Release of Gelatinase from a Novel Secretory Compartment of Human Neutrophils

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ABSTRACT Gelatinase is a metallo-proteinase that acts specifically on denatured collagen. In human neutrophils, this enzyme is localized in small, morphologically still unidentified storage organelles that are resolved from the specific and the azurophil granules upon subcellular fractionation by differential sedimentation. When neutrophils isolated from freshly drawn blood are exposed to soluble stimuli such as *N*-formyl-methionyl-leucyl-phenylalanine, zymosan-activated serum, phorbol myristate acetate, or the calcium ionophore A 23187, or are induced to phagocytose opsonized zymosan, they rapidly release gelatinase in large amounts (30–70% of the cellular content in 10 min). When neutrophils from donor blood, which had been stored for 24 h at 4°C are used, extensive release even occurs without added stimuli by simply warming to 37°C.

Gelatinase release appears to occur by secretion because it is not dependent on phagocytosis. It is paralelled by the release of specific granule contents (vitamin B_{12}-binding protein), but is more rapid and much more extensive. It is, however, dissociated from the discharge of azurophil granules (as assessed by β-glucuronidase). In addition, it was found that gelatinase release does not depend on the activation of the respiratory burst, although the two responses are often observed in parallel. Release is not due to cell damage as the cytoplasmic enzyme lactate dehydrogenase is fully retained.

The distinct subcellular distribution and kinetics of release of gelatinase reported in this paper uncover a novel, truly secretory compartment of human neutrophils, which is highly responsive to stimulation. Gelatinase and possibly other enzymes stored in this secretory organelle may be involved in the early events of neutrophil mobilization, the response to chemotactic signals and diapedesis.

INTRODUCTION

Human neutrophils contain two collagen-degrading metallo-proteinases, a specific collagenase (1, 2), and a gelatinase (3). In a recent study, we found evidence suggesting that these two metallo-proteinases do not share the same subcellular compartment. Collagenase is localized in the specific granules while gelatinase appears to be associated with particles sedimenting more slowly (4). This observation led us to test for gelatinase release by neutrophils that were exposed to a variety of stimuli, soluble and particulate, known to induce a series of responses such as increased chemokinesis, chemotaxis, activation of the respiratory burst, stimulation of the arachidonic acid metabolism, and release of granule enzymes.

Several studies have shown that agents such as phorbol myristate acetate (PMA)[1], the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), and partially purified C5a cause the selective release of contents of the specific granules while azurophil granules are retained (5, 6). We now show that under these conditions neutrophils secrete large proportions of their gelatinase. These results uncover a novel, truly secretory subcellular compartment of the neutrophil that is highly responsive to stimulation.

METHODS

Reagents used in this work were obtained from the following sources: zymosan, superoxide dismutase, ferricytochrome *c* (type III) and *p*-aminophenylmercuric acetate (Sigma Chemical Co., St. Louis, MO); A 23187, A grade (Calbiochem-Behring Corp., Calbiochem AG, Lucerne, Switzerland); fMLP (Bachem AG, Bubendorf, Switzerland); PMA (Consolidated Midland Corp., Brewster, NY); cytochalasin B (Aldrich-Europe, Janssen Pharmaceutica, Beerse, Belgium); di-isopropyl phosphorofluoridate (DFP) (Fluka, Buchs, Switzerland); cyano [^{57}Co]cobalamin, 10–20 μCi (The Radiochemical Centre, Amersham, England); [^{3}H]acetic an-

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[1] *Abbreviations used in this paper*: DFP, di-isopropyl phosphorofluoridate; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline.

hydride, 50 mCi/mmol (New England Nuclear, Dreieich, Federal Republic of Germany). Two solutions of phosphate-buffered saline (PBS) were used: (*a*) Ca^{++}, Mg^{++}-free PBS containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, and (*b*) PBS, containing in addition to the above, 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$.

Cell preparations. Neutrophils were prepared either from fresh blood samples (150 ml) obtained from healthy volunteers or from buffy coats of donor blood stored for 24 h at 4°C (Swiss Red Cross Laboratory, Berne, Switzerland). Fresh neutrophils were purified by dextran sedimentation followed by centrifugation through a layer of Ficoll-Hypaque as described by Bøyum (7). The granulocyte pellet was washed in saline, and the contaminating erythrocytes were eliminated by hypotonic shock. Preparations obtained in this manner contained 94.5±2.7% neutrophils and 4.7±2.5% eosinophils (mean±SD, n = 31). Buffy coat neutrophils were purified by dextran sedimentation followed by hypotonic lysis of contaminating erythrocytes and finally by three washing cycles in saline. Cells used for exocytosis experiments were resuspended in Ca^{++}, Mg^{++}-free PBS at a density of 2–2.5 × 10^8 cells/ml. Cells used for fractionation experiments were resuspended at the same density in 0.34 M sucrose. Mononuclear cells were obtained from fresh blood using the Ficoll-Hypaque technique mentioned above. The cells at the interface between the Ficoll-Hypaque and sample layer were collected with a pasteur pipette and were further purified by hypotonic shock and several washings in saline. Contamination by granulocytes in these preparations varied between 3 and 12%.

Exocytosis experiments. 2–2.5 × 10^7 cells suspended in 1.0 ml PBS were incubated at 37°C for 5–30 min in the presence or absence of stimuli at the indicated concentrations. Incubation was terminated by rapid cooling in ice and centrifugation (800 *g* for 10 min). The cell-free supernates were collected, and the pellets were resuspended in Ca^{++}, Mg^{++}-free PBS to twice the original volume. Enzymes and vitamin B_{12}-binding protein were assayed in the supernates and pellets and release was calculated as the percentage of the total, i.e., of the amount measured in the supernate plus pellet. For control, recoveries were calculated by comparing the amounts in the supernate plus pellet with those of the original cell suspension. Cells that were treated with cytochalasin B (5 μg/ml) were preincubated for 5 min at 37°C before addition of the stimuli. For all exocytosis experiments except those presented in Fig. 4B and the lower part of Table III, neutrophils prepared from fresh blood samples were used.

Opsonized zymosan and zymosan-treated serum were prepared by incubating 25 mg of zymosan in 5 ml serum obtained from the same donor as the neutrophils used in the particular experiment. After 30 min at 37°C, the mixture was centrifuged at 230 *g* for 4 min. The supernate (zymosan-treated serum) was saved, and the pellet (opsonized zymosan) was washed twice at 4°C with PBS and finally suspended in PBS at the concentration of 10 mg zymosan per ml.

Subcellular fractionation. Subcellular fractionation was performed by zonal sedimentation at 13,500 rpm for 15 min in a B-XIV rotor (MSE 59144) operated by a MSE SS-65 ultracentrifuge (Measuring & Scientific Equipment, Crawley, Sussex, England) or by isopycnic equilibration in a Beaufay rotor (8) according to described techniques (8, 9). As indicated in Table I, two types of density equilibration gradients were used.

Biochemical assays. Protein (10), peroxidase (8), lysozyme (11), alkaline phosphatase (12), *N*-acetyl-β-glucosaminidase (9), and lactate dehydrogenase (9) were determined by previously published methods. β-Glucuronidase was measured fluorimetrically with 4-methylumbelliferyl-β-D-glucuronide as substrate. The incubation mixture consisted of 0.05 M sodium acetate buffer, pH 4.0, 0.05% Triton X-100, 5 mM substrate, and sample in a total volume of 0.2 ml. The reaction was carried out at 37°C for 30 min and was stopped by addition of 3 ml of a glycine-NaOH buffer, pH 10.4, containing 5 mM EDTA. The unsaturated vitamin B_{12}-binding protein was assayed by a slight modification of the method of Kane et al. (13). In brief, 0.1 ml of sample was mixed with 0.4 ml 0.025% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.5, before addition of 1 ml of a solution containing 1.33 ng [^{57}Co]cyanocobalamin in 0.1 M potassium phosphate, pH 7.5. Incubation and separation of free and bound [^{57}Co]cyanocobalamin were performed as described (13).

Gelatinolytic activity was determined by a modification of the method of Harris and Krane (14). ^{3}H-Acetylated gelatin used as substrate was prepared as follows: collagen type I was isolated from the skin of young rats by extraction with 1 M NaCl in 50 mM Tris/HCl, pH 7.4, containing 10 mM EDTA, followed by ammonium sulfate precipitation (45% saturation), dialysis of the precipitate against 0.5 M NaCl in 50 mM Tris/HCl, pH 7.4, containing 10 mM EDTA, precipitation with 2.5 M NaCl, which was repeated once, and finally dialysis against 0.05% acetic acid. Gelatin was obtained from collagen by denaturation at 45°C for 1 h. To prepare ^{3}H-acetylated substrate, the gelatin solution (50 mg/25 ml) was dialyzed against 0.1 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.02 M KCl, and 0.05 M sodium acetate, and was then mixed with 0.1 mmol ^{3}H-acetic anhydride (50 mCi/mmol) in 0.5 ml acetonitrile with stirring and kept at 4°C for 1 h. The acetylated gelatin was dialyzed against three changes of 50 mM Tris/HCl, pH 7.6, containing 50 mM NaCl. Before determination of gelatinase activity all samples were treated with DFP to block serine proteinases. To this end the samples were incubated in the presence of 3 mM DFP in 50 mM Tris/HCl, pH 7.6, containing 50 mM NaCl and 0.05% Triton X-100 for 60 min at 37°C. The reaction was stopped by cooling in ice and addition of 0.1 mg/ml bovine serum albumin. Gelatinase activity was assayed in an incubation mixture containing 35 μg ^{3}H-acetylated gelatin (~30,000 cpm) and DFP-treated enzyme, 2 mM *p*-aminophenylmercuric acetate, 10 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, 0.05% Triton X-100, 39 mM Tris/HCl, pH 7.6, and 39 mM NaCl in a total volume of 0.225 ml. *p*-Aminophenylmercuric acetate was added to activate the latent form of gelatinase. The incubation was carried out at 37°C for 1 h and was stopped by addition of 25 μl bovine serum albumin (100 mg/ml) in 50 mM Tris/HCl, pH 7.6, containing 50 mM NaCl followed by 50 μl of cold 90% TCA (wt/vol). After standing in ice for 30 min the mixtures were centrifuged at 1,250 *g* for 15 min and the radioactivity was measured in 100 μl of the supernate.

Superoxide generation was measured by incubating 2 × 10^6 neutrophils in a total volume of 0.6 ml PBS in the presence of 85 μM cytochrome *c* and the appropriate stimulus at 37°C for 10 min. The reactions were terminated by addition of 2 vol of cold Ca^{++}, Mg^{++}-free PBS and rapid cooling in ice. After centrifugation at 1,200 *g* for 10 min, the cytochrome *c* reduced was determined according to Curnutte et al. (15). To correct for nonspecific cytochrome *c* reduction each assay was done in the absence and presence of 20 μg/ml superoxide dismutase.

RESULTS

Subcellular localization of gelatinase. Fractionation of postnuclear supernates by zonal sedimentation

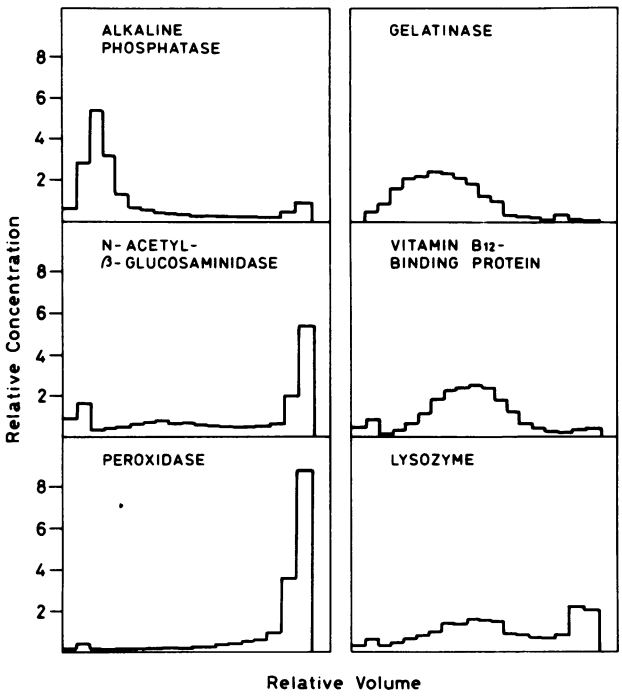

FIGURE 1 Fractionation of subcellular components of human neutrophils by zonal sedimentation at 13,500 rpm for 15 min. The graphs are normalized distribution histograms as a function of the volume collected. The radial distance increases from left to right. The ordinate is the concentration in the fraction relative to the concentration that corresponds to uniform distribution throughout the gradient. The percentage recoveries were 82 for alkaline phosphatase, 70 for *N*-acetyl-β-glucosaminidase, 99 for peroxidase, 121 for gelatinase, 110 for vitamin B_{12}-binding protein, and 73 for lysozyme. For determination of gelatinase activity the particulate material from 10-ml aliquots of each fraction was concentrated by centrifugation at 100,000 *g* for 35 min and resuspension in 0.5 ml 0.9% NaCl before treatment with DFP. In other experiments, concentrating was omitted and gelatinase activity determined in the original fractions. Closely corresponding distribution histograms were obtained under both conditions.

at 13,500 rpm yields optimal resolution of the three major particulate fractions of human neutrophils, i.e., a membrane fraction and the specific and azurophil granules (8, 9). In Fig. 1, the following markers were used for the identification of these three compartments: alkaline phosphatase for the membranes, vitamin B_{12}-binding protein for the specific granules, and peroxidase for azurophil granules. Vitamin B_{12}-binding protein has previously been shown by Kane and Peters (16) to occur exclusively in the specific granules. We have confirmed this finding in a series of fractionation experiments using both density equilibration and rate sedimentation and we consider this protein an ideal specific granule marker, unlike lysozyme, which also occurs in the azurophil granules (Fig. 1).

As shown in Fig. 1, gelatinase is almost completely recovered in the inner half of the gradient. Its distribution profile is unimodal. It is separated from that of the azurophil granule markers (i.e., peroxidase and acid glycosidases) that accumulate at the cushion and although partially overlapping, is clearly resolved from the profile of vitamin B_{12}-binding protein. Gelatinase sediments together with a minor portion of certain acid hydrolases, namely glycosidases and acid β-glycerophosphatase, which has been previously assigned to a small population of lysosomes, the C-particles, in human and rabbit neutrophils (8, 11). In Fig. 1, the C-particles are revealed by a small peak of *N*-acetyl-β-glucosaminidase in the inner half of the gradient, which coincides with the peak of gelatinase. The resolution between gelatinase and vitamin B_{12}-binding protein was confirmed in four additional experiments. As shown in Table I, the difference of the median relative sedimentation velocities of these two subcellular components is highly significant. By contrast, no resolution between gelatinase and vitamin B_{12}-binding protein was obtained in two isopycnic equilibration experiments (Table I). This is in accordance with our

TABLE I

*Resolution of Gelatinase and Vitamin B_{12}-Binding Protein by Subcellular Fractionation**

Type of fractionation	Resolution parameter	No. experiments	Gelatinase	Vitamin B_{12}-binding protein	*P*
Zonal differential sedimentation	Median relative sedimentation velocity (mean±SD)	5	0.367±0.023	0.488±0.022	$P < 0.0005$
Isopycnic equilibration	Median equilibrium density (mean)	2	1.190 (1.188; 1.192)	1.191 (1.189; 1.192)	NS

* Results are calculated and presented as described by de Duve (17). Median relative sedimentation velocity is expressed as the fractional gradient volume containing half of the total activity of marker recovered (see Methods for experimental conditions). Median equilibrium density is the density of the gradient fraction at which half of the total activity of marker is recovered. Initial conditions for the isopycnic experiments were: first experiment, 10 ml of sample ($d = 1.05$) and 34 ml of gradient (linear with respect to volume between the densities 1.08 and 1.32); second experiment, 10 ml of sample ($d = 1.10$), 30 ml of gradient (as above between the densities 1.17 and 1.29), and 5 ml of cushion ($d = 1.32$).

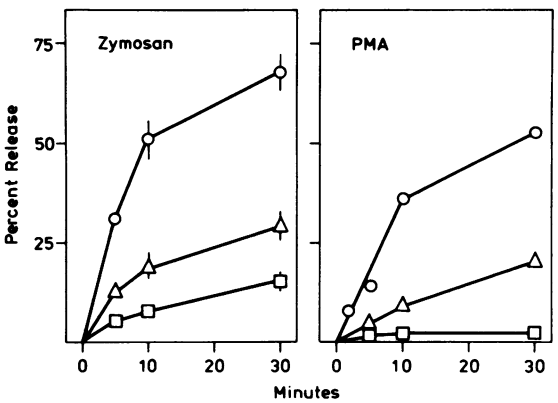

FIGURE 2 Release of gelatinase (○), vitamin B_{12}-binding protein (△), and β-glucuronidase (□) by human neutrophils during zymosan phagocytosis or after stimulation with PMA. Fresh donor neutrophils (2.5×10^7 in 1.0 ml of PBS) were incubated with 2.5 mg of opsonized zymosan or with 10 ng of PMA and the percentage of markers released into the medium were determined. Mean values±SD from seven experiments are given for zymosan phagocytosis and mean values from two experiments for PMA stimulation. Data on release from nonstimulated control neutrophil preparations are presented in Fig. 4, graph (A).

previous observations that isopycnic equilibration does not resolve specific granules and C-particles (8).

Gelatinase exocytosis. The release of gelatinase was compared with that of vitamin B_{12}-binding protein and of markers of the azurophil granules, in most cases β-glucuronidase, after challenge of the neutrophils with either opsonized zymosan or soluble stimuli (Fig. 2). Under all conditions, release of gelatinase was much faster and much more extensive than that of other markers. Within 30 min, 70% of its activity was liberated during phagocytosis of zymosan. The corresponding values for the constituents of the specific and azurophil granules, which are known to fuse rapidly with phagocytic vacuoles, were only 30 and 15%, respectively. Stimulation with PMA resulted in a similar, time-dependent release of gelatinase and vitamin B_{12}-binding protein. However, under these conditions β-glucuronidase was fully retained.

Because short periods of incubation were found to be sufficient for the release of large proportions of gelatinase from stimulated cells, most subsequent experiments were limited to 10 min. A summary of the results obtained is given in Table II. As expected in view of the data presented in Fig. 2, soluble stimuli such as PMA, the calcium ionophore A 23187, and fMLP induced a redistribution of gelatinase and vitamin B_{12}-binding protein. Increasing amounts of both subcellular markers were released with increasing concentrations of either PMA or A 23187. The percentage of gelatinase released was generally two to three times higher than that of the specific granule marker. fMLP induced some gelatinase (but no vitamin B_{12}-binding protein) release already at 1 nM. At 10 nM, the release reached ~25%, and there was little further increase up to 1 μM fMLP. A similar plateau (7–10%) was observed for vitamin B_{12}-binding protein. None of the soluble stimuli caused a significant release of β-glucuronidase, which indicates that they did not induce the discharge of azurophil granules. In some experiments peroxidase and *N*-acetyl-β-glucosaminidase were also determined giving results closely corresponding to those for β-glucuronidase. The soluble stimuli were neither cytotoxic nor cytolytic as shown by the minimal losses of lactate dehydrogenase. Zymosan-treated serum as a source of C5a (and/or its desarginine derivative) showed effects that were similar to those of fMLP. It induced the release of about one-third of the gelatinase and of a small portion of vitamin B_{12}-binding protein, but did not affect β-glucuronidase distribution. In these experiments, the serum controls induced some gelatinase exocytosis without influencing the other parameters. As already seen in Fig. 2, neutrophils that were challenged with opsonized zymosan differed from the cells treated with soluble agents solely with respect to the redistribution of β-glucuronidase, which was released into the medium of phagocytosing cells to a minor but significant extent, presumably via phagocytic vacuoles communicating to the outside (17).

Because cytochalasin B enhances neutrophil response to soluble stimuli (18, 19), fMLP was also tested on cytochalasin B-treated cells. As shown in Fig. 3, cytochalasin B increased the exocytotic response to fMLP. The release of gelatinase was nearly doubled. However, a more dramatic effect was seen with respect to vitamin B_{12}-binding protein and β-glucuronidase, which both reached values of up to 35% in only 10 min. In cells that were not pretreated with cytochalasin B, release of vitamin B_{12}-binding protein did not exceed 10% and there was no significant release of β-glucuronidase, which agrees with the data presented in Table II. Similar results were obtained when activated serum, instead of fMLP, was used as the stimulus (data not shown).

The data presented so far show that extremely mild stimuli are sufficient to induce the exocytosis of gelatinase. We therefore compared enzyme release in freshly prepared and aged neutrophils, i.e., cells obtained from buffy coats of donor blood that had been stored in the cold for 24 h. As shown in Fig. 4, incubation of the aged cells at 37°C in the absence of any stimulus was sufficient to induce a rapid and massive release of gelatinase, which was accompanied by some release of vitamin B_{12}-binding protein. Under these conditions, superoxide generation was not enhanced,

TABLE II

Effects of Stimuli on the Release of Subcellular Markers from Human Neutrophils

Stimulus		Percent release* of Gelatinase	Vitamin B_{12}-binding protein	β-Glucuronidase	Lactate dehydrogenase	No. experiments
None		5.3±2.1	3.0±1.2	2.0±1.1	1.7±1.0	(24)
PMA, *ng/ml*	2.5	8.5	4.0	1.5	2.5	(2)
	5.0	22.0±12.2‡	9.2±5.7‖	2.5±0.6	2.7±0.5	(4)
	10.0	57.1±14.5§	22.6±9.9§	1.8±1.1	2.2±1.3	(16)
	20.0	63.5	29.0	2.5	4.5	(2)
A 23187, *μM*	0.1	9.3±3.8*	3.0±1.4	2.0±0	2.8±1.0	(4)
	0.3	25.0±20.0	6.3±5.1	2.3±0.6	3.0±1.0	(3)
	0.5	43.0	12.0	3.0	5.0	(1)
	1.0	74.0±6.6§	28.8±5.8§	3.2±0.4	3.6±0.5	(5)
fMLP, *μM*	0.001	8.0±2.0‖	2.7±0.6	2.3±0.6	1.0±0	(3)
	0.01	25.6±8.6§	6.5±2.2§	2.0±1.1	1.2±0.9	(11)
	0.03	24.7±4.5§	7.2±1.5§	3.5±2.4	1.6±1.1	(6)
	0.1	34.4±10.2§	10.4±4.0§	2.6±2.2	1.5±0.9	(12)
	0.3	32.3±15.0§	8.3±1.0§	4.0±3.4	2.0±0.8	(4)
	1.0	30.3±4.8§	8.0±0.8§	3.7±3.5	2.0±0.8	(4)
Zymosan-treated serum, *ml/ml*	0.3	37.3±14.6‖	10.0±3.5‖	3.0±1.7	2.3±1.5	(3)
Normal serum, *ml/ml*	0.3	10.5	3.0	2.0	2.0	(2)
Zymosan, *mg/ml*	2.5	50.8±10.0§	18.8±5.5§	7.7±2.2‡	1.0±1.3	(6)

* Incubation of 2–2.5 × 10^7 cells/ml for 10 min at 37°C. Mean percent of marker released into the medium (Methods)±SD (calculated for three or more experiments). Statistical difference with respect to the unstimulated control was calculated according to Welch (29), using one-tailed probability tables, and is indicated as follows:

‡ $0.005 < P < 0.05$.

§ $P < 0.001$.

‖ $0.05 < P < 0.1$.

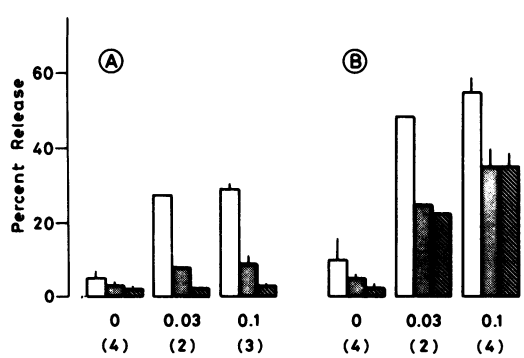

FIGURE 3 Effect of cytochalasin B on fMLP-stimulated release of gelatinase (open columns), vitamin B_{12}-binding protein (stippled columns), and β-glucuronidase (hatched columns). Neutrophils were preincubated for 5 min at 37°C either in PBS (A) or in PBS containing 5 μg/ml cytochalasin B (B). fMLP was then added at the concentrations indicated (μM), and incubation was continued for 10 min. Mean percent release ±SD, number of experiments in brackets.

suggesting that gelatinase secretion is independent from the respiratory burst.

An analysis of the possible relationship between gelatinase release and the induction of a respiratory burst is presented in Table III. Results obtained with freshly prepared and with aged neutrophils are shown. In both cases, PMA, A 23187, and fMLP induced the release of large proportions of gelatinase. In addition (see also Fig. 4), a high rate of gelatinase secretion was observed in unstimulated aged neutrophils. The difference with respect to the amounts released by unstimulated fresh neutrophils was in every case highly significant ($P < 0.0005$). By contrast, superoxide production in response to a given stimulus did not always parallel gelatinase release. PMA had the most pronounced effect, resulting in an ~20-fold enhancement of superoxide production while A 23187 and low concentrations of fMLP had only minimal effects, which in two instances (fMLP 0.01 and 0.03 μM on fresh neutrophils) were

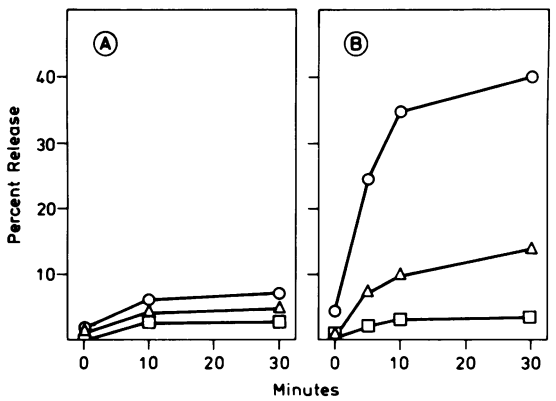

FIGURE 4 Release of gelatinase (○), vitamin B_{12}-binding protein (△), and β-glucuronidase (□) from neutrophils purified from freshly drawn blood (A) and from buffy-coats of donor blood stored at 4°C for 24 h (B). Conditions were as in Fig. 2, except that no stimulus was added. (A), mean values from nine experiments; (B), mean values from two experiments. Additional values for the 10-min time point were obtained in separate experiments with stored neutrophils. Mean percent release ±SD (n = 9) was 34.8±7.9 for gelatinase, 11.7±2.6 for vitamin B_{12}-binding protein, and 2.1±1.3 for β-glucuronidase.

not significant. In the latter cases and in unstimulated aged neutrophils, increase in gelatinase secretion was highly significant despite the lack of a respiratory burst, indicating that the two processes are independent.

In all release experiments reported here, the cell samples consisted of at least 95% granulocytes. We nevertheless tested for the possible influence of mononuclear cells, which are the major contaminants of neutrophil preparations, on the release of gelatinase and other constituents. Mononuclear cells have only minimal if any gelatinase activity. The specific activity of two mononuclear cell preparations amounted to 1 and 9% of the corresponding neutrophil preparation and could be accounted for by neutrophil contamination, which was 3 and 12%, respectively (Table IV). Fresh neutrophil and mononuclear cell preparations were incubated for 10 min at 37°C in the absence of added stimuli either alone or mixed in different proportions, and the activities liberated were measured. The results presented in Table IV show that mononuclear cells had only a minimal effect on gelatinase release, which was slightly enhanced. No effect, however, was observed on the distribution of vitamin B_{12}-binding protein, and β-glucuronidase.

DISCUSSION

The results presented show that human neutrophils rapidly secrete large proportions of gelatinase when exposed to soluble or particulate stimuli known from many studies (reviewed in reference 5) to induce the release of constituents of the specific granules. Gelatinase release, however, is faster and much more extensive than that of the specific granule marker vitamin B_{12}-binding protein. Discharge of azurophil granules did not occur with the concentrations of soluble

TABLE III

*Relationship Between Superoxide Formation and Gelatinase Release by Human Neutrophils**

Neutrophils prepared from	Stimulus		Superoxide formation	P	Gelatinase release	P
Fresh blood	none		2.8±1.9 (17)		5.3±2.2 (30)	
	PMA	10 ng/ml	43.0±4.1 (9)	<0.0005	57.1±14.5 (16)	<0.0005
	A 23187	1.0 μM	8.1±3.3 (4)	<0.05	77.7±6.9 (9)	<0.0005
	fMLP	0.01 μM	4.0±1.6 (4)	NS	26.1±8.3 (12)	<0.0005
		0.03 μM	6.5±3.7 (3)	NS	26.4±6.2 (7)	<0.0005
		0.1 μM	13.9±5.6 (9)	<0.0005	35.3±10.3 (13)	<0.0005
Blood stored at 4°C for 24 h	none		2.1±1.4 (30)	NS	34.8±7.9 (9)	<0.0005
	PMA	10 ng/ml	42.1±4.2 (30)	<0.0005	72.3±10.1 (7)	<0.0005
	A 23187	1.0 μM	5.2±2.9 (4)	<0.1	76.9 (70.2, 83.6)	
	fMLP	0.01 μM	4.9±1.8 (7)	<0.025	59.3±4.9 (3)	<0.0005
		0.03 μM	10.0±3.2 (7)	<0.0005		
		0.1 μM	19.9±5.9 (18)	<0.0005	58.3±5.5 (3)	<0.0005

* Superoxide-dependent cytochrome c reduction (nmol per 3 × 10^6 cells in 10 min at 37°C) and percentage of gelatinase released into the medium by 2–2.5 × 10^7 cells/ml in 10 min at 37°C. Mean values±SD, number of experiments in brackets. P values express the statistical significance of an increase with respect to the unstimulated control (fresh neutrophils in the absence of stimulus); NS indicates P values >0.1 or mean value lower than the value of the unstimulated control. Calculations according to Welch (29).

TABLE IV

Effect of Mononuclear Cells on the Spontaneous Release of Subcellular Markers by Neutrophils

Cell sample		Percent release		
Neutrophils ($\times 10^6$)	Mononuclear cells ($\times 10^6$)	Gelatinase	Vitamin B_{12}-binding protein	β-Glucuronidase
20	—	5.5	3.5	1.5
20	5	8.5	3.0	2.5
20	20	11.0	3.0	2.5

Mean values from two closely corresponding experiments. Average differential counts were 91.3% neutrophils, 7.5% eosinophils, 0.7% lymphocytes, and 0.5% basophils for the neutrophil preparation, and 7.7% neutrophils, 0.3% eosinophils, 55.6% lymphocytes, 33.6% monocytes, and 2.8% basophils for the mononuclear cell preparation. The respective specific activities were 24.3 and 1.3 μg per 60 min per 10^6 cells for gelatinase, 857 and 69 pg per 10^6 cells for vitamin B_{12}-binding protein, and 1.05 and 0.61 nmol/min per 10^6 cells for β-glucuronidase.

stimuli used unless the cells were pretreated with cytochalasin B. This is an important point because it shows that under mild stimulatory conditions, such as are likely to occur in a gradient of a chemotactic factor, gelatinase and possibly other constituents of the novel compartment are released in significant amounts while the acid hydrolases and in particular the neutral serine proteinases, elastase, and cathepsin G, are retained. On the other hand, some collagenase, localized in the specific granules (4), is likely to be released along with gelatinase. Discharge of gelatinase was found to be independent of the respiratory burst, although it was frequently paralleled by an increase in superoxide formation. Evidence for the dissociation of the two responses stems mainly from the observation that aged neutrophils (prepared from blood that had been stored at 4°C for 24 h) released substantial amounts of gelatinase upon warming to 37°C without any enhancement of superoxide production. Similar results were obtained when fresh neutrophil preparations were incubated with low concentrations of fMLP or A 23187. Of interest was the effect of chemotactic peptides and activated serum. In contrast to the other soluble stimuli, these agents induced the release of one-third of the cell-associated gelatinase at the most. Such a level was reached already at very low concentrations and was not enhanced significantly by greatly increased stimulus concentrations. Taken together these results reveal the presence of a novel, truly secretory neutrophil storage compartment that is mobilized by very low concentrations of soluble stimuli as well as during phagocytosis.

In view of the rapid and ready mobilization it is tempting to envisage a function for gelatinase in the chemotactic response and/or in diapedesis, e.g., by facilitating the dissociation of endothelial cell junctions or by easing the way through the basement membrane meshwork. However, like other metallo-proteinases (20, 21), gelatinase is present within the cell and is released upon stimulation in latent form. Latent gelatinase is thought to be an enzyme inhibitor complex (22), which is split by treatment with mercurials or by the action of other proteinases (4, 22). Presently, we can only speculate on the mechanisms of gelatinase activation in the microvessels or the tissues invaded by neutrophils. The latent form is activated by cathepsin G, but is inactivated by neutrophil elastase (4). It however appears unlikely that these serine proteinases play a role in the physiological activation process because they are localized in the azurophil granules and are thus retained within neutrophils that have released large amounts of their gelatinase. Plasmin, which may be formed locally by plasminogen activator (23), was reported to activate latent collagenase (24, 25). In preliminary experiments, however, latent neutrophil gelatinase could not be activated by plasmin or a combination of plasminogen and urokinase. On the other hand, activation may turn out to occur extracellularly independent of other neutrophil enzymes. It is conceivable that, being an enzyme-inhibitor complex, gelatinase may become activated when exposed to its preferred substrate (e.g., a structural protein of the basement membrane) by competition for the inhibitor binding site.

The ready availability of cellular gelatinase is also demonstrated by our experiments with neutrophils prepared from donor blood, which had been stored at 4°C for 24 h. Simple warming up of these cells to 37°C was sufficient to deplete the gelatinase compartment. Independent of their significance to the mechanism of gelatinase secretion, these results show that stored neutrophils are likely to release most of their gelatinase upon warming for infusion. Such neutrophils may be impaired in function, a point that should be considered in connection with the clinical use of white cell preparations.

In qualitative terms, the behavior of gelatinase is analogous to that of a comparatively smaller portion of the contents of the specific granules. Indeed, as pointed out by Wright and Gallin (26) on the basis of evidence from their own and several other laboratories, and as supported by our data on the release of vitamin B_{12}-binding protein, the specific granules have some properties in common with the storage granules of secretory cells. On the other hand, it is known that specific granules also fuse with phagocytic vacuoles (27, 28), which probably is their main fate. Whether the novel organelle containing gelatinase is also dis-

charged into phagocytic vacuoles or, as we tend to believe owing to its massive release by nonphagocytosing cells, is exclusively secretory remains to be established.

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