Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules

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An e.l.i.s.a. was developed using specific polyclonal rabbit antibodies against human neutrophil gelatinase. This assay, in contrast to the functional assay, is independent of activation of gelatinase, and is specific for the detection of gelatinase in both its reduced and unreduced forms. Using this assay, we were able to demonstrate a difference between the subcellular localization of gelatinase on the one hand, and the subcellular localization of vitamin B-12-binding protein, lactoferrin and cytochrome b_{558} on the other hand. The latter three co-localized in fractions of slightly higher density than gelatinase on a two-layer Percoll density gradient. Furthermore, the release of gelatinase exceeded the release of vitamin B-12-binding protein as well as lactoferrin by a factor of 3–6 following stimulation with formylmethionyl-leucyl-phenylalanine, leukotriene B_4 and other soluble stimuli. Thus, although gelatinase has previously been found to co-localize with lactoferrin on immuno-electron microscopy, we confirm the existence of gelatinase-rich and lactoferrin- and vitamin B-12-binding-protein-poor granules, that are lighter and mobilized more easily than specific granules. These gelatinase-containing granules are not the store of cytochrome b_{558} .

INTRODUCTION

The neutrophil is of prime importance in the host defence against bacteria and fungi. Its ability to kill invading micro-organisms is dependent on its conversion from a dormant state into a highly activated state [1]. Exocytosis of different granule subsets is an important aspect of activation, since it leads to supplementation of the plasma membrane with functional proteins localized in granule membranes such as adhesion molecules [2,3] and receptors for complement factors and chemotactic peptides [4], as well as membrane-bound components of the microbicidal NADPH-oxidase (cytochrome b_{558} and associated flavoprotein) necessary for respiratory burst activity [5,6]. In addition, diapedesis and migration of neutrophils are thought to be dependent on exocytosis of at least three neutral proteinases, capable of degrading the extracellular matrix [7,8]: elastase which is located in azurophil granules [9], collagenase which is located in specific granules [10,11], and gelatinase whose subcellular localization has been a matter of much debate.

The existence of a separate subset of gelatinase-containing granules, distinct from specific and azurophil granules, was first proposed by Dewald *et al.* [12]. Using zonal sedimentation, they found differences between the subcellular localization of the specific-granule marker, vitamin B-12-binding protein, and gelatinase. The latter was contained in a granule population with a slightly lower sedimentation velocity. This was further substantiated by the observation that gelatinase was found to be released much more readily than vitamin B-12-binding protein upon stimulation by weak secretagogues, e.g. formylmethionyl-leucyl-phenylalanine (FMLP) and C5a. These findings have been confirmed by others [13–15]. The importance of gelatinase-containing granules relative to specific granules has been underscored by the observation that the membrane of gelatinase-

containing granules and not of specific granules is the major reservoir of the NADPH-oxidase components cytochrome b_{558} and ubiquinone [16,17] and of the adhesion protein, Mac-1 [13,14,18,19].

The existence of gelatinase-containing granules as a separate entity has lately been questioned by Hibbs & Bainton [20]. Using a double-labelling immunogold technique, they found 80% co-localization of gelatinase and lactoferrin, another well-established marker for specific granules [21]. They argued that the extensive release of gelatinase relative to vitamin B-12-binding protein reported by others was caused by an underestimation of the gelatinase content remaining in the cells, since elastase and cytosolic factors present during the assay of gelatinase inhibit gelatinase activity.

To circumvent this problem and to elucidate further the subcellular localization and release of gelatinase, we decided to raise polyclonal antibodies against gelatinase and to develop an e.l.i.s.a. for the enzyme. With this assay, the problems of activation and inactivation that seriously affect the validity of the findings based on the gelatinolytic assay [22] are avoided. Using the gelatinase e.l.i.s.a., we were able to confirm the existence of highly mobilizable gelatinase-rich/lactoferrin- and vitamin B-12-binding-protein-poor granules with a density slightly less than that of specific granules. We further found that the b -cytochrome component of the NADPH-oxidase co-localized with vitamin B-12-binding protein and lactoferrin, distinct from the localization of peak amounts of gelatinase.

MATERIALS AND METHODS

Preparation of neutrophils

Human neutrophils were isolated either from freshly prepared buffy coats, supplied by the blood bank, or from whole blood

Abbreviations used: KRP, Krebs–Ringer phosphate buffer; PMA, phorbol myristate acetate; FMLP, formylmethionyl-leucyl-phenylalanine; PAF, platelet-activating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; DAB, diaminobenzidine tetrahydrochloride.

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donated by healthy volunteers. Whole blood was anti-coagulated in 25 mM-sodium citrate/126 mM-glucose. Erythrocytes were sedimented by adding an equal volume of 2% (w/v) Dextran T-500 (Pharmacia LKB, Uppsala, Sweden) in 0.9% (w/v) NaCl. The resulting leucocyte-rich supernatant was aspirated, and the cells pelleted at 200 *g* for 10 min. Cells were resuspended in saline and neutrophils separated by centrifugation through Lymphoprep (Nygaard, Oslo, Norway) [23] at 400 *g* for 30 min. Remaining erythrocytes were removed by hypo-osmotic lysis in ice-cold water for 30 s. Osmoticity was restored with an equal volume of 1.8% (w/v) NaCl. The neutrophils were then washed once in 0.9% (w/v) NaCl and subsequently resuspended in the desired buffer. All steps, except for dextran sedimentation (room temperature), were done at 4°C.

Release studies

Cells were resuspended at a cell density of 3×10^7 cells/ml in Krebs-Ringer phosphate buffer (KRP, 130 mM-NaCl, 5 mM-KCl, 1.27 mM-MgCl₂, 0.95 mM-CaCl₂, 5 mM-glucose, 10 mM-NaH₂PO₄/Na₂HPO₄, pH 7.4). Control cells were either kept on ice or incubated at 37°C for 20 min. For stimulation, 1 ml of cell suspension was preincubated at 37°C for 5 min followed by addition of the stimulus at the indicated concentrations. Incubation was terminated after 5–40 min by addition of 2 vol. of ice-cold buffer and immediate sedimentation of the cells by centrifugation. The supernatant, S₀, was aspirated and the cells were resuspended in 1 ml of ice-cold buffer containing 0.2% Triton X-100. Release of gelatinase, vitamin B-12-binding protein and lactoferrin was calculated as the content in the supernatant in terms of a percentage of the total content (pellet + supernatant). In some experiments, catalase (Sigma Chemical Co., St Louis, MO, U.S.A.) (2000 units/ml) was included during stimulation to avoid potential inactivation of vitamin B-12-binding protein by secreted myeloperoxidase and H₂O₂ [24]. To assess the cellular integrity, lactate dehydrogenase activity was measured using a commercial kit from Sigma.

Subcellular fractionation

Neutrophils (either control cells or cells stimulated as described above) were resuspended in 0.9% (w/v) NaCl containing 2.5 mM-di-isopropyl fluorophosphate (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) and kept on ice for 5 min. Cells were then pelleted by centrifugation at 200 *g* for 6 min, and resuspended at 5×10^7 cells/ml in disruption buffer (100 mM-KCl, 3 mM-NaCl, 2 mM-Na₂ATP, 3.5 mM-MgCl₂, 10 mM-Pipes, pH 7.2) containing 0.5 mM-phenylmethanesulphonyl fluoride (PMSF). Cells were disrupted by nitrogen cavitation as described [5]. Nuclei and intact cells were pelleted by centrifugation at 400 *g* for 15 min (P₁). A sample (10 ml) of the post-nuclear supernatant (S₁) was carefully applied to the top of a 28 ml two-layer Percoll gradient (1.05/1.12 g/ml) containing 0.5 mM-PMSF and was centrifuged at 37000 *g* for 30 min. This resulted in a gradient with three visible bands; from the bottom they were designated the α -band, containing the azurophil granules, the β -band, containing the specific granules, and the γ -band, containing plasma membrane and secretory vesicles [5,25]. The cytosol (S₂) was present above the γ -band on top of the Percoll. The gradient was collected in fractions by aspiration from the bottom of the tube. In some experiments, 27 equally sized fractions were collected (1.4 ml each); in other experiments, fractions 8–12 were further sub-fractionated into 0.46 ml fractions to obtain a better resolution of the β -band.

Assays

Myeloperoxidase, for identification of azurophil granules [26], was measured by spectral analysis using an absorption coefficient

of 75 mm⁻¹·cm⁻¹ for the 472 nm peak [27]. Cytochrome *b*₅₅₈ was quantified by dithionite-reduced-minus-oxidized difference spectra using an absorption coefficient for the 420 nm peak of 105 mm⁻¹·cm⁻¹ [28]. Specific granules were identified by vitamin B-12-binding protein [29] as described by Gottlieb *et al.* [30] and by lactoferrin [21]. Lactoferrin was measured by an e.l.i.s.a., using goat antibody raised against human lactoferrin (Nordic Immunology, Tilsburg, The Netherlands) diluted 1:500 as the catching antibody. Rabbit antibody raised against human lactoferrin [Dakopatts (A186), Glostrup, Denmark] was used as the detecting antibody in a dilution of 1:2000. Peroxidase-conjugated, affinity-purified goat anti-rabbit IgG antibody was diluted 1:1000 [Dakopatts (P448)]. Samples were diluted 500–2000-fold, except for S₀ which was diluted 10–100-fold. Purified human milk lactoferrin was used as the standard in concentrations ranging from 6.25 to 400 ng/ml. Buffers and all other procedures were as described for the gelatinase e.l.i.s.a. Plasma membranes were identified by histocompatibility locus antigen Class I in a mixed e.l.i.s.a. [31]. Secretory vesicles were quantified by latent alkaline phosphatase [25,32,33], i.e. alkaline phosphatase only measurable in the presence of detergent [Triton X-100 (0.2%)]. Protein was measured as described by Lowry *et al.* [34].

SDS/PAGE protein profile of subcellular fractions of unstimulated cells and exocytosed material

Samples (400 μ l or 150 μ l) of each fraction (as indicated in Figure legends) were centrifuged for 20 min in an Airfuge (Beckmann, Palo Alto, CA, U.S.A.) to sediment out the Percoll. The biological material was resuspended in 100 μ l of saline and mixed with 100 μ l of electrophoresis sample buffer. Aliquots (150 μ l) from each sample were applied to PAGE systems under reducing conditions on to a 5–20% gradient gel with a 3% stacking gel [35].

S₀ from approx. 3.3×10^8 cells stimulated with FMLP at 10 nM, was dialysed against H₂O and concentrated using Speedvac to 0.5 ml. A sample (50 μ l) of the supernatant was electrophoresed as mentioned. Gels were stained in 0.01% Coomassie Blue in 10% (v/v) acetic acid/25% (v/v) propanol, and were destained in 7% (v/v) acetic acid.

Anti-gelatinase antibodies

Gelatinase was purified as described by Hibbs *et al.* [36]. Purity was assessed by SDS/PAGE. The purified enzyme, diluted to approx. 1 mg of protein/ml, was added to an equal vol. of incomplete Freund's adjuvant. Five rabbits were immunized by injection of 50 μ g of protein every 14 days. The rabbits were initially bled at 2-week intervals but then only once a month 3 months after immunization. Immunoglobulins were purified as described by Harboe & Ingild [37]. The antiserum was affinity-purified on a CNBr-activated Sepharose-4B column (Pharmacia) to which 8 mg of purified gelatinase had been coupled. The column was equilibrated in phosphate-buffered saline (PBS; 139 mM-NaCl, 10 mM-NaH₂PO₄/Na₂HPO₄, pH 7.4). Antiserum (10 ml) was dialysed against PBS and subsequently applied to the gelatinase column. Bound material was eluted in 3 M-KSCN in PBS and dialysed against PBS. This affinity-purified antibody was biotinylated essentially as described in [38]. The solution was dialysed twice against 0.9% (w/v) NaCl and finally against PBS. This antibody was used in the e.l.i.s.a. as the detecting antibody.

The specificity of the antibodies was tested by immunoblotting of a post-nuclear supernatant, S₁. This showed that the antibody reacted with both gelatinase and with a 25 kDa protein. To remove antibody with specificity for the 25 kDa protein, neutrophils were stimulated with phorbol myristate acetate

Fig. 1. SDS/PAGE of purified gelatinase and Western blotting of post-nuclear supernatant (S_1) under non-reducing and reducing conditions

A sample (5 μ g) of the purified gelatinase was subjected to electrophoresis (a) under reducing (lane 1) and non-reducing (lane 2) conditions. For Western blotting (b), 10 μ l of S_1 was added to 90 μ l of saline and 100 μ l of sample buffer. An aliquot (150 μ l) of sample was subjected to electrophoresis under reducing (lane 1) and non-reducing conditions (lane 2). Blotting was performed as described in the Materials and methods section. Primary antibody was IgG-anti-gelatinase depleted of antibodies towards the 25 kDa protein (diluted 5000-fold).

(PMA; 2 μ g/ml) to exocytose granule proteins. The 25 kDa protein, present in the exocytosed material, was separated from gelatinase by gel filtration on a Sephadex G-200 column (Pharmacia) and coupled to CNBr-activated Sepharose. The anti-gelatinase antibody (IgG fraction) was depleted of antibodies against the 25 kDa protein by passage through this column. This antibody was used as the 'catching' antibody.

Gelatinase e.l.i.s.a.

Gelatinase was assayed by an e.l.i.s.a. using 96-well flat-bottom immunoplates (NUNC, Roskilde, Denmark). The plates were coated overnight with anti-gelatinase IgG depleted of antibodies towards the 25 kDa protein, diluted 1:5000 in carbonate buffer (50 mM- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.6) and washed once in buffer A (0.5 M-NaCl, 3 mM-KCl, 8 mM- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.2, 1% Triton X-100). Additional binding sites were blocked by incubation with 200 μ l of buffer B (0.5 M-NaCl, 3 mM-KCl, 8 mM- $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$, pH 7.2, 1% BSA, 1% Triton X-100)/well. Samples were then applied, followed by addition of biotinylated, affinity-purified anti-gelatinase antibody diluted 1:1000, and by addition of avidin-peroxidase [Dakopatts (P347)] diluted 1:4000. All incubations were carried out in 100 μ l/well for 1 h unless otherwise stated. Colour was developed during incubation for 40 min in 0.1 M-sodium phosphate/0.1 M-citric acid buffer,

pH 5.0, containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2 (100 μ l/well), and stopped by addition of 100 μ l of 1 M- H_2SO_4 . The plates were washed three times in buffer A between all steps, unless otherwise stated. Before colour development, an additional wash in sodium phosphate/citric acid buffer was included. Absorbance was read at 492 nm in a Multiscan Plus e.l.i.s.a. reader (Labsystems, Helsinki, Finland). A series of standards of purified gelatinase ranging from 0.25–4 ng/ml were used. In this range, the absorbance showed linearity with dilution. Samples were diluted from 100 to 40000-fold in buffer B. All steps were performed at room temperature.

Immunoblotting

Protein was transferred from SDS/PAGE slabs to 0.2 μ m nitrocellulose filters (Bio-Rad Laboratories, Richmond, CA, U.S.A.) essentially as described by Towbin *et al.* [39]. Transfer buffer was 192 mM-glycine, 25 mM-Tris, pH 8.3, 20% (v/v) methanol, and proteins were transferred in a Bio-Rad trans-blot vertical system at 60 V, 210 mA for 4 h. Additional binding sites were blocked by incubating the nitrocellulose filters in 2% (w/v) Tween-20 in PBS for 30 min. After three washes in PBS, containing 0.05% Tween-20, the blots were incubated with the primary antibody (1:1000) overnight. Primary antibodies were labelled with peroxidase-conjugated swine anti-rabbit antibody [Dakopatts (P217)] diluted 1:1000 and incubated for 2 h. The filters were then washed three times in PBS containing 0.05% Tween-20 and once in 50 mM-Tris, pH 7.6, and developed in 50 mM-Tris, pH 7.6, containing diaminobenzidine tetrahydrochloride (DAB) chromogen (Dakopatts) at a concentration of 10 mg/ml and 0.03% H_2O_2 .

RESULTS

Purification of gelatinase

SDS/PAGE of the purified gelatinase (Fig. 1a) showed a 92 kDa band under reducing conditions and three bands of 220, 135 and 92 kDa under non-reducing conditions. This is in accordance with the known molecular masses for gelatinase [36]. In addition a minor 25 kDa band is observed, but solely under reducing conditions, indicating co-purification of this protein with gelatinase.

Gelatinase e.l.i.s.a.

The specificity of the polyclonal antibodies was tested by Western blotting of a post-nuclear supernatant from nitrogen-cavitated neutrophils. Initially the antibodies reacted not only to gelatinase, but also to the 25 kDa protein described above (results not shown). After further affinity purification as described in the Materials and methods section, the antibodies proved to be specific, recognizing one 92 kDa band under reducing conditions and three bands of 220, 135 and 92 kDa respectively under non-reducing conditions (Fig. 1b). The e.l.i.s.a. was reproducible with an interday variation of less than 10% and was sensitive, with a detection limit of 0.25 ng of gelatinase/ml. The standard curve was linear over a wide range of concentrations (0.25–4 ng/ml).

Subcellular fractionation

Initial subcellular fractionation experiments, in which equally sized fractions were collected, indicated a slight difference in the localization of gelatinase and the traditional specific-granule markers, vitamin B-12-binding protein and lactoferrin. To clearly demonstrate this difference, fractions were collected in a smaller volume in the relevant region of the gradient. This showed that gelatinase peaked one fraction above vitamin B-12-binding protein and lactoferrin (Fig. 2). This was reproduced in all of six

Fig. 2. Subcellular fractionation of unperturbed neutrophils

Cells (4.6×10^8) were resuspended in disruption buffer and disrupted by nitrogen cavitation. The post-nuclear supernatant was separated on a two-layer Percoll density gradient. The gradient was collected in 35 fractions. Fractions 1–7 and 23–35 were 1.4 ml, whereas fractions 8–22 were 0.46 ml. Results of a typical experiment are given. Average recoveries were: gelatinase, 97.7%; vitamin B-12-binding protein, 85.9%; lactoferrin, 109%. Key to symbols: (a), ■, gelatinase; *, vitamin B-12-binding protein; □, myeloperoxidase (MPO); ×, latent alkaline phosphatase (AP); △, histocompatibility locus antigen (HLA). (b) *, vitamin B-12-binding protein (identical to a); +, lactoferrin; X, cytochrome b_{558} . The solid line shows the shape of the gradient [density (g/ml)] at the end of centrifugation.

experiments. Gelatinase was well resolved from azurophil granules and secretory vesicles. The recovery of gelatinase in the

Table 1. Release of gelatinase, vitamin B-12-binding protein and lactoferrin from control cells and from cells stimulated as indicated

Cells at a cell density of $3 \times 10^7/\text{ml}$ in KRP were either kept on ice (control) or stimulated for 15 min at 37°C as indicated. The cells were then pelleted by centrifugation, and the supernatant, S_0 , was aspirated. Following resuspension in disruption buffer, the cells were disrupted by nitrogen cavitation. The cavitate was centrifuged to pellet nuclei and undrupted cells, (P_1), and the post-nuclear supernatant (S_1) was applied to subcellular fractionation. Release was calculated as $S_0/(S_0+S_1+P_1)$. Release is given as a percentage $\pm$ s.d., with the number of experiments in parentheses. Abbreviations: ND, not determined; LTB_4 , leukotriene B_4 .

	Gelatinase (%)	Vitamin B-12-binding protein (%)	Lactoferrin (%)
Control	0.2 ± 0.2 (9)	0.2 ± 0.2 (8)	0.4 ± 0.1 (6)
FMLP (10 nM)	21.8 ± 3.3 (9)	5.0 ± 1.8 (8)	2.9 ± 0.4 (4)
LTB_4 (30 nM)	9.0 (1)	2.9 (1)	2.0 (1)
PMA (5 $\mu\text{g/ml}$)	74.6 ± 1.2 (3)	41.6 ± 6 (3)	ND

gradient was 98% [$\pm 18\%$ (s.d.), $n = 10$]. The demonstrated difference in subcellular localization between gelatinase on the one hand, and vitamin B-12-binding protein and lactoferrin on the other, was substantiated by the studies on exocytosis of these proteins in response to a variety of inflammatory stimuli. The calculated release of gelatinase, vitamin B-12-binding protein, and lactoferrin in a number of fractionation experiments is summarized in Table 1. In control cells negligible amounts of the markers were released. Upon stimulation with 10 nM-FMLP, the average release of gelatinase reached 21.8%, thus surpassing the release of both vitamin B-12-binding protein and lactoferrin by a factor of 4–6.

This could be confirmed visually by examination of SDS/PAGE protein profiles of subcellular fractions from control cells and exocytosed material from cells stimulated as mentioned above (Fig. 3). The gradient from control cells was collected in 27 fractions of 1.4 ml each. Samples of equal volume from each fraction were subjected to electrophoresis. In lanes 9 and 10, which contain the major part of the specific granules, the 78 kDa lactoferrin band was dominant. Above this band, a far less

Fig. 3. SDS/PAGE protein profile of subcellular fractions of unstimulated cells and material exocytosed in response to FMLP stimulation

Subcellular fractionation was performed as described in the Materials and methods section. A sample (400 μl) of each fraction (all fractions 1.4 ml) was centrifuged for 20 min to deposit the Percoll. The resuspended biological material was subjected to electrophoresis under reducing conditions. Exocytosed material from 3.3×10^8 cells stimulated by 10 nM-FMLP was dialysed against water and concentrated to 0.5 ml by Speed-Vac. Samples (50 μl) of this were electrophoresed under reducing conditions. Exocytosed material is marked S_0 .

Fig. 4. SDS/PAGE protein profile and Western blotting of subcellular fractions from the β -band of unstimulated cells and material exocytosed in response to FMLP stimulation

Subcellular fractionation of 3×10^8 cells was performed as described in the Materials and methods section. Samples ($150 \mu\text{l}$) of each fraction (fractions of the β -band collected at $460 \mu\text{l}$ each) were centrifuged for 20 min to remove Percoll. The resuspended biological material and $50 \mu\text{l}$ of S_0 (see legend to Fig. 3) was subjected to electrophoresis under reducing conditions (lower panel). For Western blotting of gelatinase (upper panel, right) and lactoferrin (upper panel, left), $10 \mu\text{l}$ and $2 \mu\text{l}$ respectively of each fraction was subjected to SDS/PAGE and subsequent Western blotting. Western blotting was performed as described, except that nitrocellulose filters were blocked in PBS containing 10% (v/v) goat serum and 3% (w/v) BSA. The middle panels show the content of lactoferrin ($\square$) and gelatinase ($\blacksquare$) in each fraction as measured by e.l.i.s.a.

intense band with a molecular mass of 92 kDa, representing gelatinase, was noticed. By examination of the exocytosed material, lanes marked S_0 , it is obvious that the above-mentioned bands were almost equal in density, indicating that the 92 kDa protein was secreted much more extensively than the 78 kDa protein. This is visualized in Fig. 4, where the fractions are reduced to $460 \mu\text{l}$ each to improve the resolution between organelles present in the β -zone. The difference in subcellular localization of lactoferrin and gelatinase observed on SDS/PAGE is corroborated by Western blotting of these fractions (Fig. 4), and indeed the intensity of the bands in the blots is in accordance with the content of gelatinase and lactoferrin as measured by e.l.i.s.a.

Examination of the distribution of gelatinase from control cells and stimulated cells on Percoll gradients did not indicate that any particular subset of gelatinase-containing granules was

selectively mobilized in response to FMLP. An even reduction in the curve to 77% of its area was observed following stimulation (Fig. 5).

In unperturbed cells, a major peak of cytochrome b_{558} co-localized with the specific-granule markers, vitamin B-12-binding protein and lactoferrin, while a minor peak was confined to the γ -band, i.e. secretory vesicles and plasma membrane (Fig. 2). The b -cytochrome content in the γ -band rose from 13.8% [$\pm 4\%$ (S.D.), $n = 7$] in control cells to 21.2% ($\pm 3.5\%$, $n = 5$) in cells stimulated with FMLP. This translocation of approx. 7% of cytochrome b_{558} from the β - to the γ -band was in accordance with the release of vitamin B-12-binding protein and lactoferrin rather than gelatinase (Table 1), thus confirming the allocation of the major proportion of cytochrome b_{558} to the specific granules [5,40]. This finding was corroborated in subcellular fractionation experiments where cells were stimulated with a

Fig. 5. Subcellular fractionation of control and FMLP-stimulated cells

Cells at 3×10^7 /ml in KRP were either kept on ice (control) or stimulated with 10 nM-FMLP for 15 min at 37 °C. Subcellular fractionation was performed as described in the Materials and methods section and in the legend to Fig. 2. The subcellular distribution of gelatinase is shown. —, Control; □, FMLP.

potent secretagogue, the calcium ionophore ionomycin. Under these conditions it is difficult to estimate the release of gelatinase and vitamin B-12-binding protein because concomitant release of potent proteases and oxidizing species results in inactivation and proteolysis of the marker proteins [24]. One cannot, therefore, compare release of gelatinase and vitamin B-12-binding protein with translocation to cytochrome b_{558} from the β - to the γ -band. This problem can be circumvented by examining the granules remaining in the β -band after stimulation, since these have not been exposed to proteases or oxidizing radicals liberated at the cell surface. It is readily observed that the β -band is selectively depleted of gelatinase relative to cytochrome b_{558} and vitamin B-12-binding protein (Fig. 6). The ratio between the content of gelatinase and cytochrome b_{558} in the β -band (i.e. fractions 8–12) dropped from 2.9 in the control gradient to 0.8 after stimulation, whereas a corresponding ratio between vitamin B-12-binding protein and cytochrome b_{558} remained unchanged (control, 0.99; ionomycin, 0.96).

Release studies

To investigate further and characterize the mobilization of gelatinase, the neutrophils were exposed to various concentrations of FMLP, leukotriene B_4 , granulocyte-macrophage colony stimulating factor, zymosan-activated serum, and platelet-activating factor. The results of these experiments are summarized in Fig. 7. In all conditions there was a significantly higher release of gelatinase compared with vitamin B-12-binding protein. This was not caused by inactivation of secreted vitamin B-12-binding protein, since the total binding capacity was the same in activated cells and control cells. The average recovery of vitamin B-12-binding protein, with reference to control cells incubated at 37 °C, was 98 % (range 82–111 %), whereas the recovery of gelatinase was 102 % (range 94–119 %). Addition of catalase (2000 units/ml) during stimulation with 10 nM-FMLP did not increase the amount of vitamin B-12-binding protein released, thus confirming that vitamin B-12-binding protein was not inactivated by myeloperoxidase-induced oxidation [24]. Release of granule proteins could not be ascribed to cell death since the content of lactate dehydrogenase in S_0 was less than 1 %.

The time course for secretion of gelatinase and vitamin B-12-binding protein was investigated by exposing cells to FMLP (10 nM) for various time intervals (Fig. 8). This revealed no major

Fig. 6. Subcellular fractionation of control and ionomycin-stimulated cells

Cells at 3×10^7 /ml in KRP were either kept on ice (control) or stimulated with 0.5 μ M-ionomycin for 15 min at 37 °C. Subcellular fractionation was performed as described in the Materials and methods section and in the legend to Fig. 2. Subcellular distributions are shown separately for gelatinase, vitamin B-12-binding protein, and cytochrome b_{558} . —, Control; □, ionomycin.

differences in the kinetics of mobilization between the two granule subsets.

We were not able to obtain reliable estimates of the release of lactoferrin by relating exocytosed lactoferrin to lactoferrin present in the solubilized cell pellet. This is probably because of an interaction between nuclei and lactoferrin in the solubilized cell pellet, causing inhibition of the assay and thus overestimation of the release. This hypothesis was tested by mixing samples of a post-nuclear supernatant, S_1 , obtained from cavitated neutrophils, with the pellet containing nuclei, P_1 . Mixing S_1 and P_1 for 15 min at 4 °C, in the presence of 0.2 % Triton X-100 to solubilize granules, caused the lactoferrin content to drop to 26 % ($n = 3$) of the value present in S_1 and P_1 incubated without detergent. By measuring lactoferrin in disrupted neutrophils, where nuclei (P_1) had been separated from post-nuclear supernatant (S_1) (Table 1), or by preparing dilutions of cell pellets for lactoferrin e.l.i.s.a. before solubilization (Table 2), the above-mentioned problem was avoided and the release of lactoferrin could be estimated. Using these procedures a series of experiments was performed to compare the release of vitamin B-12-binding protein and lactoferrin. This did not reveal any difference between the release of lactoferrin and the release of vitamin B-12-binding protein (Table 2).

Fig. 7. Release studies

Isolated neutrophils, at 3×10^7 cells/ml in KRP, were incubated either on ice (4 °C), at 37 °C without addition of any stimulus (37 °C) or at 37 °C in the presence of various concentrations of FMLP, leukotriene B_4 (LTB_4), granulocyte-macrophage colony stimulating factor (GM-CSF), PAF or zymosan-activated serum (ZAS). After 15 min the incubation was stopped by the addition of 2 vol. of ice-cold buffer. The cells were pelleted, the supernatant (S_0) aspirated and the cells resuspended to the initial volume in ice-cold KRP containing 0.2 % of Triton X-100. Release was calculated as $S_0/(S_0 + \text{pellet})$. Bars are means of at least three experiments. Error bars represent S.D. ■, Gelatinase; □, vitamin B-12-binding protein.

Table 2. Release of vitamin B-12-binding protein and lactoferrin from control cells kept at 4 °C or at 37 °C and from cells stimulated as indicated

Experiments were performed as described in the Materials and methods section and in the legend to Fig. 7, except that dilutions for lactoferrin e.l.i.s.a. were made before solubilization of the cell pellet to avoid interaction between lactoferrin and nuclei. Values for release are expressed as a percentage $\pm$ S.D. ($n = 6$ for vitamin B-12-binding protein, $n = 3$ for lactoferrin). Abbreviations used: ZAS, zymosan-activated serum; LTB_4 , leukotriene B_4 .

Incubation conditions	Vitamin B-12-binding protein (%)	Lactoferrin (%)
4 °C	0.4 $\pm$ 0.3	0.8 $\pm$ 0.2
37 °C	1.9 $\pm$ 0.8	1.8 $\pm$ 0.4
FMLP (100 nM)	5.7 $\pm$ 0.5	4.2 $\pm$ 0.4
PAF (100 nM)	4.9 $\pm$ 1.1	3.9 $\pm$ 0.7
ZAS (10 %, w/v)	5.7 $\pm$ 0.8	4.1 $\pm$ 0.6
LTB_4 (300 nM)	3.7 $\pm$ 1.2	2.2 $\pm$ 0.2
GM-CSF (200 units/ml)	3.4 $\pm$ 0.5	2.5 $\pm$ 0.3
FMLP (10 nM for 40 min)	5.7 $\pm$ 1.0	5.0 $\pm$ 1.4

DISCUSSION

We developed an e.l.i.s.a. for gelatinase that has several advantages over the functional gelatinolytic assay [22]. First, using the e.l.i.s.a., the latent enzyme does not need to be activated before assay. Thus, exposure of the enzyme to proteases and other inhibitors present in the neutrophil granules is avoided. This problem is particularly pertinent to measurements of gelatinase in whole cells and subcellular fractions where the gelatinase is exposed to high concentrations of azurophil and specific-granule contents both during the period of activation and during the gelatinolytic assay. Inaccuracies owing to inactivation or lack of activation are also eliminated using the e.l.i.s.a. Secondly, the problem of specificity of the gelatinolytic assay based on release of trichloroacetic acid-soluble gelatine fragments is eliminated.

Using the gelatinase e.l.i.s.a., we were able to show that gelatinase is contained within a granule subset of slightly lower density than specific granules. The differences were discrete with considerable overlap of the distribution profiles of gelatinase and vitamin B-12-binding protein, as has been observed in previous publications [12–15,32]. Dewald *et al.* were able to achieve separation only by applying zonal centrifugation [12]. The difference in localization of gelatinase on the one hand and the traditional specific-granule markers, lactoferrin and vitamin B-12-binding protein, on the other hand was further substantiated by release studies which showed a 3–6-fold more extensive release of gelatinase relative to lactoferrin and vitamin B-12-binding protein upon stimulation with a variety of physiological stimuli. The average release of gelatinase upon stimulation with 10 nM-FMLP reached 22.5 %, which is considerably less than the 40 % observed by Jones *et al.* [14] using the functional assay. The susceptibility of the functional assay to proteases of the β -band may explain this difference, and may also explain why Jones *et al.* found the major part of gelatinase to be located in and released from the pre- γ region, and not the β -band as we found.

Subcellular fractionation has the advantage over electron microscopy that antigens can be accurately quantitated in different fractions, and that antigenicity is not destroyed by fixation procedures. It is probable that this explains the difference between our results and those obtained by Hibbs & Bainton [20], who found co-localization between lactoferrin and gelatinase for

Fig. 8. Time course of gelatinase release upon stimulation with FMLP

Cells at a density of 3×10^7 cells/ml in KRG were either kept on ice (4 °C) or incubated at 37 °C unstimulated (37 °C) or with the addition of 10 nM-FMLP and incubated for the indicated intervals of time. Samples at 37 °C were incubated for 15 min. Bars are means of at least three experiments. Error bars represent S.D. ■, Gelatinase; □, vitamin B-12-binding protein.

80% of the antigens identified by immunogold. Differences in distribution profiles of gelatinase and lactoferrin have been observed before using a gelatinase e.l.i.s.a. applied to 240 fractions collected from a three-step Percoll gradient [15]. It is possible that specific granules should be regarded as a heterogeneous population, all containing vitamin B-12-binding protein, lactoferrin, and gelatinase, but some containing high concentrations of gelatinase and very little vitamin B-12-binding protein and lactoferrin. This subset is released more readily than the traditional specific granules and may play an important role in the migration of neutrophils through tissues. It is probable, as has been proposed by others, that important adhesion molecules reside in this compartment [13,14,18,19]. Our results regarding cytochrome b_{558} localization are in contradiction to those of Mollinedo *et al.* [16,17]. Their use of a functional gelatinase assay, and the rather poor resolution of organelles on sucrose density gradients which they achieved, may explain these discrepancies. The strict co-localization of vitamin B-12-binding protein, lactoferrin and cytochrome b_{558} , and the lack of substantial translocation of cytochrome b_{558} from the β - to the γ -band during stimulation with FMLP, indicate that gelatinase-rich granules do not participate in early activation of the respiratory burst oxidase.

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