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Research Article

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Phagocytosing Human Neutrophils Inactivate Their Own Granular Enzymes

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ABSTRACT During phagocytosis, neutrophils generate reactive oxygen metabolites and release lysosomal enzymes into the extracellular medium. We have investigated the possibility that these enzymes are inactivated by the oxygen compounds. Phagocytosing neutrophils from 12 patients with chronic granulomatous disease, which do not generate these oxygen metabolites, released two to three times more activity of lysozyme and β -glucuronidase than did normal neutrophils. This difference proved to be due to a decrease of ~20% of the total activity of these enzymes in normal neutrophils, but not in neutrophils of patients with chronic granulomatous disease. This inactivation of enzymes took place during phagocytosis of opsonized zymosan particles as well as during stimulation of normal cells with phorbol myristate acetate. The inactivation was not due to formation of inhibitors. The lysosomal enzymes were not inactivated when the neutrophils were stimulated under anaerobic conditions. Addition of catalase, superoxide dismutase, or albumin gave no protection against the oxidative damage; reduced glutathione gave partial protection. The oxidative inactivation was more pronounced in the presence of azide. Measurement of the activity and the amount of protein of acid α -glucosidase in the cells showed that the specific activity of this enzyme decreased by ~50% during 30 min of phagocytosis. This indicates that the inactivation of the lysosomal enzymes takes place in the phagolysosomes, before the enzymes have leaked into the extracellular medium.

INTRODUCTION

During phagocytosis, neutrophils degranulate, i.e., the granules in these cells fuse with the membrane around

the ingested material and discharge their contents into the phagosomes (1, 2). Because this process starts before the phagosomes are closed, a certain amount of the enzymes from the granules leaks into the extracellular medium (3, 4). Concomitantly, the neutrophils also generate highly reactive oxygen metabolites, e.g., superoxide and hydrogen peroxide (5–8). These compounds are also released into the phagosomes and, with the granular enzymes, cause the death of ingested microorganisms (9, 10).

The neutrophils themselves are also vulnerable to the reactive oxygen products, however. These cells contain superoxide dismutase, catalase, and the glutathione redox system (11–13) that protect them. Nevertheless, several recent studies have indicated that neutrophils may be damaged by their own oxidative products, leading to a decreased functioning of these cells (14–16). For instance, neutrophils from patients with chronic granulomatous disease (CGD),¹ which fail to generate the oxygen metabolites (6, 8, 17), move at a faster rate into the pores of a filter than do neutrophils from healthy donors (16).

Degranulation of neutrophils from CGD patients has been claimed to be either decreased (18), retarded (19), or normal (20–24). The present report describes a careful study with neutrophils from 12 CGD patients. In each experiment with these cells we found two to three times higher activity of granular enzymes in the extracellular medium during phagocytosis than with neutrophils from healthy controls. This difference proved to be the result of inactivation of these enzymes by oxidative products of normal neutrophils.

METHODS

Materials. Zymosan (ICN Pharmaceuticals, Inc. Cincinnati, Ohio) was opsonized by incubation in fresh normal serum

¹ *Abbreviations used in this paper:* CGD, chronic granulomatous disease; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate.

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as described (25). The serum-treated zymosan was suspended in 154 mM NaCl and used in the cell incubations at 1 mg/ml ($6-7 \times 10^7$ particles/ml).

Phorbol myristate acetate (PMA) (Consolidated Midland Corp., Brewster, N. Y.) was dissolved in dimethyl sulfoxide (1 mg/ml), diluted with 154 mM NaCl and used at 500 ng/ml in the cell incubations. The final concentration of dimethyl sulfoxide, 0.05% vol/vol, had no effect on the cells.

Rabbit anti-human acid α -glucosidase, coupled to beads of Sepharose 4-B, was prepared as described (26).

Donors. Venous blood was obtained from healthy donors and from 12 patients with CGD (4 females, 8 males). The clinical criteria for diagnosis of CGD were histories of recurrent infections of skin, lungs, and lymph nodes with predominantly *Staphylococcus aureus*. Furthermore, in most patients the presence of granulomas was documented. In vitro, the neutrophils of these patients showed normal chemotaxis towards casein; absence of increased oxygen consumption, superoxide formation, and hydrogen peroxide generation during ingestion of opsonized zymosan particles; diminished intracellular killing of *S. aureus*; and normal activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, and glutathione peroxidase. The methods used for these tests have been described in reference 27. During the study described in this paper, all CGD patients were in good clinical condition; blood was drawn during polyclinical check-ups.

Cell isolation. Neutrophils were isolated from fresh defibrinated blood as described (28). The neutrophils were suspended in a medium (pH 7.4) that consisted of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.6 mM CaCl_2 , 1.0 mM MgCl_2 , 5.5 mM glucose, and 0.5% (wt/vol) human albumin. The final cell suspension contained 95% neutrophils; the remaining cells were lymphocytes.

Enzyme assays. Lysozyme (*N*-acetylmuramide glycanohydrolase, E.C. 3.2.1.17) was measured with a modification of a method described by Parry et al. (29). *Micrococcus luteus* (Worthington Biochemical Corp., Freehold, N. J.) was used at 0.17 mg/ml in 66.7 mM sodium-phosphate buffer (pH 6.2). Lysate (50 μl) of 10^5 neutrophils or 50 μl cell-free supernatant fluid from an incubation with about 8×10^6 neutrophils/ml were added to 2.95 ml of the *M. luteus* suspension. The decrease in turbidity at 540 nm was determined during 10 min at 25°C. Values obtained with *M. luteus* only were subtracted from the values obtained with cell-derived material. The variance of this assay was 5.2%.

β -glucuronidase (beta-D-glucuronide glucuronohydrolase, E.C. 3.2.1.31) was measured according to the method described by Brittinger et al. (30). Phenolphthalein mono- β -glucuronic acid (Sigma Chemical Co., St. Louis, Mo.) was used at 0.83 mM in 100 mM sodium-acetate buffer, pH 4.4. Lysate (100 μl) of 2×10^5 neutrophils or 100 μl cell-free supernate of an incubation with 8×10^6 neutrophils/ml were added to 1.1 ml of the phenolphthalein glucuronidate solution. This mixture was incubated for 18 h at 37°C. The assay was linear with time for 24 h. The reaction was stopped with 2.5 ml of an ice-cold 200-mM glycine NaOH buffer (pH 10.5). The absorbance of the liberated phenolphthalein was measured spectrophotometrically at 550 nm. Values obtained with substrate only were subtracted from the values obtained with cell-derived material. The variance of this assay was 3.0%.

Acid α -glucosidase (alpha-D-glucoside glucohydrolase, E.C. 3.2.1.20) was measured as described by Schram et al. (26). A sample of 250 μl , containing either lysate of 5×10^5 neutrophils or cell-free supernatant fluid of an incubation with 8×10^6 neutrophils/ml, was incubated with 150 μl sodium-acetate buffer (100 mM, pH 4.0) and 100 μl p-nitrophenyl- α -D-glucoside (6.6 mM, Koch-Light Laboratories Ltd., Coln-

brook, Buckinghamshire, U. K.) for 24 h at 37°C. The assay was linear with time for 76 h. The reaction was stopped by adding 1 ml 0.3 M glycine-NaOH buffer (pH 10.6). The liberated p-nitrophenol was measured spectrophotometrically at 405 nm. Values obtained from blanks with reagents only were subtracted from values obtained with cell-derived material. The variance of this assay was 2.5%.

Lactate dehydrogenase (L-lactate:NAD oxido-reductase, E.C. 1.1.1.27) was determined according to Wacker et al. (31). A sample of 200 μl , containing either lysate of 4×10^5 neutrophils or cell-free supernatant fluid of an incubation with 8×10^6 neutrophils/ml, was added to 2.8 ml of 0.26 mM NADH and 0.52 mM sodium pyruvate in 38.3 mM sodium-phosphate buffer (pH 7.4). The decrease in absorbance at 340 nm was measured for 10 min. Values obtained from blanks with reagents only were subtracted from the values obtained with cell-derived material. The variance of this assay was 2.8%.

Precipitation of acid α -glucosidase. The amount of acid α -glucosidase protein was estimated by precipitation with Sepharose-coupled antibody to this enzyme (see Materials). Sepharose-coupled antibody reacts with active as well as with inactive (denatured) enzyme (unpublished results). Beads coated with normal rabbit serum did not absorb any acid α -glucosidase. Samples of 300 μl , containing lysate of 24×10^5 neutrophils, were incubated with various amounts of beads for 45 min at 20°C. Next, the samples were centrifuged for 2 min at 3,000 g, and the remaining acid α -glucosidase activity was measured in the supernatant fluid. The coefficient of variation of this assay was <1% (calculated from 33 duplicate samples). The between-assay variance of this method, as used in this study (with dilutions of 10–25%, Fig. 8), was ~15%.

Cell incubations. Neutrophils were preincubated at 37°C for 10 min. The stimulating compounds were then added; the suspension was thoroughly mixed, divided over several tubes, and incubated at 37°C. For each sample, a different tube was used, because taking samples from one tube introduced artifacts from cells sticking onto the tube. At different times, tubes were cooled to 0°C and centrifuged for 10 min at 250 g and 4°C. The supernatant fluid was carefully collected, and the pellet was lysed in medium with 0.2% (wt/vol) Triton X-100.

Anaerobic incubations. Medium was made oxygen poor by passing hydrated nitrogen gas through it for 1 h. Cells were spun down and the supernatant fluid was removed. Nitrogen gas was then led into the tube, and oxygen-poor medium was added 10 s later. The cells were resuspended in this medium and the whole procedure was repeated. Stimulating compounds added to the cells were also suspended or dissolved in oxygen-poor solutions. Throughout the cell incubations, nitrogen gas was led over the cell suspensions.

RESULTS

Differences between normal and CGD neutrophils. Fig. 1 shows the release of granular enzymes into the supernatant fluid of neutrophils from normal donors and eight CGD patients during a 30-min incubation at 37°C with opsonized zymosan particles. CGD neutrophils released twice as much lysozyme and three times as much β -glucuronidase activity as did normal neutrophils. Neutrophils from four additional CGD patients were only assayed at 0 and 30 min after addition of zymosan particles; with these cells, similar

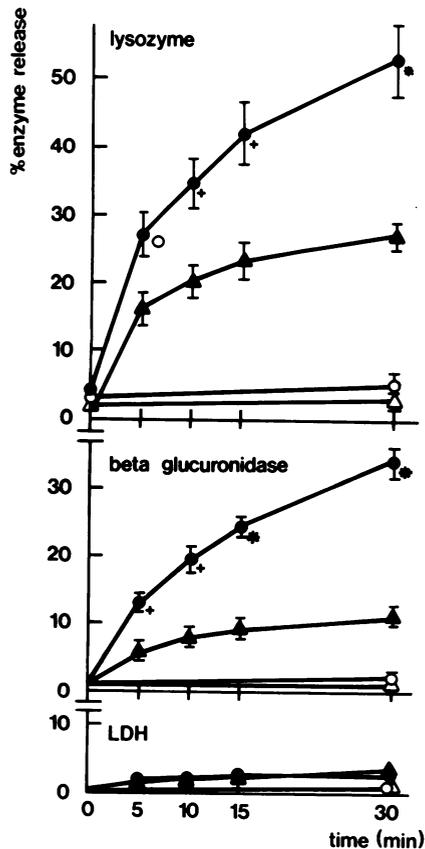


FIGURE 1 Release of enzymes during phagocytosis. The results are given as percentage of the enzymic activities in the cells at the time 0. Enzymic activity in the supernatant fluid of normal cells incubated with serum-opsonized zymosan (1 mg/ml) (▲); normal cells incubated with an equal volume of 154 mM NaCl (Δ); CGD cells incubated with serum-opsonized zymosan (1 mg/ml) (●); and CGD cells incubated with an equal volume of 154 mM NaCl (○). Results are the means of eight paired experiments \pm SEM. Statistical significance between results obtained with phagocytosing normal neutrophils and CGD neutrophils (*t* test for paired observations): *, $P < 0.001$; +, $P < 0.005$; ○, $P < 0.01$. Enzymatic activity in nonincubated, normal neutrophils (mean \pm SEM of 20 preparations): lysozyme, $56 \pm 4 \mu\text{g}/10^7$ cells; β -glucuronidase, $2.3 \pm 0.2 \text{ nmol}/\text{min}$ per 10^7 cells; LDH, $425 \pm 31 \text{ nmol}/\text{min}$ per 10^7 cells. For the eight preparations of CGD cells, these values were: lysozyme, $41 \pm 4 \mu\text{g}/10^7$ cells; β -glucuronidase, $2.0 \pm 0.3 \text{ nmol}/\text{min}$ per 10^7 cells; LDH, $485 \pm 60 \text{ nmol}/\text{min}$ per 10^7 cells.

results were obtained to those of other CGD cells (not shown). The release of lactate dehydrogenase (LDH) in these experiments was always $< 5\%$, indicating that the cells remained intact during the incubations. Resting cells released only minimal amounts of enzymes. Thus, it seems that phagocytosing neutrophils from CGD patients release more granular enzyme activity into the surrounding medium than do normal neutrophils.

In two of the above-mentioned experiments, the

remaining enzymic activity in the cells was also measured. Fig. 2 shows the results. In the CGD neutrophils, the enzymic activity that disappeared from the cells was completely recovered in the cell-free supernatant fluids. In normal neutrophils, however, the decrease in enzymic activity from the cells was not completely compensated by a similar increase in enzymic activity in the cell-free supernatant fluids. This phenomenon is also shown in Fig. 3, in which the sum of the enzymic activities in the cells and in the

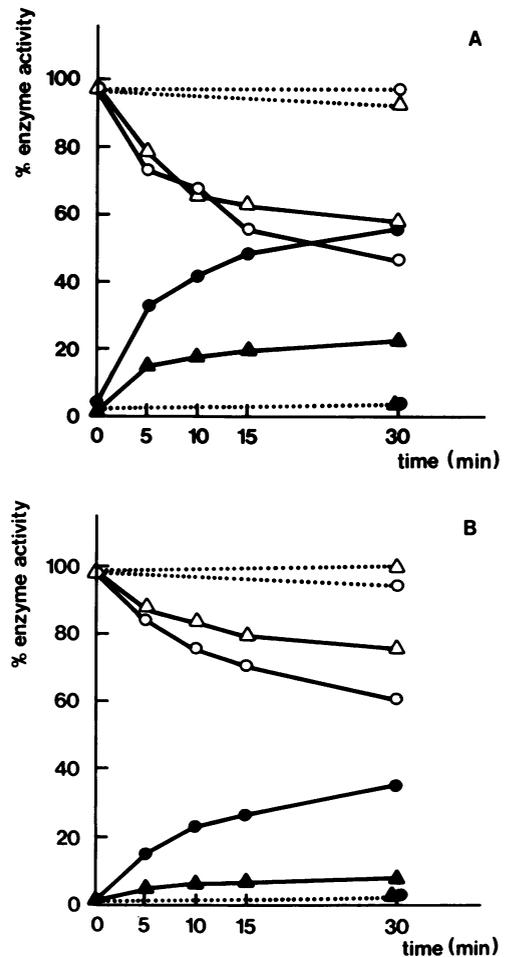


FIGURE 2 Lysozyme (A) and β -glucuronidase (B) activity in supernate and in neutrophils during phagocytosis. The results are given as percentage of the enzymic activities in the cells at time 0. At various times, samples were taken and centrifuged. The enzymic activity was measured in the cell-free supernatant fluid and in the pellet. Enzymic activity: in the pellet of normal neutrophils is (▲); in the supernatant fluid of normal neutrophils (▲); in pellet of CGD neutrophils (○); and in supernatant fluid of CGD neutrophils (●). Solid line, cells incubated with serum-opsonized zymosan (1 mg/ml); broken line, cells incubated with an equal volume of 154 mM NaCl. Results are the means of two experiments. For mean enzymic activities in nonincubated cells, see legend to Fig. 1.

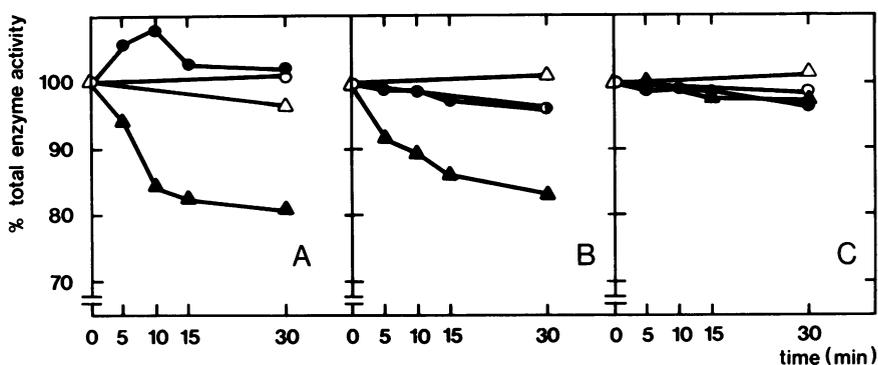


FIGURE 3 Change in total enzymic activity of phagocytosing neutrophils. The results are given as percentage of the enzymic activities in the cells at time 0. The total activity was calculated by summation of the percentage release into the medium and the percentage activity left in the cells. Total enzymic activity of: normal cells incubated with serum-opsonized zymosan (1 mg/ml) (▲); normal cells incubated with an equal volume of 154 mM NaCl (△); CGD cells incubated with serum-opsonized zymosan (1 mg/ml) (●); CGD cells incubated with an equal volume of 154 mM NaCl (○). Results are the means of two experiments. For mean enzymic activities in nonincubated cells, see legend to Fig. 1. A, lysozyme; B, β -glucuronidase; C, LDH.

medium is depicted. In CGD neutrophils, the total activity of the granular enzymes remained undiminished during the 30-min incubation, but in normal cells there was a loss of lysozyme activity of ~20% and of β -glucuronidase activity of ~17%. In CGD, as well as in normal neutrophils, the LDH activity remained constant. In control experiments with suspensions of phagocytosing cells treated with Triton X-100 at $t = 30$ min, the same decrease in total activity of granular enzymes was found (not shown), thus excluding a selective removal of enzymic activity from normal neutrophils by the separation of supernatant fluid and cell pellet. Mixing experiments did not reveal an inhibitor of granular enzyme activity in normal phagocytosing neutrophils or their supernatant fluids (not shown).

Similar results were obtained with PMA as a (soluble) stimulus for enzymic exocytosis (see Fig. 4). Although this agent induces only minimal release of enzymes from azurophilic granules (32), a profound inactivation of both lysozyme and β -glucuronidase was observed in normal neutrophils, but not in CGD neutrophils.

Mechanism of enzyme inactivation. Because it is well known that CGD neutrophils do not generate bactericidal oxygen metabolites, we investigated the possibility that granular enzymes are damaged in normal neutrophils by these reactive oxygen compounds. Table I shows that the inactivation of granular enzymes was not observed under anaerobic conditions. Under these conditions, the activities of the granular enzymes that were released into the extracellular medium were as high as those found with CGD neutrophils. We did not observe inactivation of granular enzymes from CGD neutrophils by incubation of these cells with glucose plus glucose oxidase (as a H_2O_2 -

generating system). Glucose oxidase was ineffectively coupled to opsonized zymosan particles (cyanogen bromide method) (26).

Addition of catalase (360 U/ml), superoxide dismutase (1 μ M), or a combination of these two enzymes to incubations of normal neutrophils with opsonized zymosan under aerobic conditions had no protective effect, although the enzymes retained their activity

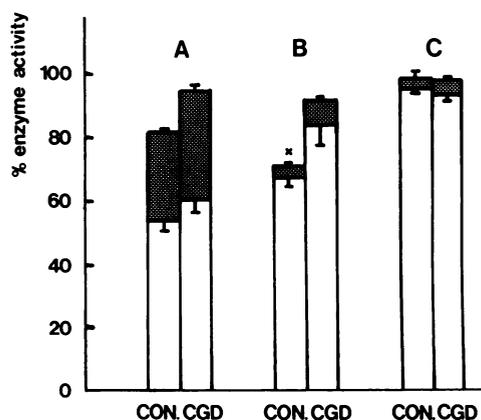


FIGURE 4 Enzyme distribution induced by PMA. Neutrophils of healthy donors (CON) and of CGD patients were incubated with 0.5 μ g PMA/ml for 30 min at 37°C. The enzymic activities of lysozyme (A), β -glucuronidase (B), and LDH (C) were measured in the supernatant fluid and in the cell pellet. Values are given as percentage of the enzymic activities in the cells at time 0. Shaded bars, enzymic activities in the supernates; open bars, enzymic activities in the pellets. Results are the mean of three experiments \pm SEM. Statistical significance between results obtained with normal neutrophils and those obtained with CGD cells (t test for paired observations): \times , $P < 0.05$. For mean enzymic activities in nonincubated cells, see legend to Fig. 1.

TABLE I
Effect of Anaerobiosis on Total Enzymic Activities

Addition	Lysozyme*		β -Glucuronidase*		LDH*	
	Air	N ₂	Air	N ₂	Air	N ₂
NaCl	98	102	100	101	100	100
STZ	84	99§	80	95§	94	98

* Total enzymic activity in supernate plus cell pellet after 30 min incubation with or without serum-treated zymosan (STZ, 1 mg/ml), in an aerobic or an anaerobic system. The values are given as percentage of the enzymic activity at time 0. Values given as means of three experiments.

§ $P < 0.005$, statistical significance between results obtained with aerobically and anaerobically incubated cells (t test for paired observations).

during these experiments (not shown). Addition of extra albumin up to 6% (wt/vol) or catalase coupled to opsonized zymosan particles had no effect either. The failure of particle-coupled catalase to protect the cells may be due to a decrease in the pH in the phagosomes to about 4–5 (33) as opposed to the pH optimum of catalase at 7.5. Indeed, the activity of zymosan-coupled catalase at a pH between 4 and 5 ranged from 6 to 50% of the optimal activity (not shown). Addition of reduced glutathione to a concentration of 5 mM gave partial protection (Fig. 5). The total enzymic activity in

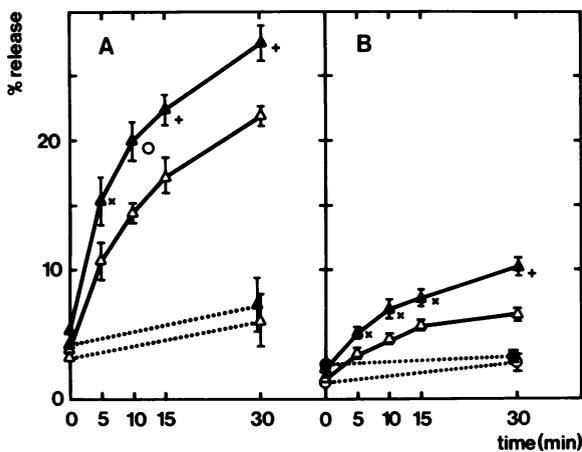


FIGURE 5 Effect of reduced glutathione on enzymic activities in supernatant fluids of phagocytosing neutrophils. The results are given as percentage of the enzymic activities in the cells at time 0. A, lysozyme; B, β -glucuronidase. Enzymic activity: in the supernate of normal neutrophils (Δ); in the presence of 5 mM reduced glutathione (\blacktriangle). Solid line, cells incubated with serum-opsonized zymosan (1 mg/ml); broken line, cells incubated with an equal volume of 154 mM NaCl. Results are the means of three paired experiments \pm SEM. Statistical significance between results obtained with and without reduced glutathione (t test for paired observations): +, $P < 0.005$; \circ , $P < 0.01$; \times , $P < 0.05$. For mean enzymic activities in nonincubated cells, see legend to Fig. 1.

the supernate plus cells was increased by glutathione from 82 to 94% (not shown). Glutathione had no significant effect on the release of enzymes from CGD cells (not shown).

Addition of 2 mM sodium azide to block endogenous heme compounds potentiated the inactivation of the granular enzymes (Fig. 6). In phagocytosing CGD cells, azide slightly decreased the granular enzyme activity in the cells, but did not affect the total enzyme activity in cells plus medium. Azide had no effect in the absence of zymosan (not shown).

Site of enzyme inactivation. The foregoing experiments suggest that the inactivation of the granular enzymes takes place in the cells, before release into the surrounding medium. This was further investigated by measuring the activity of acid α -glucosidase in normal and CGD neutrophils and in the supernatant fluids of these cells during ingestion of opsonized zymosan. Fig. 7 shows that CGD cells release $\sim 15\%$ of the total activity of acid α -glucosidase into the medium, with a concomitant and about equal decrease of the activity of this enzyme in the cell pellets. In contrast, normal neutrophils released hardly any activity into the medium, although the activity in the cell pellets de-

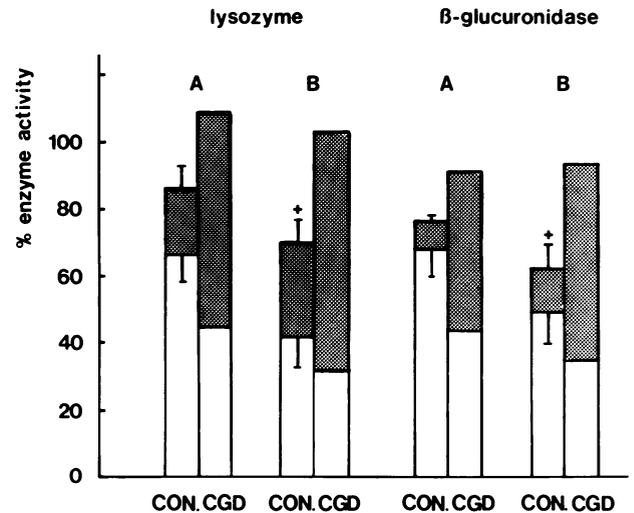


FIGURE 6 Effect of azide on enzyme distribution in phagocytosing neutrophils. Neutrophils of five healthy donors and one CGD patient were incubated with 1 mg serum-opsonized zymosan/ml, in the absence (A) and presence (B) of 2 mM sodium azide. The enzymic activities of lysozyme and β -glucuronidase were measured in the supernate and in the cell pellet. Values are given as percentage of the enzymic activities in the cells at time 0. Shaded bars, enzymic activities in the supernate; open bars, enzymic activities in the pellets. Results with normal cells are given as mean \pm SEM of five experiments. Statistical significance between results obtained with and without azide (t test for paired observations): +, $P < 0.005$. For mean enzymic activities in nonincubated cells, see legend to Fig. 1. LDH release was $< 5\%$ in each experiment (not shown).

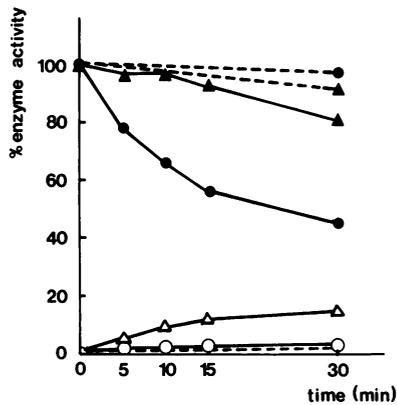


FIGURE 7 Release of acid α -glucosidase during phagocytosis. The results are given as percentage of the activity in the cells at time 0. At various times, samples were taken and centrifuged. The activity of acid α -glucosidase was measured in the cell-free supernatant fluid (open symbols) and in the pellet (solid symbols). The results were obtained with neutrophils from one healthy donor (circles) and one CGD patient (triangles). Solid line, cells incubated with serum-opsonized zymosan (1 mg/ml); broken line, cells incubated with an equal volume of 154 mM NaCl. Mean activity of acid α -glucosidase in nonincubated, normal neutrophils (mean \pm SEM of five preparations): 7.7 ± 1.5 mU/ 10^7 cells.

creased by as much as 50% during the 30-min incubation. Fig. 8 shows that the amount of acid α -glucosidase protein in the normal cells remained about constant, because the same amount of antibody was needed to remove the acid α -glucosidase activity from lysates of cells taken at time zero and from lysates of cells taken at 30 min. The same result was obtained in samples of total cell suspensions (not shown). Thus the specific activity of acid α -glucosidase in phagocytosing neutrophils decreased by $\sim 50\%$, indicating that the oxidative damage to lysosomal enzymes takes place intracellularly.

Indeed, when isolated human α -glucosidase was incubated for 30 min with zymosan-stimulated neutrophils, the specific activity of this enzyme was not affected. Similarly, isolated lysozyme was not inactivated when incubated for 30 min with 3 mM H_2O_2 , with 5 mM glucose plus 0.05 U glucose oxidase/ml (as a H_2O_2 -generating system), or with 10 mM acetaldehyde plus 0.02 U xanthine oxidase/ml (as a H_2O_2 - and O_2^- -generating system).

DISCUSSION

The degranulation of CGD neutrophils has been investigated several times before. Judged by morphological and cytochemical methods, both a decreased (18) and a normal (21) reaction were observed after bacterial ingestion. However, these techniques revealed a large variation in the extent of degranulation among neutrophils from healthy donors; hence, it is impos-

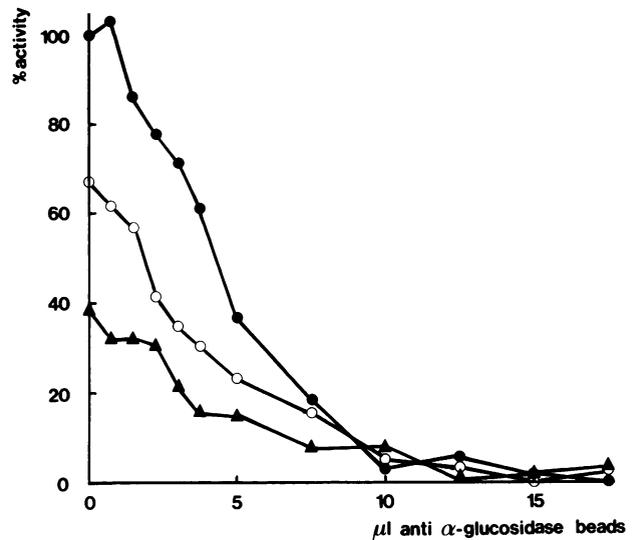


FIGURE 8 Precipitation of acid α -glucosidase with Sepharose-coupled antibody to acid α -glucosidase. Normal neutrophils (8×10^6 /ml) were incubated with serum-opsonized zymosan (1 mg/ml). At 0 and 30 min, 20 ml were centrifuged, the supernatant fluid was discarded and the pellets were lysed. A precipitation curve was then made by adding various amounts of Sepharose-coupled antibody to acid α -glucosidase. Values are given as percentage of the acid α -glucosidase activity in the cells at time 0 with no beads present. \bullet , cells at time zero; \blacktriangle , cells after 30 min of incubation with serum-treated zymosan; \circ , mixture of equal volumes of lysates from cells at time 0 and cells after 30 min of incubation with serum-treated zymosan. Values are means of duplicate determinations in one experiment. From the difference between the expected and the experimental values with the mixture, a between-assay variance of 15% was calculated. After addition of 10 μ l of beads, the results are no longer distinguishable from each other or from the abscissa.

sible to quantitate differences between patients' and normal cells (34). The change in the subcellular distribution of acid phosphatase, β -glucuronidase, and myeloperoxidase after ingestion of various particles was similar in CGD neutrophils and in normal cells (20, 23). Also after adhesion of the cells to a surface coated with aggregated human gammaglobulin, there was a normal (22) or even enhanced (24) release of granular enzymes from CGD cells. Gold et al. (19) stressed the importance of kinetic studies in this respect, because these authors observed a retarded release of granular enzymes from CGD cells in the first 15 min after contact with such surfaces.

We have now repeated these studies with neutrophils from 12 CGD patients. After phagocytosis of opsonized zymosan particles, we always found much higher activities of lysozyme and β -glucuronidase in the cell-free supernatant fluids of CGD cells than in those of the normal control cells. This was true not only after 30 min of ingestion but also after 5, 10, and 15 min. In principle, this might be due to a higher rate of phagocytosis by the CGD cells. Although we found

that phagocytosis of ^3H -labeled DNA-antiDNA complexes, ^{14}C -labeled *S. aureus* and/or ^{125}I -labeled zymosan, by neutrophils from 14 CGD patients was always in the normal range (18, 23, 35–39, and unpublished results), others have reported an enhanced uptake of lipopolysaccharide-coated paraffin-oil droplets by CGD cells (40). Nevertheless, as an alternative or additional explanation, we have now found that granular enzymes are partly inactivated in phagocytosing normal neutrophils. This observation confirms and extends previously reported loss of total granular enzyme activity during neutrophil phagocytosis (22, 41).

The retarded release of granular enzymes from CGD neutrophils adhering to a surface coated with aggregated human gammaglobulin, found by Gold et al. (19), differs from our results. It is unknown whether the adherence of CGD cells to such a surface is completely comparable to that of normal cells. If the adherence of CGD cells would be retarded, this might explain the slow release of granular enzymes. It is also unknown whether normal neutrophils exhibit as large a metabolic burst as when these cells ingest opsonized zymosan particles. If normal neutrophils would generate fewer oxygen metabolites during adherence, less damage to granular enzymes would be expected. This, too, may be an explanation for the differences between the results of Gold et al. (19) and those described in the present report.

The inactivation of granular enzymes in normal neutrophils is due to oxidative damage to these enzymes because the phenomenon was observed neither in phagocytosing CGD cells, nor in phagocytosing normal cells under anaerobic conditions. The site of inactivation is probably the phagolysosome, where the reactive oxygen metabolites and the granular enzymes meet in high concentrations. This was deduced from the decrease in the specific activity of acid α -glucosidase in normal neutrophils. Fig. 7 shows that CGD cells release $\sim 15\%$ of this enzyme into the extracellular medium. If we assume a similar release by normal neutrophils, followed by extracellular inactivation, this would indicate that still at least 35% of the activity of acid α -glucosidase is inactivated within normal cells. Thus, the major part of the inactivation of this enzyme, which is apparently more susceptible to oxidative damage than lysozyme or β -glucuronidase, takes place intracellularly. In fact, the experiment shown in Fig. 8 indicates that acid α -glucosidase protein is not released by normal cells at all; this would mean that the inactivation is a totally intracellular process. The same result was obtained with samples of total cell suspensions, excluding the possibility that inactivated enzyme in the cell pellets bound less antibody in proportion to the decreased specific activity. The antigen precipitation assay, as calculated from Fig. 8, has a between-assay variance

of $\sim 15\%$, however. Therefore, we cannot exclude the possibility that some acid α -glucosidase did leave normal phagocytosing neutrophils, to be inactivated extracellularly.

Another indication for intracellular inactivation of granular enzymes was obtained from the experiments with PMA. This agent induces hardly any release of β -glucuronidase (32), either from normal or from CGD neutrophils (Fig. 4). Nevertheless, the activity of this enzyme in normal neutrophils was decreased by about 20%. Thus, β -glucuronidase, too, is inactivated mainly in the cells and not in the medium. Intracellular inactivation of granular enzymes also explains why scavengers of superoxide or hydrogen peroxide in the extracellular medium did not protect the enzymes. Only small molecules such as reduced glutathione were able to reach the site of inactivation and partially prevent (or repair) the oxidative damage. The protecting effect of glutathione cannot be explained by an effect on degranulation, because this agent did not affect the release of enzymes from CGD cells. Furthermore, intracellular inactivation might explain why lysosomal enzymes added to phagocytosing normal neutrophils or to an H_2O_2 -generating system were not inactivated (the conditions in the phagolysosome may have been irreproducible in vitro).

As to the mechanism of inactivation, we can only conclude from the potentiating effect of azide that myeloperoxidase was not needed for this process. Azide slightly enhanced degranulation, because it decreased the granular enzyme activity in phagocytosing CGD cells (Fig. 6). Perhaps this contributed to the increased enzyme inactivation in normal neutrophils. Inhibition of myeloperoxidase and catalase by azide probably also causes a higher concentration of hydrogen peroxide in the phagolysosomes, thus increasing the oxidative damage. A direct protecting role of endogenous catalase is unlikely, because this enzyme is localized in the cytosol and in microperoxisomes of the neutrophils (42, 43), separated from the granular enzymes.²

² Recently, the release and inactivation of granular enzymes from myeloperoxidase-deficient neutrophils were measured. The results (in duplicate samples) after 30 min of phagocytosis were as follows: lysozyme supernate, 75%; lysozyme in pellet, 31%; beta-glucuronidase in supernate, 30%; beta-glucuronidase in pellet, 69%; LDH in supernate, 7%; LDH in pellet, 96%. Thus, myeloperoxidase-deficient neutrophils release as much activity of granular enzymes as do CGD cells. Moreover, there is no inactivation of these enzymes in either myeloperoxidase-deficient or CGD cells. Nevertheless, myeloperoxidase-deficient cells produce supranormal amounts of H_2O_2 (44). This indicates that, in normal neutrophils, the inactivation is caused by the combined action of H_2O_2 and myeloperoxidase, probably by the reaction described by Klebanoff (9). In the presence of azide, when myeloperoxidase is inhibited, the concentration of H_2O_2 in the phagosomes is probably raised to such high concentrations that inactivation proceeds even in the absence of active myeloperoxidase.

In summary, we have shown that granular enzymes are both released from, and inactivated by, normal neutrophils during phagocytosis. Neutrophils from CGD patients only release, but do not inactivate these enzymes. Because the decrease in the activity of lysozyme and β -glucuronidase in CGD cells during phagocytosis is at least as large as that seen with normal cells, we conclude that the degranulation process in CGD cells is normal or perhaps enhanced when compared to normal cells (24). Although the physiological meaning of the inactivation of granular enzymes in normal neutrophils is not clear, our studies indicate that the release of these enzymes from phagocytes can only be properly interpreted if the remaining enzyme activities in the cells are also taken into account.

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