THE MOBILIZATION AND EXTRACELLULAR RELEASE OF GRANULAR ENZYMES FROM HUMAN LEUKOCYTES DURING PHAGOCYTOSIS

DANIEL G. WRIGHT and STEPHEN E. MALAWISTA

From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

The importance of granular (lysosomal) enzymes from neutrophils in producing the tissue damage of acute inflammation has been suggested by much indirect and some direct evidence. This study has investigated the kinetics of release and subsequent fate of granular enzymes from phagocytizing human leukocytes. The following observations are made: (a) During phagocytosis, the granular enzyme lysozyme is released from leukocytes into the extracellular medium. (b) Release of lysozyme increases as phagocytic challenge increases, but attains a maximum. (c) Release of lysozyme accompanies phagocytosis and is not a delayed event. (d) The lack of release of a nongranular enzyme, lactic dehydrogenase, indicates that cell damage is not a necessary condition of enzyme release. (e) Like lysozyme, β-glucuronidase is released from phagocytizing leukocytes. Acid α-naphthyl phosphatase and cathepsin also appear to be released, but are not found in appreciable amounts in the extracellular medium, in part because of their lability in solution. These results support the concept that extracellular release of granular enzymes may be a useful secretory function of inflammatory leukocytes which becomes damaging to the host in certain circumstances.

INTRODUCTION

In a celebrated series of experiments, Metchnikoff recognized that inflammatory leukocytes phagocytize and digest microorganisms, and thus perform a vital function in protecting the body against infection. It is now known, however, that inflammatory leukocytes may be harmful as well as useful to the host. In acute gout or in the "immune complex" vasculitides, for example, tissue injury results from the inflammatory response itself rather than from the relatively benign stimuli that evoke it. To understand the mechanisms of tissue damage in such conditions has become an important goal of research.

Metchnikoff analyzed the problem of acute inflammatory tissue damage largely by means of military metaphors—it was the expected result of fierce battles between invading microbes and defending white cells: "As soon as the infective agents have penetrated into the body, a whole army of white corpuscles proceeds towards the menaced spot, there entering into a struggle with the microorganisms.... The leukocytes having arrived at the spot where the intruders are found, seize them... and within their bodies subject them to intra-cellular digestion. This digestion takes place in vacuoles in which is usually a weakly acid fluid which contains digestive ferments."

The "ferments" or "cytases" reported by Metchnikoff have been identified as lytic enzymes bound in the granules, or "lysosomes," of neutrophils and mobilized when these cells
phagocytize particles. Recent evidence suggests that these enzymes mediate acute inflammatory tissue damage when released into tissues. First, the presence of neutrophils in acutely inflamed tissues appears to be necessary for the occurrence of tissue damage (3–6). Second, enzymes isolated from the granules are active in degrading various tissue constituents (6–8). Finally, the tissue damage of acute inflammation has been reproduced experimentally by direct infusion of neutrophil granule extracts into tissues (6, 8–10).

A question has remained, however. How are these destructive enzymes released extracellularly to become available for damaging tissues? Mecham et al. argued that cytochrome do not escape from leukocytes during their normal function (11), and early studies on the intracellular redistribution of granular enzymes during phagocytosis supported this view (12). More recent work, however, has indicated that granular enzymes may be extruded from leukocytes during phagocytosis (13–21).

In the present experiments, we have employed lysozyme, β-glucuronidase, acid phosphatase, and cathepsin to investigate the kinetics of release and subsequent fate of granular enzymes from phagocytizing human leukocytes.

**METHODS**

**Preparation of Human Leukocytes**

Leukocytes were isolated from heparinized blood of normal volunteers by sedimentation in dextran solution, osmotic lysis of erythrocytes, and washing, as described previously (22). Isolates were resuspended in a heparinized, modified Krebs-Ringer phosphate buffer (22), and Wright-stained smears were prepared for differential cell counts. In the isolation procedure approximately 10 ml of whole blood are required to yield 1 ml of a suspension containing $3 \times 10^7$ leukocytes, of which 60–80% are neutrophils. All glassware was sterilized and made pyrogen-free by heating at 160°C for 2 hr; in addition, glassware used in isolation and incubation of leukocytes was siliconized to prevent sticking of the cells to the glass. All solutions used were sterilized by autoclaving at 13 lb. pressure for 2 hr.

**Preparation of Heat-Killed Bacteria**

A culture of *Staphylococcus albus* (originally obtained from a finger abscess) was suspended in normal saline at known concentrations, killed by autoclaving, and frozen in aliquots for subsequent use (22).

**Conditions of Incubation**

1 ml aliquots of a leukocyte suspension (containing $2.6–3.4 \times 10^7$ leukocytes per milliliter) were incubated in 25-ml Erlenmeyer flasks with 1.5 ml heparinized buffer, 0.3 ml autologous serum, and 0.2 ml saline or bacterial suspension, for up to 1 hr at 37°C in a shaking water bath (Iwatsch Corp., Ann Arbor, Mich.; 100 excursions per minute). Flasks containing 2.7 ml buffer and 0.3 ml serum were incubated concurrently as serum enzyme controls. After incubation, samples from the various leukocyte suspensions were prepared on slides for microscopic inspection, as described previously (22, 23). Leukocytes actively phagocytize bacteria in this system.

**Preparation of Cell Fractions**

After incubation, leukocyte suspensions were centrifuged at 1200 rpm (International Centrifuge, PR-2) for 10 min at 4°C. The supernatants represented the incubation media fractions. The cell buttons, representing the whole cell fractions, either were resuspended in 0.2 M sucrose to 3 ml, or else they were resuspended for preparation of the separate nuclear, granular, and cytoplasmic fractions. The preparation of these latter three fractions was accomplished by modification of a method reported previously (24). The cell buttons were resuspended to 3.7 ml, in 0.2 M sucrose, and 0.3 ml heparin (5000 units/ml) was added. The cell suspension was mixed gently with a Pasteur pipette until marked viscosity appeared, indicating cell lysis. The lyte suspensions were then centrifuged at 1200 rpm (International Centrifuge) for 10 min at 4°C. Buttons from this centrifugation, designated the “nuclear” fractions, were resuspended in 0.2 M sucrose to 3 ml; the new supernatants were transferred to high-speed Sorval tubes and centrifuged at 10,000 rpm (12,000 g, Sorval Centrifuge Superspeed SS-3) for 20 min at 4°C. The buttons from this centrifugation, designated the “granular” fractions, were resuspended in 0.2 M sucrose to 3 ml; the final supernatants represented the “cytoplasmic” fractions. All fractions were frozen and stored at −20°C for not more than 3 days until enzyme determinations could be made. Before such determinations all fractions were freeze-thawed five times in the presence of 0.1 ml 1.0% Triton-X 100 (Robins and Haas Co., Philadelphia, Pa.). When enzyme activities were measured, the values obtained for the cytoplasmic fractions were multiplied by a factor of 2 in order to adjust for dilution, values obtained for the incubation media fractions were adjusted for enzyme activities present in the serum-buffer media alone.

**Determinations of Enzyme Activity**

**LYSOZYME:** The assay for lysozyme is an adaptation of a commonly used turbidimetric method (25).
3-ml aliquots of substrate solution were warmed to 37°C. 0.5 ml aliquots of the samples to be measured were then each added to aliquots of the substrate solution, mixed, and transferred to spectrophotometer wells. Lysozyme activity, or lysis, was then added to aliquots of the substrate and lysis was mixed, and transferred to spectrophotometer wells. The sample-substrate buffer mixtures were incubated in a shaking water bath for 15 min at 37°C, after which 1.0 ml 0.5 M NaOH was added and the mixture was immediately measured for fluorescence in a FOCS spectrophotometer Mark 1, Farrand Optical Co., Valhalla, N. Y. (incident wavelength, 340 nm, emitted wavelength, 455 nm, optimal for measurement of fluorescence of free α-naphthol).

At concentrations of substrate used, the liberation of free α-naphthol was found to be linear with time up to 15 min. Acid phosphatase activity is measured as milligrams of free phosphate per 3 X 10⁶ leukocytes incubated per hr. For those experiments, serum-normal saline was used instead of serum-buffer (to avoid phosphate in the medium).

**RESULTS**

The localization of lysozyme in the incubation media and in various cell compartments, when the leukocytes have been incubated with and without bacteria for 60 min, is illustrated in Fig. 1. Active phagocytosis was demonstrable microscopically; when bacteria were incubated with leukocytes at a ratio of 20 bacteria to 1 neutrophil, most neutrophils took up more than 10 bacteria. Only neutrophils and monocytes ingested bacteria; the latter cells made up no more than 5-8% of isolated leukocytes.

In Fig. 1 it is evident that the greatest changes in distribution of lysozyme that occur with phagocytosis are in the granular and the media fractions, indicating a shift of lysozyme into the media during phagocytosis. In the four experiments shown, the differences without and with bacteria are statistically significant for cells (P < 0.05) and for media (P < 0.01) (paired t test). Almost no increase of lysozyme activity was observed in the cytoplasmic fraction after phagocytosis—a finding that differs from observations made with other granular enzymes in...
The distribution of lysozyme within cells and in media with and without phagocytosis is represented in Fig. 1. Total lysozyme activity recovered from cells and media incubated without bacteria: 8.8-6.4 μg (human lysozyme) per 10⁷ leukocytes incubated. Results represent mean values for four experiments; standard errors of the means are indicated.

Two questions followed from these initial experiments: First, how is the extracellular shift or release of lysozyme related to time and degree of phagocytosis? Second, does extracellular release of lysozyme represent injury and disruption of leukocytes during phagocytosis? Experiments were designed to answer these questions.

The relation of extracellular release of lysozyme to varying degrees of phagocytic challenge is represented in Fig. 2. In these experiments leukocytes were incubated for 60 min, as before, but with varying numbers of bacteria. Since we were interested in extracellular release of lysozyme, cellular lysozyme is represented as a whole, without separation of cellular compartments. Extracellular release of lysozyme increases with increasing phagocytic challenge. However, release of enzyme appears to approach a maximum at a ratio of about 30 bacteria to 1 neutrophil, although individual neutrophils may be observed to take up far more than 30 bacteria in this time period (60 min). In the four experiments shown, the differences without and with bacteria are all statistically significant for both cells and media (paired t test), beginning with the lowest ratio (2:1), where P < 0.05 for both cells and media.

With a constant phagocytic challenge (30 bacteria to 1 neutrophil) and variable times of incubation, extracellular release of lysozyme occurs rapidly (Fig. 3); it is half completed by 7 min and almost entirely completed by 30 min in this experimental system, phagocytosis also
occurs rapidly and is almost completed by 30 min. The results of these experiments correspond to previously reported findings which related appearance of granular enzyme extracellularly to time of phagocytosis (17, 18). These experiments demonstrate that release of lysozyme occurs during phagocytosis and not as a delayed event after phagocytosis has been completed.

If extracellular release of lysozyme during phagocytosis represents leukocytic injury, one would expect that lactic dehydrogenase, a non-granular, cytoplasmic enzyme, would also escape during phagocytosis. LDH is commonly measured clinically as a marker for cell damage. Experiments illustrated in Fig 4 demonstrate no appreciable release of LDH comparable to that of
Figure 4: Lack of release of lactic dehydrogenase during phagocytosis. Total enzyme activity recovered from cells and media incubated without bacteria—LDH: 540-710 units per 10^7 leukocytes incubated; lysozyme: 4.8-5.5 μg (human lysozyme) per 10^7 leukocytes incubated. Results represent mean values for four experiments; standard errors of the means are indicated.

Because of evidence that leukocyte granular enzymes are not packaged in uniform granules (29, 30, 33), we compared the fate of three other granular enzymes, β-glucuronidase, acid phosphatase (α-naphthyl phosphatase), and cathepsin, to that of lysozyme during phagocytosis. In experiments depicted in Fig 5, β-glucuronidase, like lysozyme, is lost from the leukocytes and appears in the extracellular medium during phagocytosis. In the 15 trials shown for this enzyme, the differences without and with bacteria are statistically significant (paired t test), for cells and for media, P < 0.0001. Both acid phosphatase and cathepsin are also lost from leukocytes during phagocytosis, but, unlike lysozyme, there are only slight, though significant, increments of enzyme activities in the incubation media.

With acid (α-naphthyl) phosphatase and cathepsin, it was thought possible that the small increments in the media may have resulted from instability of the enzymes once they were released into solution. To test this hypothesis, two supplementary sets of experiments were done. In the first set, leukocytes were disrupted (by freeze-thawing in the presence of Triton-X 100) to release the granular enzymes into solution, and the lysates were then placed in incubation media and measured for enzyme activity immediately, and after varying periods of incubation at 37°C. Representative results are indicated in the left panel of Fig 6: under these conditions, α-naphthyl phosphatase and cathepsin are clearly more unstable than are lysozyme and β-glucuronidase.

Although there is evidence in another cell type that the intragranular location of α-naphthyl
phosphatase is the same as that of β-glycerophosphatase (28), these enzymes do not appear to have similar stabilities once they are released into solution (left panel of Fig. 6).

Since the last procedure compared the behavior of enzymes in a cell soup and not that of enzymes released from intact cells, a second set of experiments was done. Since some release of acid (α-naphthyl) phosphatase and cathepsin, although small, occurred during phagocytosis, we attempted to magnify the amounts of enzymes released by incubating high concentrations of leukocytes (3.5 × 10⁷ leukocytes/ml) with bacteria (ratio 30:1) for a time period shortened to 30 min. After incubation, the cells were separated from the media by centrifugation, and the cell-free media were measured for enzyme activities immediately, and after varying periods of additional incubation at 37°C. Representative results are presented in the right panel of Fig. 6. Lysozyme and β-glucuronidase released extracellularly from leukocytes lose little activity with further incubation, whereas the acid (α-naphthyl) phosphatase and cathepsin released from the cells lose activity rapidly.

DISCUSSION

Experiments presented here demonstrate five points. (a) Extracellular release of the granular enzyme, lysozyme, during phagocytosis represents a shift from the granules to the extracellular medium, without an appreciable rise of enzyme activity in the cytoplasm (Fig. 1). (b) Extracellular release of lysozyme increases with increasing phagocytic challenge, but attains a maximum (Fig. 2). (c) Extracellular release of lysozyme occurs during phagocytosis and not as a delayed effect of phagocytosis (Fig. 3). (d) The lack of release of a nongranular enzyme, lactic dehydrogenase, indicates that cell damage is not necessary for extracellular release of granular enzymes (Fig. 4). (e) Like lysozyme, the acid hydrolase, β-glucuronidase, is released from leukocytes during phagocytosis (Fig. 5). Acid (α-naphthyl) phosphatase and cathepsin also appear to be released extracellularly during phagocytosis; however, appreciable amounts of these enzymes are not recovered from the medium, in part because of their lability in solution (Fig. 6).
It would be unwise to assume that lysozyme, or any one granular enzyme, is a model for all enzymes and active proteins that are located in leukocyte granules. As shown in our studies, lysozyme and $\beta$-glucuronidase differ from $\alpha$-naphthyl phosphatase and cathepsin in their stability once they are released into solution. Furthermore, some granular enzymes differ from one another in respect to the specific granules that contain them. In disrupted rabbit neutrophils, studied by zonal differential centrifugation, lysozyme was associated with two granule types: granules of small size with which most acid hydrolytic enzymes, including $\beta$-glucuronidase, were associated, and somewhat larger granules in which alkaline phosphatase was found (29, 30). Human neutrophils also appear to have diverse granule types (33).

Although $\alpha$-naphthyl phosphatase and cathepsin were not recovered from the incubation media to the same degree as were lysozyme and $\beta$-glucuronidase in our experiments, all four of these enzymes were lost from leukocytes during phagocytosis. Loss of $\beta$-glycerophosphatase from phagocytizing leukocytes has been reported by others (22, 34). All these results differ from those of earlier studies with rabbit neutrophils, in which there was no loss of granular enzymes from leukocytes after phagocytosis (12).

The loss of total enzyme activities (incubation media + cells) after phagocytosis as shown in our experiments has only rarely been reported by others. One study did report a loss of total $\beta$-glucuronidase after phagocytosis, and on that basis it was suggested that hydrolyases may rapidly become inactive once they are released from granules (21). Our results have provided direct evidence to support this suggestion for two hydrolyases. $\alpha$-naphthyl phosphatase and cathepsin rapidly become inactive not only in a cell lysate but also when released from intact cells during phagocytosis (Fig. 6). However, we cannot explain the small losses of total enzyme activities after phagocytosis for lysozyme and $\beta$-glucuronidase in our experimental system.

If our findings can be extended to conditions
in vivo, granular enzymes would be expected to become available for tissue damage when there is an intense phagocytic challenge to inflammatory leukocytes, or in conditions where the local rate of decay in activity of these enzymes lags far behind their rate of release. Conversely, the anti-inflammatory properties of such agents as colchicine and glucocorticoids may depend in part on the ability of these agents to lessen the release of granular enzymes during phagocytosis (1, 22).

Extracellular release of granular enzymes may represent, as some have suggested, an inadvertent event related to the mechanics of rapid phagocytosis (20). However, since there is some release of enzyme even with small phagocytic challenges (Fig. 2), release may represent in part a useful secretory function of leukocytes. Various agents have been identified in leukocyte granules which may have useful extracellular functions. For example, "basic cationic proteins" obtained from leukocyte granules have been shown to cause increased vascular permeability and chemotaxis when released into tissues (6, 35-38); at least some of these active proteins have been shown to be released from intact cells during phagocytosis (37, 38). Also, bactericidal and bacteriostatic actions of several granular products have been described (lysozyme, leukin [39], phagocytin [31], basic cationic proteins [6, 40]), and the function of these agents may not be limited to phagocytic vacuoles within cells. One may speculate that the release of such agents from leukocytes serves the useful functions of amplifying and localizing an inflammatory response, and of neutralizing or killing microorganisms extracellularly. Similarly, since infiltration of inflammatory leukocytes into injured tissues is necessary for the eventual success of wound healing (41), the controlled release of granular products may be useful for tissue repair.

The authors thank Mrs. Gretchen Flynn for technical assistance and Mrs. Elizabeth Wright for statistical analysis of the data.

This investigation is part of a thesis presented to the Yale University School of Medicine by Daniel G. Wright in partial fulfillment of requirements for the degree of Doctor of Medicine; parts of it have appeared in abstract (1).

Dr. Malawista is the recipient of National Institutes of Health Research Career Development Award AM-21669.

This investigation was supported in part by grants from the United States Public Health Service (AM-10493, AM-5639, AI-271), The Arthritis Foundation, and The John A. Hartford Foundation.

Received for publication 23 July 1971, and in revised form 22 February 1972.

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