MACROPHAGE METABOLISM: ACTIVATION OF NADPH OXIDATION BY PHAGOCYTOSIS.

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Summary.

Rabbit and guinea pig peritoneal and alveolar macrophages and rabbit polymorphonuclear leucocytes (PMN) have been tested for their capacity to oxidize NADPH and NADH. In all these cells granule-bound NADPH oxidase is much more active than NADH oxidase, thus confirming our previous observations on human blood and guinea pig PMN. If the phagocytes are challenged with bacteria, the activity of NADPH oxidase is considerably stimulated. The enhancement of the oxidase activity is due to an increase of its V_{max} and, in the case of the PMN, also to a decrease of the K_m. We conclude that NADPH oxidase might play a relevant role in the metabolic stimulation of both PMN and macrophages by phagocytosis.

The phagocytic act in polymorphonuclear leucocytes (PMN) is accompanied by a considerable stimulation of the oxidative metabolism (1,2,3). In the past decade we have provided evidence that in guinea pig PMN and human blood leucocytes an enhanced oxidation of NADPH by a particulate enzyme is the cardinal event in this stimulation (4,5,6,7). Some Authors believe, however, that the rise in oxygen uptake by intact PMN is supported by an increased enzymatic oxidation of NADH (8,9,10,11).

Such a rise in oxidative metabolism, including H_2O_2 production, occurs also in mononuclear phagocytes when they come into contact with foreign material, such as bacteria (12,13,14 15,16). With the view to find out which one of the two enzymatic mechanisms is operating in these cells, we have assayed the capacity of their subcellular particles to oxidize both reduced pyridine nucleotides. Experiments have shown that granules of peritoneal and alveolar macrophages from guinea pig and rabbit oxidize NADPH at a much higher rate than NADH. Furthermore, the process of phagocytosis induces a remarkable rise in activity of NADPH oxidase, which can be ascribed to a change of its kinetic properties.

MATERIALS AND METHODS

Reduced pyridine nucleotides were purchased from Böhringer (Germany) and Sigma (USA) and catalase from Sigma. All other chemicals were of reagent grade.

More than fifty cell preparations were examined.

Peritoneal polymorphonuclear leucocytes were prepared as previously reported (3). Peritoneal macrophages were elicited by injecting a 1.2% sterile solution of casein in 0.9% NaCl into the peritoneal cavity. Six days later, the peritoneum was rinsed with 0.9% NaCl and the exudate collected. Alveolar macrophages were prepared by two tracheobronchial lavages with 0.9% NaCl. Cells were centrifuged at 400 x g for 5 minutes and suspended in calcium-free Krebs-Ringer phosphate buffer, pH 7.4 (KRP).

Differential counts were carried out with May-Grünwald and Giemsa stained smears. More than two-thousand cells were differentiated in each count.

Acute peritoneal exudates of both animals contained 82-88% of mature PMN. On the average, chronic peritoneal exudates consisted of 88% macrophages, 4.5% lymphocytes, 5.5% neutrophils and 1.8% eosinophils. The alveolar lavages contained macrophages (76%, guinea pig and 94%, rabbit), lymphocytes (7% and 2%) neutrophils (7% and 3%) and eosinophils (9% and 0.8%).

Phagocytosis was induced by adding heat-killed B.subtilis, opsonized with homologous fresh serum, to cell suspensions kept at 37° in shaken plastic tubes (50-100 bacteria/phagocyte). After 3-5 minutes, cells were collected by centrifugation at 400 x g for 5 minutes. Microscopic examination of wet and stained specimen, revealed that both PMN and macrophages had phagocytized.

Packed cells were suspended in ice-cold 0.34M sucrose (buffered at pH 7 with $NaHCO_3$) to a concentration of 2-4 x 10^8 cells/ml and homogenized in a Potter tissue grinder with teflon pestle. Homogenization was performed for 2-3 minutes, cell disruption being checked at regular intervals by light microscopy. The homogenates were suitably diluted and centrifuged at 400 x g for 5 minutes to remove cell debris and nuclei. This fraction was resuspended in 0.34M sucrose and centrifuged again at low speed. The combined supernatants were centrifuged at 20,000 x g for 20 minutes, yielding a granule fraction and a final supernatant. All these operations were carried out at 0-4°.

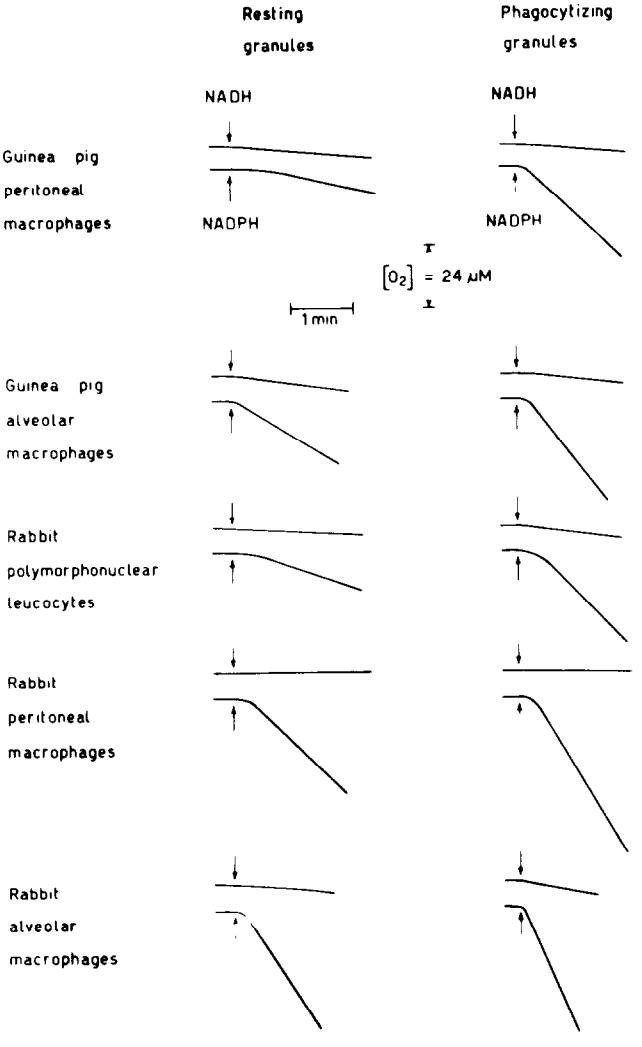
NADPH and NADH oxidases were assayed at 37° by measuring the rate of oxygen uptake with a Clark electrode (3,6,7). The assay medium consisted of 65mM

Na,K-phosphate, pH 5.5, 0.5mM $MnCl_2$, 125mM sucrose, and NADPH or NADH at the concentrations indicated in Results.

Protein was determinated with the method of Lowry et al. (17), with bovine serum albumin as standard.

RESULTS

As illustrated in the Figure, the granular fraction of all the phagocy-



Oxidation of NADPH and NADH by granules from resting and phagocytizing macrophages and PMN.

Assay medium: 65mM Na,K-phosphate buffer, pH 5.5; 125mM sucrose; 0.5mM $MnCl_2$; 1mM NADPH or 2mM NADH. Total volume: 2ml; Temperature 37°. Protein (from top to bottom): 43µg; 18µg; 34µg; 81µg; 77µg. Granules were added at the points indicated by the arrows.

tes tested efficiently oxidizes NADPH. A considerable increase of NADPH oxidation is observed with granules isolated from phagocytizing cells as respect to resting ones. On the contrary, NADH oxidation is carried out at a much lower rate, and in some types of cells the oxidation of such nucleotide is practically undetectable. Owing to this fact, it is impossible to assess the effect of phagocytosis on the activity of NADH oxidase.

In any case the 20,000 x g supernatants are virtually devoid of both NADPH and NADH oxidase activity.

Oxidation of NADPH by granules is accompanied by production of hydrogen peroxide. In fact, when an excess of catalase is added to the assay medium, after the reaction has proceeded for about two minutes, release of a fairly

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Kinetic properties of NADPH oxidase of different phagocytes.

	K_m (mM)			$\frac{V_{max}(phag.)}{V_{max}(rest.)}$
	Resting	Phagocytizing		
Guinea pig PMN*	4.0	0.4		4.0
Guinea pig alveolar macrophages	0.43±0.11	0.38±0.08	n.s.	2.4±0.6
Guinea pig peritoneal macrophages	1.36±0.45	0.71±0.18	n.s.	3.0±0.5
Rabbit PMN	1.07±0.25	0.45±0.10	$P < 0.05$	5.6±2.7
Rabbit alveolar macrophages	1.33±0.36	0.66±0.09	n.s.	1.4±0.2
Rabbit peritoneal macrophages	0.88±0.29	0.72±0.22	n.s.	1.6±0.3

K_m computed according to a "best fit" method (18). Mean values of four determinations ± S.E.. Test of significance made on paired data. n.s. = not significant.
For experimental details see the Fig. *Values taken from reference 7.

stoichiometric amount of oxygen from accumulated H_2O_2 can be observed.

The NADPH oxidase activity of granules isolated from phagocytizing cells was also assayed in the presence of 1mM KCN, to which the enzyme of phagocytizing guinea pig PMN is almost insensitive (7). NADPH oxidase of rabbit PMN is inhibited approximately 60% by cyanide, whereas the inhibition of the lung and the peritoneal macrophage enzyme is inhibited about 80% and 55% (guinea pig) or 81% and 71% (rabbit), respectively.

The K_m of NADPH oxidase of granules from resting and phagocytizing cells was assayed by computing the activity values, at different substrate concentrations (0.2-3mM), according to Wilkinson (18). The Table shows that the K_m of the mononuclear phagocytes does not change upon phagocytosis, whereas that of the PMN decreases. The oxidase V_{max} is always higher in the granules derived from phagocytizing cells.

DISCUSSION

The enzymatic mechanism of stimulation of oxidative metabolism in polymorphonuclear phagocytes challenged with foreign material has been rather controversial in the past (19). In a recent publication (7), we have provided a great body of evidence favouring an activation of an NADPH oxidase as cardinal event in this process. On the basis of the comparative analysis presented in this paper, one may conclude that, not only in PMN, but also in mononuclear phagocytes such an enzyme is likely to play a relevant role. In fact, both alveolar and peritoneal macrophages possess a particulate enzyme, which catalyzes the reaction $NADPH + H^+ + O_2 \longrightarrow NADP^+ + H_2O_2$, and becomes activated upon phagocytosis. On the contrary, an oxidation of NADH is hardly detectable both in rabbit PMN and in various rodent macrophages, at rest or under phagocytosis, thus sugesting that NADH oxidase has a negligible function in the phagocyte stimulation.

At variance with the PMN enzyme, oxidase of macrophages is almost totally inhibited by 1 mK KCN. This would explain why Vogt et al. (16) have been unable to measure the activity of such an enzyme in homogenates of alveolar macrophages in the presence of KCN.

The assumption that NADPH oxidation is actually performed by an enzyme belonging to macrophages and not to contaminant PMN is supported by the following evidence: 1) on the basis of total cell number NADPH oxidase of subcellular par-

ticles of macrophages has an activity comparable to that of PMN, even in the presence of extremely low granulocyte contaminations; 2) for each type of cells, the extent of activation of NADPH oxidase is proportional to the burst of respiration of the intact phagocyte; 3) the sensitivity to KCN of NADPH oxidase of stimulated macrophages results to be higher than that of PMN. Inhibition by cyanide of NADPH oxidase of phagocytizing cells parallels inhibition of the extrarespiration in intact cells (unpublished data).

The finding that NADPH oxidase of granules isolated from phagocytizing cells has a higher V_{max} than that of resting leucocytes is meaningful, if one considers that the granule fraction isolated from phagocytizing cells has the same protein content of the parallel fraction isolated from cells at rest and that NADPH oxidase is not released from the granules. The K_m of the macrophage enzyme is not changed upon phagocytosis, whereas that of PMN oxidase is significantly decreased. This would suggest a different mechanism of activation of NADPH oxidase of the macrophages as compared with that of PMN. In any case, the contact of both phagocytes with foreign particles would promote a removal of an enzyme inhibitor or an enzyme conformational rearrangement, as already assumed for the oxidase of guinea pig PMN (7).

Studies on the possible pathways of utilization of the extra-amounts of $NADP^+$ and H_2O_2 produced in the macrophage upon phagocytosis are in progress.

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