

SELECTIVE ENRICHMENT OF NADPH OXIDASE ACTIVITY IN PHAGOSOMES FROM GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—Recent studies have demonstrated that the activated NADPH oxidase, the enzyme responsible for the stimulation of O_2 consumption with O_2 formation during phagocytosis, is located in the plasma membrane of leukocytes. The present work deals with whether the activation induced by phagocytosis involves the enzyme of the entire membrane or only that of the portion of the membrane that interacts with the phagocytosable particle and forms the phagosome. The results presented show that the activity of the NADPH oxidase of phagosomal membrane, isolated by centrifugation of homogenates on discontinuous sucrose gradients, is increased 12.6-fold with respect to that of homogenate. In contrast, the activities of 5'-nucleotidase and of acid *p*-nitrophenyl phosphatase, enzyme markers of the plasma membrane not activated during phagocytosis and uniformly distributed on the entire membrane, are increased only about threefold with respect to that of homogenate. These results indicate that during phagocytosis the activation of NADPH oxidase is a segmentary response that involves only the enzyme that forms the phagocytic vacuole. This fact is relevant for the function of toxic intermediates of oxygen reduction that are discharged in direct contact with the engulfed agent.

INTRODUCTION

The stimulation of respiration in polymorphonuclear leukocytes (PMNs) induced by phagocytosis and by a variety of soluble membrane-perturbing agents is due to the activation of an oxidase that uses NADPH as substrate and reduces O_2 with the formation of O_2^- and, indirectly, of H_2O_2 , $OH\cdot$ and 1O_2 (1-9).

In recent years evidence has been accumulated that the oxidase is located in the plasma membrane of phagocytes (10-13). Direct measurements have been made of the activity of the NADPH oxidase in subcellular frac-

tions of homogenate from PMNs stimulated with soluble agents (10-12), which interact with the entire cell surface, and in isolated phagosomes (13). The data presented so far, however, do not indicate whether, during phagocytosis, the oxidase of the entire membrane or only that of the site of the membrane that interacts with the phagocytosable particles is activated.

We have investigated the problem by fractionating homogenates of PMNs phagocytosing PMA-coated latex particles and by isolating the phagocytic vesicles. The present paper reports that in the membranes of these vesicles the enrichment of NADPH oxidase is much higher than that of other enzyme markers of the plasma membrane, indicating that during phagocytosis only the oxidase of the portion of the membrane that forms the phagosomes changes from the inactive to active state.

MATERIALS AND METHODS

Cell Preparation, Stimulation, and Homogenization. PMNs were obtained from caseinate-induced peritoneal exudates of guinea pig (14) and resuspended in Krebs-Ringer phosphate buffer (KRP), pH 7.4. Phagocytosis was accomplished by incubating, for 3 min at 37°C, 3×10^8 PMNs in 30 ml of KRP with 9 mg of latex particles (0.81 μ m) coated with phorbol-myristate acetate (PMA), as previously described (15). As shown by light-microscopic examination, after incubation all the PMNs engulfed more than five latex beads. After two washings with ice-cold KRP, the cells were suspended in 11% (w/w) sucrose at a concentration of 1.5×10^8 cells/ml. The cells were disrupted with a Potter-type homogenizer placed in an ice bucket. The disruption of the cells was checked by light-microscopic examination.

Isolation of Phagosomes. The isolation of phagosomes was performed by flotation on sucrose gradient, substantially according to Segal et al. (16). Briefly, 5 ml of homogenate of phagocytosing PMNs, adjusted to a sucrose concentration of 46% by addition of 66% sucrose, was placed at the bottom of 17 ml polyallomer tubes and layered with 5.5 ml of 33% and 4 ml of 11% sucrose solutions (Figure 1). The tubes were centrifuged 30 min at 12,000 rpm with a Beckman SW 28 rotor (max, 25,000 g; min, 12,000 g). The material at the interface between 11% and 33% (band 1) and between 33% and 46% (band 2) was collected. The 46% sucrose band, together with the pellet at the bottom of the tubes, was diluted to 11% sucrose, centrifuged for 30 min at 100,000 g and the resulting pellet resuspended in 11% sucrose. All fractions obtained were sonicated with two 5-sec pulses at 150 watts.

Electron Microscopy. Before sonication, aliquots of the fractions were fixed for 30 min at 4°C in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, dehydrated with ethanol, embedded in Dow epoxy resin 332 (17), and double stained with uranyl acetate and lead citrate (18). Ultrathin sections were examined in a Philips 300 electron microscope.

Biochemical Assays. The enzymatic assays were performed as follows: NADPH oxidase as O_2 -forming activity by the spectrophotometric determination of the superoxide-mediated, SOD-sensitive, cytochrome *c* reduction (19); myeloperoxidase as rate of oxidation of guaiacol in presence of H_2O_2 (20); 5'-nucleotidase as release of Pi by 5'-AMP (21); acid *p*-nitrophenyl phosphatase as formation of *p*-nitrophenol from *p*-nitrophenyl phosphate (22, 23). Proteins were measured by the method of Lowry et al. (24).

Materials. Cytochrome *c* type VI, superoxide dismutase (SOD) type I, NADPH type III, and phorbol-12 myristate-13 acetate (PMA) were purchased from Sigma Chemical Co.

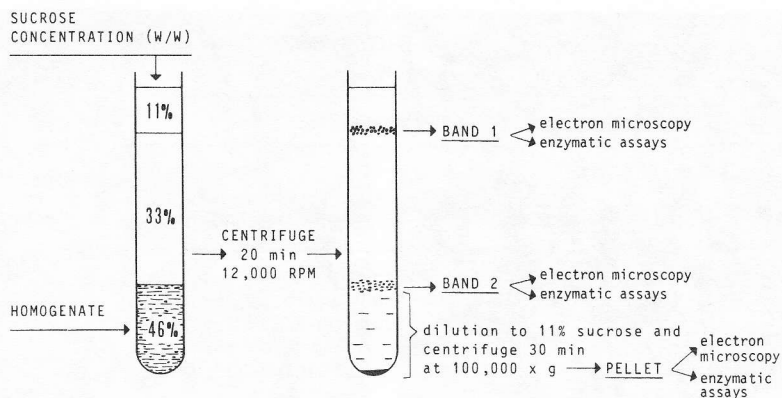


Fig. 1. Experimental procedure for the isolation of phagosomes.

(St. Louis, Missouri). Other compounds were obtained from commercial sources at highest available state of purity. All sucrose solutions contained 1 mM NaHCO_3 and 0.9% NaCl .

RESULTS

The electron microscopic pictures in Figure 2 show the composition of the fractions obtained from the homogenate of phagocytosing PMNs. The fraction at the interface between 11% and 33% sucrose (band 1, Figure 2a) is composed mainly of large vesicles containing latex beads with very low contamination by small vesicles and other organelles. Figure 2b shows (at higher magnification) the membrane that forms the wall of the phagocytic vacuoles containing latex particles and electron-dense materials discharged after fusion of granules with the phagosomes (arrow). Band 2 (Figure 2c) is heterogeneous and consists of empty vesicles of different sizes, phagosomes, broken membranes, granules, and amorphous material. The pellet (Figure 2d) contains many granules of different sizes and electron densities, some vesicles, fragments of nuclei, and other cell debris.

Table 1 reports the specific activities of acid *p*-nitrophenyl phosphatase, myeloperoxidase, and NADPH oxidase determined on the homogenates obtained from resting and phagocytosing PMNs. The specific activities of acid *p*-nitrophenyl phosphatase and myeloperoxidase, enzyme markers of the plasma membrane and azurophilic granules, respectively (23, 25), did not significantly differ in resting and phagocytosing cell homogenates, whereas the NADPH oxidase activity was markedly higher in homogenates derived from phagocytosing PMNs. This indicates that the oxidase is activated by phagocytosis and that the activation state of the enzyme is maintained after homogenization.

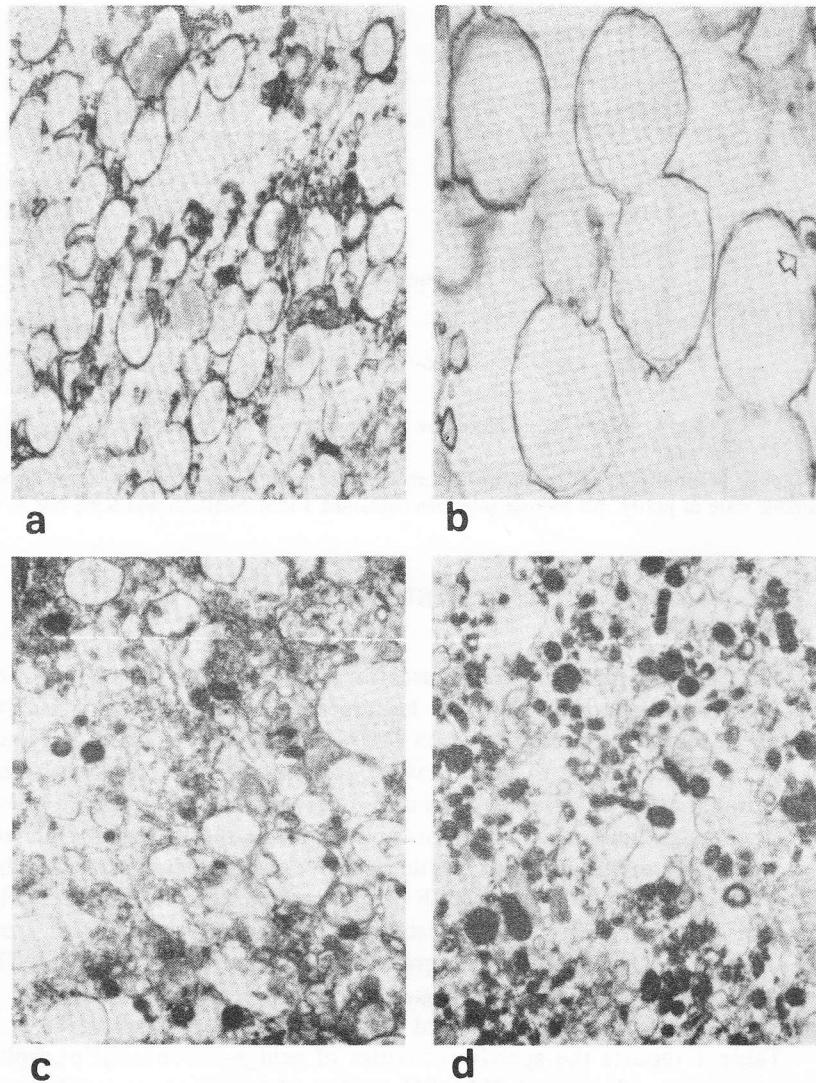


Fig. 2. Electron microscopy of the fractions isolated from homogenates of PMNs phagocytosing PMA-coated latex particles. (a) and (b), band 1; (c), band 2; (d), pellet. (a), (c), and (d), magnification $\times 7500$, (b), magnification $\times 22,500$.

Table 2 reports the specific activities of enzymes in the fractions obtained on sucrose gradients and their relative specific activities. The data show that in the phagosomal fraction (band 1) the activities of the plasma membrane markers acid *p*-nitrophenyl phosphatase and 5'-nucleotidase

Table 1. Specific Activity of Acid *p*-Nitrophenyl Phosphatase, Myeloperoxidase, and NADPH Oxidase in Homogenates of Resting and Phagocytosing Guinea Pig PMNs^a

	Resting cells	Phagocytosing cells
Acid <i>p</i> -NPPase ^b	1.75 ± 0.13	1.82 ± 0.17
Myeloperoxidase ^c	0.73 ± 0.02	1.00 ± 0.1
NADPH oxidase ^d	1.2 ± 0.2	23.6 ± 6.7

^aThe average values of five experiments ± *SEM* are reported.

^bAcid *p*-NPPase = acid *p*-nitrophenyl phosphatase; μmol *p*-nitrophenol/10 min/mg protein.

^cμmol tetraguaiacol/min/mg protein.

^dnmol O₂/min/mg protein.

are enriched about threefold while the specific activity of myeloperoxidase is lower than that in the homogenate and in other fractions. The presence of this enzyme in the phagosomal fraction can be explained both by contamination with granules or with granule material and by the discharge into the vacuole. This is in agreement with the data reported by Segal et al. concerning the very early fusion of azurophilic granules with the phagosome (16).

The specific activity of NADPH oxidase of the phagosomal fraction is very high and, with respect to that of the homogenate, it is enriched 12.6-

Table 2. Activity of Acid *p*-NPPase, 5'-Nucleotidase, Myeloperoxidase, and NADPH Oxidase in Fractions and Homogenate from Phagocytosing Guinea Pig PMNs^a

	Acid <i>p</i> -NPPase ^b	5'-Nucleotidase ^c	Myeloperoxidase ^d	NADPH oxidase ^e
Band 1				
SA	4.49 ± 1.1	6.87 ± 0.9	0.82 ± 0.18	298.3 ± 25.0
RSA	2.6	3.3	0.8	12.6
Band 2				
SA	3.59 ± 0.34	3.75 ± 0.3	1.35 ± 0.27	95.9 ± 18.2
RSA	2.0	1.8	1.35	4.1
Pellet				
SA	1.7 ± 0.34	2.11 ± 0.5	1.37 ± 0.1	24.7 ± 6.8
RSA	0.9	1.0	1.4	1.05
Homogenate				
SA	1.82 ± 0.17	2.09 ± 0.26	1.00 ± 0.1	23.6 ± 6.7

^aThe average values of five experiments ± *SEM* are reported.

SA = specific activity, RSA = relative specific activity.

^bμmol *p*-nitrophenol/10 min/mg protein.

^cμmol Pi/30 min/mg protein.

^dμmol tetraguaiacol/min/mg protein.

^enmol O₂/min/mg protein.

fold. It should be pointed out that this value of enrichment is much higher than that of *p*-nitrophenyl phosphatase and 5'-nucleotidase: 2.6- and 3.3-fold, respectively. This different behavior of NADPH oxidase with respect to that of other plasma-membrane-bound enzymes indicates that only the oxidase portion of the plasma membrane that forms the phagosome is activated.

DISCUSSION

The results presented in this paper show that by using fractionation on a discontinuous gradient of sucrose we have obtained a good separation of phagocytic vesicles from PMNs that have engulfed PMA-coated latex beads. Coating the latex beads with PMA made the particles highly effective in activating the oxidase of the PMN, as shown by the very high activity of NADPH oxidase in homogenates from phagocytosing cells, and mostly in phagosomes. The value of 298.3 nmol of O₂ produced/min/mg protein is the highest so far reported in subcellular particles of phagocytic cells of mammals. The presence of high NADPH oxidase activity in the phagosomal membrane was shown for the first time by Cohen et al. (13). However, these data do not indicate whether or not the activation of the oxidase is limited to the membrane of the phagosome. An answer to this question can be obtained by comparing the relative specific activity of NADPH oxidase with that of enzyme markers of the plasma membrane, whose activities are equally distributed in all membranes of the cell and do not vary during phagocytosis. The rationale of this comparison is the following: whether the oxidase was activated in all extensions of the plasma membrane, the value of its relative specific activity in the various fractions of homogenates should be similar to that of the other enzymes of the plasma membrane. The experimental data show that this is not the case. The relative specific activity of NADPH oxidase is much higher than that of 5'-nucleotidase and acid phosphatase. This fact is a good indication that the activation of the oxidase is limited to that portion of the cell membrane which forms the phagocytic vacuole.

The presence of the activated oxidase only in the membrane of the phagocytic vacuole is relevant for understanding the mechanisms of activation of the enzyme and the function of the respiratory burst.

It is likely that the activation of oxidase is triggered by a molecular modification of the portion of the plasma membrane that interacts with the surface of the particles during the act of engulfment. We have previously demonstrated a direct time relationship between the generation and motion of pseudopodia around the phagocytosable object and the activation of PMN respiration (26).

Therefore, by analogy with the phagocytic signals that are generated by the interaction between surface membrane and particles (27), the signals for the activation of the oxidase and the response of the enzyme are segmentary phenomena. The relationships between the mechanism of activation and the various modifications of the membrane that have been described, such as changes of polarization (28-31), changes of microviscosity (32, 33), release of bound calcium (34), protein phosphorylation (35), etc., cannot be established.

Another, more unlikely, explanation for the localized activation of the oxidase is the occurrence of an assembly of more components, located in the plasma membrane and in intracellular organelles, through the fusion between the granule membrane and the membrane that forms the phagocytic vacuole. Although some reports indicate that fusion cannot be necessary for the respiratory burst (36, 37), kinetic analyses indicate that respiratory events and fusion occur coordinately (16).

The significance of the respiratory burst is that of providing a powerful system for killing bacteria, viruses, and other phagocytosed organisms, in addition to other mechanisms, such as the discharge of enzymes and of other factors into the phagosomes. It is relevant that activated oxidase, which is the machinery for the production of intermediates of oxygen reduction, is located in the wall of the phagocytic vacuole. This is the best strategic position for the discharge of O_2^- and of H_2O_2 in direct contact with the engulfed matter and for a rapid utilization, mediated by MPO or following generation of hydroxyl radicals, for killing the phagocytosed organisms.

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