Effect of Oxygen Tension on Human Peripheral Blood Leukocytes:

Lysosomal Enzyme Release

and Metabolic Responses during Phagocytosis

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ABSTRACT   We found that nonlethal lysosomal enzyme release from human peripheral blood leukocytes during phagocytosis of opsonized zymosan in vitro was modified by the oxygen tension under which the cells were incubated; with decreasing P_{O_2}, zymosan-induced release of lysosomal enzymes was potentiated. The effect on enzyme release could not be attributed secondarily to an effect on phagocytosis, because, as others have reported, P_{O_2} had little effect on that response. Metabolic responses that accompany phagocytosis were also modified by oxygen tension. Stimulation of oxidation by way of the pentose cycle was further enhanced by increasing P_{O_2}. Conversely, anaerobic glycolysis was promoted by decreasing oxygen tension. ATP levels fell as a function of time and concentration of phagocytic stimulus, mirroring lysosomal enzyme release as modified by P_{O_2}. Cyclic AMP levels fell during phagocytosis and lysosomal enzyme release, a change that could act to facilitate lysosomal enzyme release. However, the fall in nucleotide level was greatest with highest P_{O_2} (i.e., when lysosomal enzyme release was least). The inverse relationship between oxidative metabolism and enzyme release suggested that a product of oxidative metabolism might adversely influence enzyme release. Sulfhydryl antioxidants (cysteine, glutathione) and scavengers of oxygen-derived reactants (superoxide dismutase, catalase, benzoate, hypoxanthine, xanthine, histidine, azide) all potentiated zymosan-stimulated enzyme release. These findings are consistent with the interpretation that one or more factors (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen), generated in association with the burst of oxidative metabolism which accompanies phagocytosis, acts to inhibit lysosomal enzyme release.

Neutrophils play an essential role in the pathogenesis of inflammation (1). Lysosomal enzymes released from neutrophils stimulated at sites of inflammation are capable of degrading tissue (2). Toxic by-products of oxidative metabolism may also damage cells and extracellular tissue components (3). Phagocytosis and release of lysosomal enzymes by neutrophils are associated with enhanced glucose oxidation by way of the pentose cycle and enhanced lactate production that results from anaerobic glycolysis (4). Results of experiments in which anaerobic glycolysis was suppressed by metabolic inhibitors have been interpreted as indicating that this pathway must be

intact to support both phagocytosis (4) and release of lysosomal enzymes (5). To elucidate further the interrelationships between neutrophil function and metabolism, we have altered cell metabolism by incubating cells in media with varying oxygen tension (P_{O_2}),[1] a technique that does not depend on the use of drugs or metabolic inhibitors. With this method, we

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[1] Abbreviations used in this paper: H_2O_2, hydrogen peroxide; KRP, Krebs-Ringer phosphate buffer; LDH, lactate dehydrogenase; O_2^-, superoxide anion; $OH\cdot$, hydroxyl radical; $^1O_2$, singlet oxygen; P_{O_2}, partial pressure of oxygen; SOD, superoxide dismutase.

have made correlations between functional and metabolic responses that occur during phagocytosis of zymosan.

MATERIALS AND METHODS

Incubation

Leukocytes were isolated from normal human peripheral blood as previously described (6) and suspended in Krebs-Ringer phosphate buffer (KRP) (7) containing glucose (5 mM) and gelatin (1 mg/ml). Incubation tubes containing 0.9 ml of buffer with desired additions were sealed with rubber diaphragm stoppers (Kontes Co., Vineland, N. J.) and gassed for 60 min with O_2, 48% O_2-52% N_2, air, or N_2. The gas was delivered through a 21-gauge needle, with a 25-gauge needle used as a vent. Preliminary experiments demonstrated that the P_{O_2} of the incubation medium reached a constant level by the end of this time and was sustained for at least 3 h after the addition of cells. The incubation medium contained zymosan (Sigma Chemical Co., St. Louis, Mo.), which had been boiled, opsonized by incubation with 50% fresh serum (from the cell donor), washed, and suspended to give the desired final concentration as indicated in the figure legends. Cysteine, reduced glutathione, sodium benzoate, hypoxanthine, xanthine, histidine, sodium azide, superoxide dismutase (SOD) (EC 1.15.1.1), and catalase (EC 1.11.1.6) were obtained from Sigma Chemical Co. The enzymes were heat-inactivated, where indicated, by autoclaving at 120°C for 60 min. They were diluted in buffer to yield the desired final concentration. Trace quantities of [^{14}C]inulin (1 μCi/ml) or [1-^{14}C]glucose (0.5 μCi/ml) (Amersham Corp., Arlington Heights, Ill.) were present when either [^{14}C]inulin uptake or ^{14}Co$_2$ production, respectively, was examined. To start the incubation, 0.1 ml of cell suspension was injected into each tube. The incubation mixtures contained 2.1–12.1 × 10^6 leukocytes/ml, 67–100% neutrophils.

Assay Methods

At the end of the desired incubation times, 3 ml of chilled KRP containing inulin, 1 mg/ml, was added to the tubes, which were then centrifuged at 250 g for 10 min. Supernate was saved for assay of enzyme activities, as described below. The cell pellet was washed twice with the chilled inulin solution, dissolved by standing overnight in 0.2 ml of 2 N NaOH, and diluted with 1.3 ml of water. Aliquots were taken for determination of ^{14}C by liquid scintillation counting and for measurement of protein concentration (6). The uptake of extracellular water (using ^{14}C-inulin as a marker), believed to enter pinocytic and phagocytic vacuoles, served as a measure of endocytosis (6, 8), and is expressed as microliters per milligram of cell protein.

The supernate was assayed for activities of lysosomal enzymes and lactate dehydrogenase (LDH). Lysozyme activity was measured as described by Wright and Malawista (9). The activity of β-glucuronidase was measured as previously described with the use of phenolphthalein glucuronide as substrate (10). The LDH activity, measured to estimate leakage of cytoplasmic enzymes as the result of nonspecific cell damage, was determined as previously described (11). For the determination of total cell activity of each enzyme assayed, aliquots of cell suspensions were sonified in buffer containing 0.2% Triton X-100, diluted appropriately, and assayed.

Cells were incubated in parallel for the measurement of glucose oxidation (production of ^{14}CO$_2$ from [1-^{14}C]glucose) (6, 12) and lactate production (6, 13).

When cell ATP or cyclic AMP levels were measured, the incubation was stopped by placing the tubes in a boiling water bath for 3 min, followed by centrifugation. The ATP level in the supernatant fluid was determined by the firefly luminescence method (14). Cyclic AMP was determined by a protein-binding assay (15).

Incubation medium was aspirated into 1-ml syringes for determination of oxygen tension with use of a Radiometer (Copenhagen) model pHA 927b gas monitor and E5021 microelectrode unit, with a constant-temperature water bath. The oxygen tension of cell suspensions was measured before and after incubation of cells to verify that P_{O_2} was unchanged during the incubation period.

When enzyme release, phagocytosis, ^{14}CO$_2$ production, and lactate production were determined, blanks measured on tubes removed at zero time were subtracted from other values.

RESULTS

Effect of P_{O_2} on Zymosan-stimulated Lysosomal Enzyme Release

The release of lysosomal β-glucuronidase in response to zymosan was proportional to the log concentration of particles suspended in the incubation medium (Fig. 1). This response was also dependent on oxygen tension, being greatest when

cells were incubated at the lowest P_{O_2} tested (50 mm Hg), and diminishing as the oxygen tension was raised. The release of cytoplasmic LDH was unaffected by any of the conditions of incubation, which indicated that neither zymosan nor variation in P_{O_2} nonspecifically alters cell membrane permeability to intracellular enzymes.

Oxygen tension did not affect the initial rate of release of lysosomal enzymes (Fig. 2). However, the time required to reach maximal enzyme release was inversely proportional to P_{O_2}; i.e., cells incubated at lowest P_{O_2} released enzymes for a longer period and thus to a greater extent than cells incubated at higher oxygen tension. As Fig. 2 again illustrates, the release of LDH was negligible as compared with that of the lysosomal enzymes.
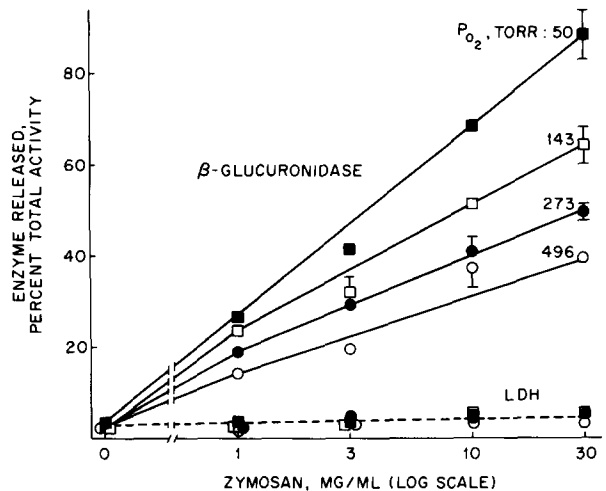

FIGURE 1 Enzyme release under varying P_{O_2} in response to zymosan. Human peripheral blood leukocytes were incubated in stoppered tubes with varying concentrations of opsonized zymosan. The tubes had been pre-gassed with 100% N_2, air, 52% N_2-48% O_2, or 100% O_2; P_{O_2} in gassed tubes was determined to be 50, 143, 273, and 496 torr, respectively. After 1 h, cells and media were separated by centrifugation and the media were assayed for activities of cytoplasmic LDH and of lysosomal β-glucuronidase. Each symbol represents the mean of triplicate determinations, the vertical bars ± one standard error of the mean. Where no error bars are shown, they lie within the symbol. See Materials and Methods for further details.
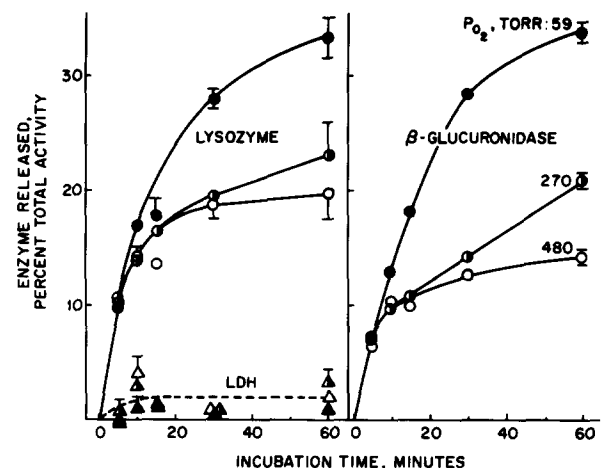

FIGURE 2 Time-course of enzyme release under varying P_{O_2} in response to opsonized zymosan (3 mg/ml). See Fig. 1 and text for further details.

Relationship of Endocytosis to Lysosomal Enzyme Release

Varying P_{O_2} had little effect on phagocytosis (as estimated by uptake of extracellular water using [^{14}C]inulin as a marker) (Fig. 3). As P_{O_2} was diminished, phagocytosis increased slightly and then declined again when cells were incubated at the lowest oxygen tension tested. As in previous experiments, the release of β-glucuronidase in response to zymosan was inversely proportional to oxygen tension.

Relationship of Cell Metabolism to Lysosomal Enzyme Release

While lysosomal enzyme release, which accompanies phagocytosis of zymosan, was promoted by decreasing P_{O_2}, the burst of pentose cycle oxidation, monitored by measuring the oxidation of [1-^{14}C]glucose to $^{14}CO_2$, was diminished as P_{O_2} was lowered. However, glycolysis in response to zymosan, estimated by measurement of lactate production is, like lysosomal enzyme release, promoted by decreasing P_{O_2} (Fig. 4).

Because it has been suggested that ATP generated by anaerobic glycolysis furnishes energy for lysosomal enzyme release (5), we measured the effect of P_{O_2} on cell ATP levels as lysosomal enzymes were released during phagocytosis of zymosan. The ATP level fell as zymosan concentration was increased. ATP fell to a greater extent at the lower P_{O_2} tested, mirroring the release of β-glucuronidase activity into the incubation medium (Fig. 5). The time-course of the changes in ATP levels of cells incubated with zymosan under varying oxygen tension also mirrored the time-course of release of lysosomal β-glucuronidase (Fig. 6).

Cyclic AMP levels, after a slight initial rise, also fell during phagocytosis and lysosomal enzyme release (Fig. 7). Oxygen tension had little effect on cyclic AMP metabolism, although the nucleotide fell to slightly lower levels in cells incubated under the higher oxygen tension tested.
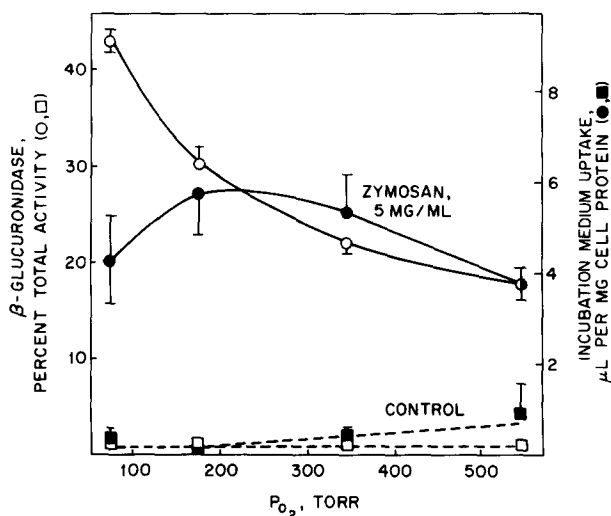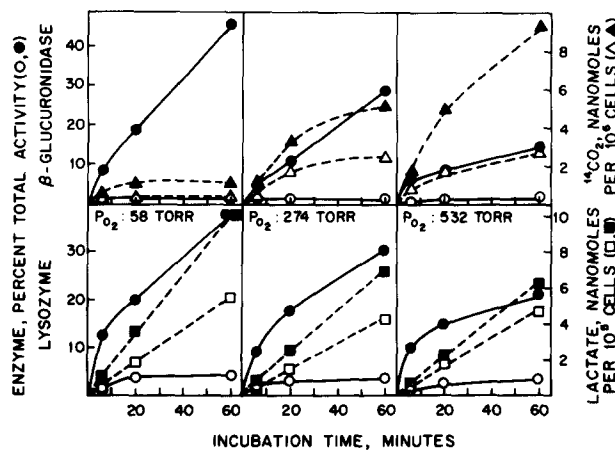

FIGURE 4   Time-course of lysosomal enzyme release, enhanced glucose oxidation, and enhanced lactate production in response to zymosan under varying P_{O_2}. Cells were incubated with (solid symbols) or without (open symbols) zymosan, 3 mg/ml. Each symbol represents the mean of results from duplicate incubations. See Fig. 1 and text for further details.
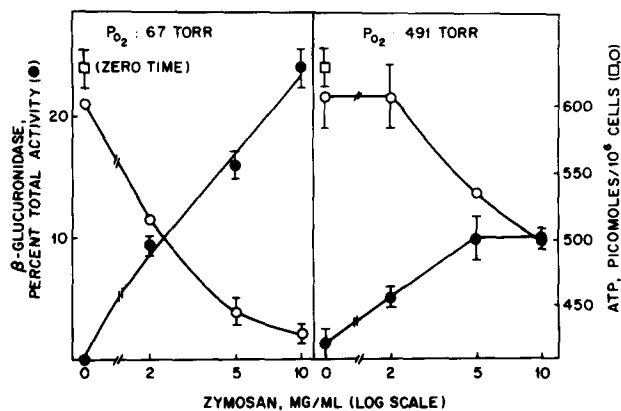

FIGURE 5   Modification by P_{O_2} of effect of zymosan on β-glucuronidase release and cell ATP level. Tubes containing leukocyte suspensions were incubated for 30 min. See Fig. 1 and text for further details.

Effects of Sulfhydryl Antioxidants and Scavengers of Oxygen-derived Reactants on Lysosomal Enzyme Release

The time-course of lysosomal enzyme release from cells stimulated by zymosan under varying oxygen tensions and the inverse relationship between glucose oxidation and release of lysosomal enzymes suggest that a product or products dependent on oxidative metabolism could exert a negative feedback influence on lysosomal enzyme release. Oxygen-derived reactants such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), and singlet oxygen ($^1O_2$), produced by stimulated neutrophils, could be responsible for such an effect. The sulfhydryl-containing antioxidants cysteine and glutathione each potentiated zymosan-induced lysosomal enzyme release (Table I). These agents had no significant effect on glucose oxidation, lactate production, or change in ATP levels in response to phagocytic stimuli. Scavengers of O_2^- (SOD), H_2O_2 (catalase), OH· (benzoate, hypoxanthine, xanthine) and $^1O_2$ (hypoxanthine, xanthine, histidine, azide) were also tested. Catalase, Na-benzoate, xanthine, histidine, and Na-azide each also potentiated zymosan-induced lysosomal en-

FIGURE 3   Phagocytosis and β-glucuronidase release under varying P_{O_2}. Leukocytes were incubated with (circles) or without (squares) zymosan, 5 mg/ml, for 1 h. A trace amount of [^{14}C]inulin was present in all tubes. Phagocytosis (solid symbols) was estimated by the uptake of extracellular water into the cell pellets, using [^{14}C]inulin as a marker. β-Glucuronidase activity (open symbols) in incubation media was measured as an index of lysosomal enzyme release. See Fig. 1 and text for further details.

zyme release (Table II). The effect of SOD was small and dependent upon the concentration of the scavenger enzyme. In low concentration, SOD inhibited enzyme release to a slight degree (Table II). At higher concentrations, SOD potentiated enzyme release slightly (data not shown).

DISCUSSION

We have studied the relationships between functional and metabolic responses of polymorphonuclear leukocytes to experimental stimuli. Cell metabolism was altered by a relatively innocuous means, i.e., by varying the oxygen tension under which cells were incubated. Incubation of cells under reduced oxygen tension enhanced release both of lysozyme (found in both specific and azurophilic granules) and of β-glucuronidase (confined to azurophilic granules). None of the experimental conditions listed resulted in enhanced release of cytoplasmic LDH which would be expected if enzyme release were a nonspecific event resulting from cell injury (16). Lactate production was linear over the incubation periods used in these studies, providing further evidence that neither alteration in P_{O_2} nor stimulation with zymosan affected cell viability.
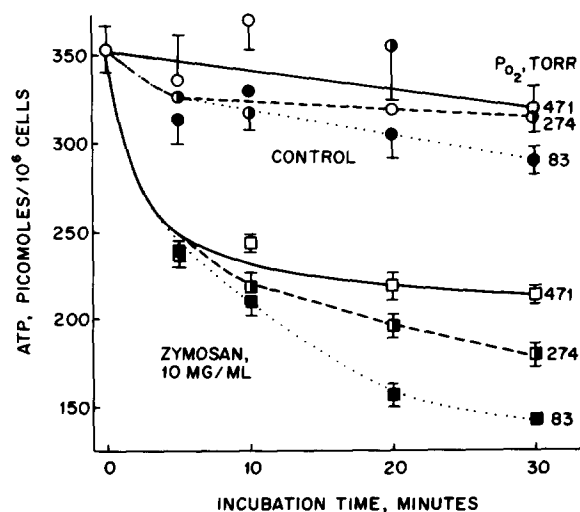
The observed effect of oxygen tension on release of lysosomal enzymes in response to stimulation with zymosan cannot be attributed secondarily to an effect of oxygen tension on phagocytosis. Phagocytosis of latex particles (17) and phagocytosis of bacteria and bacterial killing (18) by polymorphonuclear leukocytes are as effective when cells are incubated under anaerobic conditions as when aerobic conditions of incubation are used. In accord with these reports, we observed only a minimal effect of oxygen tension on endocytosis (Fig. 3).

Agents that promote a rise in cell cyclic AMP levels inhibit lysosomal enzyme release (19). One could postulate that cyclic-AMP-dependent processes act to restrain enzyme release from resting cells. A fall in cell cyclic AMP level and release from the putative restraint might then be expected to accompany lysosomal enzyme release. We did observe a fall in the level of cyclic AMP in cells stimulated by zymosan (Fig. 7) which was preceded by a slight rise, as others have also observed (20). This fall in cyclic AMP may facilitate lysosomal enzyme release. There is little difference, however, between the cyclic AMP responses of cells incubated under high and those of cells incubated under low oxygen tension. Thus, these findings do not support the view that the effect of oxygen tension on lysosomal enzyme release is secondary to an effect on cyclic
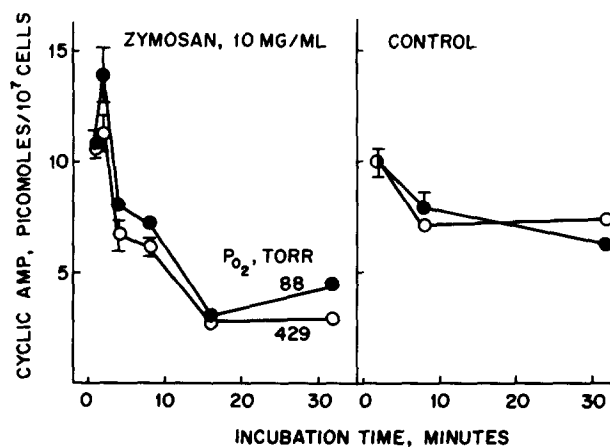

FIGURE 6   Time-course of change in cell ATP level in response to zymosan under varying P_{O_2}. See Fig. 1 and text for details.

FIGURE 7   Time-course of change in cell cyclic AMP level in response to zymosan under varying P_{O_2}. See Fig. 1 and text for details.

TABLE I

Effects of Cysteine and Glutathione on Zymosan-induced Lysosomal Enzyme Release

	Enzyme released, percent total activity		
	β-Glucuronidase	Lysozyme	LDH
Exp 1			
Control	1.1 ± 0.2	4.9 ± 0.9	7.1 ± 0.6
Cysteine, 0.3 mM	2.6 ± 0.2	6.1 ± 0.7	6.4 ± 0.6
Cysteine, 3 mM	1.4 ± 0.1	3.5 ± 2.4	3.4 ± 0.2
Zymosan, 1 mg/ml	6.8 ± 0.9	17.9 ± 2.4	4.6 ± 1.4
Zymosan + Cysteine, 0.3 mM	9.2 ± 1.3	27.2 ± 4.2	5.3 ± 0
Zymosan + Cysteine, 3 mM	17.4 ± 1.0	37.5 ± 0.7	2.2 ± 0.6
Exp 2			
Control	0.5 ± 0.1	5.1 ± 1.4	1.1 ± 0.8
Glutathione, 0.1 mM	2.2 ± 0.4	8.0 ± 1.7	0.2 ± 0.7
Glutathione, 1 mM	1.2 ± 0.7	10.1 ± 0.3	2.5 ± 0.7
Zymosan, 1 mg/ml	8.4 ± 1.3	24.0 ± 1.3	1.0 ± 1.0
Zymosan + Glutathione, 0.1 mM	11.3 ± 0.2	26.1 ± 2.1	−1.8 ± 0.5
Zymosan + Glutathione, 1 mM	20.2 ± 1.5	47.5 ± 1.4	3.9 ± 1.2

Human peripheral blood leukocytes were incubated under an atmosphere of air for 1 h at 37°C. Zymosan, when present, had been opsonized by preincubation with fresh serum. Results are expressed as mean ± one standard error of the mean of triplicate determinations. See Fig. 1 and text for further details.

TABLE II

Effects of Scavengers of Oxygen-derived Reactants on Zymosan-induced Lysosomal Enzyme Release

	Enzyme released, percent total activity					
	β-Glucuronidase		Lysozyme		LDH	
	Resting	Stimulated*	Resting	Stimulated	Resting	Stimulated
Control	−0.3 ± 0.2	9.7 ± 0.8	4.0 ± 0.2	20.8 ± 1.6	0.9 ± 0.4	1.9 ± 0.1
SOD, 290 U/ml	0.7 ± 0.2	8.3 ± 0.2	1.6 ± 0.3	16.7 ± 2.4	1.2 ± 0.4	2.0 ± 0.3
SOD, heated	0.2 ± 0.1	11.9 ± 0.4	1.0 ± 0.1	24.5 ± 0.7	1.5 ± 0.3	1.7 ± 0.3
Catalase, 6,400 U/ml	2.1 ± 0.3	18.0 ± 0.9	1.6 ± 0.2	26.4 ± 0.8	1.4 ± 0	2.9 ± 0.1
Catalase, heated	0 ± 0.2	11.7 ± 1.1	2.4 ± 0.2	23.4 ± 0.9	1.9 ± 0.6	2.4 ± 0.3
Na-benzoate, 10 mM	0.3 ± 0.2	12.1 ± 0.6	1.4 ± 0.8	24.5 ± 2.1	2.1 ± 0.1	2.7 ± 0
Xanthine, 1 mM	0.4 ± 0.1	15.3 ± 0.5	3.6 ± 0.2	30.3 ± 0.4	3.7 ± 0.1	2.7 ± 0.1
Histidine, 10 mM	0.5 ± 0.2	12.7 ± 0.2	4.0 ± 0.6	25.0 ± 0.2	2.8 ± 0.1	3.3 ± 0
Na-azide, 10 mM	2.7 ± 0.3	17.9 ± 0.8	9.9 ± 0.3	29.1 ± 1.6	3.1 ± 0.3	3.0 ± 0.5
Exp 2						
Control	1.5 ± 0.1	17.4 ± 0.5	2.4 ± 0.3	24.0 ± 0.6	2.3 ± 0.1	1.8 ± 0
Hypoxanthine, 4 mM	1.0 ± 0.3	27.2 ± 1.2	1.9 ± 0.4	30.8 ± 0.9	2.2 ± 0.5	3.9 ± 0.1
Xanthine, 1 mM	1.3 ± 0.3	19.6 ± 0.2	3.1 ± 0.6	26.6 ± 0.3	2.4 ± 0	2.3 ± 0.2
Xanthine, 4 mM		27.5 ± 0.9		29.5 ± 0.5		3.5 ± 0.1
Xanthine, 8 mM	2.0 ± 0.1	30.4 ± 1.1	4.0 ± 0.4	30.1 ± 0.5	3.7 ± 0.1	3.8 ± 0.1
Exp 3						
Control	1.3 ± 0.4	9.6 ± 1.7	2.9 ± 0.4	22.5 ± 7.1	2.2 ± 0.3	1.7 ± 0.2
Na-azide, 3 mM	3.5 ± 0.3	17.5 ± 0.9	9.4 ± 4.4	45.8 ± 1.6	1.9 ± 0.4	1.7 ± 0.5
Na-azide, 10 mM		19.5 ± 1.1		46.7 ± 1.9		2.8 ± 0.8
Na-azide, 30 mM	2.7 ± 0.2	20.7 ± 0.3	7.3 ± 4.0	44.6 ± 0.7	2.6 ± 0.5	1.8 ± 0.3

* Zymosan, 1 mg/ml. See Table I and text for further details.

AMP metabolism.

Henson and Oades (5) reported that agents that inhibit anaerobic glycolysis also inhibit exocytosis and suggested that energy derived from this metabolic process was required for enzyme release. Consonant with their suggestion, we observed concordant changes when anaerobic glycolysis (monitored by measuring lactate production) and lysosomal enzyme release in response to zymosan were modified by alterations in oxygen tension. Changes in ATP levels mirrored lysosomal enzyme release as modified by oxygen tension (Figs. 5 and 6). These findings suggest that ATP may supply energy for lysosomal enzyme release. As anaerobic glycolysis provides the energy in neutrophils for phosphorylation and generation of high-energy compounds (4), this pathway may be enhanced to replete energy stores. The precise way in which ATP metabolism is coupled to exocytosis remains to be elucidated.

Variation in P_{O_2} had little effect on the initial phase of the time-course of lysosomal enzyme release in response to opso- nized zymosan. As P_{O_2} was increased, however, maximal ly- sosomal enzyme release was achieved at an earlier time and therefore to a lesser final extent. These findings suggest that a product dependent on oxidative metabolism by neutrophils exerts a negative feedback influence on lysosomal enzyme release. Oxygen-derived reactants, such as O_2^-, H_2O_2, $OH\cdot$, and $^1O_2$, could be responsible for this effect. The observation that the sulfhydryl antioxidants cysteine and glutathione and scavengers of oxygen-derived reactants potentiate release of lysosomal enzymes from zymosan-stimulated cells is consistent with this hypothesis.

Indirect evidence suggests that O_2^- per se is not responsible for inhibition of lysosomal enzyme release. Scavengers of H_2O_2, $OH\cdot$, or $^1O_2$ would be expected to have no effect on the level of O_2^-. Further, SOD at low concentration slightly inhibited zymosan-stimulated lysosomal enzyme release, while only higher concentrations of the enzyme potentiated enzyme

release along with the other scavengers tested. Although the chemical interaction among the oxygen-derived reactants is complex, these relationships can be operationally described as follows (21). O_2^- is generated by reduction of molecular oxygen and then reacts with H^+ to form H_2O_2, the latter reaction being facilitated by SOD. H_2O_2 then reacts with O_2^- to form $OH\cdot$ and possibly $^1O_2$. It is possible that, at low levels of SOD, O_2^- is not completely converted to H_2O_2 and that an amount sufficient to react with H_2O_2 to form $OH\cdot$ and $^1O_2$ remains. Thus, SOD in low concentration may actually facilitate the generation of $OH\cdot$ and $^1O_2$. At high enzyme concentrations, the removal of O_2^- may be complete so that production of $OH\cdot$ and $^1O_2$, possibly more reactive in our system, is minimal.

There are a number of sites at which oxygen-derived reac- tants could act to inhibit enzyme release. It has been suggested that microtubule assembly, which occurs as an early event after neutrophil stimulation (22), is required for enzyme release. H_2O_2, which accumulates under conditions of glutathione per- oxidase deficiency, promotes "capping" of neutrophils by con- canavalin A, a phenomenon associated with impairment of assembly of microtubules (23). Glutathione, which, along with H_2O_2, serves as a substrate for glutathione peroxidase, could facilitate reduction of H_2O_2 by the enzyme in addition to interacting directly with oxygen-derived reactants.

Lipid peroxidation or other oxidative damage of neutrophil membrane components could result in impaired neutrophil responses. Upon prolonged incubation, after initial exposure to phagocytic stimuli, viability of neutrophils is impaired. Cell death is retarded by scavengers of oxygen-derived reactants (24). Although we did not observe overt cell death in the short period of incubation we employed, the observed impairment of function (blunted lysosomal enzyme release) may herald autotoxicity. Receptors for C3b may be altered by autooxida- tive damage (25); our results are compatible with the hypothesis that a membrane receptor with which opsonized zymosan

interacts to trigger enzyme release could be damaged in a similar manner. It is also possible that the stimulus to enzyme release could be altered by oxidative damage. C5a, which interacts with specific receptors on neutrophil membranes, is inactivated by the myeloperoxidase-H_2O_2-halide system (26). IgG or an activated component of complement, which is adherent to zymosan and which interacts with a neutrophil membrane receptor, could be similarly inactivated. Regardless of the precise mode of action, our results are compatible with the interpretation that oxygen-derived reactants act to impede release of lysosomal enzymes from zymosan-stimulated neutrophils.

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