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LYSOSOMAL ENZYME RELEASE FROM POLYMORPHONUCLEAR LEUKOCYTES IN PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE: THE EFFECT OF HYDROGEN PEROXIDE ON RELEASED ENZYME ACTIVITIES*)

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ABSTRACT

Lysosomal enzyme release from PMN exposed to STZ was examined using PMN from normal and CGD donors. Normal PMN showed an increase of extracellular lysosomal enzyme activity and a marked reduction of totol (extra-plus intra-cellular) enzyme activity after phagocytosis. On the other hand, in PMN from CGD patients, such a reduction of total enzyme activity was not observed and much more enzyme than normal was released extracellularly. When the supernatant from PMN from CGD patients after phagocytosis of STZ was incubated with an artificial H_2O_2 generating system, glucose plus glucose oxidase, the enzyme activities were greatly suppressed as the amount of H_2O_2 rose. A similar result was obtained with the addition of glucose oxidase during phagocytosis in PMN from CGD patients. These findings suggest that the presence of H_2O_2 might suppress lysosomal enzyme activities and result in inhibition of lysosomal enzyme release.

INTRODUCTION

Polymorphonuclear leukocytes (PMN) selectively discharge lysosomal constituents not only into phagocytic vesicles but also into extracellular environment when exposed to phagocytosable stimuli[1-5]. The secretory events are usually coupled with the activation of oxidative metabolism in PMN, termed the respiratory burst, with the formation of highly reactive oxygen derivatives[6-8]. These cellular responses are essential for the intracellular microbicidal activity of PMN and also contribute to the process of inflammation. PMN from patients with chronic granulomatous disease (CGD) ingest particles and degranulate normally, but fail to generate the normal respiratory burst associated with phagocytosis[9,10].

The present study was designed to investigate the relationship between lysosomal enzyme release and the respiratory burst during phagocytosis by using normal and CGD PMN. The results suggest that H_2O_2 produced during phagocytosis might inhibit lysosomal enzyme release.

MATERIALS AND METHODS

Reagents: Zymosan A (Sigma Chemical

*) 小林正夫，田中丈夫，臼井朋包：慢性肉芽腫症患者好中球よりのリソゾーム酵素放出について

Co.) was boiled, washed, and opsonized with autologous serum for 30 min. at 37°C and thoroughly washed with Hanks' balanced salt-solution (HBSS, pH 7.4) with 5.5 mM glucose (HBSSG). Glucose oxidase (GO, type I, Boehringer Mannheim Biochemicals) was diluted in HBSS. Heat-inactivated GO was autoclaved for 20 min. at 120°C.

Preparation and incubation of PMN: Heparinized venous blood was obtained from healthy adult volunteers and six patients with CGD (three males and three females) diagnosed according to previously described criteria[9,10]. PMN with 98% purity were prepared by centrifugation on Ficoll-diatrizoate sodium gradients followed by dextran sedimentation and lysis of erythrocytes with Tris-buffered ammonium chloride (0.83%). The prepared PMN were washed twice with HBSSG and suspended at a concentration of 5×10^6 cells/ml. PMN were then incubated in HBSSG alone or in the presence of serum-treated zymosan (STZ) as phagocytosable particles. The reaction was stopped by immediate cooling in an ice bath. After centrifugation (250*g*, 4°C), the supernatant was decanted. The cells were resuspended in HBSSG containing 0.1% (w/v) triton X-100 and ultrasonicated (20 kHz) in an ice bath for 30 sec. using a pulser cycle (Cell disruptor w-225 : Heat system-Ultrasonic Inc.). Portions of each fraction were then assayed for enzymes.

Determination of enzyme activities: The conditions for measurement have been reported previously[11,12]. Acid phosphatase (E. C. 3.1.3.2), N-acetyl-β-glucosaminidase (E. C. 3.2.1.30), β-glucuronidase (E. C. 3.2.1.31), α-fucosidase (E. C. 3.2.1.51), and α-mannosidase (E. C. 3.2.1.24) were assayed with 4-metylumbelliferil (4MU) compounds (Koch-Light Labs. Ltd.) as substrates and were determined by incubating 100 μl of samples with 100 μl aliquots of the following substrates: 0.2 mM 4MU-phosphate in 0.2 M acetate buffer, pH 5.0, 0.2 mM 4MU-2-acetamide-2-deoxy-β-D-glucopyranoside in 0.2 M citrate buffer, pH 4.5, 0.2 mM 4MU-β-D-glucuronide in 0.2 M acetate buffer, pH 3.5, 0.2 mM 4MU-α-L-fucopyranosids in 0.2 M acetate buffer, pH 4.5. The 4MU formed was measured fluorometrically in Hitachi 204-R spectrofluorometer. Standard assays contained 0.05 mM 4-methylumbelliferone. Lysozyme (E. C. 3.2.1.17) activity was determined by measuring turbidimetrically the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp.) at pH 6.2. Crystalline hen egg-white lysozyme (Worthington Biochemical Corp.) was used as a standard[13]. Lactate dehydrogenase (LDH) was measured by the method of Wacker et al.[14] Intra-and extracellular enzymes were expressed as a percentage of total enzymatic activities measured in simultaneously run cell incubations without STZ. A ratio of cells to STZ of 1 : 20 was found to result in stable enzyme release without cell damage from preliminary experiments. At the concentrations employed, none of the agents affected PMN viability as tested by trypan blue exclusion and LDH levels in extracellular fluid.

RESULTS

There were no differences in total enzyme activities between normal and CGD PMN before phagocytosis of STZ (Table 1). Fig. 1 shows the observed intracellular and extracellular enzyme levels during phagocytosis. With normal PMN, extracellular lysosomal enzyme activities increased as phagocytosis progressed. However, there was a marked reduction of total (intracellular plus extracellular) enzyme activity: 25 to 50% less acid phosphatase, N-acetyl-β-glucosaminidase, β-glucuronidase, and α-fucosidase. The losses of α-mannosidase and lysozyme activities were small. On the other hand, PMN from CGD patients showed no

Table 1. Lysosomal enzyme activities in PMN

	Normal PMN (n=22)	CGD PMN (n=6)
Acid phosphatase[1]	2108±706[3]	2071±714
N-acetyl-β-glucosaminidase[1]	904±295	814±226
β-Glucuronidase[1]	778±240	670±171
α-Fucosidase[1]	204±67	162±49
α-Mannosidase[1]	190±81	206±49
Lysozyme[2]	5.2±2.2	5.7±1.5

[1] Enzyme activities are expressed as picomoles of substrate hydrolyzed per min. per 5×10^6 cells.
[2] Lysozyme activity of 5×10^6 cells is expressed in equivalents of micrograms of egg-white lysozyme standard.
[3] Mean±SD

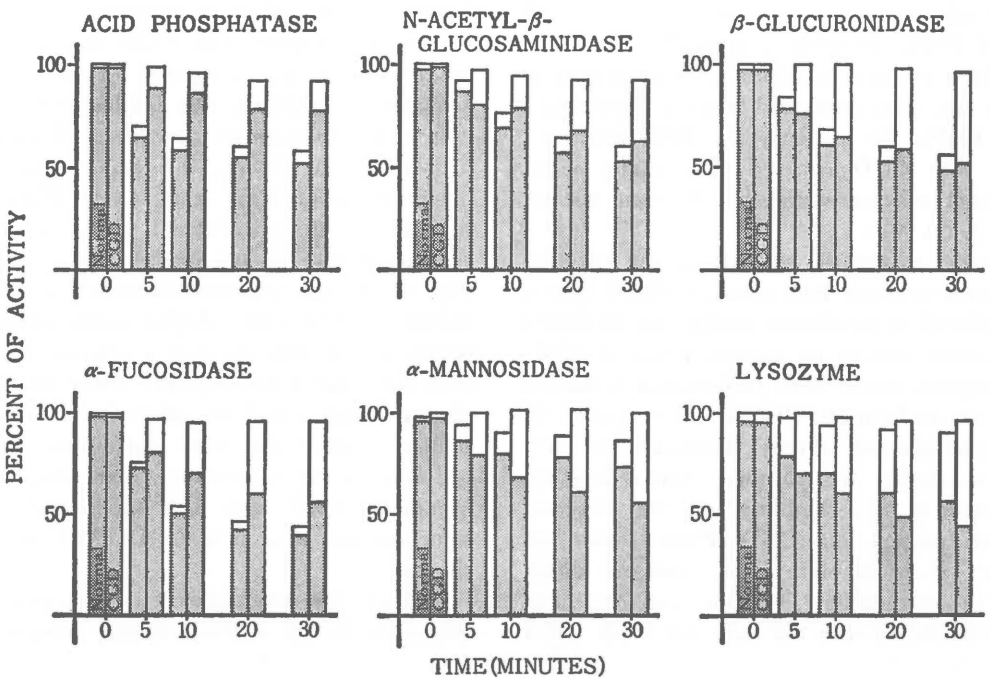

Fig. 1. Intracellular and extracellular enzyme levels during phagocytosis
Intracellular (shaded bars) and extracellular (open bars) enzyme levels are expressed as the percentage of total enzyme activities in PMN. Each bar represents the average of the results from two normal (left bar) and two CGD (right bar) donors. The conditions were described in the text.

Table 2. Intracellular and extracellular enzyme activities after phagocytosis[1]

	% of total activity					
	Acid phosphatase		N-acetyl-β-glucosaminidase		β-glucuronidase	
	Intra[2]	Extra[2]	Intra	Extra	Intra	Extra
Normal PMN (n=22)	63.3±6.6[2]	9.2±2.2	53.5±10.8	12.1±4.3	60.7±13.0	7.7±3.6
CGD PMN (n=6)	70.0±3.9	20.1±4.0[3]	56.0±6.7	40.1±9.0[3]	65.2±12.0	38.2±12.0[3]
	% of total activity					
	α-Fucosidase		α-Mannosidase		Lysozme	
	Intra	Extra	Intra	Extra	Intra	Extra
Normal PMN (n=22)	37.8±10.9	8.9±5.1	66.0±8.1	14.2±4.9	56.1±6.0	38.4±7.0
CGD PMN (n=6)	51.3±8.9	41.7±10.0[3]	58.5±7.0	40.5±10.1[3]	31.7±5.1	64.0±4.9[3]

[1] The ratio of PMN to STZ was 1:20 and the incubation time was 20 minutes.
[2] Intracellular (Intra) and extracellular (Extra) enzyme levels are expressed as the percentage of total enzyme activities in PMN (Mean±SD).
[3] $p<0.001$ for test of equality with extracellular level in normal PMN.

reduction of total enzyme activity and seemed to extrude more of these enzymes than did normal PMN. In table 2, intracellular and extracellular enzyme activities after phagocytosis for 20 min. were compared between normal and CGD PMN. For all enzymes, PMN from patients with CGD released significantly more lysosomal enzyme extracellularly than normal PMN ($p<0.001$).

In order to test the stability of the enzymes that were released into solution, PMN lysates were placed in incubation media and measured for enzyme activity at various times at 37°C. All enzymes except acid phosphatase remained stable for an hour in HBSSG (pH 7.4) at 37°C. Acid phosphatase activity decreased by 25% after incubation for 20 min. There was no difference in the stability of the enzymes between normal and CGD PMN (data not shown). It is therefore likely that the difference in extracellular enzyme activity after phagocytosis between normal and CGD PMN is not due to the enzyme stability. Mixtures of the supernatants after phagocytosis and PMN lysates from normal and CGD patients were examined for the presence of an enzyme activator or inhibitor, but neither was found.

In order to evaluate the effect of oxidative metabolism of PMN, the supernatant after phagocytosis of STZ from CGD PMN was incubated with an artificial H_2O_2 generating system, glucose-glucose oxidase. As shown in Fig. 2, enzyme activities decreased as the amount of H_2O_2 rose. Alpha mannosidase and lysozyme activities showed a smaller decrease than the other four enzymes. Heat-inactivated glucose oxidase had no effect on enzyme activities. Under the same conditions, enzyme activities in the supernatant after phagocytosis of normal PMN were also suppressed in the same manner as seen in CGD PMN (data not shown).

Furthermore, when glucose oxidase was added externally to the medium during phagocytosis

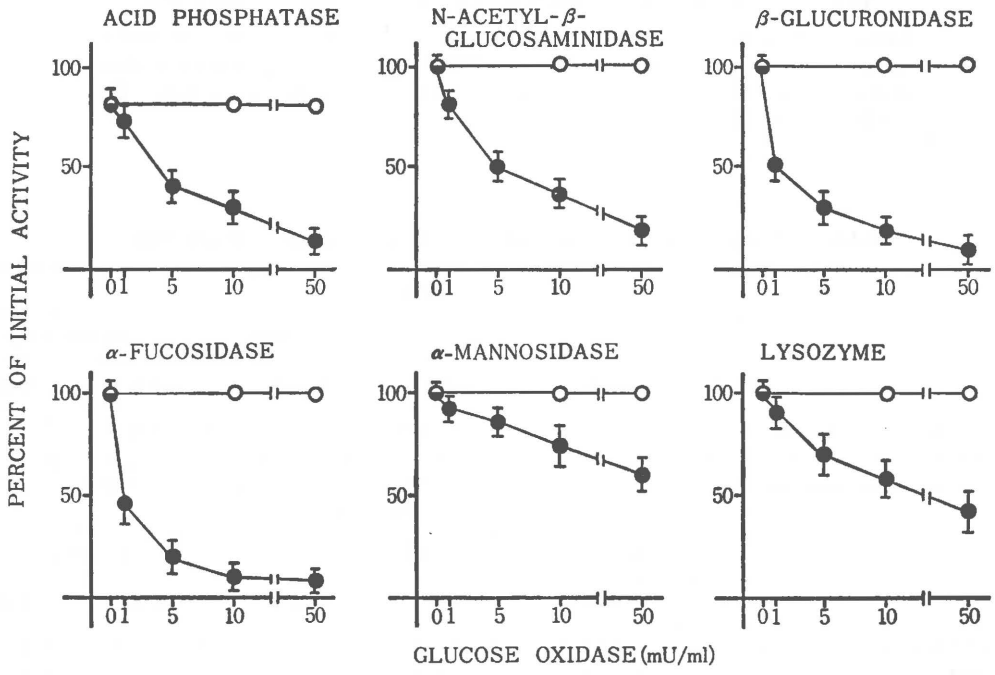

Fig. 2. Effect of H_2O_2 produced from glucose-glucose oxidase on enzyme activities in the supernatant after phagocytosis in CGD PMN

The reaction mixture contained the supernatant in HBSSG (glucose 5.5 mM) and either glucose oxidase (●) or heat-inactivated glucose oxidase (○). The data are expressed as a percentage of the initial enzyme activity of the supernatant and mean ± SD of four experiments.

Table 3. Effect of H_2O_2 produced from glucose-glucose oxidase on lysosomal enzymes in CGD PMN during phagocytosis[1]

	% of total activity					
	Acid phosphatase		N-acetyl-β-glucosaminidase		β-Glucuronidase	
	Intra[2]	Extra[2]	Intra	Extra	Intra	Extra
Case 1 (C. I.) PMN+STZ	70[2]	24	67	28	61	36
+GO (5 mU)	71	13	68	7	60	5
Case 2 (N. I.) PMN+STZ	68	21	56	39	63	35
+GO (5 mU)	67	12	53	13	60	7

	% of total activity					
	α-Fucosidase		α-Mannosidase		Lysozyme	
	Intra	Extra	Intra	Extra	Intra	Extra
Case 1 (C. I.) PMN+STZ	60	38	64	38	40	60
+GO (5 mU)	50	5	60	25	40	25
Case 2 (N. I.) PMN+STZ	50	45	58	40	38	60
+GO (5 mU)	46	7	57	25	36	28

[1] The ratio of PMN to STZ was 1 : 20 and the incubation time was 20 minutes.
[2] Intracellular (Intra) and extracellular (Extra) enzyme levels are expressed as the percentage of total enzyme activities in PMN.

in PMN from CGD patients, decreases of extracellular lysosomal enzyme activities and of total enzyme levels were observed (Table 3).

DISCUSSION

It is well known that the contents of both azurophil and specific granule constituents of PMN are released not only into phagocytic vesicles but also into extracellular space[1-5]. In addition to the increase of extracellular enzyme activity, a decrease of total enzyme levels after phagocytosis has been reported, but the cause of this phenomenon is not yet clear[2]. The present study showed a remarkable difference in the levels of released lysosomal enzymes between normal and CGD PMN after phagocytosis. As shown in Fig. 1, the decrease in enzyme levels in normal PMN was due mostly to a loss of released enzyme. PMN from patients with CGD showed higher extracellular levels of enzyme activity than normal PMN and unchanged levels of total enzyme activity after phagocytosis. PMN from patients with CGD are characterized by a lack of the respiratory burst despite normal phagocytosis[9,10]. From the evidence that PMN from CGD patients maintained released enzyme activities, it may be postulated that oxidative metabolism associated with phagocytosis is related to the loss of total enzyme activity in normal PMN. Stossel et al. have demonstrated normal degranulation and normal specific activity of lysosomal enzymes in phagocytic vesicles in PMN from CGD patients, but they did not measure the exocytic enzyme activity[15].

In order to evaluate the effect of oxidative metabolism, we used a H_2O_2 generating system, glucose plus glucose oxidase, which is more closely analogous to continuous H_2O_2 formation by phagocytosing PMN and has improved the bactericidal activity in PMN from CGD patients[16,17]. Under these conditions, released lysosomal enzyme activities from PMN from CGD patients were greatly suppressed. Furthermore, the addition of glucose oxidase during phagocytosis in PMN from CGD patients resulted in lower extracellular enzyme activities and loss of total enzyme levels as was seen in normal PMN. H_2O_2 had less of an effect of α-mannosidase and lysozyme than on the other four enzymes. H_2O_2 has been able to generate a number of active oxygen species by interaction with myeloperoxidase and halides or with the superoxide anion[8,18]. Although the precise

mechanism of suppression of lysosomal enzyme activities and the difference in susceptibility to H_2O_2 among these enzymes are unknown, this result parallels the degree of the total enzyme level decrease during phagocytosis as shown in Fig. 1. It is therefore possible that the suppression of enzyme activity may be dependent in part) on H_2O_2 production.

Recently, Newburger et al. reported that not only CGD peripheral PMN but also cultured cells from CGD bone marrow showed supranormal degranulation accompanying phagocytosis of STZ[19]. They suggested that this was due to the lack of the toxic autooxidative effect on cellular function[20] that might limit degranulation in normal PMN[19]. Our results suggest in addition that H_2O_2 produced during phagocytosis has the capability to suppress the activity of released enzymes. These two possibilities may help to explain the differences of released enzyme activities between normal and CGD PMN.

Thus, although there are many unexplained and delicate mechanisms in the interaction of lysosomal enzyme release and oxidative metabolism associated with phagocytosis, the balance between these two functions of PMN may have an important role at sites of inflammation.

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