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Research Article

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Amino Acid Oxidase of Leukocytes in Relation to H_2O_2-Mediated Bacterial Killing

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ABSTRACT D-Amino acid oxidase and L-amino acid oxidase have been measured in sucrose homogenates of polymorphonuclear leukocytes (PMN) obtained from guinea pigs and humans. Subcellular distribution patterns and studies on latency indicate that these oxidases are soluble enzymes. Their hydrogen peroxide-generating capacity was verified. Chronic granulomatous disease PMN, which lack a respiratory burst and fail to generate H_2O_2 during phagocytosis and do not kill catalase positive bacteria, had peroxide-generating amino acid oxidase activity equal to that found in PMN homogenates from patients with bacterial infections. The precise metabolic and bactericidal role of amino acid oxidases in PMN remains uncertain.

INTRODUCTION

The metabolic and bactericidal importance of hydrogen peroxide generation by leukocytes during phagocytosis has become evident. Klebanoff demonstrated that peroxide, myeloperoxidase, and halide ion comprise a potent bactericidal system within polymorphonuclear (PMN)[1] leukocytes (1, 2). Peroxide is generated by PMN from molecular oxygen and a source of hydrogen atoms catalyzed by one or more oxidative enzymes (3). Phagocytizing PMN display increased oxygen consumption which is unaffected by mitochondrial inhibitors such as cyanide (4); increased glucose utilization provides reduced pyridine nucleotides (NADH and NADPH) that can act as substrates for oxidase-mediated peroxide-forming reactions. Three cyanide-insensitive oxidases, NADH oxidase (5), NADPH oxidase (6), and D-amino acid oxidase (7) have been found in PMN. NADH oxidase activity sufficient to explain the respiratory burst has been described in guinea pig and human PMN (8), and was deficient in leukocytes of five children with chronic granulomatous disease (CGD) (9). The phagocytizing PMN from CGD patients lack a repiratory burst (10), fail to generate peroxide (11), and do not kill bacteria unless they produce peroxide (12). The CGD PMN can be corrected metabolically 13) and improved bactericidally (14) by the in vitro insertion of a peroxide generating system into the cell. NADPH oxidase was present in granule fraction of guinea pig peritoneal PMN (15) and human PMN (16) but activities were insufficient to explain the respiratory burst (18).

Recently, Cline and Lehrer found D-amino acid oxidase activity in the granule fraction of human and guinea pig PMN homogenate (7). We have found L-amino acid oxidase, as well as D-amino acid oxidase in PMN from both species. Attention was directed to the oxygen consuming activity of each oxidase, their subcellular distribution and latency, the concentration of substrate amino acid within the PMN, and the potential role of amino acid oxidase activity as a source of hydrogen peroxide within the cell. Because of the metabolic and bactericidal importance of hydrogen peroxide, we determined the peroxide generating capacity of D- and L-amino acid oxidase by means of a sensitive method for measuring small quantities of hydrogen peroxide spectrophotometrically as described by Nakano, Tsutsumi, and Danowski (16). Since the CGD PMN do not generate peroxide, their homogenates were assayed for peroxide formation by D- and L-amino acid oxidase and compared with homogenates from patients with infections.

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[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; PMN, polymorphonuclear leukocytes; WBC, white blood cell count; NBT, nitro blue tetrazolium.

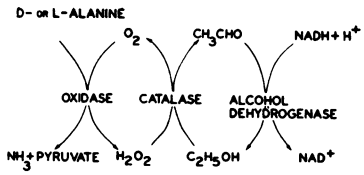

FIGURE 1 Principle of the spectrophotometric assay for hydrogen peroxide produced during oxidation of amino acid by amino acid oxidase (Method of Nakano et al. [16]).

METHODS

Preparation of leukocyte suspensions. Guinea pigs, (Hartley strain) weighing 300–350 g, were injected intraperitoneally with 15 ml sterile 13.5% solution of sodium caseinate (Nutrose)[2] 16–18 hr before being sacrificed, using a previously described method (17). An exudate rich in polymorphonuclear leukocytes (95%) was harvested in 0.9% sodium chloride, centrifuged at 250 *g* for 5 min at 4°C. A 0.5–1.0 ml pellet of packed leukocytes was obtained from each guinea pig.

50 cc of peripheral blood was collected in heparin by venipuncture from 10 control human subjects and 3 patients (2 males and 1 female) with chronic granulomatous disease (CGD) previously identified by the quantitative nitro blue tetrazolium (NBT) test (18). Control subjects were young adults with acute bacterial infections. The proportion of granulocytes was approximately 80% of the total white blood cell count (WBC). The blood was processed by a previously described method (19).

The leukocyte pellet obtained from guinea pigs and humans was washed once in Krebs-Ringer phosphate buffer at pH 7.4 (20) and was centrifuged for 10 min at 250 *g* at 4°C. The pellet was resuspended and washed once in cold 0.34 M sucrose (pH 7.4 with sodium bicarbonate) and then centrifuged for 10 min at 250 *g* at 4°C. The supernatant was discarded and the leukocyte button was resuspended in 0.34 M sucrose to a concentration of 12.5–25.0% of the total volume. The suspension of guinea pig leukocytes was homogenized for 3 min and that of the humans for 5 min in a glass cylinder with a Teflon pestle[3] at 4°C using a motor-driven homogenizer.[4]

Some samples were tested after being dialyzed against 2000 ml of 0.34 M sucrose (pH 7.4) overnight in a cold room at 4°C.

Preparation of subcellular fractions. Exudates of PMN from four guinea pigs were pooled and made into one whole sucrose homogenate as described above. The exact volume of the whole homogenate was recorded, and a portion was removed for determination of enzyme recovery from the subcellular fractions. The homogenate was centrifuged for 10 min at 250 *g*, 4°C. The button containing nuclear debris, cellular membranes, and some unbroken cells, was washed thrice in 0.34 M sucrose (pH 7.4). The residual button was resuspended in 0.34 M sucrose and was the "debris" fraction. The supernatant fluids obtained from the washes were combined and centrifuged for 20 min at 30,000 *g*, 4°C. The button was washed twice in 0.34 M sucrose and resuspended in 0.34 M sucrose. This was the "granule" fraction. The supernatant fluids were combined and centrifuged for 1 hr at 100,000 *g*, 5°C. The supernatant was considered the "soluble" fraction and the button resuspended in 0.34 M sucrose, was the "microsome" fraction. The volumes of all fractions were recorded.

Enzyme assay. D-Amino acid oxidase and L-amino acid oxidase were determined by measuring the rate of oxygen consumption at 37°C with a Yellow Springs oxygen membrane electrode[5] equipped with a scale expansion recorder so that a change of 20% oxygen saturation of the incubation mixture in air produced full scale deflection. A final volume of 3.0 ml contained 1.0 ml of enzyme homogenate and a final concentration of D-alanine or L-alanine of 5×10^{-3} M, 3×10^{-3} M flavin adenine dinucleotide[6] (disodium salt grade II) and 0.02 M sodium pyrophosphate buffer pH 8.3.

In addition, hydrogen peroxide generation by D-amino acid oxidase and L-amino acid oxidase was determined by the method of Nakano et al. (16). The basis of this spectrophotometric assay is indicated in Fig. 1. Hydrogen peroxide, produced from oxygen, and substrate amino acid catalatically oxidizes ethanol to acetaldehyde, which in turn is reduced in a coupled oxidation reaction with NADH catalyzed by alcohol dehydrogenase. Consumption of NADH was measured at OD_{340} in a 1 cm light path with a Gilford model 2400 recording spectrophotometer using a 3 ml reaction mixture which contained at final concentrations the following: 0.1 M sodium phosphate buffer, pH 7.9, 1×10^{-4} M NADH, 5×10^{-3} M D-alanine or 5×10^{-3} M L-alanine (optical rotation $+14.4°$),[7] 40 μg crystaline beef liver catalase, 0.10 mg alcohol dehydrogenase.[6] Minor modifications of the assay included addition of 0.01 ml absolute ethanol instead of 1:3 (v/v) dilution of 95% ethanol to the reaction mixture and the incubation was at 37°C instead of room temperature. The reaction was initiated by addition of 0.1 ml enzyme homogenate to duplicate mixtures. Appropriate substrate blanks, i.e., all components except the enzyme, and enzyme blanks, i.e., enzyme in buffer without substrates, were performed with each duplicate analysis. In order to verify our methods, standard hog kidney D-amino acid oxidase[6] and standard snake venom L-amino acid oxidase[6] were examined by both O_2 uptake and H_2O_2 production. Contamination of the amino acid substrate was checked using the above standard amino acid oxidases with each amino acid substrate, i.e., D- or L-amino acid.

Latency studies. In order to show that amino acid oxidase lacked latent activity and that our homogenation procedure maintained the latency of granule enzymes, myeloperoxidase (21), and amino acid oxidase activities were assayed on portions from fresh sucrose homogenate of leukocytes either frozen-thawed three times, or after addition of 0.01% (w/v) Triton X-100.[8] Previous studies have demonstrated that granule myeloperoxidase demonstrates latency (22). Protein was determined by the method of Lowry, Rosenbrough, Farr, and Randall (23).

RESULTS

Results of amino acid oxidase assays. As noted in Table I, D-amino acid oxidase, as well as L-amino acid oxidase, expressed as mμmoles O_2 consumed hr^{-1} mg^{-1} protein were present in PMN homogenates of guinea pigs and humans. The differences in mean activities for both enzymes between the two species were statistically sig-

[2] Difco Labs, Detroit, Mich.
[3] Arthur H. Thomas, Co., Philadelphia, Pa.
[4] Talboys Instrument Corp., Emerson, N. J.
[5] Yellow Springs Instrument Co, Yellow Springs, Ohio.
[6] Sigma Chemical Co., St. Louis, Mo.
[7] Calbiochem, Los Angeles, Calif.
[8] Rohm and Haas Co., Philadelphia, Pa.

TABLE I
Amino Acid Oxidase Activity

Substrate	Fresh‡		Fresh*			Frozen-thawed*		
	GP	HC	GP	HC	CGD	GP	HC	CGD
	(5)	(6)	(9)	(4)	(3)	(5)	(5)	(2)
D-amino acid	119 ±10	71 ±34	34 ±8	21 ±5	25 ±2	36 ±10	18 ±3	24 ±8
	$P = 0.01$		$P = 0.01$	$P > 0.05$		$P < 0.01$		$P > 0.05$
L-amino acid	120 ±12	72 ±29	34 ±8	19 ±7	24 ±3	35 ±7	17 ±4	20 ±0
	$P = 0.01$		$P = 0.01$	$P > 0.05$		$P < 0.01$		$P > 0.05$

‡ Oxygen electrode method; results expressed as mumoles O_2 consumed $hr^{-1}mg^{-1}$ protein.
* Spectrophotometric method; results expressed as mumoles NADH $hr^{-1}mg^{-1}$ protein.
GP means guinea pig, HC means Human Control, CGD means chronic granulomatous disease patients
Number of subjects is indicated in parenthesis.
Results are mean ±SD. P values based upon Students t test.

nificant at $P = 0.01$ level. Results of peroxide generating activity by amino acid oxidase expressed as mμmoles NADH oxidized $hr^{-1}mg^{-1}$ protein are also indicated in Table I. Species differences are evident but there was no statistical difference in activities for either D- or L-amino acid oxidase between the human controls and three patients with CGD ($P > 0.05$). Freezing and thawing of the samples did not significantly alter the activities of both enzymes in the three groups tested. The relationship between oxygen consumed by the O_2 electrode determination and peroxide available for NADH oxidation in the spectrophotometric reaction was approximately 30%, i.e., for human control samples, 71 ±34 mμmoles oxygen consumed $hr^{-1}mg^{-1}$ for D-amino acid oxidase and 21 ±5 mμmoles of NADH oxidized $hr^{-1}mg^{-1}$. Similar results were obtained for L-amino acid oxidase. These results cannot be explained by the slightly different conditions used for each type of assay since the spectrophotometric assay had a broad pH optimum between pH 7.9 and pH 8.3 and the oxygen electrode assay did not require FAD for maximal activity by human PMN homogenate. The results correspond well with the enzymatic tests conducted on the standard enzymes indicated in Table II. These results showed that oxygen consumption was three to fivefold greater than H_2O_2 produced by purified commercial D- and L-amino acid oxidase.

Substrate specificity of spectrophotometric assay. As shown in Table III assay of D- and L-amino acid oxidase was only about 75% efficient when absolute ethanol and alcohol dehydrogenase were omitted from the incubation mixture. An optimum rate was achieved when both the latter components were included in the assay. However, if catalase was omitted, the reaction ceased. When pyruvate was added to the system lacking catalase, no increase in activity was achieved. Thus, NADH was not directly oxidized either by the hydrogen peroxide generated through D- or L-amnio acid oxidase with D- or L-amino acid, respectively, or by pyruvate produced by oxidation and deamination of the amino acid, but depended upon the presence of catalase in the reaction mixture. Figs. 2 and 3 show that assay of enzyme activity utilizing either the oxygen electrode method or the

TABLE II
*Comparison of O_2 Consumed Using the Oxygen Electrode Assay with NADH Oxidized Using the H_2O_2 Spectrophotometric Assay for Measuring Amino Acid Oxidase Activity**

Enzyme	Substrate	Oxygen electrode assay	H_2O_2 spectrophotometric assay
		μmoles $hr^{-1}mg^{-1}$ protein	
D-amino acid oxidase*	D-alanine	440.3	80.5
L-amino acid oxidase‡	L-alanine	26.7	8.6

* D-amino acid oxidase was derived from hog kidney and ‡ L-amino acid oxidase from snake venom, both purchased from Sigma Chemical Co., St. Louis, Mo.

FIGURE 2 Relationship of D-amino acid oxidase activity to commercial purified D-amino acid oxidase concentration. OD_{340}/hr means the rate of decreased absorbance observed during NADH oxidation. See text for further details.

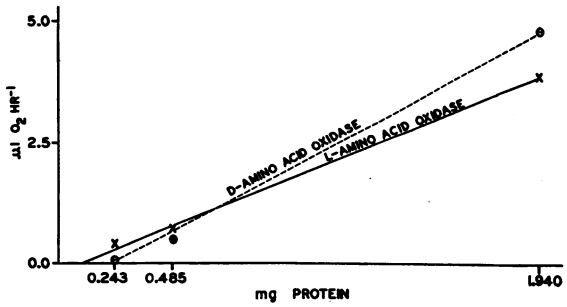

FIGURE 3 Relationship of amino acid oxidase activity to concentration of leukocyte homogenate protein. See text for details.

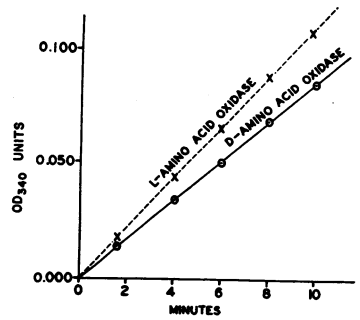

FIGURE 4 Linearity of the D- and L-amino acid oxidase reaction as determined by the spectrophotometric assay of Nakano. See text for details.

spectrophotometric method was directly proportional to the concentration of both standard enzyme and PMN homogenate protein. Fig. 4 shows that both D- and L-amino acid oxidase activities determined by the spectrophotometric assay were linear for at least 10 min.

Enzyme stability. Both D- and L-amino acid oxidase were reasonably stable in whole cell sucrose homogenates at 4°C, maintained for over 22.5 hr. In fact, L-amino acid oxidase lost no activity over that time period, whereas D-amino acid oxidase lost 30% of its activity during the first 8 hr of storage time and remained stable thereafter. No loss of activity was noted for either enzyme after the leukocyte homogenates were dialyzed overnight at 4°C.

Subcellular distribution. Figs. 5a and 5b show subcellular distribution patterns for D- and L-amino acid oxidase in sucrose homogenates of guinea pig PMN. The specific activity of the enzyme was highest in the microsome fraction (Fig. 5a). Most of the enzyme activity was in the soluble fraction, (Fig. 5b).

Latency studies. As also shown in Table I, there was no difference in the activity found for D- and L-amino acid oxidase whether they were tested in fresh or frozen-thawed homogenates of guinea pig and human leukocytes. This was true even when 0.01% Triton X-100 was added. These studies indicate that amino acid oxidases are soluble enzymes within the cell and that it is unlikely that their activity was released from granules during homogenation since another granule enzyme, myeloperoxidase demonstrated latent activity in the same homogenate (22). Almost twice as much myeloperoxidase activity was obtained after freezing-thawing the fresh homogenate. Addition of 0.01% Triton X-100 to the frozen thawed portion produced more than seven times the original activity (Fig. 5). Sucrose homogenates of human leukocytes were then tested for D- and L-amino

TABLE III
*Components of the H_2O_2 Spectrophotometric Assay Activity**

Components	Specific activity
	μmoles NADH consumed $hr^{-1}mg^{-1}$ protein
D-amino acid oxidase + D-alanine + catalase + alcohol dehydrogenase + ethanol	92.3
D-amino acid oxidase + D-alanine + catalase	69.1
D-amino acid oxidase + D-alanine	1.5
D-amino acid oxidase + D-alanine + pyruvate	1.4
L-amino acid oxidase + L-alanine + catalase + alcohol dehydrogenase + ethanol	6.3
L-amino acid oxidase + L-alanine + catalase	4.8
L-amino acid oxidase + L-alanine	0.1
L-amino acid oxidase + L-alanine + pyruvate	0.1

* The activity was determined on standard D-amino acid oxidase from hog kidney and on standard L-amino acid oxidase from snake venom purchased from Sigma Chemical Co., St. Louis, Mo. D-alanine and L-alanine, respectively, were used as the substrates.

acid oxidase activity under the identical conditions. No increase in activity for either enzyme was obtained (Fig. 6).

DISCUSSION

D-Amino acid oxidase, as well as L-amino acid oxidase, have been found in rat and mice kidney (24, 25) liver, and spleen (26) tissues. Recently, Cline and Lehrer (7) reported D-amino acid oxidase activity in PMN obtained from guinea pigs and humans. They reported that 75–90% of the activity was found in the 27,000 × *g* granule fraction which also contained 85–90% of myeloperoxidase activity. We found amino acid oxidase activity in sucrose homogenates of guinea pig and human leukocytes using either D-alanine or L-alanine as substrate. It is unlikely that D- and L-amino acid oxidase activity can be ascribed to one enzyme since it is well-known (27) that each enzyme has optical specificity for its substrate. Our substrates are pure since hog kidney D-amino acid oxidase did not effectively catalyze a reaction with L-alanine, i.e., only 5.8% activity was obtained, and snake venom L-amino acid oxidase did not effectively catalyze a reaction with D-alanine, i.e., only 7.2% activity was obtained. However, the activity for both amino acid oxidases in each species of PMN homogenates were almost identical and no enhanced activity was obtained when both L- and D-amino acids were added together. This suggests that a racemic amino acid oxidase may be present in PMN homogenates.

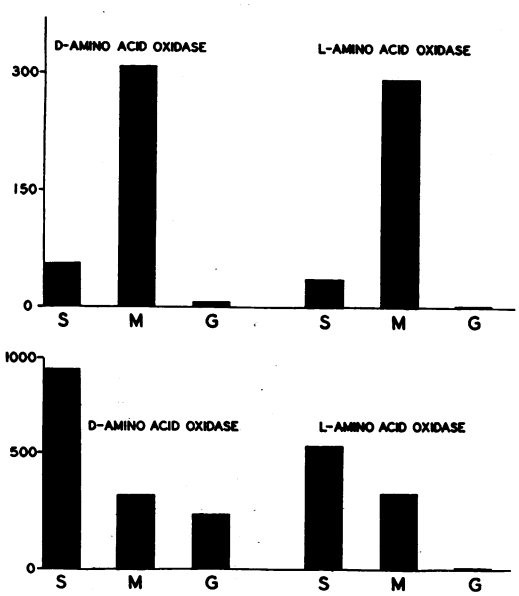

FIGURE 5 Subcellular distribution patterns of amino acid oxidase for guinea pig PMN. S indicates soluble fraction. M is the microsome fraction and G is the granule fraction. Determination of percentage of recovery showed that 98% of protein was recovered. Results expressed as specific activity, i.e., mμmoles NADH oxidized $hr^{-1}mg^{-1}$ protein in Fig. 5a (top) and as total activity, i.e., mμmoles NADH oxidized hr^{-1} in Fig. 5b (bottom).

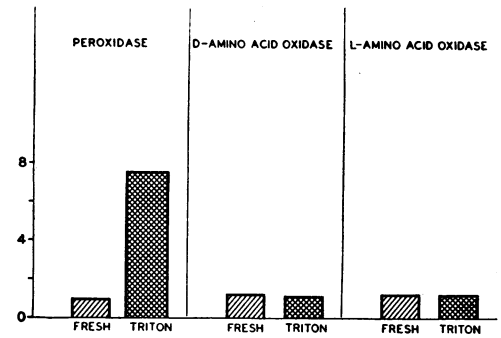

FIGURE 6 Latent activity for myeloperoxidase and absence of latent activity for D- and L-amino acid oxidase in fresh homogenates from human leukocytes, before and after the addition of 0.01% Triton X-100 to frozen-thawed homogenates. See text for further details.

In contrast to granule myeloperoxidase which demonstrated a sevenfold increase of enzyme activity after the PMN homogenate was treated with 0.01% Triton, a surfactant that disrupts cell and granule membranes, neither D- nor L-amino acid oxidase displayed such latent activity. Since latency is a property of membrane-bound lysosomes, amino acid oxidases of PMN are not granule lysosomal enzymes. Subcellular distribution studies confirmed that amino acid oxidase was not concentrated in the 30,000 × *g* lysosomal fraction, but was in the post 100,000 × *g* supernatant fraction. Nakano has found L-amino acid oxidase in the soluble fraction of rat kidney cells (28). However, Baudhuin, Beaufay, and De Duve (29) have found in rat liver D-amino acid oxidase and catalase in a special group of cytoplasmic particles called peroxisomes or microbodies. Our previous observation that catalase is soluble in human PMN (19) coupled with our present finding that amino acid oxidase is also soluble in human and guinea pig PMN would suggest that either our method of homogenation selectively disrupted certain granules containing catalase and amino acid oxidase and left other granules, such as peroxidase, intact or that the former enzymes indeed are soluble in vivo in PMN in contrast to liver and kidney cells (24).

Comparison of the rate of oxygen consumed to the rate of hydrogen peroxide produced by amino acid oxidase demonstrated that only about 30% of the latter product was available for catalatic oxidation reactions. We previously pointed out (8) that only about 3% of the calculated hydrogen peroxide produced during the burst of oxygen consumption by phagocytizing PMN was available for catalatic oxidation of formate-^{14}C. This implies that most of the peroxide either leaks out

of the cell or is dissipated in the cell and cell homogenates via other metabolic pathways. At least three such pathways seem to exist in the PMN (30); (*a*) the rapid catalatic oxidation of hydrogen peroxide to water and oxygen (31), (*b*) the oxidation of reduced glutathione by hydrogen peroxide with the stimulation of the hexose monophosphate shunt through a coupled oxidation-reduction reaction of oxidized glutathione and NADPH catalyzed by glutathione reductase (32), (*c*) the participation of hydrogen peroxide with myeloperoxidase and halide ion to form a potent bactericidal system within the PMN (1). Cline and Lehrer (7) have proposed such an antimicrobial system linked to D-amino acid oxidase, whereby, substrate D-amino acids are provided by the cell wall of ingested bacteria. McMenamy, Lund, Neville, and Wallach (33) have determined that free L-alanine is present at 3.0 mM concentrations in human leukocytes which is within the range of 5.0 mM L-alanine that we used for our assay. Skarnes had proposed a bactericidal system involving L-amino acid oxidase (34). Our study documents peroxide generation by both D- and L-amino acid oxidase utilizing a sensitive spectrophotometric method described by Nakano et al. (16) to measure small quantities of free hydrogen peroxide. Leukocyte homogenates from guinea pigs contained significantly greater activity of both peroxide producing amino acid oxidases compared with human PMN homogenates. This finding might have been expected since guinea pig PMN demonstrate larger resting and phagocytizing oxidative activities and have greater activities of NADH oxidase than do human PMN (8). Hydrogen peroxide–dependent formate-^{14}C oxidation is also greater in guinea pig PMN than in human PMN (8).

Since human PMN contain high concentrations of L-alanine and other L-amino acids (32), could L-amino acid oxidase qualify as the primary respiratory enzyme responsible for the cyanide-insensitive respiratory burst in PMN during phagocytosis? In addition, could the hydrogen peroxide produced by amino acid oxidase be responsible for bacterial killing in PMN? The rate of oxygen consumed by L-amino acid oxidase, a cyanide insensitive flavoprotein, compares favorably with the rate of the respiratory burst in PMN during phagocytosis, i.e., 72 ±29 mμmoles oxygen consumed $hr^{-1}mg^{-1}$ for L-amino acid activity of the whole cell homogenate and 63 mμ moles oxygen $hr^{-1}mg^{-1}$ (8) for the respiratory burst. However, our studies of the PMN from three children with CGD argue against both possibilities. CGD PMN lack a respiratory burst and fail to generate peroxide during phagocytosis; they do not kill nonperoxide-forming bacteria which are the source of repeated infections in these patients. Homogenates of PMN obtained from two males and one female patient with CGD demonstrated normal generation of hydrogen peroxide from L-amino acid oxidase as well as D-amino acid oxidase. On the basis of these findings, it seems likely that neither enzyme is the primary source of H_2O_2 generation during phagocytosis.

The findings reported in this paper while documenting the presence of amino acid oxidase activity in PMN, lend further support to the theory that NADH oxidase is the principle enzyme responsible for the respiratory burst and peroxide-mediated bacterial killing in PMN.

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