NADPH OXIDIZING ACTIVITY IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES: LOCALIZATION IN AZUROPHILIC GRANULES

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Summary. Rabbit polymorphonuclear leukocyte granules were submitted to zonal fractionation through a discontinuous sucrose gradient. Distribution of azurophilic and specific granules, enzymatically characterized by peroxidase and alkaline phosphatase respectively, was as reported by others. NADPH oxidizing activity was associated with azurophilic granules. 3-Amino-1H-1, 2,4-triazole stimulated NADPH oxidation by azurophilic granules and inhibited peroxidase. Relationships between peroxidase and NADPH oxidizing activity are discussed.

INTRODUCTION

It is well established that phagocytosis in polymorphonuclear leukocytes (PMN) is accompanied by increases in the cyanide insensitive respiration, in hexose monophosphate shunt (HMS) activity and in H_2O_2 production (1-7). The enzymatic mechanism of such increases is controversial. Oxidation of reduced nicotinamide adenine dinucleotide (NADH), coupled either with a NADPH-linked lactate dehydrogenase (7-10) or with the glutathione cycle (11), has been proposed by Karnovsky's laboratory. However experimental evidence has been provided by our and other laboratories indicating that an increased oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), performed by an enzyme system tightly bound to leukocyte granules, is the critical step involved in the stimulation of oxidative metabolism (12-21).

Two enzymatically different populations of rabbit PMN leukocyte granules, azurophils and specifics, have been recently described, the former characterized by peroxidase and the latter by alkaline phosphatase (22-25). In the present study we sought to determine whether NADPH

oxidizing activity was also localized in a particular granule fraction of rabbit PMN leukocytes.

MATERIALS AND METHODS

Polymorphonuclear leukocytes were obtained from heparinized rabbit peritoneal exudates induced aseptically with 1% casein. Briefly, each rabbit received 200 ml of casein solution at 7 p.m. and the following morning an additional 200 ml, containing 4000 IU of heparin, were injected. Exudates were collected a few minutes after the second injection and an aliquot subjected to differential counts in stained smears. The PMN leukocyte content averaged 95 to 98 percent. After pelleting by centrifugation, cells were suspended in hypotonic saline (0.2%) and hemolyzed until any trace of red blood cell contamination was removed. Cells were harvested by centrifugation, washed in 0.25 M sucrose, centrifuged, and resuspended in 0.34 M sucrose (5×10^8 cells/ml). Homogenization was carried out with a Potter-type homogenizer equipped with a motor-driven teflon pestle. The homogenate was diluted to 15 ml with 0.34 M sucrose and centrifuged at 250 x g x 7 minutes. The pellet was washed with 15 ml of 0.34 M sucrose, the two supernatant fluids pooled and centrifuged again at 250 x g x 7 min to remove clumped granules and residual nuclei. Twenty ml of the resulting supernatant represented the starting sample for zonal centrifugation procedures. Fractionation was accomplished following the method outlined by Baggiolini et al. (22) with the following modifications: a) an A MSE zonal rotor instead of the B-XIV Spinco rotor was used, b) the rotor was loaded and emptied while spinning at 1600 rpm at a rate of 25 ml/min in a Mistral 6L MSE centrifuge, c) the sample was centrifuged at 5000 rpm for 15 minutes. Following centrifugation, the gradient was displaced from the spinning rotor with 2.2 M

sucrose and 20 ml fractions were collected. The sucrose concentration of individual fractions was determined with an Abbe refractometer (Officine Galileo, Milan). Alkaline phosphatase was assayed by the method of Bessey et al. (26) with p-nitrophenylphosphate as substrate and 0.025% Triton X-100 in the assay medium. Myeloperoxidase activity was assayed as the rate of tetraguaiacol formation from guaiacol, according to the method of Schultz et al. (27) with the cationic detergent cetyl-trimethylammonium bromide (0.02% final) in the medium and 0.33 mM H_2O_2. NADPH oxidizing activity was measured as the rate of oxygen uptake at pH 5.5 with 0.5 mM $MnCl_2$ and 1 mM NADPH as previously described (19). Protein was determined by the Lowry method (28). Glucose-6-phosphate dehydrogenase was determined by the method of Lohr and Waller (29).

RESULTS AND DISCUSSION

Table 1 summarizes the activities of alkaline phosphatase, marker enzyme for specific granules, peroxidase, marker enzyme for azurophilic granules and "NADPH oxidase" in homogenates of rabbit PMN leukocytes.

Fractionation of rabbit PMN leukocyte granules yielded the distribution pattern shown in Figure 1. The supernatant of PMN homogenates centrifuged at 250 x g was used as starting material for zonal centrifugation instead of isolated granules, since the clumping which occurred in pure granule suspensions hindered the resolution. The 250 x g supernatant contains, besides the granule-bound enzymes, portions of the same enzymes that might have been released from granules, in soluble form, during homogenization procedures. It was necessary therefore to establish preliminarily whether and to what extent the enzymes chosen as marker of granule subpopulations were present in the soluble phase. In order to do that, distribution of the soluble

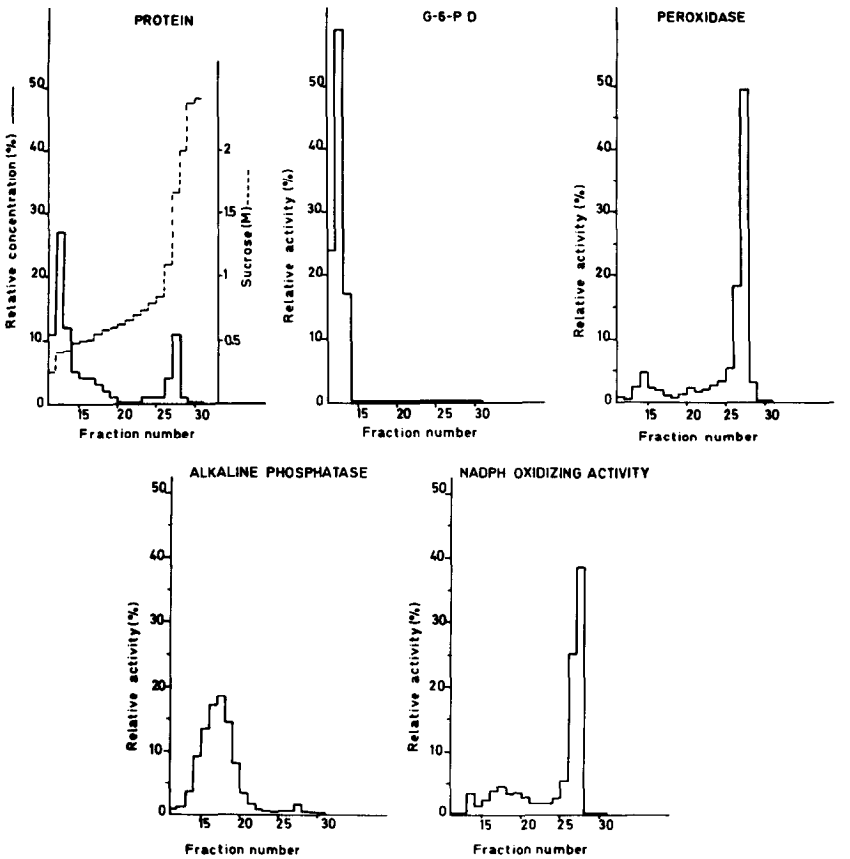

Figure 1. Fractionation of subcellular components of rabbit PMN leukocytes by zonal differential centrifugation. Distribution of protein, glucose-6-phosphate dehydrogenase (G-6-PD) and of granule-bound alkaline phosphatase, peroxidase and NADPH oxidizing activities among fractions collected from the discontinuous sucrose density gradient. Percent recoveries were 89 for protein, 86 for G-6-PD, 98 for alkaline phosphatase, 88 for peroxidase and 119 for NADPH oxidizing activity.

enzyme glucose-6-phosphate dehydrogenase (G-6-PD) was determined. The activity was confined to fractions 11, 12 and 13. After centrifugation of these three fractions at 100,000 $\underline{x}$ g, activity was recovered in the supernatant fluids. These supernatants did not contain appreciable NADPH oxidizing activity, whereas the percentages of soluble alkaline phosphatase and peroxidase activities were 20 and 6, respectively. The soluble portion was not taken into account in plotting the distribution of the two enzymes.

TABLE 1. ACTIVITIES OF ENZYMES IN HOMOGENATES OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES.

Alkaline phosphatase (u moles p-nitrophenol/min/10^8 cells)	0.6 ± 0.16	(6)
Peroxidase (u moles tetraguiacol/min/10^8 cells)	2.6 ± 0.33	(5)
NADPH oxidizing activity (u moles oxygen/min/10^8 cells)	0.132 ± 0.039	(4)

Assay for alkaline phosphatase: 25 mM glycine buffer pH 10.5, 0.25 mM $MgCl_2$, 2.75 mM Na-p-nitrophenylphosphate and 0.025% Triton X-100. Volume 1 ml. Temperature 37°. Reaction was stopped by adding 5 ml of 0.04 M NaOH. Optical density was measured at 405 nm.
Assay for peroxidase: 0.1 M phosphate buffer pH 7.0, 13.3 mM guaiacol, 0.33 mM H_2O_2 and 0.02% cetyl trimethylammonium bromide. Volume 3 ml. Temperature 37°. Reaction was started with H_2O_2 and formation of tetraguaiacol recorded at 470 nm. Assay for NADPH oxidizing activity: 65 mM phosphate buffer pH 5.5, 0.17 M sucrose, 0.5 mM $MnCl_2$ and 1 mM NADPH. Volume 2 ml. Temperature 37°. Oxygen uptake was recorded with a Clark oxygen electrode. Results are given as mean ± SEM. Number of experiments in parenthesis.

TABLE 2. EFFECT OF 3-AMINO-1H-1,2,4-TRIAZOLE (AMT) ON PEROXIDASE AND NADPH OXIDIZING ACTIVITIES OF RABBIT PMN AZUROPHIL GRANULES.

	Peroxidase activity μmoles tetraguaiacol/min/mg protein	NADPH oxidizing activity μmoles NADPH oxidized/min/mg protein
None	0.702	0.479
AMT 5 mM	0.162	3.444

For enzyme assays see Table 1.

Therefore, Figure 1 shows the distribution of true granule-bound alkaline phosphatase, peroxidase and NADPH oxidizing activities among fractions collected from the gradient. Alkaline phosphatase and myeloperoxidase assumed unimodal distributions consistent with those reported by other

authors (22-25, 30). NADPH oxidizing activity was consistently associated with the myeloperoxidase-containing granules.

The association of NADPH oxidizing activity with azurophil granules brings about again the problem of whether the so-called "NADPH oxidase" is an individual enzyme or NADPH oxidation is performed by myeloperoxidase, as Roberts and Quastel have suggested (13).

It is known, indeed, that several purified peroxidases oxidize pyridine nucleotides under various experimental conditions (13, 31-38). However, other experimental findings suggest that "NADPH oxidase" and peroxidase are distinct enzymes: a) NADPH oxidation in phagocytizing leukocytes is cyanide insensitive (14, 15, 17, 19, 20), whereas peroxidase is fully inhibited by cyanide, b) myeloperoxidase-deficient leukocytes show a normal metabolic response to phagocytosis (39, 40), and c) aminotriazole (3-amino-1H-1, 2, 4-trizole) inhibits peroxidase activity and stimulates NADPH oxidizing activity in resting leukocytes (Table 2).

Recent studies suggest that myeloperoxidase exists as six dimeric molecules originating from three distinct monomeric subunits (41). It has been shown also that the two moieties of pig leukocyte peroxidase have different affinities for cyanide (37).

Therefore, although several types of data suggest that NADPH oxidizing activity and peroxidase activity are due to distinct enzymes, the additional possibility should be considered that the two activities may be due to different isozymes or to distinct catalytic sites on the same molecule.

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