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Formation of reactive oxygen species by phagocytic cells: functions and dysfunctions

D Roos[1], BGJM Bolscher[1], RS Weening[1,2] AJ Verhoeven[1]

[1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam; [2]Pediatric Clinic, Academic Medical Center, University of Amsterdam; Amsterdam, The Netherlands

Neutrophilic granulocytes (in short: neutrophils), also called polymorphonuclear leukocytes (PMN), defend our body against invading micro-organisms. About 13×10^{10} neutrophils are produced each day in the bone marrow of a healthy, 70-kg human individual. These cells take 14 days to completely differentiate into functionally mature neutrophils, which then leave the bone marrow and pass through the blood on their way to the tissues. Here, the neutrophils survive only a few days before being destroyed by local macrophages.

During infections, small molecules are produced both by the invading micro-organisms and by complement activation (e.g. C5a). These molecules diffuse into the surroundings of the infected area and posses the capacity to attract neutrophils (and monocytes) to the site of infection. This is the process of chemotaxis (directed movement). Other molecules, such as the complement fragment C3a, cause histamine release from mast cells, thus inducing blood vessel dilatation. As a result, circulating antibodies leak into the infected area and cover the invading micro-organisms. Complement fragments C3b and C3bi also attach to the micro-organisms. Together, this is called the process of opsonization ("make fit for eating").

Thus, when the neutrophils move in, they encounter opsonized micro-organisms. Contact between the neutrophils and the opsonized micro-organisms is then established through Fc and C3 receptors. This contact initiates three independent processes in the neutrophils. Phagocytosis results in complete internalization of the attached micro-organisms in closed vesicles (phagosomes) within the neutrophils. Second, fusion of neutrophil granules with the phagosomal membranes causes liberation of microbistatic and microbicidal proteins into the phagosomes, in close proximity to the phagocytized micro-organisms. And thirdly, activation of a membrane-bound oxidase enzyme starts the generation of reactive oxygen compounds, also within the phagosome. These three processes cooperate in the intracellular killing and degradation of the invading micro-organisms.

This communication will now be restricted to the characteristics of the oxidase enzyme.

Chronic granulomatous disease

The oxidase enzyme should actually be called NADPH: O_2 oxido-reductase, because it catalyzes the reduction of oxygen to superoxide ($O_2^{\cdot-}$) with reducing equivalents derived from NADPH. Much information regarding this enzyme has been obtained from studies with neutrophils from patients with chronic granulomatous disease (CGD). Such patients lack NADPH oxidase activity in their phagocytic leukocytes, and as a result suffer from recurrent, serious infections of the skin, the respiratory and gastro-intestinal tract, the lymph nodes, the liver, the bones and sometimes of other organs. Granuloma formation may lead to obstructions, especially of the gastro-intestinal and the urinary tract. Thus, a clear relation exists between the occurrence of such infections and a dysfunction of the NADPH oxidase.

CGD is a clinical syndrome caused by different molecular defects. This has been known for a long time already from the occurrence of an X-linked as well as an autosomal form of the disease [4]. By cell fusion experiments with monocytes from pairs of different CGD patients, we have shown complementation of NADPH oxidase activity [10]. This proved the existence of two genetically different forms of CGD. Subsequently, we have discovered a third complementation group with CGD [22]. This means that the activity of NADPH:O_2 oxidoreductase depends on at least three different gene products.

Cytochrome b_{558}

NADPH:O_2 oxidoreductase is a multi-component enzyme. This was indicated already by the experiments mentioned above, although the complementation studies do not differentiate between structural components of the oxidase, proteins involved in the activation of the enzyme, or proteins involved in post-translation modifications of the enzyme. Work from many laboratories has recently proven, however, that at least three structural oxidase components exist.

The best characterized of these is cytochrome b_{558}. This heme protein is present in large amounts in all cell types that express NADPH oxidase activity. In the phagocytes from patients with the X-linked form of CGD, however, the heme spectrum of this protein is undetectable [18]. In the phagocytes of most patients with the autosomal form of CGD, this heme spectrum *is* detectable, but the cytochrome in these cells is not reduced upon anaerobic stimulation of these cells (as it is in normal phagocytes) [20]. This points to cytochrome b_{558} as a component of the NADPH oxidase.

Cytochrome b_{558} has been purified and cloned. This protein consists of a heterodimer, with an alpha chain (non-glycosylated) of 23 kDa and a beta chain (heavily glycosylated) of

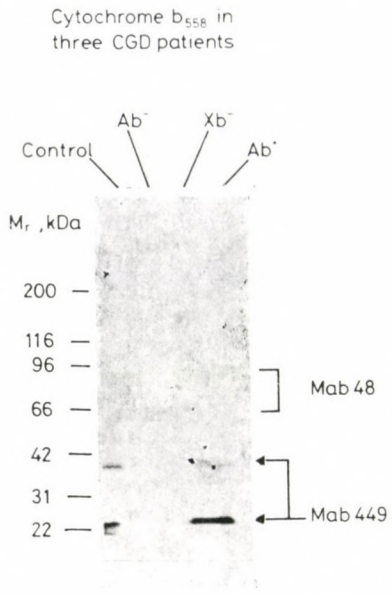

FIG. 1. Immunoblot of cytochrome b_{558} from three CGD patients. Neutrophils were extracted with 1% Triton X-100 in the presence of protease inhibitors. The extract was centrifuged and the supernatant was mixed 1 : 1 with an equal volume of Laemmli sample buffer containing 20% SDS and incubated for 20 mint at 60 °C. A sample of the mixture was subjected to SDS-PAGE (5—15%). Next, the proteins were blotted onto nitrocellulose and incubated with a mixture of McAb 449 (anti-α-chain of cyt. b_{558}) and McAb 48 (anti-β-chain of cyt. b_{558}) followed by staining with horse-radish-peroxidase--conjugated goat-anti-mouse-Ig

91 kDa [13, 15—17]. The beta chain is a transmembrane protein; the alpha chain might be the heme-carrying protein. The primary defect in X-linked CGD lies in the gene that encodes the beta chain of cytochrome b_{558} [8, 21]. In the rare, autosomal, cytochrome-b_{558}-negative form of CGD [22], the primary defect may be located in the gene that encodes the alpha chain of cytochrome b_{558} [14].

Recently, we have succeeded in raising monoclonal antibodies against the two peptide chains of cytochrome b_{558}. One of these monoclonals, McAb 449, reacts with the alpha chain of cytochrome b_{558}, as shown by its reaction with a 23—26 kDa band in a protein blot of control neutrophils and of purified cytochrome *b* after SDS--polyacrylaminde gelelectrophoresis (SDS-PAGE). The other monoclonal, McAb 48, reacts with the beta chain of cytochrome b_{558} (reaction with a 68—92 kDa smear in a protein blot after SDS-PAGE). With these monoclonals, we have investigated the presence of the two cytochrome-b_{558} subunits in the various forms of CGD. Fig. 1 shows that both subunits are present in the neutrophils from healthy donors and in those from patients with the common, autosomal, cytochrome-b_{558}--positive (Ab^+) form of CGD. Both subunits are lacking, however, in the rare, autosomal, cytochrome-b_{558}-ne-

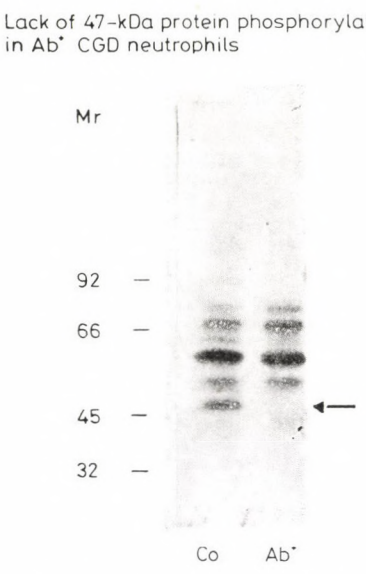

FIG. 2. Lack of 47-kDa protein phosphorylation in Ab+ CGD neutrophils. Cytosol from control (Co) or Ab+ CGD neutrophils was incubated for 5 min at 30 °C with ^{32}P-gamma--ATP and liposomes containing phosphatidylserine, phosphatidylcholine and phorbol--myristate acetate to activate the endogenous protein kinase C in the neutrophil cytosol. Thereafter, the reaction was stopped by addition of Laemmli sample buffer and incubated at 80 °C for 10 min. SDS-PAGE (5—15%) was followed by autoradiography. The results show a lack of phosphorylation at the 47-kDa position in the Ab+ CGD neutrophil cytosol. A similar defect is seen when intact neutrophils are loaded with ^{32}P-ortho-phosphate and stimulated with an activator of the NADPH oxidase. Subsequent experiments have ruled out a lack of protein-kinase activity in Ab+ CGD neutrophils

gative (Ab−) form of CGD as well as in the X-linked, cytochrome-b_{558}-negative (Xb−) form of CGD. This indicates that the two subunits need each other for stabilization, and confirms the results obtained with polyclonal antisera [14]. Moreover, Fig. 1 also shows the presence of a small amount of alpha chain protein in Xb− CGD neutrophils. This is in accord with the primary defect of beta chain synthesis in these cells [8, 21]: as a result, alpha chains cannot form a stable complex with beta chains and are probably degraded more rapidly than in normal cells. Thus, only little alpha chain material is detectable in Xb− CGD neutrophils. In reverse, Ab− CGD neutrophils might contain some beta chain protein, but this was not detectable with our present staining technique of McAb 48. Finally, Fig. 1 also shows that McAb 449 reacts not only with a protein band at 23—26 kDa, but also with an additional band at about 40 kDa. Whether this last protein represents an immature form or a dimer of alpha chains is not known.

The 47-kDa phosphoprotein

As mentioned above, the phagocytes of Ab+ CGD patients do contain cytochrome b_{558}, but this component

does not function in the NADPH: O_2 oxidreductase. Moreover, it has been found that Ab^+ CGD phagocytes show a defect in the phosphorylation of a 47-kDa protein during cell activation [12, 19]. When normal phagocytes are treated with an agent that activates the NADPH oxidase, a number of proteins are phosphorylated. In Xb^- and in Ab^- CGD phagocytes, this reaction proceeds normally, despite the lack of subsequent oxidase activation. In Ab^+ CGD phagocytes, however, the phosphoprotein pattern lacks a band at 47 kDa (Fig. 2).

The question arises whether this 47-kDa protein is also a structural component of the NADPH: O_2 oxidoreductase. We have studied this problem by means of the so-called cell-free activation system. In this system, a membrane fraction and a cytosol fraction of neutrophils are combined with NADPH (200 μM) and SDS (100 μM). As a result, the mixture starts to generate superoxide [2]. The physiological relevance of this system is proven by the lack of superoxide generation in fractions derived from CGD neutrophils [3]. With this system, the defect in Xb^- and Ab^- neutrophils has been localized in the membrane fraction of these cells, whereas Ab^+ CGD neutrophils show a defect in the cytosol fraction [5, 7]. Fig. 3 exemplifies these results by oxygen consumption measurements.

The cell-free activation system can also be used to study more closely the cytosolic component(s) involved in the NADPH oxidase activity. We have fractionated neutrophil cytosol over carboxymethyl (CM) Sepharose, and tested the activity of the fractions in a system with normal neutrophil membranes, NADPH and SDS. Fig. 4 shows that we obtained two protein peaks, each with very little activity in the SDS system. Combination of protein that did not bind to CM Sepharose with protein that eluted at 0 – 200 mM NaCl from the CM Sepharose resulted in NADPH oxidase activity in the SDS system. This indicates that at least two cytosolic components are involved in the NADPH oxidase of human neutrophils. We called these two proteins Soluble Oxidase Component (SOC) I and SOC II, respectively. The peak of SOC-II activity eluted at about 125 mM NaCl from the CM Sepharose.

Subsequently, SOC I and SOC II were analyzed with an in-vitro phosphorylation assay [12]. This assay involves treatment of the fractions with purified protein kinase C, a liposome mixture of phosphatidylserine, phosphatidylcholine and phorbol-myristate acetate to activate the protein kinase, and ^{32}P-gamma-ATP. Thereafter, the mixture was subjected to SDS-PAGE and autoradiography. The results showed that SOC II contained a 47-kDa phosphoprotein. Similar analysis of SOC II from Ab^+ CGD neutrophils revealed a lack of activity and a lack of 47-kDa phosphoprotein in this fraction. Moreover, the defect in cytosolic NADPH oxidase activity of Ab^+ CGD neutrophils was overcome by addition of SOC II, but not by addition of SOC I from normal neutrophils (Table I). Thus, our re-

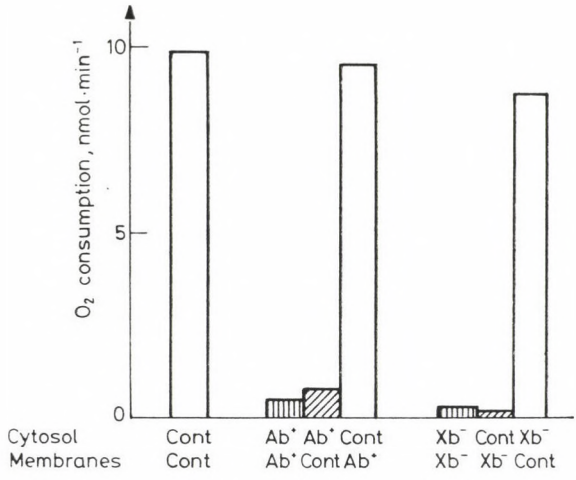

FIG. 3. NADPH oxidase activity in a cell-free reconstitution system. NADPH oxidase activity was measured with an oxygen electrode in a mixture of neutrophil membranes (2×10^6 cell equivalents), neutrophil cytosol (2×10^6 cell equivalents), NADPH (200 μM) and SDS (100 μM). Membranes and cytosol were obtained from neutrophils of either healthy individuals (cont), Ab^+ CGD patients (Ab^+) or Xb^- CGD patients (Xb^-). Similar results as those shown with Xb^- CGD neutrophil fractions were obtained with Ab^- CGD neutrophil fractions

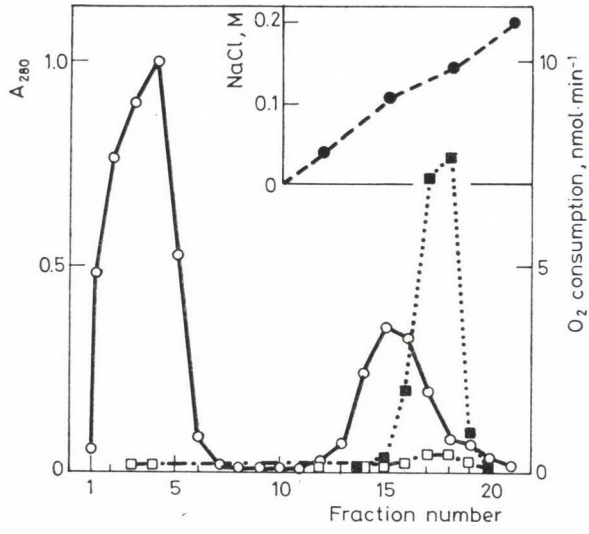

FIG. 4. Fractionation of neutrophil cytosol on carboxymethyl Sepharose. Cytosol from 3×10^9 neutrophils was dialyzed against elution buffer (MES buffer of pH 6.8, containing protease inhibitors) and applied to CM Sepharose equilibrated with the same elution buffer. A linear gradient of 0—200 mM NaCl was applied (●—●). Fractions of 8.8 ml were collected. The fractions were assayed for protein content at 280 nm (○—○) and for NADPH oxidase activity in a system with membranes from normal neutrophils (3.5×10^6 cell equivalents), NADPH (200 μM) and SDS (100 μM). □—□ O_2 consumption of 75 μl of the fractions tested in this system; ■—■, O_2 consumption of 75 μl of the fractions tested in this system in the presence of 75 μl of fraction 4

TABLE I

Effect of SOC I and SOC II on NADPH-dependent O_2 consumption in a reconstituted system of normal neutrophil membranes and cytosol from Ab+ CGD neutrophils

Source of membranes (2×10^6 cell eq.)	Source of cytosol (2×10^6 cell eq.)	Addition (2×10^6 cell eq.)	NADPH-dependent O_2 consumption (nmol/min/2×10^6 cell eq.)
Control PMN	Control PMN	none	8.8 ± 0.7
Control PMN	Ab+ CGD PMN	none	0.1 ± 0.7
Control PMN	Ab+ CGD PMN	SOC I	0.4 ± 0.2
Control PMN	Ab+ CGD PMN	SOC II	7.7 ± 0.8
Control PMN	none	SOC I	0.1 ± 0.1
Control PMN	none	SOC II	0.4 ± 0.2

Values are mean ± S.D. obtained with cytosol from neutrophils of three unrelated donors and three unrelated CGD patients. SOC I and SOC II were derived from normal neutrophils.

sults indicate that of the two (or more) cytosolic NADPH oxidase components of human neutrophils, only one is defective in Ab+ CGD; this component is probably identical with the 47-kDa protein that fails to be phosphorylated upon NADPH oxidase activation of Ab+ CGD neutrophils. Most likely, this protein is also a structural component of the NADPH: O_2 oxidoreductase, although its function in this enzyme remains to be established.

CONCLUSIONS

Table II summarizes the present ideas about the molecular defects that lead to the syndrome of CGD. From these and other studies a picture of the NADPH:O_2 oxidoreductase of human phagocytic leukocytes emerges as a multicomponent system. Its components are localized in different compartments of the cells when the NADPH oxidase is "at rest". During activation by particulate or soluble

TABLE II

Molecular characterization of chronic granulomatous disease

CGD type	NADPH oxidase activity	Cell-free system		Phosph. 47-kDa protein	Cytochrome b_{558}			
		cytosol activity	membrane activity		α subunit		β subunit	
					protein	mRNA	protein	mRNA
Xb−	−	+	−	+	±	+	−	−*
Ab+	−	−	+	−*	+	+	+	+
Ab−	−	+	−	+	−	−*?	−	+

* indicates the primary defect leading to the defect in NADPH oxidase activity

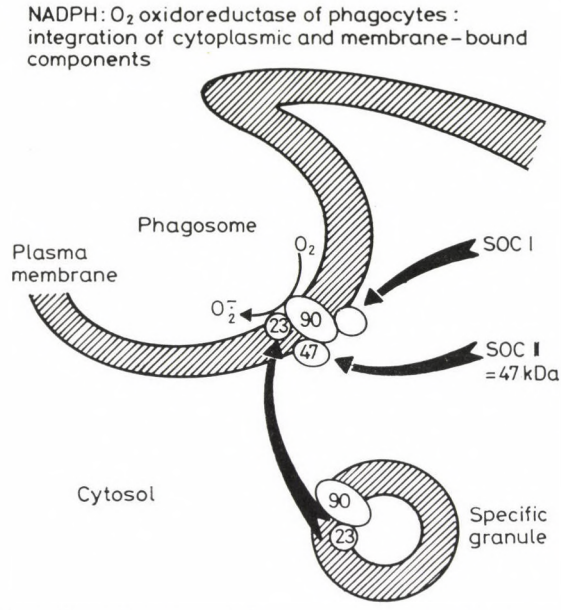

FIG. 5. NADPH: O_2 oxidoreductase of phagocytes: integration of cytoplasmic and membrane-bound components. Schematic representation of translocation of components of the NADPH oxidase during activation of phagocytes. Cytochrome b_{558} (alpha chain of 23 kDa and beta chain of about 90 kDa) are translocated from the specific granules to the membrane that constitutes the developing phagosome. The cytosolic components SOC I and SOC II translocate from the cytosol to the phagosomal membrane. Together, these components form an active NADPH oxidase complex that accepts electrons from NADPH at the cytosolic side of the membrane and reduces O_2 to O_2^- at the phagosomal side of the membrane

stimuli, translocation of these components take place, and an active, superoxide-generating NADPH oxidase is assembled in the plasma membrane and/or the phagosomal membrane. Fig. 5 shows this concept in a schematic way. Formation of an active NADPH oxidase in the membrane had been shown earlier [6, 9, 11] by superoxide formation in a cell-free system with activated membranes and NADPH, without the need of cytosolic components. Translocation of cytochrome b_{558} from the specific granules in resting neutrophils to a membrane fraction in activated cells had been shown earlier by Borregaard et al. [1]. Our results indicate that at least two cytosolic components are also involved and need to be translocated from the cytosol to the membrane for the formation of an active NADPH oxidase. Altogether this constitutes a unique biochemical system with an essential function in the host defense against invading micro-organisms.

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