADPH oxidase. To address the source of the O_2^- - positive compartments, Kobayashi and colleagues used native and cationic ferritin in conjunction with O_2^- localization. Ferritin was used to mark plasma membrane-derived endocytic vesicles. Sites of O_2^- production were distinct from ferritin-positive vesicles, indicating that these sites of O_2^- production were not derived from the plasma membrane. However, the O_2^- - positive compartments and the ferritin-positive compartments did fuse and their contents mix at later times. This illustrates the complex patterns of intracellular membrane trafficking in activated neutrophils. The Kobayashi study also combined O_2^- cytochemistry and cerium-based cytochemistry for the detection of alkaline phosphatase. This was possible since the electron-dense reaction products generated with these two cytochemical reactions were distinct in their morphology. It was found that these two cytochemical reactions occurred in the same compartment. Moreover, the kinetics of phorbol ester-induced O_2^- generation (as measured biochemically) and the up regulation of alkaline phosphatase activity to the cell surface (as measured biochemically) occurred with a similar time course. This further supports the contention that intracellular generation of O_2^- is associated with the alkaline phosphatase-positive compartment. Previous work had shown that alkaline

phosphatase was unregulated to the cell surface in response to chemoattractants (Borreagaard et al. 1987). Subsequently it was shown that alkaline phosphatase resides in unique rod-shaped structures that are in lower abundance than azurophil or specific granules and in response to chemoattractants these structures appeared to fuse into longer tubular compartments that also fused with the plasma membrane (Kobayashi and Robinson 1991; Robinson et al. 1999).

Immunocytochemical localization of components of the NADPH oxidase

Immunocytochemical localization of NADPH oxidase components has been important in understanding the assembly of the NADPH oxidase. The rates of ROS generation by neutrophils during phagocytosis, with two different types of particles, were measured biochemically and compared to the kinetics of the association of cytochrome b_{558} and $\text{p}47^{\text{phox}}$ and $\text{p}67^{\text{phox}}$ with phagosomes as determined by immunofluorescence microscopy (DeLeo et al. 1999). It was found that cytochromes b_{558} and $\text{p}47^{\text{phox}}$ and $\text{p}67^{\text{phox}}$ are associated with phagosomes and that $\text{p}47^{\text{phox}}$ and $\text{p}67^{\text{phox}}$ subsequently dissociated from the phagosome; this later event correlated with the end of the oxidase activity. This same group, using immunofluorescence microscopy, showed in neutrophils from patients with X-linked CGD that $\text{p}47^{\text{phox}}$ and $\text{p}67^{\text{phox}}$ accumulated in the periphagosomal region but did not remain when phagocytosis was completed (Allen et al. 1999). In contrast, phagocytosis in normal neutrophils was accompanied by a more stable assembly of $\text{p}47^{\text{phox}}$ and $\text{p}67^{\text{phox}}$ at the phagosome membrane. These data suggested that cytochrome b_{558} , non-functional in X-linked CGD, was required for more stable association of these cytosolic components with the phagocyte membrane. Immunofluorescence microscopy was used to show that $\text{gp}91^{\text{phox}}$ and cytosolic phospholipase A co-localize to the plasma membrane in stimulated PLB-985 cells (a neutrophil surrogate) and that in the absence of $\text{gp}91^{\text{phox}}$ phospholipase A does not translocate to the plasma membrane (Shmelzer et al. 2003). The movement of $\text{gp}91^{\text{phox}}$ in PMA stimulated neutrophils has been described with a set of monoclonal antibodies using immunofluorescence microscopy; one of these antibodies appeared to bind preferentially to the active form of cytochrome b_{558} (Campion et al. 2007). Biochemical and immunofluorescence microscopy experiments were used to study the interactions of $\text{p}47^{\text{phox}}$ and interleukin-1 receptor-associated kinase-4 (IRAK-4) in neutrophils treated with lipopolysaccharide (LPS) (Pacquelet et al. 2007). The authors report co-localization of $\text{p}47^{\text{phox}}$ and IRAK-4 at sites near the plasma membrane in LPS stimulated neutrophils. Comparison of the sub-cellular

localization of cytochrome b in neutrophils and macrophages has been carried out using immunofluorescence microscopy. Resting neutrophils showed a primarily intracellular granular labeling pattern while macrophages did not display an intracellular labeling pattern but instead showed a cell surface pattern (Johansson et al. 1995). While not a component of the NADPH oxidase, myeloperoxidase is important in the formation of downstream ROS and for participating in microbicidal activity of neutrophils. Fluorescence microscopy and flow cytometry have been used to analyze binding of quantum dots conjugated with anti-myeloperoxidase (Hoshino et al. 2007). The Hoshino study reports that these quantum dots bind neutrophils stimulated with proinflammatory cytokines but not control cells.

Immunolocalization of components of the NADPH has also been carried out at the electron microscope level. Neutrophils that were cryofixed and prepared by molecular distillation drying were used in conjunction with antibodies to components of the cytochrome b_{558} ; it was found that $\sim 20\%$ of the immunolabel was associated with the plasma membrane with the remainder associated with cytoplasmic granules (Jesaitis et al. 1990). When ultrathin cryosections were used as the labeling substrate for a monoclonal antibody to the 22kD component of cytochrome b_{558} , there was little labeling associated with the plasma membrane with the label being primarily associated with the specific granules (Ginsel et al. 1990). In a subsequent immunoelectron microscopy study, cytochrome b_{558} co-localized to a significant extent with albumin (Calafat et al. 1993). Albumin was previously shown to be a marker for the matrix of the secretory vesicle of neutrophils (Borregaard et al. 1992). This secretory vesicle is the same structure as the alkaline phosphatase-positive vesicle we described using enzyme cytochemistry (Kobayashi and Robinson 1991; Robinson et al. 1999). Cytochrome b_{558} has been reported as a component of the specific granule, the gelatinase granule, and secretory vesicles of the human neutrophil (reviewed in Borregaard and Cowland 1997). The bacterium *Anaplasma phagocytophilum* invades neutrophils but appears to repress the respiratory burst. Immunoelectron microscopy showed that both subunits of cytochrome b_{558} were significantly reduced in phagosomes containing *A. phagocytophilum* when compared to *E. coli* containing phagosomes and thus could be part of the explanation for the reduced respiratory burst (IJdo and Mueller 2004). Combined immunoelectron and immunofluorescence microscopy of neutrophils infected with *A. phagocytophilum* also demonstrated a low association of cytochrome b_{558} subunits with phagosomes containing *A. phagocytophilum* (Carlyon et al. 2004). These two studies with *A. phagocytophilum* illustrate the importance

of immunolocalization in understanding the interaction of particular microbes and the neutrophil phagosome.

Immunolocalization experiments and particularly double or triple localization preparations can be very valuable since low affinity interactions that may be detected by immunolocalization techniques may not survive the harsher conditions associated with cell-fractionation studies. For example, as noted above, transient recruitment of $p47^{phox}$ and $p67^{phox}$ to phagosomes in neutrophils from X-linked CGD was reported (Allen et al. 1999); cell fractionation and cell-free activation systems from these cells did not show translocation of these components (Heyworth et al. 1991).

Fluorescence-based localization of ROS

The detection of ROS by fluorescence microscopy is an attractive option since it permits evaluation of single-living phagocytes. Endogenous autofluorescence associated with NADPH and NADH can be monitored and a change in the distribution of NAD(P)H at phagosomes has been observed (Liang and Petty 1992). It is noteworthy that polarized substratum-attached neutrophils produce bursts of O_2^- production (determined using an exogenous fluorescent probe for ROS) that correlated with waves of NAD(P)H [determined by monitoring the autofluorescence of NAD(P)H] that traveled from the rear to the front of crawling cells (Kindzelskii and Petty 2002). In this case, cells were attached to quartz microscope slides. Production of O_2^- occurred in control cells as well as in cells stimulated with a chemotactic peptide. The difference between the NAD(P)H wave and the release of O_2^- in the two conditions was the periodicity; it was ~ 22 s in the absence of the chemotactic peptide and ~ 11 s in its presence (Kindzelskii and Petty 2002). It has long been known that in standard biochemical assays for monitoring O_2^- production in populations of cells in suspension, that these cells are quiescent (see Fig. 1). How are these two observations to be reconciled? In the case of crawling neutrophils, they have undergone a dramatic shape change. On the other hand, neutrophils assayed in suspension have essentially the same rounded shape that they have in the bloodstream. However, when intact neutrophils, in suspension, are challenged with a stimulus (e.g., phagocytizable particles, phorbol myristate acetate, the chemotactic peptide *f*-Met-Leu-Phe, arachidonic acid, or retinoids) they too undergo changes in shape and reorganization of intracellular compartments (e.g., Badwey et al. 1984; Robinson et al. 1987; Kobayashi and Robinson 1991). The degree to which shape change itself modulates the phagocyte NADPH oxidase is not clear. Nathan (1987) has shown that tumor necrosis factors did not induce production of H_2O_2 in neutrophils in suspension but when

these cells were attached to plastic surfaces coated with proteins (e.g., serum, fibronectin), tumor necrosis factors induced massive release of ROS. It was also observed in that study that neutrophils attached to surfaces in the absence of tumor necrosis factors also produced low levels of ROS. Another study of adherent neutrophils also found low levels of ROS release in the absence of additional stimuli (Suchard and Boxer 1994). It is interesting to note that rabbit synovial fibroblasts that had undergone integrin-mediated cell shape change had activated Rac1 which was correlated with the generation of ROS by these cells (Kheradmand et al. 1998). As already noted above, Rac is a component of the assembled NADPH oxidase. Taking all of these factors into account, studies directed toward understanding, in a detailed manner, how shape change itself plays a role in generation of ROS and how shape change modulates ROS production in response to stimulation in phagocytic leukocytes seem in order.

Exogenous fluorescence probes used for monitoring ROS production have been reviewed (Gomes et al. 2005). Exogenous fluorescent probes have been used in several studies of phagocytic leukocytes. The utility and the limitations of current exogenous fluorescent probes for use with phagocytes has been reviewed (Yeung et al. 2005). Important considerations for use of the exogenous fluorescent probes, such as dihydroethidine and dihydrorhodamine 123 include mode of entry into cells and the retention of the fluorescent product at the site of ROS generation as well as the linearity of the response. A few examples of the use of exogenous fluorescence probes with neutrophils will be presented. Kobayashi et al. (2000) have used fluorescent probes for ROS detection [succinimidyl ester of dichlorodihydrofluorescein diacetate (H₂DCFDA)] and endocytosis [TRITC-dextran] to monitor intracellular trafficking in phorbol myristate acetate-activated neutrophils. They found intracellular sites of ROS production and a complex pattern of the interaction of the ROS-positive compartments and endocytic vesicles. A reduction in H₂DCFDA was noted in the presence of fungal gliotoxin (Li et al. 2004). Streptolysin-O permeabilized neutrophils were tested for production of ROS using H₂DCFDA. In this study, the ROS-induced fluorescence was monitored by flow cytometry and fluorescence microscopy (Brown et al. 2003). It was found that fluorescence was associated with intracellular structures with a granule-like labeling pattern in the permeabilized neutrophils.

An alternative strategy to applying the fluorescent probes directly to cells consists of covalent attachment of the fluorescent probe to phagocytizable particles. In this way, the ROS-induced changes in fluorescence will be restricted to the phagosome (Yeung et al. 2005). These same authors discuss the potential of using ROS-sensitive fluorescent proteins to monitor ROS production. These proteins could

be expressed in phagocytic cells, in some cases, or in the target microorganism. This is an attractive prospect, however, the harsh conditions encountered in the phagosome should be taken into account in the design of environmentally-sensitive fluorescent proteins. Expression of fluorescent proteins in primary phagocytes, such as human neutrophils, is problematic so the use of microorganisms expressing the ROS-sensitive fluorescent protein as targets for phagocytosis would be preferable in this instance.

Fluorescence microscopy has been used in conjunction with a micromanipulator in order to move phagocytic targets to human neutrophils, thus allowing the study of changes in cytosolic Ca²⁺ and the respiratory burst associated with a single phagocytic event at a defined site in individual cells (Dewitt et al. 2003). The fluorescent probes Fura-2 and dichlorodihydrofluorescein were used to monitor changes in cytosolic Ca²⁺ and production of ROS, respectively. Two Ca²⁺ peaks were observed to be associated with phagocytosis but only the second peak correlated with initiation of the respiratory burst.

An enzyme cytochemical experiment is shown to illustrate the dramatic nature of the morphological alterations neutrophils can display during activation. Cytochalasin B, a compound that disrupts filamentous actin in cells, has been widely used in conjunction with stimuli such as *f*-Met-Leu-Phe to enhance the release of ROS and the exocytosis of intracellular granules (e.g., Zurier et al. 1973; Honeycutt and Niedel 1986; Mukherjee et al. 1994). It has also been shown that cytochalasin B reduces the cortical tension and cytoplasmic viscosity in neutrophils (Tsai et al. 1994). We have studied the effects of cytochalasin B and *f*-Met-Leu-Phe stimulation on the morphology of human neutrophils. Cerium-based cytochemistry for the detection of alkaline phosphatase has been used in conjunction with “thick section” electron microscopy. In resting cells, in suspension, the alkaline phosphatase is present in rod-shaped granules that can be detected by this cytochemical method only if the cells are permeabilized (Fig. 7). Following stimulation, these cells undergo a dramatic alteration in their organization. The cells display an elaborate system of tubular structures that are positive for alkaline phosphatase. Moreover, the cytochemical reactivity does not require cell permeabilization showing that the tubular structures are in continuity with the extracellular medium (Fig. 7). The use of “thick sections” in electron microscopy enables the appreciation of how dramatic the alterations are when neutrophils are stimulated with cytochalasin B and *f*-Met-Leu-Phe. The full extent of these changes were not observed either with conventional thin section electron microscopy or with light microscopy (not shown). These results indicate dramatic modifications in cell shape and organization that neutrophils are capable of performing.

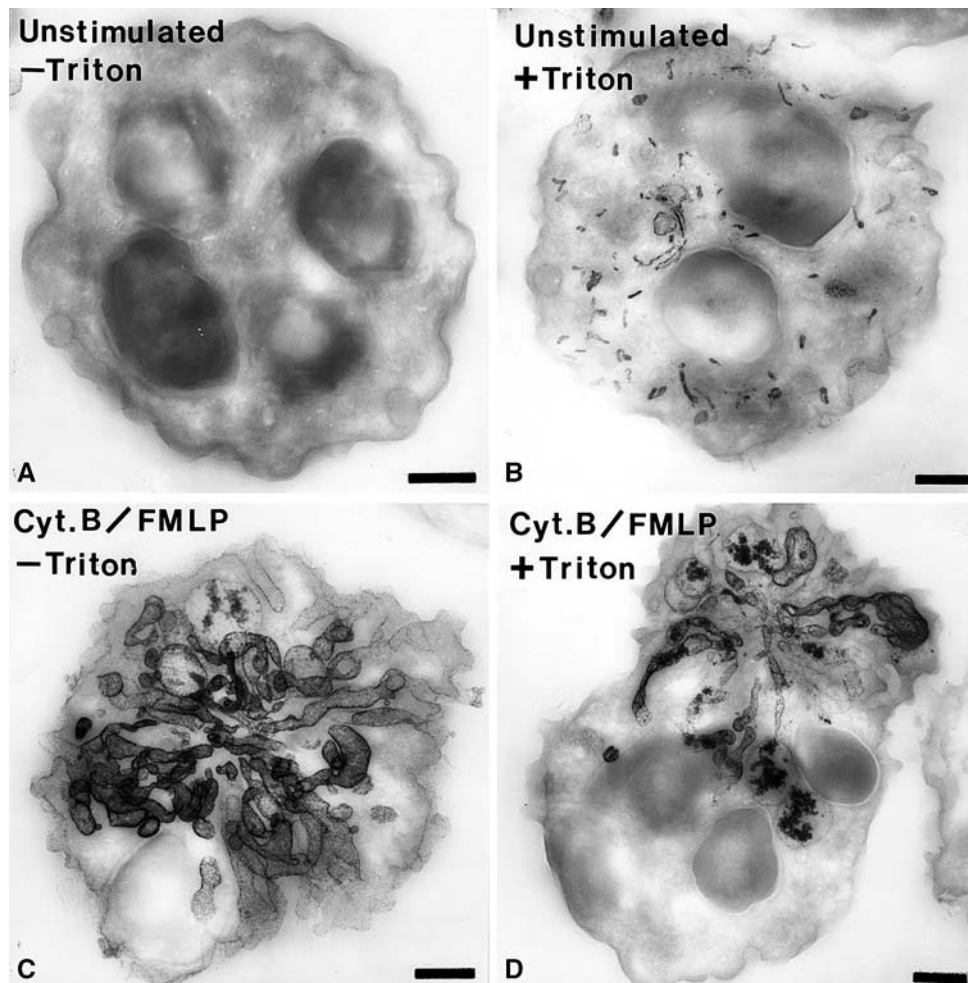


Fig. 7 Electron micrographs of thick sections (0.35 μm thickness) of human neutrophils incubated for the cytochemical detection of alkaline phosphatase. **a** An unstimulated cell that was incubated for the detection of alkaline phosphatase activity in the absence of cell permeabilization with Triton X-100. Note that there is no reaction in the absence of permeabilization. **b** A cell that was incubated for the detection of alkaline phosphatase activity following cell permeabilization. In this case, numerous small rod-shaped organelles containing reaction product are evident. **c** A cell stimulated with *f*-Met-Leu-Phe (FMLP) and cytochalasin B (Cyt.B) that was incubated for the detection of alkaline phosphatase activity in the absence of cell

permeabilization. Note the dramatic rearrangement of the alkaline phosphatase compartments into elaborate tubular structures. These tubular structures appear to be in continuity with the extracellular space since permeabilization was not required to detect the enzyme activity. **d** A cell stimulated with *f*-Met-Leu-Phe (FMLP) and cytochalasin B (Cyt.B) that was incubated for the detection of alkaline phosphatase activity in the presence of cell permeabilization. This cell has a appearance similar to the cell treated in the same manner but without permeabilization. The cytochemical reaction was carried out as we have described previously (Kobayashi and Robinson 1991). Bars: 1 μm

Biophysical methods for study of the phagocyte NADPH oxidase

A chemiluminescence signal was detected during phagocytosis and was thought to be associated $^1\text{O}_2$ generation (Allen et al. 1972). Luminol was incorporated into these studies later to enhance the chemiluminescence signal (Allen and Loose 1976). Subsequently, real-time visualization of chemiluminescence associated with the respiratory burst was reported in a series of studies (Suematsu et al. 1987, 1989; Suematsu and Tsuchiya 1991). For example, rabbit neutrophils were used to study

exocytosis associated with formation of the phagosome in the presence of luminol (Suzaki et al. 1997). These studies required the use of a very sensitive photon-counting camera. Most of the recorded photons were in the vicinity of particles in the phagosomes. It has been proposed that chemiluminescence has advantages for imaging ROS generation in living cells when compared to fluorescence microscopy (Suematsu and Tsuchiya 1991). The suggested advantages are: (1) since the light is derived from the chemical reaction itself (i.e., chemiluminescence), excitation illumination is not required (excitation illumination may alter structure and function in living cells); (2)

photobleaching does not occur; and (3) generation of ROS may be dissected further with specific chemiluminescent probes.

The above arguments notwithstanding, fluorescence microscopy in its various forms has been very important in cell biological studies, including those with phagocytic leukocytes. This is particularly so for live-cell imaging of stimulated leukocytes. Imaging procedures to monitor the endogenous autofluorescence signal from NAD(P)H during activation of the NADPH oxidase have been developed (Liang and Petty 1992). This procedure, when coupled with monitoring ROS generation by fluorescence microscopy, has provided intriguing data on metabolic waves in these cells and was discussed in an earlier section of this review (Kindzelskii and Petty 2002). Other oscillations, ranging from structural to biochemical, have been observed in neutrophils (reviewed in Petty 2001). Also as already noted, this same group used Soret band (absorption contrast) transmitted light to monitor changes in hemoglobin in phagocytosed erythrocytes (Petty et al. 1992).

Using high-speed fluorescence imaging (shutter speeds ~ 100 ns), it was possible to make “stop action movies” of signaling events such as calcium waves in neutrophils that had phagocytosed (Petty 2006). In this case, a calcium wave moves around the periphery of the cell and then jumps to the phagosome when it moves nearby. Rapid image acquisition methodology enabled these observations and should continue to provide new insights into cellular behavior in future work.

Other advanced imaging modalities have been employed for studying the phagocyte NADPH oxidase (reviewed in Van Manen et al. 2006). The distribution of cytochrome b_{558} has been imaged in resting and phagocytosing neutrophils at the single cell level by confocal resonance Raman microscopy (van Manen et al. 2003). In this case, Raman spectroscopy was used to visualize the cytochrome b_{558} directly rather than with a labeling procedure as in immunocytochemistry. While the cytochrome b_{558} accumulates in the region of ingested particles, the spatial resolution was rather low and thus does not provide definitive localization. The authors noted that individual specific granules were not resolved using this imaging modality. Refinements in this instrumentation may improve spatial resolution. The immunofluorescence localization of gp91^{phox} in hematopoietic stem cells mobilized by granulocyte-colony stimulating factor was determined by near field scanning optical microscopy (NSOM) (Frassanito et al. 2008). With NSOM imaging, they report the presence of “specialized clusters” and also that “protein domains” in the 300–600 nm size range could be detected. The precise nature of these small protein domains remains to be determined.

The use of cell lines that display certain properties of primary neutrophils have advantages over freshly isolated neutrophils for some studies. The cultured cells (e.g., PLB-985 and HL-60 cells) can be manipulated in ways that are not readily applicable to primary neutrophils. For example, they can be transfected to express components of the NADPH oxidase that are tagged with fluorescent proteins such that these components can be monitored in living cells under various conditions with real-time fluorescence microscopy. An analysis of this expanding area is beyond the scope of this review; however, some examples are cited to illustrate the potential for these types of experiments. Fluorescence fusion proteins have been used to study the interaction of p67^{phox} and Rac2 with the phagosome and the role of gp91^{phox} in these interactions (Van Bruggen et al. 2004). A fluorescent fusion protein form of p40^{phox} was used to monitor the association of p40^{phox} with phagosomes in cultured cells (Suh et al. 2006). The ability to express fluorescent forms of components of the NADPH oxidase opens up the possibility of applying advanced optical techniques for the study of the NADPH oxidase in living cells.

Other considerations

There is some controversy as to whether intracellular site of NADPH oxidase activity, not associated with phagosomes or the plasma membrane, normally exists. This is an important point in understanding the regulation of the oxidase and generation of ROS. Therefore, it is important to pay close attention to details of the methodology employed in the various studies that have addressed this point. It should be noted that biochemical assays of production of ROS or the consumption of oxygen in activated neutrophils are typically done with cells in suspension (e.g., Robinson et al. 1984; Dahlgren et al. 2007). Additionally, cell fractionation studies used to track the movement of oxidase components using biochemical assays have neutrophils in suspension as the starting material (reviewed in Nauseef 2004). To further complicate matters, it has been reported that standard centrifugation methods used to isolate neutrophils from blood led to the surface expression of molecules that were not detected at the surface of neutrophils in whole blood (Kuijpers et al. 1991). However, most immunocytochemical studies as well as live cell microscopy studies where ROS production is localized occur in cells attached to a substratum (e.g., cover slips, microscope slides, or plastic dishes that may also be coated with a variety of proteins) (e.g., DeLeo et al. 1999; Kindzelskii and Petty 2002). As we have already noted, it has been shown that attachment of neutrophils (and other cells) to surfaces alters their shape; in addition, attachment can modulate their activity with regard to ROS production (e.g., Nathan 1987; Suchard and Boxer

1994; Kheradmand et al. 1998; Kindzelskii and Petty 2002). Indeed, oscillations in the respiratory burst have been correlated with fluctuations in neutrophil cell shape (Wymann et al. 1989). Petty and colleagues (Petty and Kindzelskii 2000; Kindzelskii and Petty 2002; Petty 2004, 2006) have advocated the use of high-speed imaging to study metabolic waves and cellular signaling in living neutrophils. Neutrophils were monitored during the process of cell adhesion to a substratum and it was shown that there were waves of NAD(P)H autofluorescence and cytosolic pH changes associated with attachment (Petty and Kindzelskii 2000). They did not monitor whether ROS production occurred during attachment in that study. However, in polarized neutrophils (crawling cells) NAD(P)H waves were associated with production of ROS (Kindzelskii and Petty 2002). These authors stated: “We have previously noted the presence of longitudinal NAD(P)H waves in elongated neutrophils (Petty et al. 2000). Thus, cell shape (spherical vs. elongated) correlates with the dissipative metabolic patterns observed (circular vs. longitudinal)” (Petty and Kindzelskii 2000). These observations emphasize further the need to compare cells activated in suspension and those activated attached to a surface and to study the role of cell shape in neutrophil function.

In cytochemical preparations carried out with neutrophils in suspension, intracellular sites of ROS production have been observed (Kobayashi et al. 1998; Brown et al. 2003). We have shown, as have others, that the intracellular trafficking of membrane-bounded compartments in activated neutrophils can be very dramatic and complex (Kobayashi and Robinson, 1991; Robinson et al. 1999). This point is illustrated further herein (see Fig. 7). Therefore caution should be used when comparing neutrophils (and other cells as well) activated in suspension to those activated when attached to a substratum. Membrane trafficking events occurring with cells in suspension may not be exactly replicated with attached cells and vice versa. It would be useful for this issue to be addressed in a formal manner such that the intracellular trafficking patterns for membrane compartments of individual neutrophils activated in suspension or when attached to a substratum could be determined and compared.

Reactive oxygen species in other cell types

While this review has focused narrowly on phagocytic leukocytes and neutrophils in particular, it must be mentioned that ROS have assumed considerable importance beyond phagocytes. Indeed, ROS production is wide spread throughout the animal and plant kingdoms. The literature on this topic has grown considerably over the past 10 years or so and illustrates the pervasiveness of ROS production in biological systems and the importance of ROS in various

biological functions. A review of this area, no matter how cursory, is well beyond the scope of this paper. However, there are numerous excellent review articles available on these topics (e.g., Geiszt and Leto 2004; Lambeth 2004; Terada 2006; Bedard and Krause 2007; Bedard et al. 2007; Lambeth 2007; Brandes and Schröder 2008).

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