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The Involvement of Oxygen Radicals in Microbicidal Mechanisms of Leukocytes and Macrophages

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Summary. Phagocytic leukocytes generate large amounts of reactive oxygen compounds during and after phagocytosis of micro-organisms. These compounds are essential for the killing of a wide variety of microbes. The enzyme responsible for this process is NADPH:O_2 oxidoreductase (NADPH oxidase), which utilizes the reduction equivalents of NADPH to reduce atmospheric oxygen to superoxide (O_2^-). Subsequently, superoxide is converted by the leukocytes to other reactive compounds, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and N-chloramines (R-NCl). Each of these compounds has potent microbicidal properties. Under resting, non-phagocytizing conditions, phagocytes do not produce reactive oxygen compounds. However, within 15–30 sec after binding of micro-organisms to cell surface receptors, superoxide generation starts. This phenomenon is called the respiratory burst. The activation of the NADPH oxidase is caused by the assembly of components of this enzyme into an active complex. Under resting conditions, at least three components reside in the cytoplasm and at least two are located in the plasma membrane. Activation of the NADPH oxidase results in translocation of cytosolic components to the plasma membrane and formation of an active enzymatic complex in the plasma membrane.

Key words: Phagocytosis – NADPH oxidase – Oxygen radicals – Cytochrome b_{558} – Chronic granulomatous disease

Phagocytic leukocytes (neutrophilic granulocytes, eosinophilic granulocytes, monocytes and macrophages) are effector cells in our defense against microbial pathogens. Phagocytes kill various micro-organisms (bacteria, fungi, yeasts, mycoplasmata) by ingesting and attacking them intracellularly with hydrolytic enzymes and reactive oxygen products. This line of defense can be divided into several stages.

For ingestion of micro-organisms, it is often necessary that this material is covered with specific antibodies and/or complement fragments. As a result of attachment of opsonized micro-organisms to the phagocyte surface receptors, three processes are initiated: phagocytosis, degranulation and formation of reactive oxygen species. Phagocytosis proceeds by increased actin filament formation, leading to extension of pseudopods around the micro-organism. In this way, an increasing number of receptors on the phagocyte surface can bind to opsonins on the micro-organisms (Fig. 1). When the pseudopods have completely folded around the micro-organism, they fuse and form a closed, membrane-surrounded vesicle (phagosome) inside the phagocyte. Killing of the micro-organism takes place within this phagosome.

During the process of degranulation, the granules in the phagocyte, which contain a large array of bactericidal proteins, fuse with the developing phagosome and deliver their contents in the small space between the micro-organism and the surrounding phagosome membrane. Thus, a high concentration of these proteins is reached in the immediate vicinity of the micro-organism. Together with reactive oxygen species, these enzymes then kill and degrade the phagocytized micro-organism.

The third process triggered by phagocyte receptor occupation is the generation of reactive oxygen metabolites. This reaction is mediated by an en-

Abbreviations: CGD = chronic granulomatous disease; R-NCl = N-chloramines; SDS = sodium dodecylsulfate; Xb^- = X-linked cytochrome b_{558}-negative; Ab^- = autosomal cytochrome b_{558}-negative; Ab^+ = autosomal cytochrome b_{558}-positive; SOC = soluble oxidase component

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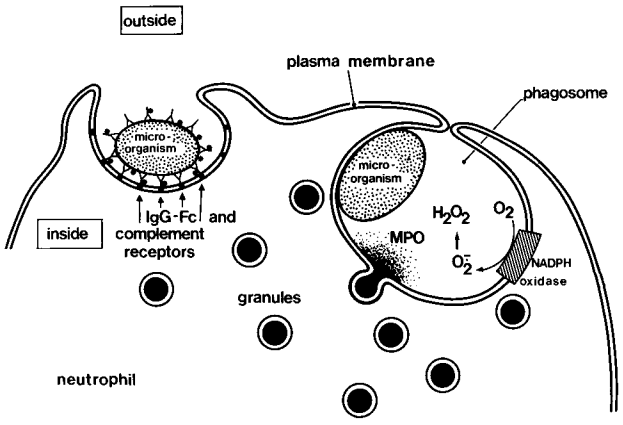

Fig. 1. Schematic representation of phagocytosis, degranulation and generation of oxygen radicals. Micro-organisms opsonized with specific IgG antibodies and complement fragments C3b/iC3b (*) attach to Fc-gamma receptors and complement receptors, respectively. This attachment induces phagocytosis, fusion of intracellular granules with the phagosome membrane and activation of the NADPH oxidase. Superoxide generated by the NADPH oxidase is spontaneously dismuted into hydrogen peroxide (H_2O_2). One of the enzymes released into the phagosome is myeloperoxidase (MPO), which catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions. Illustration from: D. Roos, The respiratory burst of phagocytic leukocytes. Drug Invest [Suppl 2] 3:48, 1991

zyme located in the plasma membrane of phagocytes. It receives electrons from NADPH in the cytosol and transfers these to molecular oxygen on the other side of the membrane. Therefore, this enzyme is called NADPH:O_2 oxidoreductase or – in short – NADPH oxidase. The primary product of this enzyme is superoxide (O_2^-), which is delivered into the phagosome. Its bactericidal potential is low, but this is substantially increased by conversion of superoxide into other reactive oxygen compounds, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and N-chloramines (R-NCl). Inside microbial targets, hydroxyl radicals ($\cdot OH$) may also be formed.

NADPH Oxidase – Biological Importance

About 25 years ago it was discovered that the leukocytes from patients with so-called chronic granulomatous disease (CGD), who suffer from recurrent, often fatal bacterial infections, fail to consume oxygen [1] and generate superoxide [2, 3] during phagocytosis of bacteria. Subsequently, it became clear that this failure to produce oxygen radicals is the cause of CGD: the defective leukocytes also fail to kill ingested bacteria, but this defect is corrected in vitro when the leukocytes are allowed to co-ingest an artificial oxygen-radical producing system [4].

Because CGD is a rare disease (incidence about 1:250000), it has been remarked that more people study the disease than suffer from it. However, these studies have also greatly increased our general knowledge about the working mechanisms of phagocytic leukocytes, knowledge that has proven of immense value for understanding and treating such seemingly unrelated clinical conditions as chronic inflammations, acute respiratory distress syndrome, septic shock, reperfusion injury after hypoxia and even ageing.

One general lesson learned from these studies is the beneficial importance of oxygen radicals generated by phagocytic leukocytes in our defence against microbial pathogens. Another lesson is the realization that overproduction of oxygen radicals may lead to severe tissue injury, and even contribute to multi-organ failure and death. Thus, it is a major challenge to clinicians, laboratory scientists and pharmaceutical companies to devise ways and means to check overproduction of oxygen radicals without endangering the host defense against micro-organisms.

Cytochrome b_{558}

The CGD studies soon showed that different genetic patterns of transmission of this disease exist: X-linked as well as autosomal. This indicates that the NADPH oxidase may consist of more than one component, encoded by genes located either at the X chromosome or at an autosome. Subsequently, it was discovered that the X-linked form of CGD corresponded with the absence of a heme protein, called cytochrome b_{558}, in the phagocytes from these patients [5]. This protein has a low redox potential [6], and is therefore considered to be the NADPH oxidase component that donates electrons directly to molecular oxygen. The idea emerged that cytochrome b_{558} is the X-chromosome encoded component of the NADPH oxidase and that one or more other components might be autosome encoded. The truth proved to be more complicated.

First, we discovered a family in which CGD was apparently transmitted in an autosomal fashion although the cells from the three affected children (one boy, two girls) were practically devoid of cytochrome b_{558} [7]. Fusion of monocytes from these patients with those of CGD patients with the X-linked or the (usual) autosomal, cytochrome b_{558}-positive form of the disease resulted in heter-

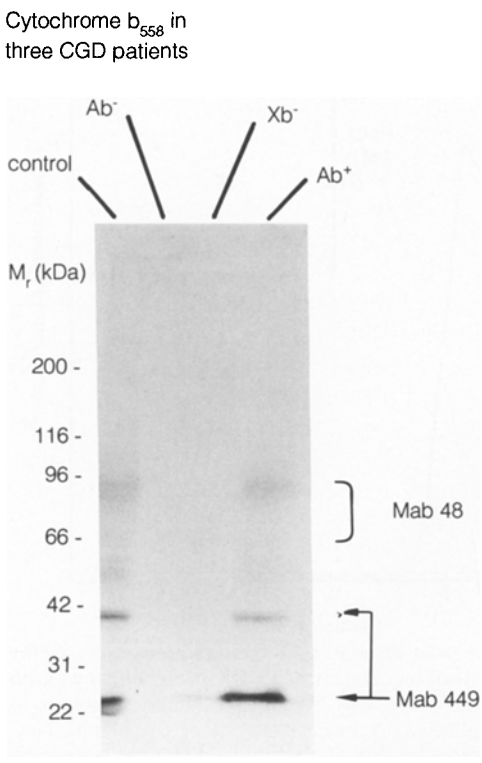

Fig. 2. Western blot of neutrophil extracts incubated with monoclonal antibodies against cytochrome b_{558} subunits. Neutrophil extracts were loaded on an SDS-polyacrylamide gel and after electrophoresis blotted into nitro-cellulose membrane. Subsequently, the membrane was incubated with the monoclonal antibodies and alkaline phosphatase – conjugated goat-anti-mouse-immunoglobulin. Technical details can be found in ref. 12. The picture shows that neutrophils from normal donors and from an Ab^+ CGD patient (who lacks p47-*phox*) contain both the alpha (23 kD) and the beta (75–93 kD) subunits cytochrome b_{558}. In contrast, neutrophils from an X-linked CGD patient and from an Ab^- CGD patient (both without cytochrome b_{558} heme spectrum) lack both subunits. The bands at about 40 kD and at 50–60 kD may be aggregates, precursors or degradation products of these subunits

okaryons with restored NADPH oxidase activity. Monocytes from the latter two groups fused with each other also showed this NADPH oxidase complementation [8]. These studies proved that at least three different gene products are involved in NADPH oxidase activity.

Subsequently, Segal [9] and Parkos et al. [10] discovered that cytochrome b_{558} is a protein composed of two different peptides, each of which is missing in X-linked as well as in autosomal, cytochrome b_{558}-negative CGD [11, 12] (Fig. 2). Both subunits have now been cloned and sequenced [13–16]. The sequence of these peptides does not provide information which of the two subunits carries the heme. As expected, X-linked (Xb^-) CGD is caused by defects in one of these subunits (the beta chain of cytochrome b_{558}) [13] and autosomal, cytochrome b_{558}-negative (Ab^-) CGD by defects in the other subunit (the alpha chain) [17]. The fact that both peptides are missing in either form of CGD may be caused by decreased stability of single subunits as compared to the alpha-beta heterodimer.

Cell-Free Activation System

In resting, non-phagocytizing leukocytes, the NADPH oxidase is inactive: these cells do not generate oxygen radicals. However, upon binding of opsonized micro-organisms to cell surface receptors, or upon activation of the cells with any of a number of soluble stimuli, the NADPH oxidase is rapidly (within 15–30 seconds) activated. In activated phagocytes, the NADPH oxidase activity is confined to the (cell surface or phagosome) membrane-containing fraction [18]. Until a few years ago, this activation was only possible in intact phagocytes. An important step in the elucidation of the oxidase was made when it was discovered that fractions made from resting phagocytes could be induced to generate oxygen radicals by low concentrations of sodium dodecylsulfate (SDS), arachidonic acid or other amphiphilic agents. In particular, this so-called cell-free activation system required the presence of plasma membranes, cytosol, NADPH, GTP and an amphiphile [19–23].

When this system was applied to CGD cells, it was found that these cells did not display any activity, thus proving that this assay measures real, physiological NADPH oxidase activity [22]. Moreover, Xb^- CGD and Ab^- CGD cells showed a defect in their membranes, whereas autosomal, cytochrome b_{558}-positive (Ab^+) CGD cells had a defect in their cytosol [24] (Fig. 3). The membrane defect in Xb^- and Ab^- CGD can easily be explained by the defect in the (membrane-bound) cytochrome b_{558}. The cytosol defect in the third type of CGD indicated that a distinct cytosolic protein is involved in NADPH oxidase activity, either by confering activity to membrane-bound oxidase components, or by integrating with membrane components into an active enzyme complex.

Cytosolic Components

The value of the cell-free activation system is that it enables testing of various cytosol fractions for oxidase activity (e.g. oxygen consumption or superoxide production) in the presence of phagocyte

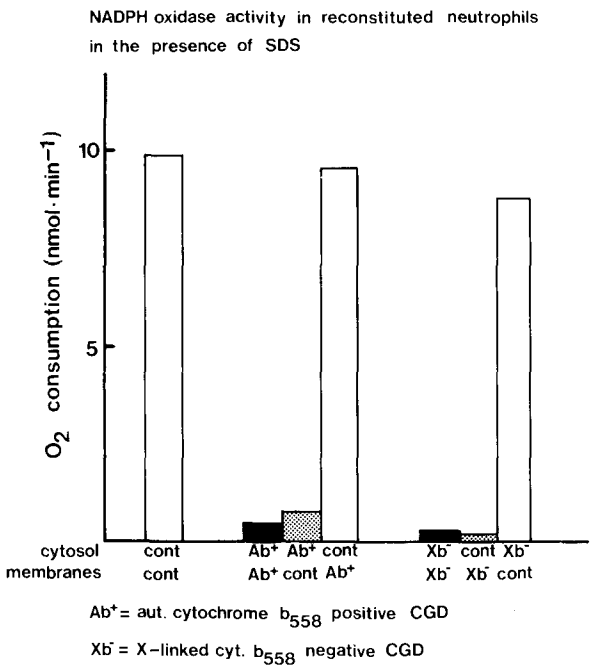

Fig. 3. NADPH oxidase activity in the cell-free activation system. A mixture of neutrophil membranes (2×10^6 cell equivalents), neutrophil cytosol (2×10^6 cell equivalents), SDS (100 μM), NADPH (200 μM) and GTP-gamma-S (10 μM) was incubated at 27° C, and the oxygen consumption was measured. Membranes and cytosol were obtained from neutrophils of either healthy individuals (cont), Ab^+ CGD patients who lack p47-*phox* or Xb^- CGD patients who have a defect in gp91-*phox* synthesis (the beta-subunit of cytochrome b_{558}). Similar results as those shown with Xb^- CGD neutrophil fraction were obtained with neutrophil fractions from Ab^- CGD patients who have a defect in p22-*phox* synthesis (the alpha subunit of cytochrome b_{558}). Illustration from: Verhoeven AJ, Bolscher BGJM, Roos D (1991) The superoxide-generating enzyme in phagocytes: physiology, protein composition and mechanism of activation. In: Vigo-Pelfrey C (ed) Membrane lipid oxidation, vol II, CRC Press, Boca Raton, FL, USA, pp 41–63

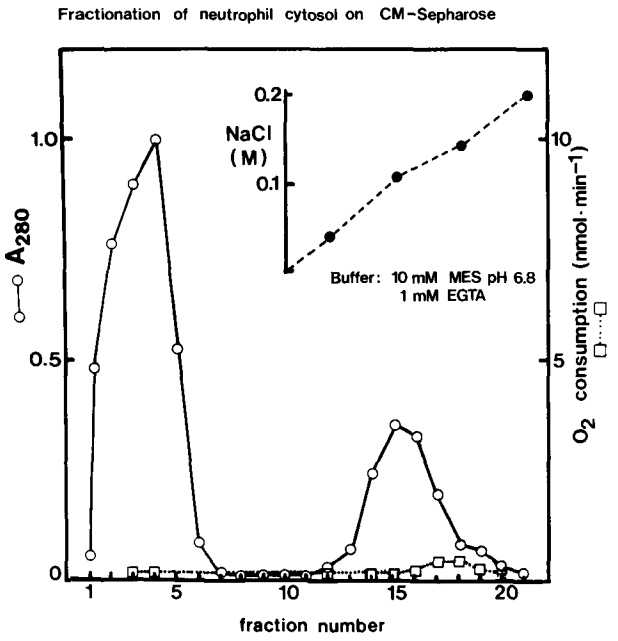

Fig. 4. Fractionation of neutrophil cytosol on carboxy-methyl Sepharose. Cytosol (equivalent of 3×10^9 neutrophils) was dialyzed and applied to a CM Sepharose column. Fractions of 8.8 ml were collected. A linear gradient of 0–200 mM NaCl was applied after collection of fraction 10. Fractions were tested for cytosol oxidase activity as described in Fig. 3. ○, light absorbance at 280 nm; ●, NaCl gradient; □, NADPH-dependent O_2 consumption of 75 μl of the fractions; ■ NADPH-dependent O_2 consumption of 75 μl of the fractions in the presence of 75 μl of fraction 4. Thus, NADPH oxidase activity is only obtained in the presence of both the flowthrough fraction and the eluate, indicating that at least two separate NADPH oxidase components are present in neutrophil cytosol. Illustration from ref. 23

membranes, NADPH, GTP and SDS. Several groups discovered that not one but several cytosolic proteins appeared to be necessary for NADPH oxidase activity [23, 25–28]. Fig. 4 shows an example from our own laboratory.

At present, there is firm evidence for two cytosolic oxidase proteins, because CGD patients with defects in these proteins are now known [25, 26, 29]. These proteins have been cloned and sequenced [30–32], and found to have a molecular mass of 47 and 67 kD, respectively. Unfortunately their sequence does not provide a clue as to their function. However, each of these proteins carries two regions 18–40% homologous with so-called A regions in non-receptor tyrosine kinases (e.g. *src*). Similar regions are present in non-erythroid alpha-spectrin, phospholipase C gamma, GTPase-activating protein and myosin I of yeast. Because all of these proteins are cytosolic proteins that move to the plasma membrane or cytoskeleton upon cell activation, these regions are regarded as important for binding to structural cell proteins. Indeed, it has been shown that both the 47-kD and the 67-kD component of the NADPH oxidase also translocate from the cytosol to the plasma membrane upon activation of intact phagocytes or upon amphiphile addition to the cell-free system [33, 34]. The (cytosolic) C-terminus of the cytochrome b_{558} beta subunit is required for this translocation [35]. Moreover, careful titration has shown that both proteins are needed in stoichiometric amounts with cytochrome b_{558} for oxidase activity [23, 26]. Therefore, p47-*phox* and p67-*phox*[1] probably integrate with cytochrome b_{558} in the formation of an active NADPH oxidase complex.

[1] According to agreement among phagocyte investigators, the NADPH oxidase components are abbreviated as follows: cytochrome b_{558} alpha chain, p22-*phox*; cytochrome b_{558} beta chain, gp91-*phox*; 47-kD protein, p47-*phox*; 67-kD protein, p67-*phox*. In this nomenclature, p = protein, gp = glycoprotein, *phox* = phagocyte oxidase

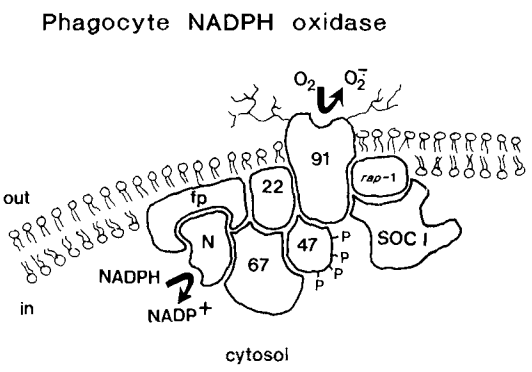

Fig. 5. Schematic model of the phagocyte NADPH oxidase. In resting cells, p47-*phox* (47), p67-*phox* (67), SOC I (a small G protein?) and the NADPH-binding protein (N) are located in the cytosol. After NADPH oxidase activation, these components translocate to the plasma membrane and integrate with the membrane-bound components gp91-*phox* (91), p22-*phox* (22), *rap*-1 (a small G protein) and a flavoprotein (fp). This results in formation of an active NADPH oxidase complex, which accepts electrons from NADPH at the NADPH-binding protein and transmits these through the flavoprotein to cytochrome b_{558} (p22-*phox*+gp91-*phox*). At the other side of the plasma membrane, cytochrome b_{558} donates these electrons to molecular oxygen, thus generating superoxide

Other Oxidase Components

Most probably, the NADPH oxidase contains more than these three proteins. For instance, the NADPH-binding component has not yet been identified. Experiments with reagents that block NADPH-binding sites have provided strong evidence that this protein is located in the cytosol and also translocates to the plasma membrane upon phagocyte activation [37].

Another cytosolic protein needed for oxidase activity is called Soluble Oxidase Component (SOC) I or Sigma 1 [28, 33]. This protein needs GTP for its translocation to the plasma membrane, but apparently loses GTP requirement upon purification (E. Pick, personal communication). Its function has not yet been elucidated.

Kakinuma et al. [39] have provided evidence for the existence of a flavoprotein in neutrophil membranes that forms a semiquinone radical upon cell activation and has a redox potential in between that of NADPH/NADPH and O_2/O_2^-. Thus, this flavoprotein may be involved in electron transport from NADPH to oxygen, but the protein has not been characterized.

Finally, it has been shown that a 22-kD *ras*-like G protein is intimately associated with the cytochrome b_{558} alpha-beta complex [40]. This small G protein, called *rap*-1 may also be needed for oxidase activity [41].

Taken together, Fig. 5 shows a schematic representation of the phagocyte NADPH oxidase complex. I speculate that p47-phox and p67-phox are needed to bring the cytosolic and membrane components together but do not take part in electron transfer themselves. The small G-proteins may have similar, activity-inducing properties. Electron flow may proceed via the cytosolic NADPH-binding protein to the membrane-bound flavoprotein, from there to cytochrome b_{558} and finally to oxygen. In this complex, the two electrons derived from each NADPH must be delivered to two separate oxygen molecules. This may be achieved either in sequence or simultaneously to the two hemes that are probably located between the two subunits of cytochrome b_{558}. Evidently, much more biochemical studies are needed to fully analyze this important enzyme.

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