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Respiratory response of phagocytes: terminal NADPH oxidase and the mechanisms of its activation

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Abstract. The chemical composition, properties and activation mechanism of the O_2^--forming NADPH oxidase of phagocytes were investigated, using partially purified enzyme preparations. Highly active NADPH oxidase was extracted as an aggregate of high M_r from the membranes of neutrophils and macrophages. The enzyme complex contained phospholipids and cytochrome b_{-245}, very little FAD and almost no quinones or NAD(P)H-dye reductase activity. The purification of a polypeptide with a relative molecular mass of 31 500 strictly paralleled the purification of NADPH oxidase, suggesting that this polypeptide is a component of the enzyme. This protein was identified as cytochrome b_{-245} after dissociation of the proteolipid complex and purification of the cytochrome moiety. The 31 500 M_r protein was phosphorylated in enzyme preparations from activated but not from resting cells. The results indicate that: (1) cytochrome b_{-245} is a major component of NADPH oxidase; (2) the involvement of NAD(P)H dye reductases in the O_2^--forming activity is questionable; (3) the cytochrome b_{-245}: FAD ratio in the enzyme complex is much higher than that indicated in crude preparations; (4) the M_r of pig neutrophil cytochrome b_{-245} is 31 500; (5) the activation of the O_2^--forming system involves a process of phosphorylation of cytochrome b_{-245}.

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The activation of respiration and production of toxic oxygen metabolites that is usually called the respiratory burst is one of the main functional responses by which phagocytes kill invading organisms and tumour cells. This response is induced by phagocytosis and by the interaction of the phagocytes with a number of soluble factors such as complement components, lectins, chemotactic peptides, phorbol esters, calcium ionophores, leukotrienes and platelet-activating factors (Babior 1978, Rossi et al 1982).

It is now generally agreed that the respiratory burst is due to the activation of an NADPH oxidase which we described about 20 years ago (Rossi & Zatti 1964). The active enzyme is located in the plasma membrane and it catalyses

the one-electron reduction of oxygen to superoxide (O_2^-), using the reducing equivalents provided by NADPH, which in turn is formed by the dehydrogenases of the hexose monophosphate shunt for glucose oxidation. The reaction stoichiometry is the following: $NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2H^+ + 2O_2^-$. Two molecules of superoxide can then dismutate to hydrogen peroxide (H_2O_2) according to the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, which occurs either spontaneously or catalysed by the enzyme superoxide dismutase (EC 1.15.1.1.). Reaction of H_2O_2 with O_2^- can generate the very reactive hydroxyl radical ($OH^\cdot$), probably through an Fe^{2+}-catalysed Haber–Weiss reaction: $H_2O_2 + O_2^- \rightarrow OH^\cdot + OH^- + O_2$. This paper deals with recent investigations on the nature of the NADPH oxidase in granulocytes and macrophages and with findings related to its activation.

Nature of oxidase: the candidate components

The information now available seems to indicate that the oxidation of NADPH involves various components that form an electron transport chain in the plasma membrane. The candidate components are a flavoprotein, a *b*-type cytochrome, and perhaps other cofactors, including quinones. The evidence that these components are involved is indirect because so far attempts to purify NADPH oxidase have been unsuccessful.

Involvement of a flavoprotein

The evidence that a flavoprotein (with FAD as prosthetic group) forms part of the NADPH oxidase system is as follows: (1) O_2^--forming activity by membranes solubilized with Triton X-100 is enhanced by the addition of FAD to the assay system (Gabig & Babior 1979); (2) the FAD analogue 5-carbadeaza-FAD inhibits the O_2^--forming activity of the solubilized enzyme (Light et al 1981); (3) leucocytes of patients affected by chronic granulomatous disease (CGD), a condition in which there is no NADPH oxidase activity, are deficient in FAD (Cross et al 1982a, Gabig 1983); (4) membrane extracts prepared from neutrophils contain flavoproteins which are reduced by NADPH in anaerobiosis (Cross et al 1984, Gabig & Lefker 1984).

It has been suggested that the flavoprotein moiety of this oxidase is an NAD(P)H-dye reductase, that is an NAD(P)H dehydrogenase which can give electrons to an artificial electron acceptor (Green et al 1983, Wakeyama et al 1983, Sakane et al 1984, Gabig & Lefker 1984).

Involvement of cytochrome b_{-245}

The involvement of a cytochrome *b* first discovered about 20 years ago by Hattori (1961) and Shinagawa et al (1966) has been investigated by Segal's and Jones's groups (Segal 1983). The evidence suggesting that this haemoprotein, which has an unusually low midpoint potential ($E_0 = -245$ mV), plays a part in the NADPH oxidase activity of phagocytes is as follows: (1) patients with the X-linked form of CGD have no cytochrome b_{-245} (Segal 1983); (2) this cytochrome behaves as a typical component of a terminal oxidase, since it is reduced when the cells are activated in anaerobic conditions, is rapidly reoxidized by oxygen, and binds carbon monoxide, although with low affinity (Shinagawa et al 1966, Cross et al 1982b, Bellavite et al 1983a); (3) during cell differentiation, the cytochrome appears in parallel with the development of the ability to perform the respiratory burst (Roberts et al 1982); (4) cytochrome b_{-245} can reconstitute the oxidase activity that is lacking in cells from patients with CGD. It has been shown that the hybridization of monocytes from a cytochrome b_{-245}-negative X-linked male CGD patient with monocytes from a cytochrome b_{-245}-positive male CGD patient of unknown genetic background restores the O_2^--forming activity (Hamers et al 1984).

In spite of the above positive evidence, there are experimental findings that raise doubts about the role of cytochrome b_{-245} in NADPH oxidase activity. According to Babior (1983), the large discrepancy observed between the rate of cytochrome b_{-245} reduction needed to account for O_2^- production by stimulated neutrophils and the rate of cytochrome b_{-245} reduction by NADPH forces the conclusion that this haemoprotein cannot be an obligatory electron carrier in the O_2^--forming reactions of leucocytes. However, the rate of O_2^- production by stimulated neutrophils has been measured aerobically, while NADPH-dependent cytochrome b_{-245} reduction has been measured anaerobically. It has been suggested that the presence of oxygen might affect the affinity of cytochrome b_{-245} for its electron donor (Tauber et al 1983) or that the removal of oxygen grossly distorts the bioenergetic circumstances under which cytochrome b_{-245} must function (Segal 1983). It is worth pointing out that the oxygen requirement for the rapid reduction of cytochrome b_{-245} by NADPH in solubilized enzyme preparations has been demonstrated recently by Cross et al (1985).

An alternative explanation can be advanced for the necessity of oxygen for the reduction of cytochrome b_{-245}. Assuming that cytochrome b_{-245} is reduced by a flavin semiquinone, in the absence of oxygen this semiquinone could not be formed from the one-electron reduction of O_2 by the fully reduced flavin. A branched NADPH oxidase system which forms O_2^- through both the flavin + O_2 and the flavin + cytochrome b_{-245} + O_2 pathways, as suggested

by Light et al (1981) and by Morel & Vignais (1984), would be compatible with the fact that cytochrome b_{-245} in anaerobiosis is slow.

Involvement of quinones

A role of quinones, namely of ubiquinone-50, has been proposed by Cunningham et al (1982) and by Crawford & Schneider (1982, 1983). The main evidence is the following: (1) a substantial content of ubiquinone-50 in various neutrophil fractions, including plasma membranes and phagolysosomes, has been found (Crawford & Schneider 1983); (2) ubiquinone-5 and duroquinone stimulate O_2 consumption by intact resting neutrophils and, in the presence of NADPH and NADH, by disrupted cells (Crawford & Schneider 1982). This stimulated O_2 consumption by intact neutrophils is inhibited by the ubiquinone analogues, quercetin and juglone, which also inhibit the respiratory burst induced by latex particles and the succinoxidase of rat liver mitochondria; these inhibitors act between ubiquinone and cytochrome *b* of the mitochondrial respiratory chain (Crawford & Schneider 1982); (3) NAD(P)H-dye reductase activities solubilized from guinea-pig polymorphonuclear leucocytes are enhanced by menadione and the enhancement is accompanied by the formation of superoxide anion (Sakane et al 1983, 1984). The enhancing effect of mendione has been explained by a mechanism in which menadione mediates the electron transfer from NAD(P)H to cytochrome *c* or molecular oxygen by undergoing cyclic oxidation–reduction between the quinone and semiquinone forms (Sakane et al 1983).

In our opinion these effects of quinones on neutrophil metabolism, shown about 20 years ago by our group (Rossi & Zatti 1964) and more recently by Gallin et al (1982), are independent of NADPH oxidase, and the mechanism of inhibition of the quinone analogues is not specific. Against the role of quinone is the demonstration that its very low content in neutrophils is exclusively associated with the mitochondrion (Cross et al 1983), a subcellular organelle that has nothing to do with the respiratory burst. Recently Lutter et al (1984) found no ubiquinone-50 in human neutrophils.

The electron-transport chain

From the findings so far presented, the more likely hypothesis is that NADPH oxidase is formed by a flavoprotein enzyme, which contains the substrate binding site and catalyses the transfer of electrons from NADPH to the flavin, and by cytochrome b_{-245}, which transfers the electrons from the reduced flavin

to oxygen (see Fig. 1). The flavin:cytochrome b_{-245} ratio measured in leucocyte membranes or in crude extracts varies from 2:1 (Cross et al 1982a), to 1:1 (Gabig 1983) or 1:2 (Borregaard & Tauber 1984, Lutter et al 1984).

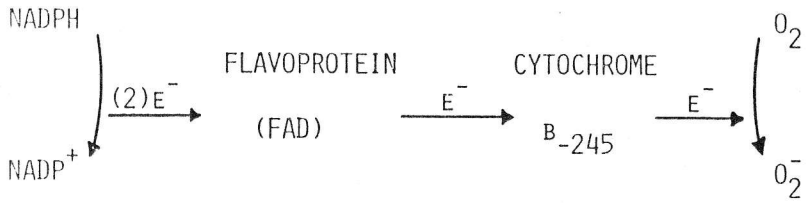

FIG. 1. Simplified hypothetical scheme for O_2^--forming NADPH oxidase.

Assuming that this is the composition of NADPH oxidase, two main problems remain to be investigated: the mechanism of the transformation of this simple electron transport chain from the inactive to the active state, and the mechanisms of electron transport between the various components. For the second problem, the main points to be investigated are (a) the presence of metal atoms (e.g. Fe, Mo, Cu) or of other groups as part of the structure of the flavoprotein or of cytochrome b_{-245}; (b) the type of oscillation of flavin between the oxidized and the reduced forms (e.g. $FAD \rightleftharpoons FADH_2$, $FAD \rightarrow FADH_2 \rightarrow FADH \rightarrow FAD$, $FAD \rightleftharpoons FADH$, $FADH \rightleftharpoons FADH_2$), which is relevant for the mechanisms of NADPH oxidation and oxygen reduction; (c) the actual FAD:cytochrome b_{-245} ratio; (d) the mechanism of oxidation of the reduced flavin (e.g. by cytochrome b_{-245} and oxygen, or by cytochrome b_{-245} only); (e) the requirement of phospholipids, suggested by Gabig & Babior (1979).

Partial purification of NADPH oxidase

In order to clarify the nature of the oxidase, the mechanism of its activation and the pathway of O_2^- formation, some years ago we began a series of attempts to extract and purify the enzyme. In common with other groups we experienced many difficulties because the enzyme has proved to be extremely unstable. Nonetheless, in a first group of experiments on guinea-pig neutrophils we obtained some relevant findings (Bellavite et al 1983a,b, 1984a,b, Serra et al 1984).

Experiments on guinea-pig neutrophils

Subcellular particles were solubilized from phorbol myristate acetate (PMA)-activated guinea-pig neutrophils with deoxycholate in the presence of glycerol

as a stabilizing agent, followed by chromatography through Ultragel AcA22 equilibrated with buffer containing glycerol but not detergents. We obtained an extract containing aggregates of high M_r formed by several proteins, including cytochrome b_{-245} and phospholipids, and endowed with high NADPH-dependent O_2^--forming activity. The extract was then further purified by centrifuging it on a glycerol density gradient and partially disaggregating it with 0.4 M-NaCl. The electrophoretic pattern of this purified preparation showed the presence of a major band of 32000 M_r and of other protein bands. By comparing the electrophoretic analyses of preparations at different purification steps we found that only the protein of M_r 32000 progressively increased as purification proceeded. Since the specific activities of NADPH oxidase and cytochrome b_{-245} also increased in the same preparations, we suggested that this protein belonged to NADPH oxidase and that it could be cytochrome b_{-245}.

Biochemical studies of these extracts from guinea-pig neutrophils allowed the oxidase to be characterized better and we were able to distinguish NAD(P)H-cytochrome *c* and NAD(P)H-dichlorophenol indophenol reductases (here called dye-reductases) from NADPH-dependent O_2^--forming activity. The main properties of leucocyte NADPH oxidase are shown in Table 1. It is worth noting that the properties of neutrophil and macrophage NADPH oxidase are similar, the only difference being that the kinetic parameters (K_m for NADPH) of macrophage oxidase can be modulated by the different activation states of these cells (Berton et al 1985).

The evidence in favour of the distinction between NADPH oxidase-forming O_2^- and dye reductase activities is as follows (Bellavite et al 1984a): (1) the dye reductase activities are similar in preparations from resting and activated

TABLE 1 Properties of leucocyte NADPH oxidase

(1) Catalyses the reaction
$NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2O_2^- + 2H^+$
(2) Works on the plasma membrane, releasing O_2^- (and perhaps H^+) at the external side of the membrane and in the phagosome
(3) Non-active in resting cells, activated by phagocytosis and by some soluble agents. During phagocytosis the enzyme in contact with the particle is selectively activated
(4) The activated state of the enzyme in intact cells is reversible upon removal of the stimulatory agent
(5) Optimum pH: 7.0–7.5
(6) K_m for NADPH: 0.03–0.05 mM, K_m for NADH: 0.5–1.0 mM; the K_m for NADPH can change in various types of macrophages
(7) Inhibited by parachloromercuribenzoate, quinacrine, batophenanthroline sulphonate, trifluoperazine, nordihydroguaiaretic acid, cibacron blue, strong detergents, high ionic strengths, 5-carba-deaza FAD
(8) Optimum activity of solubilized enzyme requires phospholipids

cells; (2) the dye reductase activities use NADH as the preferred substrate whereas the O_2^--forming enzyme uses NADPH; (3) the dye reductase activities have a K_m for NADPH (5 μM) that is different from the K_m for NADPH of the O_2^--forming enzyme (30–50 μM); (4) unlike the dye reductases, the 'true' NADPH oxidase does not give electrons to artificial electron acceptors in either aerobiosis or anaerobiosis. The finding that molecular oxygen is the preferred and perhaps the obligatory acceptor suggested two considerations. The first is that, since practically all flavin dehydrogenases can be oxidized by artificial electron acceptors, the behaviour of our enzyme indicates that the role of flavin must be reinvestigated. The second is that NADPH oxidase is 'protected' from the withdrawal of electrons which are completely utilized for the formation of O_2^- and H_2O_2, the actual weapons of the bactericidal and tumoricidal oxygen-dependent system of phagocytes.

Experiments with pig neutrophils and guinea-pig macrophages

In subsequent experiments using pig neutrophils we obtained better results with purified membranes as the starting material instead of nuclei-free homogenates, with a different mixture of detergents (deoxycholate plus Lubrol PX instead of deoxycholate), and with different chromatographic procedures (Ultragel AcA34 chromatography eluted in the presence of detergents) (Bellavite et al 1984b, 1985). First, we confirmed with these procedures that a protein of M_r 31 500 progressively copurified with the NADPH oxidase activity and with cytochrome *b*. This protein band was present in enzyme preparations from both PMA-activated and resting neutrophils. Secondly, after gel filtration chromatography we obtained two distinct peaks (Fig. 2). The first protein peak contained practically all the NADPH oxidase activity and cytochrome *b*. This peak corresponded to the void volume of the column, indicating that it contained aggregate material of $M_r > 350\,000$. This material also contained phospholipids (0.27–0.3 mg/mg protein) and was totally devoid of quinones. The second protein peak was almost devoid of cytochrome *b* and NADPH oxidase but contained most of the FAD and NAD(P)H-dye reductase activity. Similar results have been also obtained with macrophages from guinea-pig peritoneal exudates (G. Berton et al, unpublished work 1985).

The above results clarify the following points: (1) quinones are not components of the NADPH oxidase system; (2) NAD(P)H dye reductases are different from NADPH oxidase and therefore the hypothesis that the first component of the proposed electron transport chain is an NAD(P)H dye reductase is untenable; (3) cytochrome b_{-245} is probably a true component of the NADPH oxidase system; (4) most of the flavin contained in the membrane extract does not belong to NADPH oxidase. This last finding requires

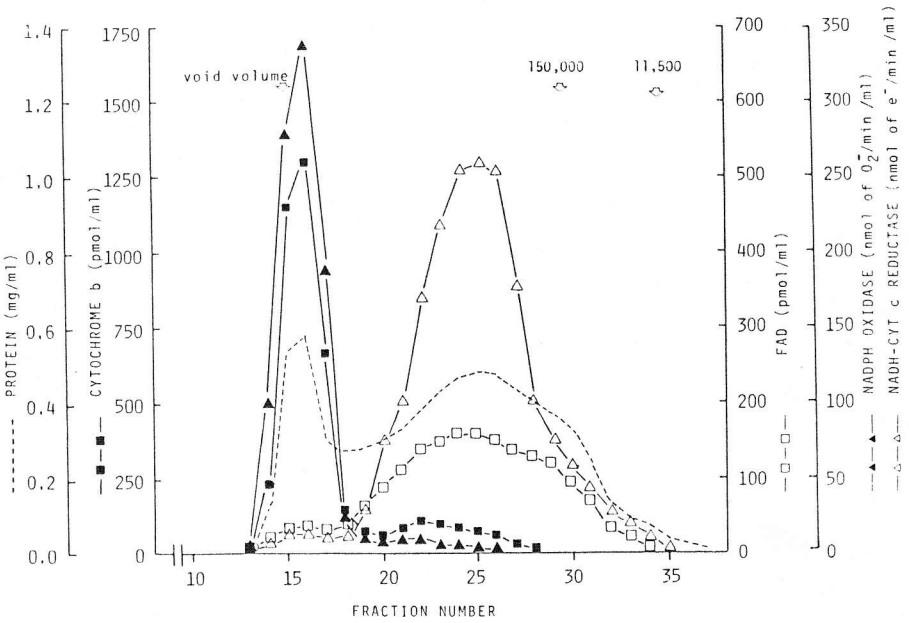

FIG. 2. Gel filtration chromatography of the solubilized extract from plasma membranes of phorbol myristate acetate (PMA)-activated pig neutrophils. Membranes were solubilized with 0.4% Lubrol PX and 0.4% deoxycholate in the presence of 20% glycerol and centrifuged at 100 000 *g* for 60 min; 4 ml of the supernatant (3.3 mg protein/ml; 2.31 nmol cytochrome b_{-245}/ml; 1.03 nmol FAD/ml; NADPH oxidase: 601 nmol O_2^- min^{-1} ml^{-1}; NADH cytochrome *c* reductase: 475 nmol cytochrome *c* reduced min^{-1} ml^{-1}) was chromatographed through an Ultragel AcA 34 column (LKB Produkter, Bromma, Sweden) (1.6 × 30 cm) equilibrated with phosphate buffer containing 20% glycerol, 0.15% sodium deoxycholate and 0.15% Lubrol PX. Fractions of 1.5 ml were collected at a flow rate of 18 ml/h. Assays were carried out as described by Bellavite et al (1984b). The void volume was determined with blue dextran, standards were alcohol dehydrogenase (M_r 150 000) and cytochrome *c* (M_r 11 500).

a revision of the cytochrome b_{-245}: FAD ratio in true NADPH oxidase, which in non-purified preparations resulted in a range between 2:1 and 1:2 (Cross et al 1982a, Gabig 1983, Borregaard & Tauber 1984, Lutter et al 1984).

Analysis of the NADPH oxidase, cytochrome *b* and flavin content during the various steps of purification (Table 2) points to some important observations. First, the findings confirm the previous results obtained with guinea-pig neutrophils, i.e. that cytochrome *b* copurifies with NADPH oxidase, since the cytochrome b_{-245}: NADPH oxidase ratio remains similar in all the preparations. Secondly, the ratio between NADPH oxidase activity and flavin content, which remains similar in the postnuclear supernatant, membranes and

TABLE 2 NADPH oxidase activity, cytochrome b_{-245} and FAD content of preparations at different stages of purification

	Postnuclear supernatant	*Washed membranes*	*Solubilized extract*	*First protein peak (AcA34)*
NADPH oxidase (nmol O_2^- min^{-1} mg protein $^{-1}$)	30.8	210	182	572
Cytochrome b_{-245} (pmol/mg protein)	70.3	617.2	702	1915
Flavin (FAD) (pmol/mg protein)	35.6	290	312.5	50
NADPH oxidase: cytochrome *b* ratio	0.4	0.3	0.3	0.3
NADPH oxidase: FAD ratio	0.9	0.7	0.6	11.4
Cytochrome *b*: FAD ratio	2.0	2.1	2.2	38.3

The postnuclear supernatant from PMA-activated pig neutrophils was centrifuged on sucrose gradient and the plasma membrane fraction was collected. After being washed with 0.3 M-NaCl, membranes were treated with 0.4% Lubrol PX and 0.4% deoxycholate and centrifuged at 100 000 *g* for 1 h. The supernatant (solubilized extract) was chromatographed through an Ultragel AcA34 column (see Fig. 2) and the fractions were assayed as described by Bellavite et al (1984b)

first crude extract, enormously increases in the first peak of AcA34 chromatography, due either to an increase in the specific activity of the enzyme or (mostly) to a decrease in the flavin content. The decrease in the flavin content in the first peak is not due to removal of flavin from the flavoprotein of the enzyme complex because if this happened the enzymic activity would also decrease. Finally, the cytochrome b_{-245}:FAD ratio, which is about 2:1 in the postnuclear supernatant, progressively increases and becomes about 40:1 in the first peak from gel filtration chromatography. A similar ratio has been found in preparations from resting cells. All these findings strongly suggest that the content of flavin in NADPH oxidase is much lower than that previously reported in studies with crude enzyme preparations. On this basis, kinetic analysis of flavoprotein reduction and oxidation in crude membrane extracts (Gabig & Lefker 1984, Cross et al 1984) also becomes questionable, since other flavoproteins not related to the oxidase system could affect it.

As regards the small amount of flavin associated with cytochrome b_{-245} and NADPH oxidase activity, two explanations can be advanced. The first is that NADPH oxidase is an electron-transport system composed of one flavoprotein and many cytochrome b_{-245} molecules. The manner of the functional association of the flavoprotein with many cytochromes remains to be investigated. A non-equimolecular concentration of flavoprotein and cyto-

chrome P-450 has been found in liver microsomes (Peterson et al 1976). The second explanation is that the small amount of flavin found in the first protein peak of gel filtration represents a contaminant belonging to the NAD(P)H-dye oxidoreductases or to other enzymes. If this is so, NADPH oxidase does not contain a flavoprotein. The finding that NADPH oxidase does not give electrons to artificial acceptors is compatible with either the latter explanation or with the molecular structural arrangement required by the high cytochrome b_{-245}: flavoprotein ratio.

Further purification of components of the NADPH oxidase system

Attempts to purify further the components of the NADPH oxidase system have been made by dissociating the proteolipid complex by procedures such as high concentrations of Triton X-100, deoxycholate or Lubrol PX, or extensive sonication, treatment with 1 M-NaCl and/or 1 M-KCl, or treatment with 8 M-urea. Unfortunately, all these procedures caused a complete loss of activity. In spite of this, we decided to identify some components of the oxidase.

Partially purified enzyme complexes from the first peak of AcA34 were loaded on a glycerol gradient (22–32%) and centrifuged overnight at 131 000 g. The protein peak resulting from the gradient fractionation and containing NADPH oxidase and cytochrome b_{-245} was concentrated, treated with 1% Triton X-100, 0.5% deoxycholate and 1 M-KCl, and centrifuged for 3 h at 100 000 $\times g$. The resulting supernatant contained most of cytochrome b_{-245}, indicating that the complex had been dissociated. In fact, when the supernatant was re-chromatographed through an Ultragel AcA34 column eluted in the presence of 1% Triton X-100, 0.5% deoxycholate and 1 M-KCl, the cytochrome b_{-245} appeared in a peak in the included volume, with an apparent M_r of 170 000–230 000 (Fig. 3). During the course of this experiment, NADPH oxidase activity was progressively lost. On the other hand, cytochrome b_{-245} was more resistant to the experimental manipulations, and its overall purification compared to the starting material (postnuclear supernatant) was 147-fold. The cytochrome b_{-245} content of the most purified preparation was 10.3 nmol/mg protein.

Analytical SDS-polyacrylamide gel electrophoresis of the 147-fold purified cytochrome b_{-245} showed only one major band of 31 500 M_r with minimal contaminants (Fig. 4). This is a direct demonstration that this protein band, previously indicated as a component of the oxidase, corresponds to cytochrome b_{-245} or to a subunit of the cytochrome dissociated by SDS. The oxidized and reduced spectra of purified cytochrome b_{-245} from pig neutrophils are shown in Fig. 5. Table 3 summarizes the main properties of phagocyte cytochrome b_{-245}, as found in our laboratory and elsewhere. Harper et al

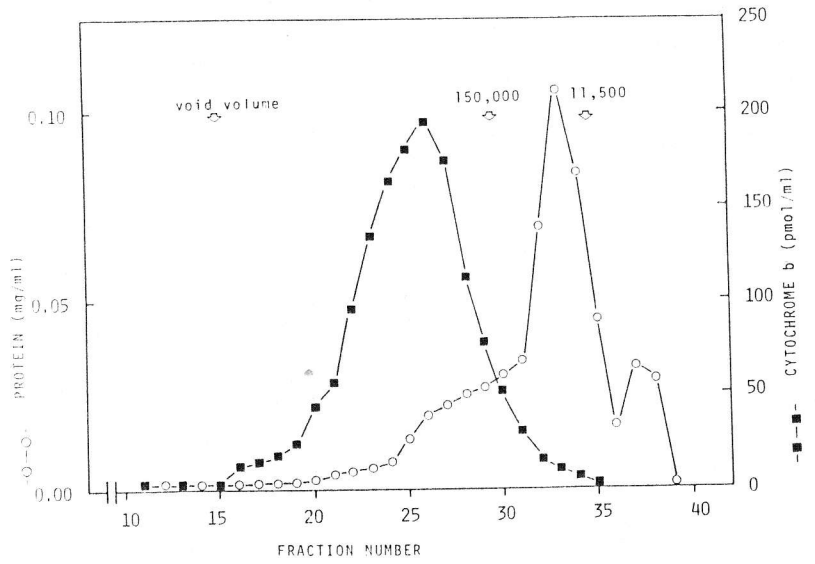

FIG. 3. Isolation of cytochrome b_{-245} by gel filtration chromatography. Enzyme complexes from AcA34 chromatography and subsequent glycerol gradient (see text) were dissociated with 1% Triton X-100, 0.5% deoxycholate and 1M-KCl. The extract (2 ml; 0.44 mg protein/ml; 1.035 nmol cytochrome b_{-245}/ml) was chromatographed through the same Ultragel AcA column as was used in the experiments illustrated in Fig. 2, but eluted with phosphate buffer containing 1% Triton X-100, 0.5% deoxycholate and 1 M-KCl. Fractions of 1.5 ml were collected.

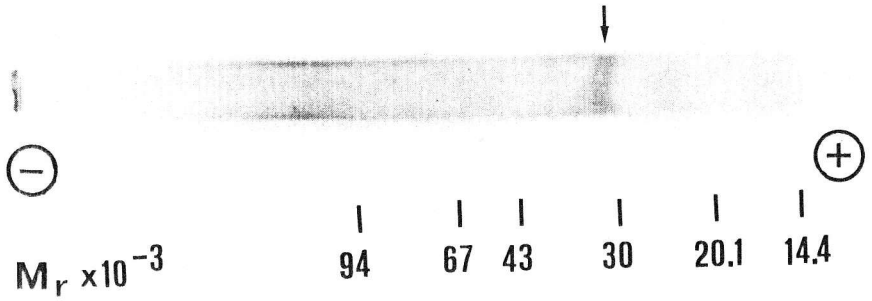

FIG. 4. SDS-polyacrylamide gel electrophoresis of 147-fold purified cytochrome b_{-245} from pig neutrophils. Cytochrome b_{-245} isolated by gel filtration chromatography (see Fig. 3) was rechromatographed through Ultragel AcA34 eluted in the absence of detergents in order to remove the excess of Triton X-100, deoxycholate and KCl. The eluted cytochrome was pelleted by centrifugation for 16 h at 131 000 *g* and then treated with 2% SDS and 2% 2-mercaptoethanol for 1 h at 50°C in the presence of 2 mM phenylmethanesulphonyl fluoride (PMSF). Electrophoresis was performed with a slab gel consisting of a linear polyacrylamide gradient (4–20%), in 40 mM-Tris/HCl buffer, pH 8.0, containing 1% SDS, 1 mM-EDTA and 20 mM-sodium acetate; 7.3 μg protein were loaded on the gel. Arrow: 31 500 M_r.

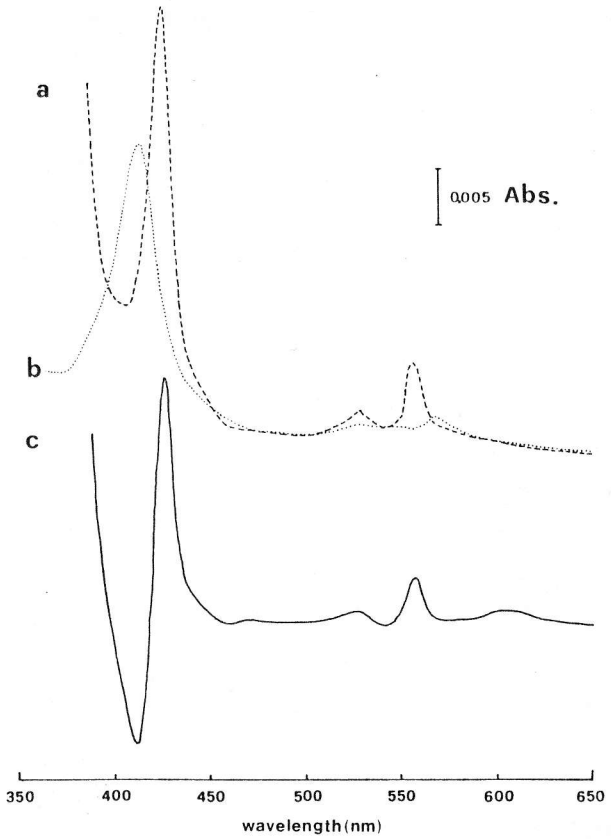

FIG. 5. Dithionite reduced (a), oxidized (b), and reduced–oxidized (c) absorption spectra of neutrophil cytochrome b_{-245}. Protein concentration, 0.017 mg/ml.

TABLE 3 Properties of leucocyte cytochrome b_{-245}

(1) Absorption peaks: oxidized 412 nm; reduced–oxidized 559 nm, 528 nm, 427 nm
(2) Difference absorption coefficient at 559–540 nm: 21.6 mM^{-1} cm^{-1}
(3) Low oxidation–reduction midpoint potential ($E_{m\,7.0}$): −245 mV
(4) Reduced by NADPH, slowly in anaerobiosis and fast in aerobiosis
(5) Reduced in intact phagocytes activated under anaerobic conditions
(6) Oxidized by oxygen with a $t^{\frac{1}{2}}$ of 4.7 ms
(7) Binds CO, although with lower affinity than O_2
(8) Present in neutrophils, eosinophils, monocytes, macrophages; not present in lymphocytes
(9) Copurifies with NADPH oxidase activity
(10) Relative molecular mass: 31 500 (Rossi's group); 68 000–78 000 (Segal's group); 17 000–14 000–11 000 (Pember's group)

(1984) have recently reported an M_r of 68 000–78 000 for human neutrophil cytochrome b_{-245}, whereas Pember et al (1984) indicated that three proteins of 14 000, 12 000 and 11 000 M_r were components of cytochrome purified from bovine neutrophils. The reason(s) for these discrepancies remain(s) to be investigated.

Hypotheses of the structure of NADPH oxidase

We are now working on two main hypotheses concerning the chemical composition of NADPH oxidase.

Hypothesis 1

NADPH oxidase is a two-component system made by a flavoprotein dehydrogenase (different from the NAD(P)H-dye reductase) and by many cytochrome b_{-245} molecules. The structural and functional relationship between flavoprotein and the cytochromes remains to be clarified. This clarification must take into account (a) that the ratio cytochrome b_{-245}:FAD is similar in extracts from resting and activated cells, and (b) the lack of quinones that could carry electrons between the components in the lipid milieu of the membrane.

Hypothesis 2

NADPH oxidase is made by only one molecular species, the haemoprotein with a cytochrome *b*-like spectrum, not containing flavin. This molecule should have a substrate binding site facing the cytoplasmic side and a haem group for the reduction of oxygen on the other side. The following points have yet to be clarified: (a) the enhancing effect of FAD on enzymic activity, and (b) how a two-electron donor (NADPH) reduces a one-electron acceptor (the haem group). This latter point could be explained if the haemoprotein contains other electron carriers such as metal atoms (e.g. Fe, Mo, Cu). The presence of non-haem iron is suggested by our finding that batophenanthroline sulphonate inhibits oxidase activity (Bellavite et al 1983b). Furthermore, the ESR spectra presented by Shinagawa et al (1966) could be indicative of some non-haem metal protein, and Pember et al (1984) mentioned the existence of an EPR spectrum atypical of haem proteins in purified cytochrome b_{-245}, suggesting the existence of another paramagnetic centre.

The above hypotheses do not consider the importance of SH-groups (Bellavite et al 1983a) and of phospholipids (Gabig & Babior 1979) for O_2^--generating activity. These properties of the oxidase are compatible with both models.

Phosphorylation of the 31 500 M_r protein

A fundamental question that remains open is the mechanism by which the NADPH oxidase system is turned on in activated cells. In the last few years the hypothesis has been advanced that the transduction mechanism responsible for many leucocyte responses to external stimuli involves the activation of a calcium- and phospholipid-dependent protein kinase (protein kinase C) and of protein phosphorylation (Sha'afi et al 1983, McPhail et al 1984). Protein kinase C may be activated either directly, for example as it is by PMA (Castagna et al 1982), or indirectly, via formation of diacylglycerol by breakdown of phosphoinositides (Grzeskowiak et al 1985). We have therefore investigated the possibility that phagocyte activation is associated with phosphorylation of some identified component of the NADPH oxidase system.

Granulocytes labelled with $^{32}P_i$ were divided in two aliquots, one of which was stimulated with PMA. The activated and resting cells were processed until partial purification of NADPH oxidase and cytochrome b_{-245} was achieved by gel filtration chromatography. Electrophoresis of these preparations, followed by autoradiography, showed that the 31 500-M_r protein was markedly phosphorylated in the enzyme obtained from activated but not from resting cells (Fig. 6). Other minor protein bands of about 26 500 and 41 000 M_r also appeared to be phosphorylated in preparations from activated cells.

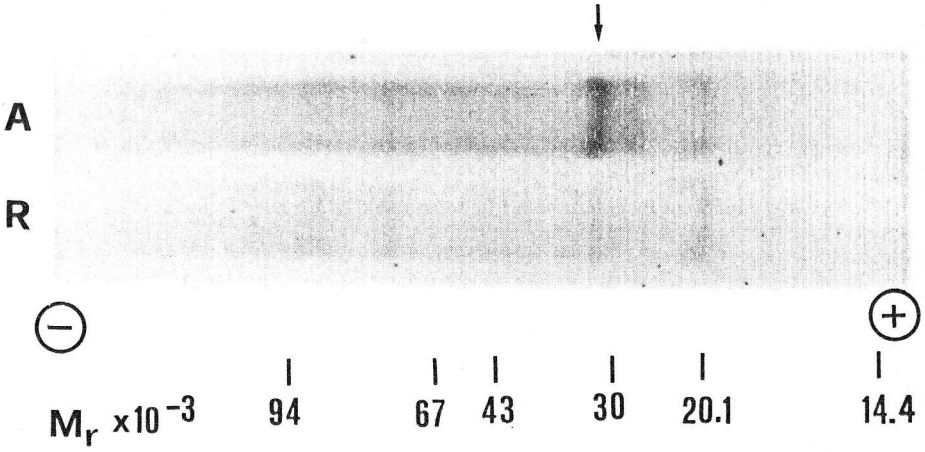

FIG. 6. Autoradiography of phosphorylated proteins present in resting (R) and PMA-activated (A) partially purified NADPH oxidase. Neutrophils were labelled with $^{32}P_i$ and divided in two parts, one of which was treated with PMA. NADPH oxidase was extracted from membranes and partially purified through Ultragel AcA34 chromatography (see Fig. 1). Fractions of the first protein peak were pooled, concentrated and subjected to SDS electrophoresis on a linear polyacrylamide gradient (4–20%) slab gel. The gel was desiccated and exposed to Kodak X OMAT R film for two days at 70 °C; 40 μg protein from each sample was subjected to electrophoresis. The arrow indicates the position of the 31 500 M_r band.

However, these proteins probably do not belong to the oxidase system because they are not enriched during the various steps of NADPH oxidase purification.

The finding that the 31 500 M_r protein which has been identified as cytochrome b_{-245} becomes phosphorylated in the extract from PMA-activated neutrophils is relevant. In fact this could be the evidence that the activation of protein kinase C is involved in the transduction mechanism for cell stimulation. If this is the case, it represents the identification of the target of protein kinase, phosphorylation of which is essential to activation of the respiratory burst. However, phosphorylation of cytochrome b by a kinase other than protein kinase C cannot be ruled out.

The mechanism by which phosphorylation of the 31 500 M_r protein changes the state of the NADPH oxidase system remains to be explained. Phosphorylation may induce a conformational modification of the haemoprotein, facilitating its functional relation with other components of the oxidase, the transfer of electrons, or—if the haemoprotein is the oxidase—the interaction with substrate NADPH. Alternatively, phosphorylation may remove or change the interaction with a regulatory factor.

It remains to be established whether phosphorylation is the only mechanism for activation of NADPH oxidase or whether other processes are involved in different conditions of stimulation. The problem will be clarified by experiments on the phosphorylation of the 31 500 M_r protein in cells stimulated by chemotactic peptides or other stimulants and on the activation of NADPH oxidase in cell-free systems.

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DISCUSSION

Roos: There are now four reports from groups who have purified cytochrome *b* from phagocytic cells, and there are four different M_r values. This may be due to species differences, but it is hard to believe that species differences alone would account for the difference between the M_r we find (127000) and Dr Bellavite's protein of 31500 M_r. It might be argued that yours is a subunit of our protein from human neutrophils, but we cannot obtain a lower M_r by SDS treatment, in either reducing or non-reducing conditions.

Another possibility is that proteins of lower M_r have been partly degraded, because neutrophils are loaded with proteinases. So I would be very careful about calling your preparation intact cytochrome *b*. If purified cytochrome *b* can be incorporated into the chronic granulomatous disease cell, and if it restores the function, that would show that it is a protein that is still working. It would also prove that the cytochrome is part of the oxidase system.

Bellavite: Pember et al (1984) reported that cytochrome *b* from bovine neutrophils showed three protein bands with M_r of 14000, 12000 and 11000. Harper et al (1984) and you (D. Roos, personal communication), using human neutrophils, get 68000 and 127000 respectively. It is tempting to suggest that the three M_r bands that Dr Pember gives come to a value not far from the M_r we indicated, 31500, which is close to half of 68000, which in turn is about half of 127000 . . . As you suggest, this may be a problem of the aggregation of different subunits. Segal indicates that there is a strong tendency for cytochrome *b* to aggregate, even in the presence of SDS. We took many precautions against proteolysis. We pretreated the cells with diisopropylphosphorofluoridate and we always included phenylmethanesulphonyl fluoride in the buffer. So in our case it is not a problem of proteolysis but perhaps one of aggregation in different conditions. Finally, the existence of marked species differences seems to be excluded because we found the same M_r of cytochrome *b* in pig guinea-pig neutrophils (Serra et al 1984).

Roos: An important point is that we find one haem per protein molecule of 127000, which indicates that our preparation is an intact protein.

Bellavite: Your preparation seems less dissociated than ours but we cannot estimate the haem: protein ratio in our preparation because it is not 100% pure. We indicated the M_r of cytochrome *b* by looking at the relative increase in the 31 500 band during purification. With very sensitive methods we can find other bands but these cannot be cytochrome *b* because their intensity decreases during purification. Our procedure takes three to four days and some loss of haem would be expected in that time. Taking these limitations into consideration I think that in our cytochrome *b* preparation the haem:protein ratio approaches 1:1.

Whaley: What are the specific functional activities of these two proteins? Dr Roos, have you tried limited proteolytic digestion of your protein and examined the relative molecular mass of the digests?

Roos: Once we have purified cytochrome *b* we don't find any activity. We have given up trying to purify the oxidase activity. When we solubilize the oxidase we lose most of the activity, and we didn't want to draw conclusions from the 2–3% of the activity we can really purify. Therefore, we purified one of the components that we believe is part of the oxidase system and concentrated our efforts on that. From there, we want to go back to the oxidase system with chemical cross-linkers and try to identify the other possible components.

Whaley: What happens after proteolytic digestion?

Roos: We did that but for completely other reasons. We had a very pure protein so we wanted to make digests, do amino acid sequence analyses and try to make a DNA probe to go to the gene level. It turned out that trypsin treatment generated an enormous amount of small peptides. Using cyanogen bromide, we found two peptides of about equal M_r.

Springer: Dr Bellavite, you are isolating oxidase from plasma membranes of PMA-activated cells. What are the normal storage sites of the oxidase and the cytochrome *b*? Is there a translocation process during activation?

Bellavite: Fractionation studies indicate that a significant proportion of cytochrome *b* is in specific granules in the resting cells but is translocated to the membrane after the activation process. However, nobody demonstrated that this is the actual mechanism by which the oxidase is activated. The translocation is not necessary for the activation of neutrophil cytoplasts, which are practically devoid of specific granules and contains cytochrome *b* on the membrane only (Roos et al 1983).

Another piece of evidence against the role of translocation is that the release of the granules can be blocked by suitable inhibitors without affecting activation of the respiratory burst (Korchak et al 1980).

Finally, we have some data indicating that a lot of cytochrome *b* is present on the membrane in resting cells. If we treat macrophages with a small amount of a reducing agent, sodium dithionite, practically all the cytochrome *b* is reduced without reduction of intracellular chromophores such as mitochondrial and

mitosomal cytochromes and peroxidase. The membrane of the cell therefore seems to be impermeable to the reducing agent. This indicates that cytochrome *b* is available on the surface of the cells.

Roos: We found that cytochrome *b* in the plasma membrane is a transmembrane antigen, because we can iodinate it from the outside and monoclonal antibodies against cytochrome *b* bind to the inside of the plasma membrane. For this last reaction, we have to spin the cells on a coverslip, so they become permeable to antibodies. This indicates that cytochrome *b* sticks out of the membrane on both sides.

Singer: But does that require the active site to be externalized?

Roos: I don't know whether the cytochrome needs a flavoprotein to form an active oxidase complex, but I think the active site is inside the cells because it somehow has to receive its electrons from NADPH, either directly or via a flavoprotein. On the other hand, cytochrome *b* also has to react with oxygen, and it does that on the outside of the cells or maybe in the membrane.

Singer: What about ubiquinone?

Roos: If you purify the neutrophils very carefully hardly any ubiquinone is present. We suspect that most of the ubiquinone that has been reported as part of the oxidase system has been derived from platelet-contaminated neutrophils.

Cohn: What about the localization of NADPH oxidase?

Bellavite: The active oxidase works on the membrane. In particular conditions, for example in the presence of cytochalasin B, practically all the superoxide produced is released outside the cell. The production of free radicals takes place on the membrane but obviously during phagocytosis free radicals and hydrogen peroxide are also released inside the cell.

Nathan: In your review of the evidence for the involvement of flavin, the only strong point is the genetic evidence that in some patients with chronic granulomatous disease the membrane proteins lack flavin fluorescent spectral signals. Have you confirmed that, in CGD neutrophils, membrane flavins are absent in the cytochrome *b*-containing population?

Bellavite: CGD is a very complex disease and many other defects have been described besides cytochrome *b* deficiency. For example there are also defects of the red cell antigens. The demonstration of the lack of flavin is not a direct demonstration that flavin is involved in the oxidase. Secondly, I wonder how a cell can be completely free of flavin, as described by one of these reports (Gabig 1983). The activity of cytochrome *c* reductase, diaphorase enzymes and so on suggests that other flavoprotein dehydrogenases are present in these cells. It is a bit contradictory.

Roos: We have looked at cells from eight CGD patients: X-linked, autosomal and a third group. Using cytoplasts for simplicity, we found completely normal amounts of non-covalently bound flavin in the membrane fraction and

in the soluble fraction of the cytoplasm. That does not completely invalidate the published results but I have been wondering why such large deficiencies have been found in whole membranes and even in whole cells.

Nathan: It is difficult to prove, by either time or quantity, that phosphorylation of cytochrome *b* represents an activation step. Couldn't it either be unrelated to activation or represent deactivation? We know that the oxidase begins to be suppressed shortly after it starts to be activated.

Bellavite: The phosphorylation of cytochrome *b* is not due to deactivation, as the preparations where we observed phosphorylation were fully active. Certainly we cannot be sure that this is the only mechanism of NADPH oxidase activation. For example activation may consist of phosphorylation of cytochrome *b*, which could allow interaction with other factors necesary for the activity. This does not mean that activation affects only cytochrome *b* molecules *per se*.

Nathan: Have you looked for any other covalent modifications after stimulating the cell, such as acylation, for example?

Bellavite: No.

Dean: Is the system a self-inactivating one? In other words, if electron transport occurs, do enzymes in the system have to be progressively inactivated? It looks as if that is what happens with electron transport in chloroplasts, in relation to the 32K protein, and perhaps also in mitochondria (Dean & Pollak 1985).

Bellavite: The system is not self-inactivating. Oxidase activity can be turned on and off but this is due to the activation mechanism of the cell. For example, we can turn off the function of the oxidase by destroying the activator factor. If we activate the cells with arachidonic acid and then add albumin, this binds to the arachidonic acid and stops activation immediately. This is due to the removal of the stimulant that should be continuously present if activation is to be maintained. Addition of new stimulants can reactivate the oxidase.

Dean: So after a burst of activity you would expect the system to activate equally well again?

Bellavite: Yes.

Nathan: That is not what happens in macrophages. Once they are triggered they are refractory for at least three days, which is as long as anyone has looked, although they are viable (Murray 1982).

Dean: Is there any evidence that during the refractory period there are still components which are active and reconstitutable?

Nathan: No one has tried that.

Bellavite: The refractory period depends on the function of the receptor, because if you add a different stimulus the cells are responsive in terms of the respiratory burst.

Gordon: With large doses of PMA there is total deactivation to all stimuli.

Singer: Wouldn't this have to be a very unusual cytochrome to be in the plasma membrane? Most electron transport chains are in the endoplasmic reticulum. Does this suggest that the same cytochrome might be present in other cells but is for some reason stuck in the endoplasmic reticulum and never gets to the cell surface, so never expresses this kind of activity? Or is it a unique cytochrome that is characteristic of neutrophils and is not present in any other cells? Dr Roos, have you shown that your monoclonal antibody that reacts with this cytochrome is on the cytoplasmic face of the plasma membrane by *in situ* labelling?

Roos: Those experiments are being done now.

Singer: Is it present in homogenates of other cells?

Roos: Segal has shown that it is present in monocytes, some macrophages, eosinophils and neutrophils, that is in all the real phagocytes that are able to produce superoxide and hydrogen peroxide (Segal et al 1981).

The term cytochrome is perhaps a bit misleading. It was given the name because of the light absorbance spectrum, which is reminiscent of *b* type cytochromes. However, we think that it is a protein of high M_r and we prefer to call it a haem-containing protein. Indeed, as Dr Bellavite said, there may be other active centres in the same protein.

*Bellavite:*It should be stressed that this 'cytochrome *b*' has a very low mid-point potential, which is completely different from the potential of other *b* cytochromes present in other systems. This is necessary so that the reaction with oxygen can generate superoxide.

Unkeless: Does the monoclonal antibody that binds to the macrophages correlate with the capacity to evoke superoxide in, say, cells stimulated with IFN-γ, or is that a different component?

Roos: Those experiments are also on the programme.

Stanley: Is the protein of M_r 127000 phosphorylated in response to PMA?

Roos: Yes, and also in response to serum-opsonized zymosan. We have not tried other stimuli yet.

Stanley: Do you use solubilized membranes, or what?

Roos: Several groups have already shown that when the neutrophils are stimulated dozens of proteins are phosphorylated, giving different patterns with different stimuli. We took the simplest system that we could think of, the phagosomes from neutrophil cytoplasts. We used Oil Red 0 to form phagosomes and then floated them up in a gradient. We looked at the protein bands in the homogenate from the phagosomes. The 127000 M_r protein was phosphorylated.

Stanley: Were other proteins phosphorylated as well?

Roos: Yes, a couple of others.

Gordon: With G. Berton, H. Rosen and other collaborators we used the almost final stage of purified guinea-pig 'oxidase' to raise monoclonal anti-

bodies. We produced rat hybridomas which were screened for the ability to trigger a respiratory burst on intact guinea-pig neutrophils. Most of the antibodies do not trigger a respiratory burst but we have isolated two which dramatically stimulate a burst. This requires the presence of cytochalasin B. If we treat the cells with intact antibody there is an efficient respiratory burst after a very short lag period. We can shorten the lag period slightly by preincubation with antibody. We can also trigger a burst by taking $F(ab')_2$ fragments of the antibody and cross-linking it with a second $F(ab')_2$ antibody. The $F(ab')_2$ fragment by itself does not trigger the burst any more, although the cells are still responsive to PMA. The second antibody, OX-12, is a mouse anti-rat hybridoma which also does not trigger the burst by itself.

Both monoclonal anti-'oxidase' antibodies see the same antigen, which is a novel proteolipid molecule found in macrophages and neutrophils only. It is present in the preparation originally used for immunization and is found in lysates and on the cell surface. It is very hydrophobic. We can extract with chloroform–ethanol and get rid of most of the other cellular proteins. In addition to the respiratory burst it also triggers degranulation. Although it is physically associated with the respiratory burst oxidase preparation, it may be more generally involved in signal transduction. Although we can trigger the burst in intact neutrophils, we cannot trigger it in macrophages even with cross-linking.

Cohn: Does its relative molecular mass fit in with the others we have heard about?

Gordon: It is smaller than 10000 M_r but it has very distinctive properties. It is very hydrophobic, and extremely resistant to proteolysis even if we boil it in SDS.

Roos: Why didn't you get enough cross-linking with the Fab_2 fragments? Would you get better cross-linking with the intact antibody?

Gordon: We don't understand the role of Fc on the first antibody. The two antibodies we have used have different effects. With one antibody the intact antibody will trigger but $F(ab')_2$ fragment will not until we cross-link. In the other case neither the intact antibody nor the $F(ab')_2$ will trigger unless we cross-link with the second antibody. We have done controls to exclude that the Fc fragment of the intact immunoglobulin could be involved directly in stimulation. We assume that aggregation of the antibody and antigen within the membrane is facilitated by the intact molecule and not the $F(ab')_2$. That is a bit of a puzzle.

Springer: There are antibodies to a human neutrophil component, *N*-acetyl lactosamine (X-hapten). This carbohydrate moiety is present in both glycolipids and glycoproteins. Antibodies to the moiety can trigger a respiratory burst on human neutrophils. The density of this antigen on the cell surface is much greater than that of any other antigen that we looked at during the Second

International Leukocyte Workshop. Cross-linking of this high density antigen may non-specifically trigger the respiratory burst. What is the density of your antigen? Could you be looking at a glycolipid?

Gordon: We have purified the antigen by HPLC and find protein. We don't know if there is sugar as part of the proteolipid as well.

Singer: Was that measured by immunoprecipitation?

Gordon: When we blot we pick up a single band which is very characteristic. We looked at several other antibodies and they don't do this.

Cohn: There is no evidence that the antigen is part of the oxidase synthesis, is there?

Gordon: The antigen is physically associated with the oxidase preparation. It cofractionates with it in detergent-solubilized membranes until activity is lost.

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