Isolation and Characterization of Gelatinase Granules From Human Neutrophils

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We recently confirmed the existence of gelatinase granules as a subpopulation of peroxidase-negative granules by double-labeling immunogold electron microscopy on intact cells and by subcellular fractionation. Further characterization of gelatinase granules has been hampered by poor separation of specific and gelatinase granules on both two-layer Percoll gradients and sucrose gradients. We have developed a three-layer Percoll density gradient that allows separation of the different granules and vesicles from human neutrophils; in particular, it allows separation of specific and gelatinase granules. This allows us to characterize these two granule populations with regard to their content of membrane proteins, which become incorporated into the plasma membrane during exocytosis. We found that gelatinase granules, defined as peroxidase-negative granules containing gelatinase but lacking lactoferrin, contain 50% of total cell gelatinase, with the remaining residing in specific granules. Furthermore, we found that 20% to 25% of both the adhesion protein Mac-1 and the NADPH-oxidase component cytochrome b_{558} is localized in gelatinase granules. Although no qualitative difference was observed between specific granules and gelatinase granules with respect to cytochrome b_{558} and Mac-1, stimulation of the neutrophil with FMLP resulted in a selective mobilization of the least dense peroxidase-negative granules, ie, gelatinase granules, which, in concert with secretory vesicles, furnish the plasma membrane with Mac-1 and cytochrome b_{558}. This shows that gelatinase granules are functionally important relative to specific granules in mediating early inflammatory responses.

HUMAN NEUTROPHILS are equipped with a battery of proteins that contribute to the bactericidal potential of the cell. Many of these proteins, including adhesion proteins, receptors for chemotactic substances and complement factors,[1-4] potent proteases,[5] and the cytochrome b_{558} component of the NADPH-oxidase,[6,7] are localized in intracellular stores. These substances are brought into contact with their respective targets only when the granules/vesicles in which they reside are exocytosed, as occurs in response to mediators of inflammation.

The existence of two neutrophil granule subpopulations has been known for decades, namely, peroxidase-positive azurophil granules and peroxidase-negative specific granules.[8] However, lately, the structure of the neutrophil has proven more complex. We have demonstrated the existence of a very easily mobilizable compartment, the secretory vesicles, which is important during early neutrophil activation.[3,9] Secretory vesicles are identified by latent alkaline phosphatase, ie, alkaline phosphatase that can only be measured in the presence of detergent. They constitute a reservoir of the adhesion protein Mac-1,[3,10] the NADPH-oxidase component cytochrome b_{558},[10,11] and the receptor for the chemotactic peptide FMLP.[12]

Dewald et al[13] proposed the existence of yet another granule population, the gelatinase-containing granules. This been confirmed by others, and Mac-1, cytochrome b_{558}, and the receptor for FMLP have been allocated to these granules.[4,7,14,15] These findings, which are based on the measurement of gelatinase by a functional assay, have been questioned by double-labeling immunogold electron microscopy, which demonstrated gelatinase to be a component of specific granules.[16] We have recently resolved this discrepancy by demonstrating that the 135-kD form of neutrophil gelatinase is a complex of 92-kD gelatinase and a 25-kD protein that we designated NGAL.[17] The major part of NGAL is localized in specific granules not associated with gelatinase.[18] Using antibodies specific for gelatinase, we could demonstrate that peroxidase-negative granules exist as a continuum from the most dense granules containing lactoferrin, vitamin B_{12}-binding protein, and NGAL but no gelatinase to the lightest granules containing gelatinase but no lactoferrin, vitamin B_{12}-binding protein, or NGAL.[18-20] The latter granules, which constitute approximately 25% of all peroxidase-negative granules, are the most easily mobilized and our data indicate that they contain the majority of the gelatinase within the cell. These granules are defined as gelatinase granules. The remaining peroxidase-negative granules, which all contain lactoferrin and of which 80% also contain gelatinase, are defined as specific granules.[20]

Although gelatinase granules are lighter than specific granules, their respective densities do not differ very much. Previously, only Graves et al[15] have obtained a separation of a subset of granules described as specific granules containing gelatinase but lacking vitamin B_{12}-binding protein (corresponding to "our" gelatinase granules) from specific granules containing both vitamin B_{12}-binding protein and gelatinase. They used a three-layer sucrose density gradient specifically designed for this purpose.[15] The use of other sucrose density gradients,[4,7,14] zonal sedimentation,[13] and Percoll density gradients[3,19] has resulted in a considerable overlap between the distribution profile of lactoferrin and

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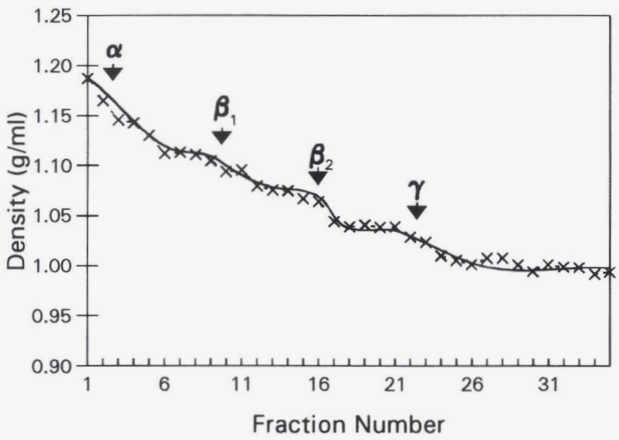


Fig 1. Density profile of three-layer Percoll density gradient. Density gradients were prepared as described in Materials and Methods by applying the postnuclear supernatant on top of a 3 × 9 mL Percoll gradient (1.12/1.09/1.05 g/mL) followed by centrifugation at 37,600*g* for 30 minutes. The location of the visible bands is indicated.

vitamin B_{12}-binding protein on the one hand and gelatinase on the other. This has precluded analysis of the distribution within the peroxidase-negative granule subpopulations of different granule-associated proteins such as Mac-1,[3,4,14] cytochrome b_{558},[7,19] and the receptor for the chemotactic peptide FMLP.[12] We now present a three-layer Percoll density gradient that offers separation of specific granules and gelatinase granules. This enables us to further characterize the gelatinase granules regarding ultrastructure, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile, and content of the membrane-associated proteins cytochrome b_{558} and Mac-1.

MATERIALS AND METHODS

Isolation of neutrophils. Neutrophils were isolated from blood donated by healthy volunteers. Blood was anticoagulated in 25 mmol/L sodium citrate, 126 mmol/L glucose. Red blood cells were allowed to sediment for 45 minutes by the addition of equal amounts of 2% (wt/vol) Dextran (Pharmacia, Uppsala, Sweden) in saline. The leukocyte-rich supernatant was centrifuged on Lymphoprep (Nygaard, Oslo, Norway) at 400*g* for 15 minutes.[21] Remaining erythrocytes were lysed by hypotonic shock in ice-cold water for 30 seconds and tonicity was restored by the addition of 1.8% NaCl. Cells were washed once in saline and resuspended in the desired buffer. All steps except dextran sedimentation were performed at 4°C.

Stimulation of neutrophils. For stimulation, the cells were resuspended in Krebs Ringer phosphate (KRP; 130 mmol/L NaCl, 5 mmol/L KCl, 1.27 mmol/L $MgSO_4$, 0.95 mmol/L $CaCl_2$, 5 mmol/L glucose, 10 mmol/L NaH_2HPO_4/Na_2HPO_4, pH 7.4) at 3×10^7 cells/mL and preincubated for 5 minutes at 37°C. After the addition of the stimulus (10 nmol/L FMLP or 2 μg/mL phorbol myristate acetate [PMA]) cells were incubated for 15 minutes. The incubation was stopped by centrifugation at 200*g* for 6 minutes. The supernatant, termed S_0, was aspirated and the cells were resuspended in saline for subsequent subcellular fractionation. Release of granule markers was calculated as content in S_0 as a percentage of the content in $S_0 + S_1 + P_1$ (see below).

Subcellular fractionation. After incubation of neutrophils with diisopropylfluorophosphate (DFP; 5 mmol/L; Aldrich Chemical Co, Milwaukee, WI) for 5 minutes and centrifugation at 200*g* for 6 minutes, neutrophils were resuspended at 3 to 5×10^7/mL in disruption buffer (100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L $ATPNa_2$, 3.5 mmol/L $MgCl_2$, 10 mmol/L PIPES, pH 7.2) containing 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by nitrogen cavitation (pressurized for 5 minutes) as described.[6] Nuclei and intact cells were pelleted by centrifugation at 400*g* for 15 minutes (P_1). Ten milliliters of the postnuclear supernatant (S_1) was applied on top of a 3×9 mL three-layer Percoll gradient (1.05/1.09/1.12 g/mL) containing 0.5 mmol/L PMSF and centrifuged at 37,000*g* for 30 minutes. This resulted in a gradient with 4 visible bands, from the bottom designated the α-band, the β_1-band, the β_2-band, and the γ-band. The cytosol was present above the γ-band on top of the Percoll. The gradient was collected in fractions of 1 mL each by aspiration from the bottom of the tube. All fractions were assayed for markers as described below.

Marker assays. Myeloperoxidase (azurophil granules), lactoferrin (specific granules), gelatinase (gelatinase granules), albumin (secretory vesicles[22]), and HLA (plasma membranes) were all measured by enzyme-linked immunosorbent assay (ELISA) as described.[3] Specific granules were also identified by vitamin B_{12}-binding protein as described by Gottlieb et al[23] and Kane and Peters.[24] Secretory vesicles were also identified by latent alkaline phosphatase, ie, alkaline phosphatase only measurable in the presence of detergent (0.2% Triton X-100).[9,25] Furthermore, fractions were assayed for β_2-microglobulin and for the α subunit CD11b of Mac-1, both assessed by ELISA.[3,26] General procedures for the ELISAs were as described for NGAL ELISA below, except that samples for the CD11b ELISA were solubilized by 25 mmol/L N-octyl glucoside and 0.2% cetyltrimethylammonium bromide (CTAB) before further dilution.[3] The content of cytochrome b_{558} was quantitated by dithionite-reduced-minus-oxidized difference spectra using an

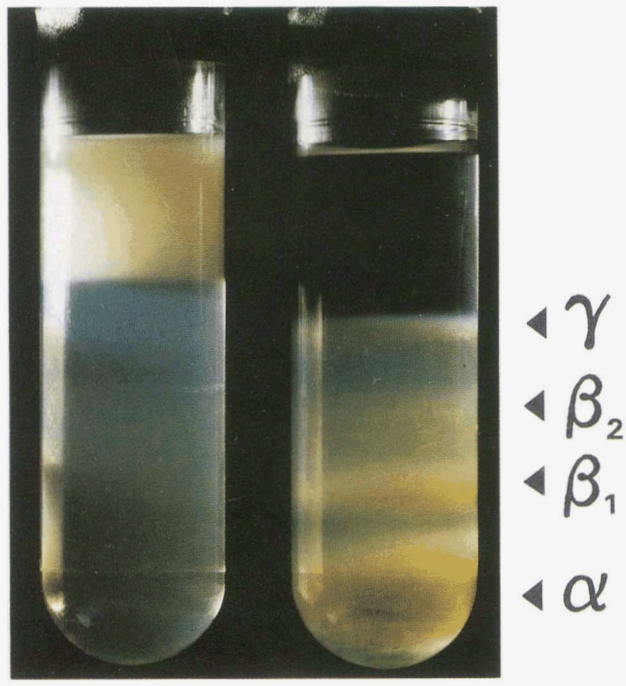


Fig 2. Photo of three-layer Percoll density gradient before and after centrifugation of postnuclear supernatant. The resulting bands, the α-band, the β_1-band, the β_2-band, and the γ-band, are readily visible.

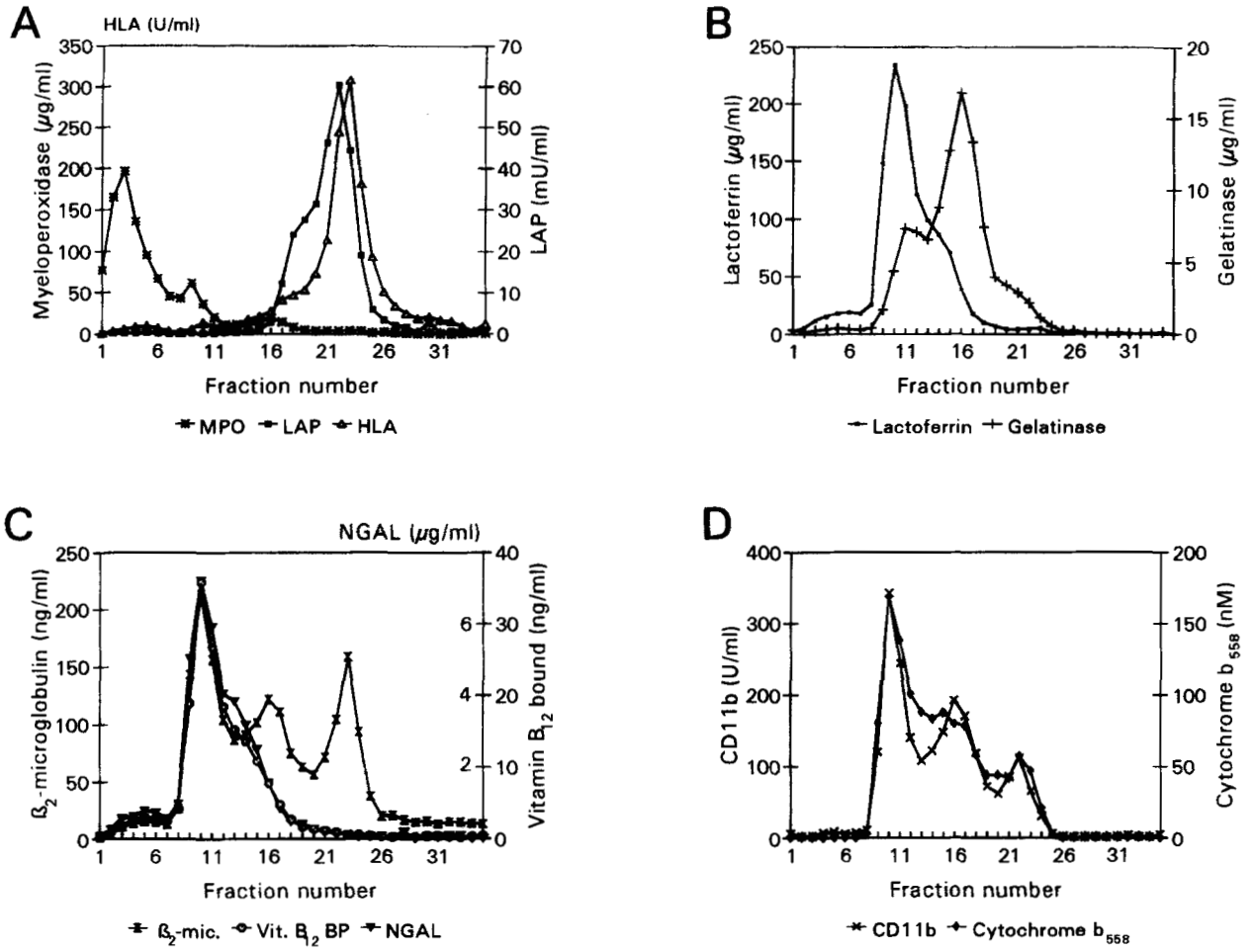


Fig 3. Subcellular fractionation of unperturbed neutrophils on three-layer Percoll density gradients. Neutrophils at 3 to 5 × 10^7 cells/mL were resuspended in cavitation buffer and disrupted by nitrogen cavitation followed by subcellular fractionation on a three-layer Percoll density gradient as described in Materials and methods. The gradient was fractionated by aspiration from the bottom of the tube into 37 fractions of 1 mL each. The lower the fraction number, the denser the fraction. The content of various markers was measured in each fraction as described. (A) The distributions of myeloperoxidase (MPO), latent alkaline phosphatase (LAP), and HLA. (B) The distributions of lactoferrin and gelatinase. (C) The distributions of vitamin B_{12}-binding protein (vit. B_{12} BP), NGAL, and β_2-microglobulin ($\beta_2$mic). (D) The distributions of cytochrome b_{558} and CD11b. The results are average distributions in 3 to 7 subcellular fractionation experiments (the actual number for each marker is shown in Table 1).

absorption coefficient for the 426 nm peak of 105 $mmol/L^{-1} \cdot cm^{-1}$.[27]

NGAL was measured by ELISA.[18] Antibodies against NGAL were obtained as described in Kjeldsen et al.[17] NGAL-ELISA was performed using 96-well flat-bottom immunoplates (Nunc, Roskilde, Denmark). The plates were coated overnight with anti-NGAL antibodies, diluted 1:2000 in carbonate buffer (50 mmol/L $Na_2CO_3/NaHCO_3$, pH 9.6). Additional binding sites were blocked by incubation with 200 μL/well of dilution buffer (0.5 mol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na_2HPO_4/KH_2PO_4, pH 7.2, 1% bovine serum albumin [BSA], 1% Triton X-100). Samples were then applied, followed by the addition of biotinylated anti-NGAL antibody diluted 1:2,000 and by the addition of avidin-peroxidase (Dakopatts [P347]) diluted 1:2,000. All incubations were performed in 100 μL/well for 1 hour unless otherwise stated. Color was developed during 30 minutes of incubation in 0.1 mol/L sodium phosphate, 0.1 mol/L citric acid buffer, pH 5.0, containing 0.04% o-phenylenediamine and 0.03% H_2O_2 (100 μL/well), and stopped by the addition of 100 μL 1 mol/L H_2SO_4. The plates were washed three times in washing buffer (0.5 mol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na_2HPO_4/KH_2PO_4, pH 7.2, 1% Triton X-100) between all steps. Before color development, an additional wash in sodium phosphate citric acid buffer was included. Absorbance was read at 492 nm in a Multiscan Plus ELISA-reader (Labsystems, Helsinki, Finland). A standard of purified monomeric NGAL ranging from 0.086 to 5.5 ng/mL was used. Samples were diluted from 100- to 10,000-fold in dilution buffer. All steps were performed at room temperature.

SDS-PAGE. SDS-PAGE was performed essentially as described by Laemmli, using 5% to 20% gradient gels with a 3% stacking gel.[28]

Electron microscopy. Subcellular fractions were centrifuged on an Airfuge (Beckmann, Palo Alto, CA) to sediment the Percoll. The biologic material from each fraction was aspirated and resuspended and fixed for 3 hours in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.3. After washing twice in cacodylate buffer, pellets were incubated for 5 hours at room temperature in 1% osmium tetroxide in 0.1 mol/L cacodylate buffer, pH 7.3. The pellets were subsequently dehydrated in ethanol and soaked in 100% epoxypropane

Table 1. Distribution of Biochemical Markers After Subcellular Fractionation of Unperturbed Neutrophils on Three-Layer Percoll Density Gradients

	α-band	β_1-band	β_2-band	γ-band	Cytosol	Recovery
Myeloperoxidase	73.4 (4.2)	19.5 (3.3)	4.5 (0.8)	2.2 (1.0)	0.4 (0.3)	99.7 (8.5)
Lactoferrin	8.2 (3.0)	83.3 (4.6)	6.6 (1.3)	1.8 (1.0)	0.1 (0.1)	102.1 (10.6)
Gelatinase	2.1 (1.4)	47.1 (4.7)	40.0 (3.1)	10.3 (2.0)	0.5 (0.5)	91.6 (14.8)
Latent alkaline phosphatase	0.4 (0.7)	1.4 (1.6)	21.1 (4.2)	74.4 (3.7)	2.8 (2.2)	97.2 (13.5)
Albumin	1.4 (1.3)	8.1 (2.0)	28.7 (3.3)	59.8 (4.3)	2.1 (1.5)	95.8 (9.6)
HLA	2.6 (2.6)	5.2 (2.2)	10.5 (4.5)	71.6 (6.8)	10.1 (4.5)	82.9 (11.1)
Vitamin B_{12}-binding protein	8.0 (1.9)	78.3 (4.2)	9.5 (1.3)	2.8 (2.0)	1.4 (1.1)	93.7 (8.9)
NGAL	9.5 (2.1)	79.6 (1.7)	8.0 (0.7)	2.1 (0.4)	0.9 (0.5)	105.4 (7.5)
β_2-Microglobulin	3.5 (0.7)	44.7 (1.0)	17.9 (0.8)	26.5 (1.9)	7.5 (0.7)	90.9 (3.3)
Cytochrome b_{558}	0	61.1 (6.2)	21.9 (2.7)	17.0 (5.6)	0	ND
Mac-1	2.0 (1.2)	55.5 (6.4)	24.7 (4.0)	17.0 (3.3)	0.8 (1.1)	ND

Neutrophils at 3 to 5 $\times$ 10^7 cells/mL were disrupted by nitrogen cavitation followed by centrifugation to pellet nuclei and unbroken cells. The supernatant, S_1, was applied on three-layer Percoll density gradients and centrifuged as described in Materials and Methods. This resulted in a gradient with four visible bands, from the bottom designated the α-band (corresponding to fractions no. 1 through 7), the β_1-band (corresponding to fractions no. 8 through 15), the β_2-band (corresponding to fractions no. 16 through 19), and finally the γ-band (corresponding to fractions no. 20 through 26). The remaining fractions represent the cytosol. The content of the various markers in the regions defined above is expressed as the content recovered in the region as a percentage of the total content recovered in the gradient. The recovery of each marker is calculated as total content recovered on the gradient in percentage of content in S_1. Values are in percentages and are the mean of seven experiments (except for Mac-1, NGAL, and for β_2-microglobulin, which represent 6, 4, and 2 experiments, respectively) with the SD given in parentheses.

Abbreviation: ND, not determined.

and left overnight in a 50% mixture (vol/vol) of epoxypropane and Epon before a final embedding in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope (Philips, Eindhoven, Holland).

Morphometrical analysis of peroxidase-negative granules was performed by measuring the diameters of granules displaying double membrane configuration. Only granules from the β_1-band and β_2-band were evaluated.

RESULTS

Subcellular fractionation on two-layer Percoll density gradients (1.05/1.12 g/mL) results in generation of three vis-

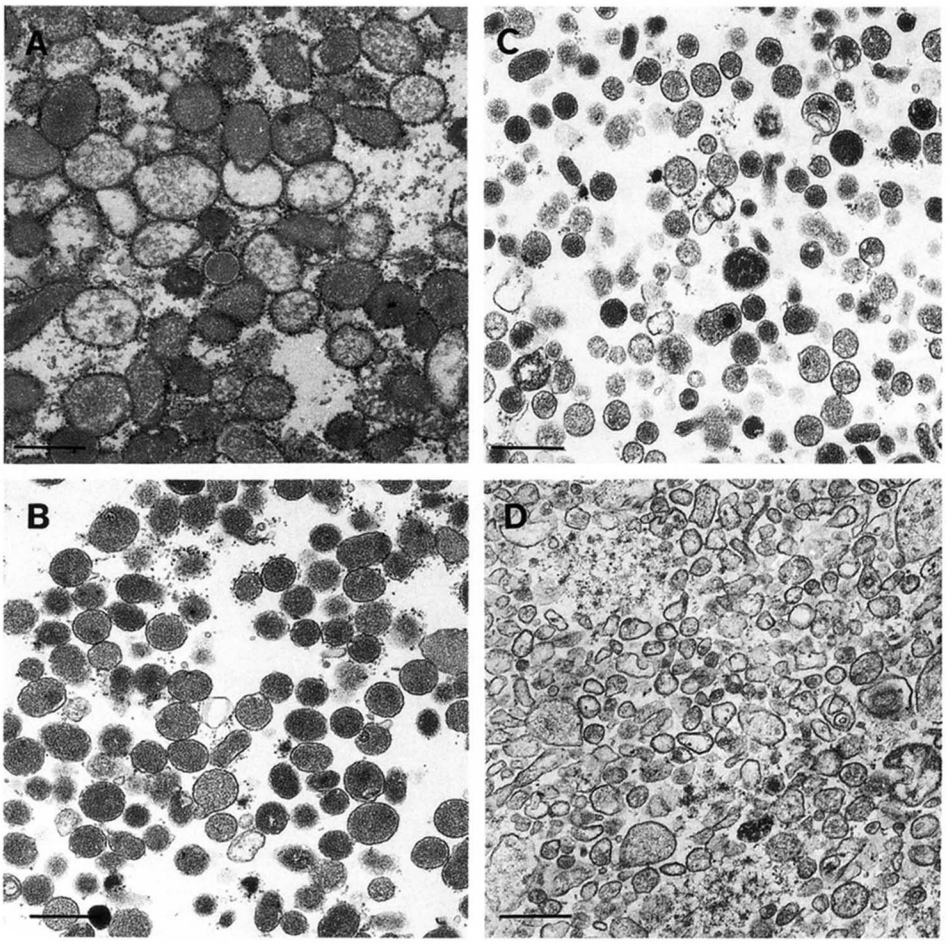
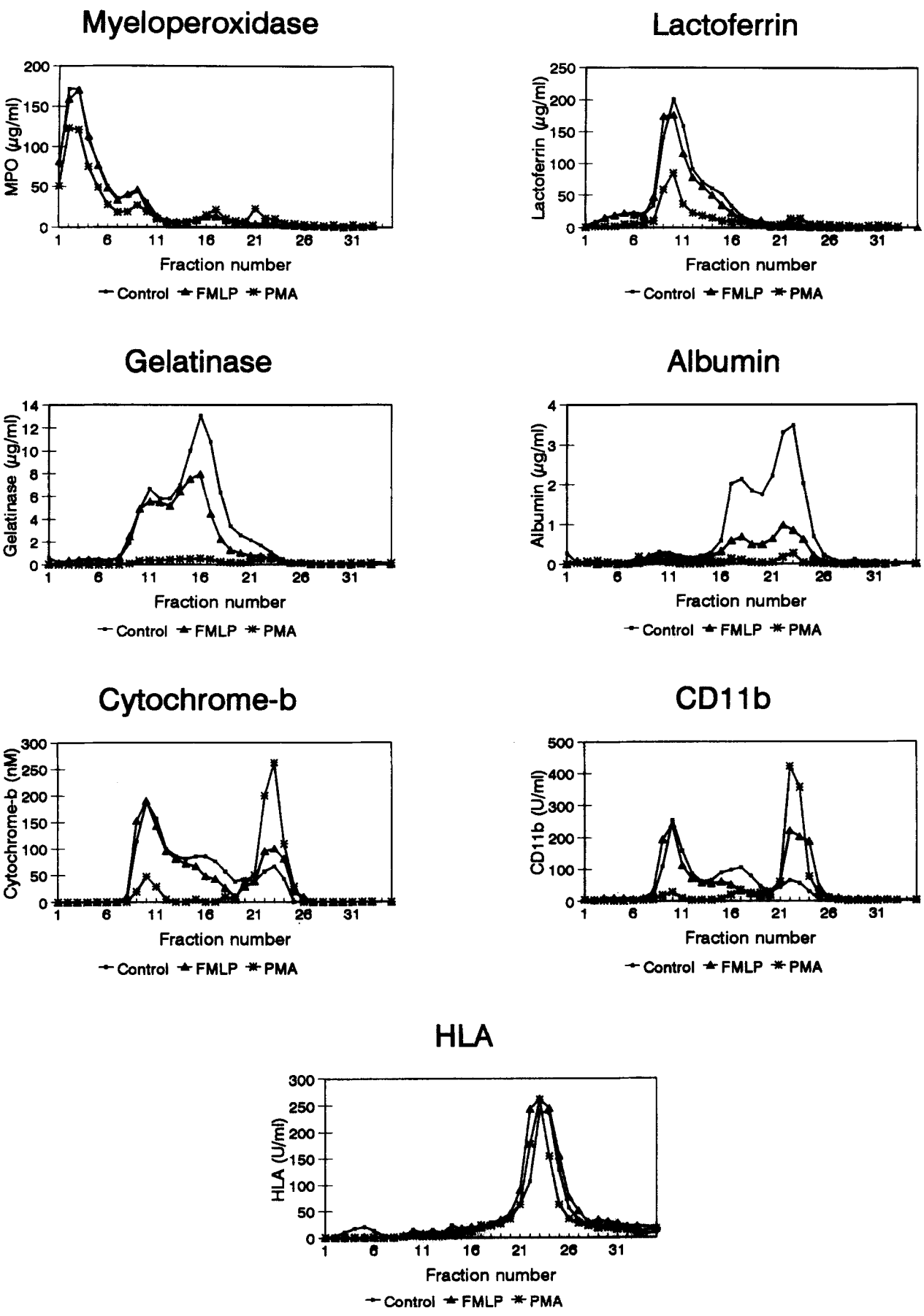


Fig 4. Ultrastructure of material from α-, β_1-, β_2-, and γ-band isolated on Percoll density gradients. Four hundred microliters of each fraction was centrifuged in an airfuge to remove Percoll. The biologic material from fractions 2 and 3 (α-band), 10 and 11 (β_1-band), 16 and 17 (β_2-band), and 23 and 24 (γ-band), respectively, was pooled and processed for electron microscopy as described in Materials and Methods. The micrographs show (A) α-band, (B) β_1-band, (C) β_2-band, and (D) γ-band. Bars: 0.5 μm.



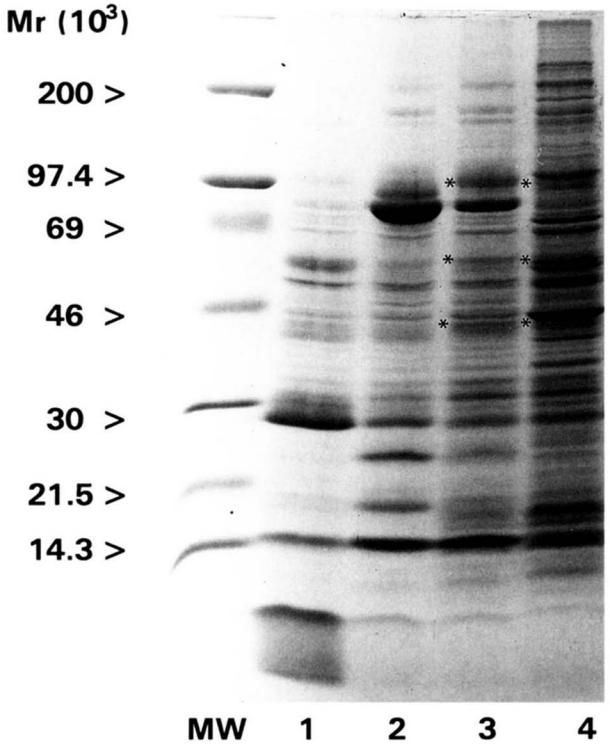


Fig 5. SDS-PAGE protein profile of fractions from Percoll gradient. Four hundred microliters of fractions containing peak amounts of myeloperoxidase (lane 1), lactoferrin (lane 2), gelatinase (lane 3), and latent alkaline phosphatase/HLA (lane 4), respectively, was centrifuged in an airfuge to pellet the Percoll. The biologic material was collected and resuspended in 100 μL of saline. Sixty-six microliters of the resulting material was mixed with 34 μL of saline and 100 μL of Laemmli sample buffer containing mercapto-ethanol. After boiling for 3 minutes, 150 μL of each fraction was applied to electrophoresis on a 5% to 20% gradient gel with a 3% stacking gel. The lane marked MW shows molecular weight markers. Bands that are enriched in gelatinase granules are marked (*) on either side in lane 3.

ible bands, as previously described.[6] The α-band contains azurophil granules, the β-band specific and gelatinase granules, and the γ-band light membrane structures, including secretory vesicles and plasma membranes.[9] The density profile within the β-band is rather steep.[6] Introducing a third layer of Percoll with a density of 1.09 g/mL leads to a less-steep density profile (Fig 1), resulting in generation of 4 visible bands, from the bottom designated the α-band, the β_1-band, the β_2-band, and the γ-band, with the cytosol on top of the Percoll (Fig 2). This gradient was fractionated by aspiration from the bottom of the tube into 37 fractions of 1 mL each. Figure 3A and B shows the distribution profiles of markers for the different compartments of the neutrophil, and Table 1 gives the distribution of these markers in the different regions of the gradient. The gradient is largely unchanged compared with the two-layer Percoll gradient regarding the content of the α- and the γ-band (Fig 3A and Table 1). The α-band contains the majority of the azurophil granule marker myeloperoxidase, whereas the γ-band contains secretory vesicles identified by latent alkaline phosphatase and plasma membrane identified by HLA. The characteristic "shoulder" in the profile of latent alkaline phosphatase extends into the β_2-band (21% of latent alkaline phosphatase activity is contained within the β_2-band; Table 1). The β_1-band contains the majority of the specific granule marker lactoferrin (83%), whereas the majority of gelatinase is located in the β_2-band and in the γ-band, which both contain very little lactoferrin (Fig 3B and Table 1). We have identified a subpopulation of peroxidase-negative granules that on double-labeling immunogold electron microscopy are positive for gelatinase but lack lactoferrin; we have defined these as gelatinase granules.[20] The remaining peroxidase-negative granules all contain lactoferrin; although 80% contain gelatinase as well, these are defined as specific granules. Thus, a total separation of gelatinase from lactoferrin is impossible because a significant portion of gelatinase resides in lactoferrin-containing granules. Although there is a gradual transition from specific to gelatinase granules, we here arbitrarily define gelatinase granules as all granules contained in fraction no. 16 and higher, albeit 6% of lactoferrin is contained within these fractions (Table 1). According to this definition, gelatinase granules contain 50.3% of total cell gelatinase (40.0% in the β_2-band, 10.3% in the γ-band).

The ultrastructure of the different bands was investigated by electron microscopy. Figure 4A through D shows electron micrographs of material from the α-, β_1-, β_2-, and γ-band, respectively. The ultrastructure of the α-band and the γ-band is in agreement with previous publications.[6,29] The ultrastructures of the β_1- and β_2-bands, respectively, are very similar, because both contain electron-dense rounded granules, although the β_2-fraction appears more heterogeneous, possibly because of contamination with light membrane structures. From morphometric analysis of 84 granules from the β_1-band and 79 granules from the β_2-band, it appears that the β_2 granules are slightly but significantly smaller than the β_1 granules (average diameter of β_2 granules 187 nm compared with 236 nm of β_1 granules, $P < .00001$, two sample t-test [unpaired]). This is in agreement with previous findings using double-labeling immunogold electron microscopy on whole cells, which showed gelatinase gran-

Fig 6. Subcellular fractionation of unperturbed, FMLP-stimulated, and PMA-stimulated neutrophils on three-layer Percoll density gradients. Neutrophils at 3 × 10^7 cells/mL were either kept on ice (control) or preincubated at 37°C followed by stimulation for 15 minutes with 10 nmol/L FMLP or 2 μg/mL PMA. After pelleting of the cells, the supernatant (S_0) was aspirated and the cells were incubated with DFP and subjected to subcellular fractionation as described in Materials and Methods. The gradients were fractionated by aspiration from the bottom of the tube into 37 fractions of 1 mL each. The lower the fraction number, the denser the fraction. Fractions were assayed for myeloperoxidase (MPO), lactoferrin, gelatinase, albumin, HLA, cytochrome b_{558}, and CD11b. Results are means of three experiments of control, FMLP-stimulated, and PMA-stimulated cells fractionated in parallel.

Table 2. Distribution of Biochemical Markers After Subcellular Fractionation on Three-Layer Percoll Density Gradients of Unperturbed Neutrophils or Neutrophils Stimulated With FMLP or PMA

	Myeloperoxidase		Lactoferrin			Gelatinase			Albumin			Cytochrome b_{558}			Mac-1		
	Control	FMLP	PMA	Control	FMLP	PMA	Control	FMLP	PMA	Control	FMLP	PMA	Control	FMLP	PMA	Control	FMLP	PMA
α-Band	75.7 (0.8)	75.2 (1.3)	64.6 (7.0)	11.0 (1.9)	11.1 (1.6)	3.0 (1.6)	3.2 (1.5)	2.2 (0.6)	0 (—)	1.6 (1.7)	1.0 (1.4)	0.7 (0.8)	0 (—)	0 (—)	0 (—)	2.7 (1.2)	4.0 (2.8)	1.9 (2.5)
β_1-Band	17.0 (0.6)	16.7 (0.3)	14.5 (1.4)	79.1 (3.7)	79.5 (2.6)	34.1 (9.9)	48.5 (4.9)	46.6 (7.1)	3.4 (2.6)	7.1 (1.5)	3.8 (1.5)	1.8 (1.8)	61.7 (3.9)	62.5 (5.3)	13.1 (1.5)	57.7 (4.9)	47.1 (4.1)	6.8 (1.6)
β_2-Band	4.4 (0.6)	4.6 (0.6)	8.0 (3.1)	7.2 (1.4)	5.9 (0.8)	3.8 (1.2)	37.9 (2.8)	18.7 (3.4)	1.9 (1.4)	28.2 (0.7)	6.2 (2.0)	1.0 (0.7)	19.5 (2.1)	9.3 (3.4)	2.0 (1.9)	22.7 (3.5)	7.1 (1.8)	6.2 (6.4)
γ-Band	1.9 (0)	2.3 (0.6)	8.9 (2.3)	2.5 (0.9)	1.0 (0.8)	6.2 (0.9)	9.4 (1.6)	4.3 (1.3)	2.9 (2.1)	60.3 (3.5)	12.0 (1.2)	2.4 (1.7)	18.9 (2.0)	28.2 (2.0)	84.9 (2.7)	15.6 (2.2)	38.6 (9.9)	83.2 (7.2)
Cytosol	0.5 (0.2)	0.3 (0.2)	1.1 (0.7)	0.1 (0.2)	0.2 (0.2)	1.3 (0.9)	0.7 (0.5)	0.4 (0.6)	1.0 (0.8)	1.0 (1.7)	0.2 (0.2)	0.1 (0.2)	0 (—)	0 (—)	0 (—)	1.4 (1.4)	3.2 (3.2)	1.9 (2.6)
S_0	0.4 (0.2)	0.9 (0.2)	2.9 (1.4)	0.2 (0.1)	2.3 (0.5)	51.6 (9.0)	0.3 (0.12)	27.8 (2.0)	90.4 (6.8)	1.9 (0.9)	77.0 (2.8)	93.9 (4.3)	0 (—)	0 (—)	0 (—)	0 (—)	0 (—)	0 (—)
Release	0.3 (0.3)	0.8 (0.2)	2.6 (1.0)	0.2 (0.1)	2.2 (0.6)	47.3 (11.8)	0.3 (0.2)	26.0 (2.1)	86.2 (8.7)	1.7 (0.8)	71.8 (3.9)	93.3 (2.5)	0 (—)	0 (—)	0 (—)	0 (—)	0 (—)	0 (—)
Recovery	98.9 (1.9)	101.3 (18.1)	103.5 (14.9)	100.3 (6.5)	102.6 (5.6)	100.3 (26.6)	96.6 (19.1)	95.6 (6.6)	86.0 (8.9)	93.1 (6.4)	84.0 (4.6)	ND	ND	ND	ND	ND	ND	ND

Neutrophils at 3×10^7 cells/mL were either kept on ice (control) or preincubated at 37°C for 5 minutes followed by stimulation for 15 minutes with 10 nmol/L FMLP or 2 μg/mL PMA. After pelleting of the cells, the supernatant (S_0) was aspirated. The cells were resuspended in disruption buffer and cavitated by nitrogen cavitation followed by centrifugation to pellet nuclei and unbroken cells (P_1). The supernatant, S_1, was applied on three-layer Percoll density gradients and centrifuged as described in Materials and Methods. The α-band, and β_1-band, the β_2-band, the γ-band, and cytosol are defined as described in the legend to Table 1. The content of the various markers in S_0 or in the bands is expressed as the content recovered in S_0 or the appropriate band as a percentage of the total content recovered in the gradient + S_0. Release is calculated as $S_0/(S_0 + S_1 + P_1)$ and expressed as a percentage. The recovery of each marker is calculated as total content recovered on the gradient in percentage of content in S_1. Numbers are the mean of three experiments of control, FMLP-stimulated, and PMA-stimulated cells fractionated in parallel, with SD given in parentheses.

Abbreviation: ND, not determined.

ules to be smaller than specific granules.[20] Furthermore, these investigations showed that gelatinase granules comprise approximately 25% of all peroxidase-negative granules. This implies that the concentration of gelatinase is much higher in gelatinase granules than in specific granules. This is also obvious from the SDS-PAGE protein pattern of the different regions of the gradient, as shown in Fig 5. In lane 3, which shows the protein profile of fraction no. 16, one observes a very intense gelatinase band at 92 kD compared with lane 2 (specific granules, fraction no. 10). Apart from gelatinase, two bands at 55 kD and 40 kD are also relatively enriched in the gelatinase granule fraction and may therefore be constituents primarily residing in this granule subset. The nature of these proteins is not known. All other proteins visually identified in gelatinase granules are more prominent in either specific granules (lane 2) or light membranes (lane 4).

The separation of specific granules and gelatinase granules on this three-layer Percoll gradient allowed us to reinvestigate the subcellular localization of different granule proteins previously allocated to specific granules. From Fig 3C and Table 1 it is observed that another well-established specific granule marker, vitamin B_{12}-binding protein, strictly colocalizes with lactoferrin and is therefore a specific granule marker. We previously published that β_2-microglobulin[25] and NGAL[17] both reside in specific granules. Although NGAL strictly colocalizes with lactoferrin in specific granules, it is apparent from Fig 3C and Table 1 that some β_2-microglobulin is contained within gelatinase granules. In addition, a third peak of β_2-microglobulin in the γ-band colocalizes with the plasma membrane marker HLA measured by ELISA. This is not surprising because this assay measures the HLA-β_2-microglobulin complex.[30]

The subcellular localization of the NADPH-oxidase component, cytochrome b_{558}, and the adhesion protein, Mac-1, is of considerable interest. Although recent investigations from our laboratory indicate that both proteins are mainly located in the specific granules, these conclusions were hampered by the lack of separation between the profiles of gelatinase and lactoferrin on the Percoll gradient hitherto used.[3,19] From Fig 3D and Table 1 it is observed that the distribution of cytochrome b_{558} and Mac-1 is quite similar, the majority being located to specific granules (61% and 55%, respectively). Twenty percent to 25% is located in gelatinase granules, with the remaining 15% to 20% confined to the γ-band. Previous investigations have shown that most of cytochrome b_{558} and Mac-1, contained within the γ-band of unperturbed neutrophils, reside in secretory vesicles.[3,10,11] This indicates that there are three reservoirs of both cytochrome b_{558} and Mac-1 in unstimulated neutrophils, namely, specific granules, gelatinase granules, and secretory vesicles.

It has been demonstrated by several investigators that both Mac-1 and cytochrome b_{558} translocate from internal stores to the plasma membrane on activation of neutrophils.[6,11,31,32] The contribution of the different granule/vesicle subsets to the upregulation of cytochrome b_{558} and Mac-1 in the plasma membrane on stimulation can now be investigated by subcellular fractionation of neutrophils

stimulated with various secretagogues. Figure 6 and Table 2 show the distribution of myeloperoxidase, lactoferrin, gelatinase, albumin (a matrix marker for secretory vesicles), cytochrome b_{558}, Mac-1, and HLA in control cells and in cells stimulated with 10 nmol/L FMLP or 2 μg/mL PMA before subcellular fractionation. It is evident that stimulation of neutrophils with FMLP hardly mobilizes any specific granules (release of 2.2% of lactoferrin), whereas 26% of gelatinase is exocytosed. It is observed that gelatinase is selectively depleted from the lightest peroxidase-negative granules, ie, gelatinase granules residing in the β_2-band (reduced from 40% to 18.7%) and in the γ-band (reduced from 10.3% to 4.3%). Likewise, the content of both cytochrome b_{558} and Mac-1 in the β_2-band is reduced from 22% and 25% to 7.1% and 9.2%, respectively, with a parallel increase in the γ-band content of these two proteins. Furthermore, as previously shown,[3,10,11] a translocation of cytochrome b_{558} and Mac-1 occurs within the γ-band from secretory vesicles to the plasma membrane, because secretory vesicles are almost fully mobilized on stimulation with FMLP (as visualized by disappearance of albumin after FMLP stimulation; Fig 6). This translocation to the plasma membrane of approximately 15% of total cell cytochrome b_{558} and Mac-1 is overlooked if one only focuses on the total content of these proteins in the γ-band. On stimulation with PMA, one observes a further upregulation of cytochrome b_{558} and Mac-1 in the plasma membrane, with the major contribution coming from specific granules (Fig 6 and Table 2). In contrast to gelatinase granules, specific granules are not fully mobilized on stimulation with PMA, because 34% of lactoferrin remains in the β_1-band.

DISCUSSION

We describe here a novel method for subcellular fractionation of human neutrophils on three-layer Percoll density gradients. In contrast to previous fractionation protocols, this protocol offers separation of all established secretory organelles of the neutrophil, in particular, separation of gelatinase granules from specific (lactoferrin containing) granules. The present results are very much in line with our data obtained by double-labeling immunogold electron microscopy[20] and with the findings of Graves et al[15] and Jones et al[14] and confirms the recent assumptions of the existence of heterogeneity among peroxidase-negative granules. These granules constitute a continuum from granules rich in lactoferrin and vitamin B_{12}-binding protein but devoid of gelatinase and scarcely mobilized by inflammatory mediators to granules rich in gelatinase but devoid of lactoferrin or vitamin B_{12}-binding protein and responsive to stimulation of the cells by inflammatory mediators. These latter granules, the gelatinase granules (equivalent to "specific granules containing gelatinase but lacking vitamin B_{12}-binding protein," as designated by Graves et al[15]), should therefore be considered a subpopulation of peroxidase-negative granules. They contain approximately 50% of total cell gelatinase, with the remaining 50% being localized together with lactoferrin in specific granules. It should be stressed that we reserve the term specific granules for peroxidase-negative granules containing lactoferrin and vitamin B_{12}-binding protein.

The separation of gelatinase granules from specific granules enables us to estimate the relative distribution within peroxidase-negative granule subpopulations of both Mac-1 and cytochrome b_{558}, whose subcellular localizations have been a matter of debate during recent years. It was concluded, based on colocalization with lactoferrin in double-labeling immunogold electron microscopy, that specific granules were the intracellular reservoir of leukocyte adhesion receptors (including Mac-1[1]) and cytochrome b_{558}.[33] On the other hand, Mollinedo et al claimed both Mac-1 and cytochrome b_{558} to be located mainly within gelatinase granules.[4,7] We find the two proteins to be located in both specific and gelatinase granules, but find only 20% to 25% of Mac-1 and cytochrome b_{558} residing in gelatinase granules, whereas the majority (55% and 61%) is located in specific granules. The localization of Mac-1 in both specific and gelatinase granules is in line with the results obtained by Jones et al[14] and Graves et al,[15] although they found less Mac-1 in specific granules (32% and 26%, respectively) and more in gelatinase granules (35% and 44%, respectively). Furthermore, the fact that the majority of Mac-1 within the γ-band of unperturbed cells is not located in the plasma membrane but rather in secretory vesicles[3] was not considered in those studies. The quantitative differences in distribution of Mac-1 within peroxidase-negative granules may be caused by the use of different solubilization procedures. Only after including n-octyl glucoside and CTAB in the solubilization protocol in the CD11b ELISA were we able to measure all Mac-1 present in specific granules.[3]

The heterogeneity within peroxidase-negative granules may reflect differential but overlapping synthesis and packaging of different granule proteins during granulopoiesis. Ultrastructural studies on neutrophil precursors have indicated that lactoferrin is synthesized and packaged at the early myelocytic stage preceding the packaging of gelatinase.[16] Although hypothetical, it is possible that the synthesis of gelatinase continues after lactoferrin synthesis has ceased. The content of different peroxidase-negative granules could therefore simply reflect the actual proteins that are synthesized at the time of granule formation. Furthermore, the possibility exists that a constant synthesis of both Mac-1 and cytochrome b_{558} occurs during the time of specific granule formation and later during gelatinase granule formation. This would result in an equal density of cytochrome b_{558} and Mac-1 in the membrane of gelatinase granules and specific granules, respectively, but given the lower number and smaller size/surface of gelatinase granules, the total content of these two proteins will be much less in these granules than in specific granules.

The biologic significance of storing Mac-1 and cytochrome b_{558} in three different secretory organelles is very intriguing. We have recently shown that a strict hierarchy exists regarding both calcium sensitivity and kinetics of mobilization of the different organelles within the neutrophil.[34] Secretory vesicles are mobilized faster and at lower cytosolic Ca^{2+} levels than gelatinase granules, which again are mobilized faster and at lower cytosolic Ca^{2+} levels than specific granules. This could imply that secretory vesicles are rapidly exocytosed, as the neutrophil reaches inflamed endothe-

lium, leading to upregulation of surface Mac-1 and subsequent firm adhesion of the cell. Later, in the inflammatory response, a further upregulation of Mac-1 from gelatinase granules ensures a persistent firm attachment of the neutrophil,[35] with a concomitant secretion of gelatinase, allowing the cell to traverse the basement membrane of the endothelium. The parallel partial translocation of cytochrome b_{558} and subsequent activation of the NADPH-oxidase could be of importance in activation of latent gelatinase before diapedesis,[36] and could mediate expression of ICAM-1 on the surface of the endothelial cells.[37] Considering the harmful effects to the host of reactive oxygen species, it seems reasonable to store the majority of cytochrome b_{558} in specific granules, because specific granules are unlikely to be mobilized by weak stimuli involved in neutrophil adhesion and diapedesis.

The use of the three-layer Percoll density gradient presented here makes it possible to further characterize the structure and content of gelatinase granules with the potential of elucidating the mechanisms underlying the graded exoytosis of peroxidase-negative granule subpopulations.

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