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Enzymes of Oxidative Metabolism in the Human Eosinophil[1] (40241)

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Although the existence of the eosinophil has been recognized for a century, the function of this cell remains controversial (1). Recent studies have suggested that the eosinophil is a cell of considerable metabolic potential (2–4). Membrane stimulation by phagocytosis or by membrane-active agents such as phorbol myristate acetate causes a respiratory burst which is comparable to that of neutrophils, and in many respects, greater in magnitude (2–4). Stimulated eosinophils demonstrate marked increases in oxygen consumption, hexose monophosphate shunt activity, hydrogen peroxide production, superoxide production, reduction of nitroblue tetrazolium dye, and generation of chemiluminescence (4). Cellular enzymes active in the respiratory burst of neutrophils have been studied extensively; however, difficulties in procuring sufficient quantities of pure eosinophils has limited the study of these enzymes in eosinophil leukocytes (1). As the respiratory burst of eosinophils differs quantitatively from that seen in neutrophil leukocytes, the study of the enzyme activities associated with the oxidative metabolism of the eosinophil would provide insights into the mechanisms of these distinctive responses. We have recently examined a patient with hypereosinophilia whose eosinophils were shown to provide oxidative responses similar to those reported previously and whose cells could be obtained in over 90% purity with few contaminating neutrophil leukocytes (4). This provided the opportunity to examine the enzyme activities associated with the oxidative metabolic burst of the eosinophil leukocyte.

Materials and Methods. Preparation of cells. Eosinophils were isolated from the peripheral blood of a patient with long-standing eosinophilia (at least 5 years duration) of unknown etiology; a description of this patient has been previously published (4). Neutrophils were isolated from normal control donors whose peripheral eosinophil count was less than 5%, as determined by Wright stain. Cells were isolated by sedimentation with plasma gel (HTI Corp., Buffalo, NY) as previously described (5). The leukocytes were collected by centrifugation at 160*g* for 10 min at 4°. They were washed once with Dulbecco's phosphate–buffered saline (PBS), and contaminating red cells were removed by hypotonic lysis. The cells were resuspended in PBS and smears were made of the isolated cells using a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). These were stained with Wright's stain and differential counts obtained. The eosinophil preparations always yielded at least 80% eosinophils with less than 10% neutrophilic contamination; neutrophil preparations always contained at least 80% neutrophils with less than 7% eosinophil contamination. The cells were disrupted by brief sonication and stored frozen until use in enzyme assays; eosinophil and neutrophil preparations were always prepared and assayed in exact parallel to allow meaningful comparisons.

Enzyme assays. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed on a recording spectrophotometer by the increase in absorbance at 340 nm due to the reduction of NADP (6). Reactions were performed in 3 ml cuvettes with the following final concentrations: 8.33 m*M* glycylglycine buffer, pH 7.40; 0.016 *M* $MgCl_2$, 0.83 m*M* glucose-6-phosphate or 6-phosphogluconate, and 0.113 m*M* NADP. Activity was calculated using 6.22 $cm.^2/10^{-6}$ mol as the extinction coefficient for NADPH.

Glutathione peroxidase was determined by a modification of the method of Paglia and Valentine (7). Leukocyte sonicate was added

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to 2.50 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.005 *M* EDTA, 3.75 m*M* sodium azide, 5 m*M* reduced glutathione (GSH), 0.28 m*M* NADPH, and 0.05 ml of yeast glutathione reductase (20 U/ml). The mixture was allowed to equilibrate and continuous recording of the change in absorbance was made at 340 nm for 4 min. The reaction was initiated by the addition of 0.10 ml of 2.2 m*M* H_2O_2 to the cuvette. A reagent blank was included in each assay in which the leukocyte sonicate was replaced by an equal volume of PBS; all rates were corrected for nonenzymatic reaction before calculation of specific activity.

Glutathione reductase was assayed by the rate of disappearance of NADPH at 340 nm following the addition of oxidized glutathione (GSSG) and leukocyte sonicate to the reaction mixture (8). The mixture contained in a final volume of 3.0 ml: 0.067 *M* phosphate buffer, pH 7.40, 0.16 m*M* NADPH, 1.0 m*M* GSSG and sonicate; controls were run with all reagents but no added sonicate.

Catalase was assayed by monitoring the disappearance of H_2O_2 spectrophotometrically at 240 nm (6). The reaction mixture consisted of 0.30 ml of 0.053 *M* H_2O_2 prepared in 0.05 *M* phosphate buffer, pH 7.0. This was diluted with deionized water to give a final volume of 1.0 ml. The reaction was initiated by the addition of sonicate; a blank cuvette in which sonicate was omitted was run simultaneously and experimental values were corrected for this blank.

Superoxide dismutase was measured spectrophotometrically by its ability to inhibit the superoxide-mediated reduction of ferricytochrome *c*. Xanthine-xanthine oxidase was employed as the superoxide generating system (9). The incubation mixture consisted of 20 m*M* sodium carbonate buffer, pH 10.0 containing: 0.1 m*M* EDTA, .01 mM horse heart cytochrome *c*, 0.05 m*M* xanthine and the appropriate sonicate, where required. The reaction was initiated by the addition of 1.8×10^{-8} *M* buttermilk xanthine oxidase and the reduction of cytochrome *c* monitored at 418 nm in the presence and absence of sonicate.

Reduced pyridine nucleotide oxidases were assayed in the presence of 2.0 m*M* cyanide by a fluorometric assay procedure which has been previously described in detail (10). Eosinophilic NADPH oxidase has previously been shown to be activated by phagocytosis (4) and thus assays were performed on cells which had been allowed to phagocytize opsonized zymosan for 3 min as previously described (10). They were then disrupted by homogenization and fractions were obtained by differential centrifugation as described in Results. Protein determinations on the sonicates were performed by the method of Lowry *et al.* (11).

Oxidase activities were performed on phagocytizing samples while other enzymes were assayed with resting preparations. This is not unreasonable, however, since the activation phenomenon in human neutrophils appears to be relatively specific for the oxidases. The activity of a number of other neutrophilic enzymes, including acid and alkaline phosphatase, lysozyme, Mg^{2+}-ATPase, β-glucuronidase, and myeloperoxidase has been shown to be unaffected by phagocytosis (12).

All biochemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, MO, except the reduced pyridine nucleotides which were of fluorometric grade obtained from Boehringer-Mannheim Corp., Indianapolis, IN. All other chemicals were the highest grade obtainable from Fisher Scientific Co., Raleigh, NC.

Results. Previous work has suggested that the respiratory burst in human neutrophils is stimulated by activation of a reduced pyridine nucleotide oxidase which is located in a granule fraction of the cell and preferentially utilizes NADPH (10, 13). Accordingly, we assayed for a similar activity in eosinophils. In these experiments, the cells were disrupted by homogenization rather than sonication in order to examine the fractions by differential centrifugation (10). The disrupted cells were first centrifuged at 500*g* for 10 min and the supernatant was decanted and centrifuged at 27,000*g* for 15 min. The 27,000*g* pellet was resuspended in PBS and the various fractions examined for oxidase activity toward both NADH and NADPH. Four separate experiments were performed. There was substantial variation in the absolute levels of activity detected in the various experiments, and so results could not be averaged. This was probably due to variations in donors as well as in

slight variations in procedure from day to day and has previously been noted in neutrophils by both ourselves (10) and others (14). Similarly, the per cent of eosinophilic oxidase activity which sedimented in the 500*g* pellet varied from 30 to 55% in the four experiments; this was likely due to slight variations in the homogenization procedure. In spite of this variability, certain generalizations were observed in all four experiments; these are illustrated in a representative experiment in Table I. In the neutrophil, slight oxidase activity toward either NADH or NADPH was occasionally observed in the 500*g* pellet fraction, but the great bulk of the activity was always found in the 27,000*g* pellet fraction. Activity towards NADPH was always much greater than that towards NADH, and the distribution of the activity towards the two nucleotides was parallel in the various fractions. Overall, the eosinophil always contained substantially more (three to fivefold) oxidase activity than the neutrophil. This activity was consistently found in both the 500*g* pellet fraction and the 27,000*g* pellet fraction, in contrast to the neutrophil. The activity towards NADPH was always greater than that toward NADH, but the difference in the two substrates was not as marked as in the neutrophil. No significant activity toward either substrate was observed in the 27,000*g* supernate from either the neutrophil or the eosinophil.

TABLE I. REDUCED PYRIDINE NUCLEOTIDE OXIDASES IN FRACTIONS DERIVED FROM HUMAN NEUTROPHILS AND EOSINOPHILS.

	Specific activity of oxidase[a]			
	Neutrophil		Eosinophil	
Sample	NADH	NADPH	NADH	NADPH
500*g* pellet	1.2	29.6	183	241
27,000*g* pellet	11.8	223	353	637
27,000*g* supernate	0.0	0.0	0.0	13.0

[a] Specific activity is calculated as nmol of NAD(P)H oxidized/30 min/mg protein. Results represent the average of triplicate determinations; the data are from a single experiment which is representative of four separate experiments.

Other enzymes thought to be involved in the oxidative metabolism of phagocytes were examined in sonicated preparations of human neutrophils and eosinophils. Results from a number of such experiments are presented in Table II. The specific activities of the hexose monophosphate shunt enzymes appeared to be about twice as great in the eosinophil as in the neutrophil. The enzymes of glutathione metabolism, GSH peroxidase and GSSG reductase, were also present in the eosinophil and were, if anything, somewhat more active in that cell than in the neutrophil. Catalase activity was present and was approximately three times as active in the eosinophil preparation as in the neutrophil sonicate. Although superoxide dismutase was present in both cells, there appeared to be no significant difference in enzyme activity.

Discussion. Recent work has suggested that the respiratory burst in neutrophils is triggered by activation of a reduced pyridine nucleotide oxidase or oxidase(s) (10, 13). The enzyme(s) involved appear to preferentially utilize NADPH over NADH and seem to be localized in a dense granule of the cell (10). The present data support the hypothesis that this same mechanism might apply to the human eosinophil. The fact that the total eosinophilic oxidase activity is greater than the neutrophilic oxidase correlates well with the

TABLE II. ENZYMES OF LEUKOCYTE OXIDATIVE METABOLISM.*

Enzyme	Neutrophil		Eosinophil	
Catalase[a]	128 ± 16	(5)	342 ± 30	(5)
Superoxide dismutase[b]	0.235	(2)	0.188	(2)
Glucose-6-phosphate dehydrogenase[c]	0.0489 ± 0.0055	(5)	0.0984 ± 0.0166	(4)
6-Phosphogluconate dehydrogenase[c]	0.0120	(2)	0.314	(2)
GSH peroxidase[c]	0.0299 ± 0.0048	(4)	0.0365 ± 0.0057	(4)
GSSG reductase[c]	0.0172 ± 0.0039	(4)	0.0232 ± 0.0010	(5)

* Descriptions of the assays are given in the Methods Section. The values are expressed as the mean ± SE, with the number of experiments in parentheses. Each experiment was performed at three levels of protein and the results averaged. A different cell preparation was employed in each separate experiment.

[a] Expressed as μmol H_2O_2 reduced/min/mg protein.

[b] Expressed as Δ OD/min/mg protein.

[c] Expressed as μmol NADP(H) reduced or oxidized/min/mg protein.

observations that the respiratory burst in eosinophils is, in many respects, greater in magnitude than in neutrophils (2–4), under conditions where phagocytosis might even be less. The fact that a substantial amount of eosinophilic oxidase activity sediments at 500*g* argues for a granule localization, since the granules of the eosinophil are reported to be more dense than those of the neutrophil (1). Finally, the greater activity observed with NADPH suggests that this nucleotide is the physiological substrate while the similar distribution of the two activities in differential centrifugation is consistent with the hypothesis that a single enzyme might be involved which can act on either nucleotide (10). The distribution of NADPH oxidase in the present study is consistent with that observed previously using an isotopic assay which was specific for that nucleotide (4); the present study confirms those results with a different assay and extends the observation to NADH oxidation as well.

A reduced pyridine nucleotide oxidase would catalyze the following reaction in the cell.

$$NAD(P)H + O_2 \rightarrow NAD(P) + H_2O_2$$

It is probable that an intermediate in this reaction is superoxide anion (14). The generation of such highly reactive compounds as H_2O_2 and O_2^- should be deleterious to the cell, and the cell might be expected to possess mechanisms for detoxifying these substances. The enzyme, superoxide dismutase, is present in both neutrophils (14) and in eosinophils. It serves as a sink for superoxide anions by catalyzing their dismutation to H_2O_2 and molecular oxygen.

The eosinophil, like the neutrophil, possesses several mechanisms for processing excess H_2O_2 generated during phagocytosis. Catalase causes the H_2O_2 to break down to H_2O and oxygen; the observation that catalase was more active in the eosinophils than in the neutrophils is consistent with the observations that eosinophils generate more H_2O_2 during phagocytosis than neutrophils (1–3).

A second mechanism for detoxifying H_2O_2 involves the intervention of the glutathione cycle as proposed by Reed (15).

$$2\,GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP$$

These reactions, catalyzed by the enzymes glutathione peroxidase and glutathione reductase respectively, are at least as active in our eosinophil preparations as in the neutrophils and thus represent another very likely protective mechanism to guard the cell against oxidative damage.

Finally the existence of relatively high levels of the hexose monophosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, is consistent with the generally high activity of this pathway observed in both resting and phagocytizing eosinophils (2–4). Because these enzymes serve to generate NADPH, they might serve a dual function in assuring an adequate supply of substrate for the initiating oxidase, as well as to regenerate NADPH which was oxidized by glutathione reductase in the detoxification of excess H_2O_2.

Summary. Human eosinophils exhibit reduced pyridine nucleotide oxidase activity toward both NADH and NADPH. This activity is more active toward NADPH and appears in both the 500*g* pellet and 27,000*g* pellet upon differential centrifugation; activity toward both substrates is greater in eosinophils than neutrophils, consistent with the greater respiratory burst observed in eosinophils upon phagocytosis. Other enzymes involved in detoxification of oxygen metabolites are at least as active in eosinophils as in neutrophils, and in many cases appear to be more active. These include catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. Thus, the enzyme content of the eosinophil would prepare the cell for a role which involves generation of active oxidizing agents.

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