Subcellular Localization and Dynamics of Mac-1 ($\alpha_m\beta_2$) in Human Neutrophils

Henrik Sengeløv,* Lars Kjeldsen,* Michael S. Diamond,‡ Timothy A. Springer,‡ and Niels Borregaard*
**Granulocyte Research Laboratory, Department of Hematology L, University Hospital, Rigshospitalet, DK-2100 Copenhagen, Denmark; and ‡The Center for Blood Research, Harvard Medical School, Boston, Massachusetts 02115*

Abstract

The subcellular localization of Mac-1 was determined in resting and stimulated human neutrophils after disruption by nitrogen cavitation and fractionation on two-layer Percoll density gradients. Light membranes were further separated by high voltage free flow electrophoresis. Mac-1 was determined by an ELISA with monoclonal antibodies that were specific for the α-chain (CD11b). In unstimulated neutrophils, 75% of Mac-1 colocalized with specific granules including gelatinase granules, 20% with secretory vesicles and the rest with plasma membranes. Stimulation with nanomolar concentrations of FMLP resulted in the translocation of Mac-1 from secretory vesicles to the plasma membrane, and only minimal translocation from specific granules and gelatinase granules. Stimulation with PMA or Ionomycin resulted in full translocation of Mac-1 from secretory vesicles and gelatinase granules to the plasma membrane, and partial translocation of Mac-1 from specific granules. These findings were corroborated by flow cytometry, which demonstrated a 6–10-fold increase in the surface membrane content of Mac-1 in response to stimulation with FMLP, granulocyte-macrophage colony stimulating factor, IL-8, leukotriene B_4, platelet-activating factor, TNF-α, and zymosan-activated serum, and a 25-fold increase in response to Ionomycin. Thus, secretory vesicles constitute the most important reservoir of Mac-1 that is incorporated into the plasma membrane during stimulation with inflammatory mediators. (*J. Clin. Invest.* 1993. 92:1467–1476.) Key words: secretory vesicles • gelatinase granules • specific granules • CD11b • free-flow electrophoresis

Introduction

Human neutrophils constitute the primary mobile cellular defense against intruding microorganisms. Essential components of their defense mechanisms are stored in membranes of intracellular mobilizable granules and recruited to the surface during degranulation (1). Mac-1, the most abundant of the β_2 integrins in neutrophils, is essential for neutrophil adherence to endothelium, chemotaxis, and diapedesis, as well as phagocytosis of complement opsonized objects (2–5). Previous reports have demonstrated that the majority of Mac-1 in resting cells is localized in membranes of mobilizable intracellular granules or vesicles (6, 7). FACS® analysis (Becton Dickinson Immunocytometry Systems, Mountain View, CA) has demonstrated that the Mac-1 content in plasma membranes increases considerably when neutrophils are manipulated during isolation from blood, and by stimulation of neutrophils with inflammatory mediators such as FMLP, leukotriene B_4 (LTB_4)[1], TNF-α, and C5a (8). Paradoxically, subcellular fractionation data have indicated that Mac-1 is localized in specific granules (6), although these granules are not mobilized to any significant degree by the manipulations that apparently result in full upregulation of Mac-1 in the plasma membrane (9). Thus, it has been debated whether more easily mobilizable intracellular organelles exist that may furnish the surface membrane with adhesion proteins. We have discovered such a compartment, the secretory vesicle, which is identified by latent alkaline phosphatase and plasma proteins (10, 11). Secretory vesicles have recently been separated from plasma membranes (12), and their role in neutrophil activities can now be investigated.

Gelatinase granules have been claimed to exist as a separate entity distinct from specific granules (13). On the other hand, a later study showed colocalization between the specific granule marker lactoferrin and gelatinase by immunoelectron microscopy (14). The reason for these contradictory observations may be that neutrophil gelatinase, previously considered pure, is in part a complex of 92 kD gelatinase and a novel 25-kD protein termed neutrophil gelatinase–associated lipocalin (NGAL), giving rise to a 135-kD form gelatinase in addition to the 92-kD form (15). Antibodies raised against "pure" gelatinase recognized both gelatinase and NGAL, but affinity purification resulted in monospecific antibodies against gelatinase and NGAL, respectively. Using affinity-purified antigelatinase antibodies, we have demonstrated that gelatinase is mainly localized in granules with a slightly lower density than lactoferrin-containing specific granules, and that gelatinase is mobilized more readily than lactoferrin (Kjeldsen, L., D. F. Bainton, H. Sengeløv, and N. Borregaard, manuscript submitted for publication, and reference 16). On the contrary, NGAL was found to colocalize with lactoferrin in specific granules, and only a small fraction of NGAL is complexed with gelatinase in granules with a density and mobility intermediary between specific granules and gelatinase granules (Kjeldsen, L., D. F. Bainton, H. Sengeløv, and N. Borregaard, manuscript in preparation). Having introduced secretory vesicles and gelatinase granules as highly mobilizable granules, we found it relevant to readdress the question of the intracellular reservoir of Mac-1 in human neutrophils and developed an ELISA for CD11b.

Address correspondence and reprint requests to Henrik Sengeløv, M.D., Granulocyte Research Laboratory, Department of Hematology L-4041, Rigshospitalet, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark.

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1. *Abbreviations used in this paper:* CTAB, cetyltrimethylammonium bromide; KRP, Krebs-Ringer phosphate; LTB_4, leukotriene B_4; MFI, mean fluorescence intensity; NGAL, neutrophil gelatinase-associated lipocalin; PMA, phorbol myristate acetate; ZAS, zymosan-activated serum.

Methods

Human neutrophils were isolated from freshly drawn blood as previously described (1). Sedimentation of red cells was induced by Dextran T-500 (Pharmacia, Uppsala, Sweden), and the leukocyte-rich supernatant was centrifuged (Lymphoprep; Nygaard, Oslo, Norway). Residual erythrocytes were lysed by hypotonic shock, and the neutrophils were resuspended in buffer as indicated. All procedures were carried out at 4°C, except for the sedimentation of red cells.

Stimulation of neutrophils was performed at 37°C at a cell concentration of 3×10^7 cells/ml in Krebs-Ringer phosphate (KRP): 130 mM NaCl, 5 mM KCl, 1.27 mM $MgSO_4$, 0.95 mM $CaCl_2$, 5 mM glucose, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4. Stimulation was initiated after a 5-min preincubation period and terminated after 15 min by dilution with 2 vol of ice-cold buffer and centrifugation at 200 *g* for 10 min. The pelleted cells were resuspended in ice-cold buffer, and samples were taken for FACS® analysis, determination of granule markers, and subcellular fractionation. Release of granule content was determined as marker protein present in supernatant as a percent of marker protein present in supernatant plus pellet.

Subcellular fractionation was performed as previously described (1). In short, neutrophils were resuspended in 15 ml disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 0.5 mM PMSF, 10 mM Pipes, pH 7.2) at 3×10^7 cells/ml and disrupted by nitrogen cavitation. Nuclei and unbroken cells were sedimented by centrifugation at 400 *g* for 15 min (P_1), and 10 ml of the postnuclear supernatant (S_1) was loaded on top of a 28-ml two-layer Percoll density gradient (1.05/1.12 g/ml) and centrifuged as described (1). This resulted in generation of four separate regions that could be visually identified in the gradient: the bottom band (α band) containing azurophil granules, the intermediate band (β band) containing specific granules and gelatinase granules, the top band (γ band) containing plasma membranes and secretory vesicles, and the clear supernatant (S_2) containing cytosol. Gradients were aspirated from the bottom through a peristaltic pump attached to a fraction collector set to deliver 1.4 ml in each fraction.

Separation of plasma membranes from secretory vesicles was performed as described (12). In short, Percoll was removed from the γ band by ultracentrifugation. The γ band was resuspended in chamber buffer (270 mM sucrose, 5 mM triethanolamine, 5 mM acetic acid, pH 7.4, conductivity: 0.42 $m\Omega^{-1}$), and treated with neuraminidase to modify the charge of plasma membrane vesicles. The material was then applied to free-flow electrophoresis at 5°C on an Elphor Vap 22 from Bender & Hobein (Munich, Germany). It was ascertained that neuraminidase treatment did not interfere with detection of Mac-1 by ELISA.

Marker proteins. Azurophil granules were identified by myeloperoxidase measured by ELISA using rabbit antimyeloperoxidase antibody (A 398; Dakopatts; Glostrup, Denmark) as catching antibody diluted 1:10,000. The same antibody was biotinylated at 1 mg/ml (17) and was used as detecting antibody diluted 1:10,000. Myeloperoxidase, purified from isolated azurophil granules, was used as standard. Specific granules were identified by lactoferrin measured by ELISA (16) and by vitamin B_{12}-binding protein (1). Gelatinase granules were identified by gelatinase and measured by ELISA (18). Secretory vesicles were identified by alkaline phosphatase (19) that could be measured only in the presence of 0.2% (vol/vol) Triton X-100 (latent alkaline phosphatase) (10, 20) and by albumin measured by ELISA (11). Plasma membranes were identified by HLA class I assayed by a mixed ELISA (21) and by alkaline phosphatase activity measured in the absence of detergent (nonlatent alkaline phosphatase) (10).

Quantitation of Mac-1 was done by ELISA: CBRM1/23, an IgG2a mouse monoclonal antibody against the COOH-terminal extracellular region of the α chain of Mac-1 (22) was purified from ascites fluid by protein A Sepharose affinity chromatography, diluted 1:1,000 from a 2.3 mg/ml stock, and used for coating immunoplates. LM2/1, an IgG1 mouse monoclonal antibody against the I domain of the α chain of Mac-1 (22, 23) was purified from ascites fluid by protein A Sepharose affinity chromatography and biotinylated at a concentration of 1 mg/ml (17). This was used as detecting antibody at a 1:1,000-fold dilution. Serial dilutions of γ band isolated from neutrophils stimulated with 2 μg/ml phorbol myristate acetate (PMA) at 37°C for 15 min were used as standards. A stock of γ band from 5×10^8 cells in disruption buffer (0.9 mg/ml protein) was prepared and used for all experiments. This stock was diluted 16-fold in buffer B (see below) and used as the highest standard, defined as 64 arbitrary U/ml. Samples for CD11b ELISA were solubilized by addition of 25 μl 250 mM *N*-octyl glucoside (Sigma Chemical Co., St. Louis, MO), and 25 μl 2% cetyltrimethylammonium bromide (CTAB) (BDH Chemicals LTC, Poole, United Kingdom) to 200 μl sample followed by incubation on ice for 1 h. The samples were then diluted with 750 μl 500 mM NaCl, 3 mM

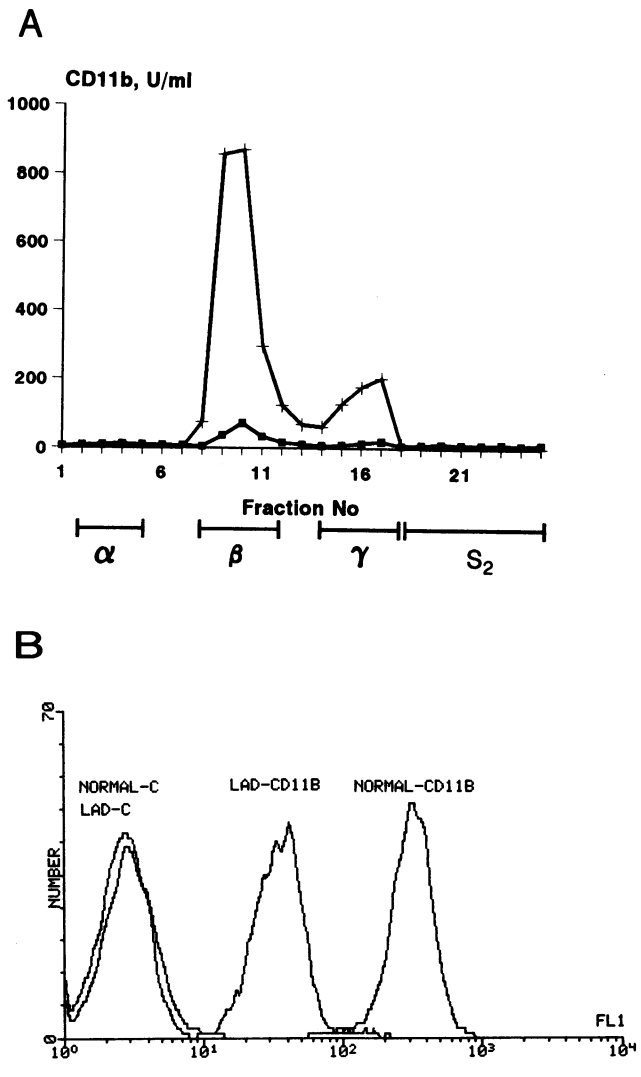


Figure 1. Mac-1 in normal and LAD neutrophils. CD11b in normal (3×10^8 cells) and LAD (2.25×10^8 cells) neutrophils was determined by ELISA in subcellular fractions (*A*) and by FACS® analysis on intact cells (*B*). In *A*, the localization of the gradient bands are depicted below the figure. The α band contains azurophil granules, the β band contains specific and gelatinase granules, and the γ band contains secretory vesicles and plasma membrane, S_2 is cytosol. Normal control, – + –; LAD patient, – ■ –. Abscissa in B is log. fluorescence intensity. The cells were stimulated with 10^{-8} M FMLP before FACS® analysis to optimize detection of surface bound Mac-1. C denotes isotype-specific nonimmune control antibody. Exocytosis of albumin, gelatinase and lactoferrin from LAD neutrophils in response to FMLP was normal (not shown).

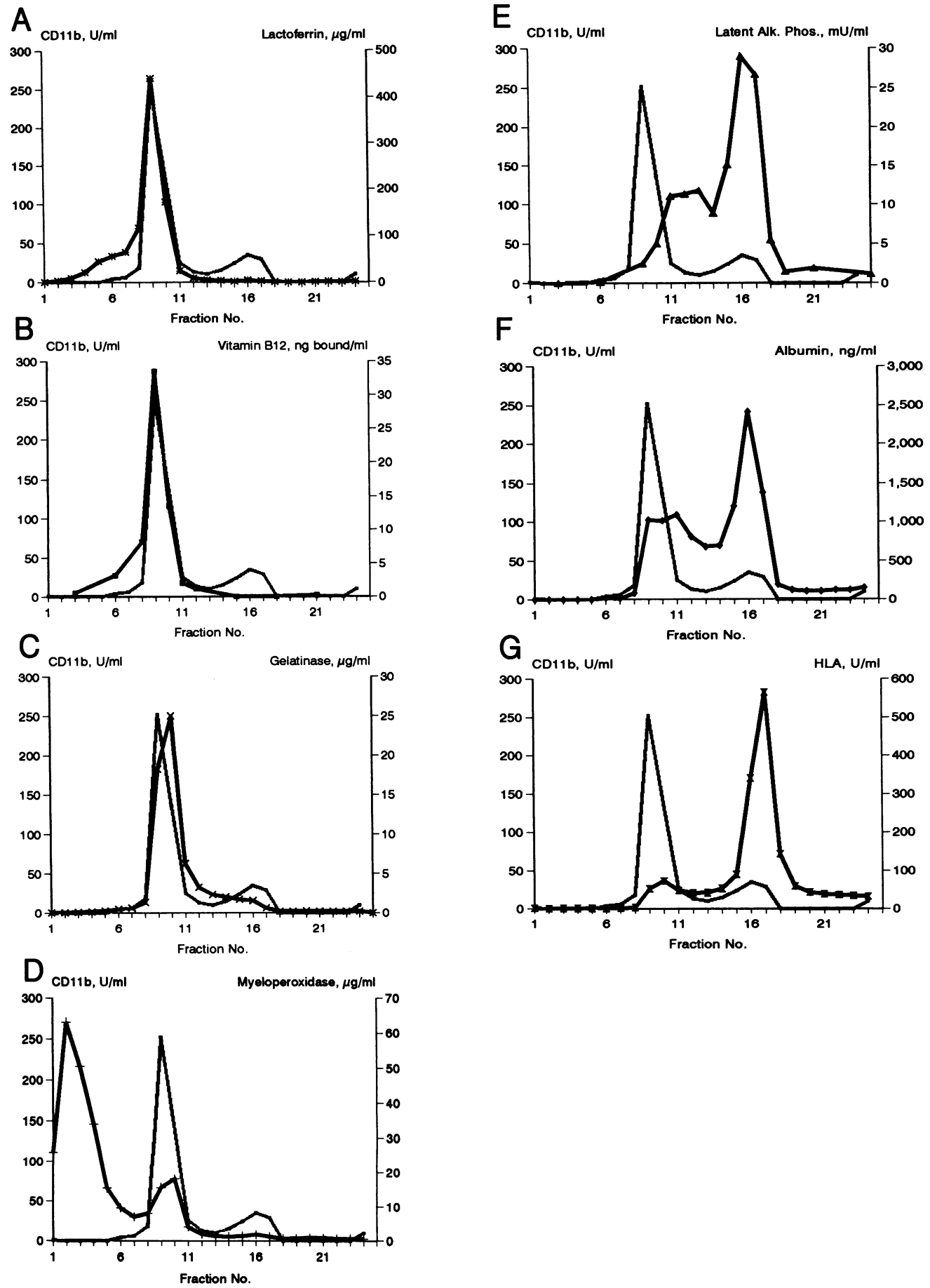
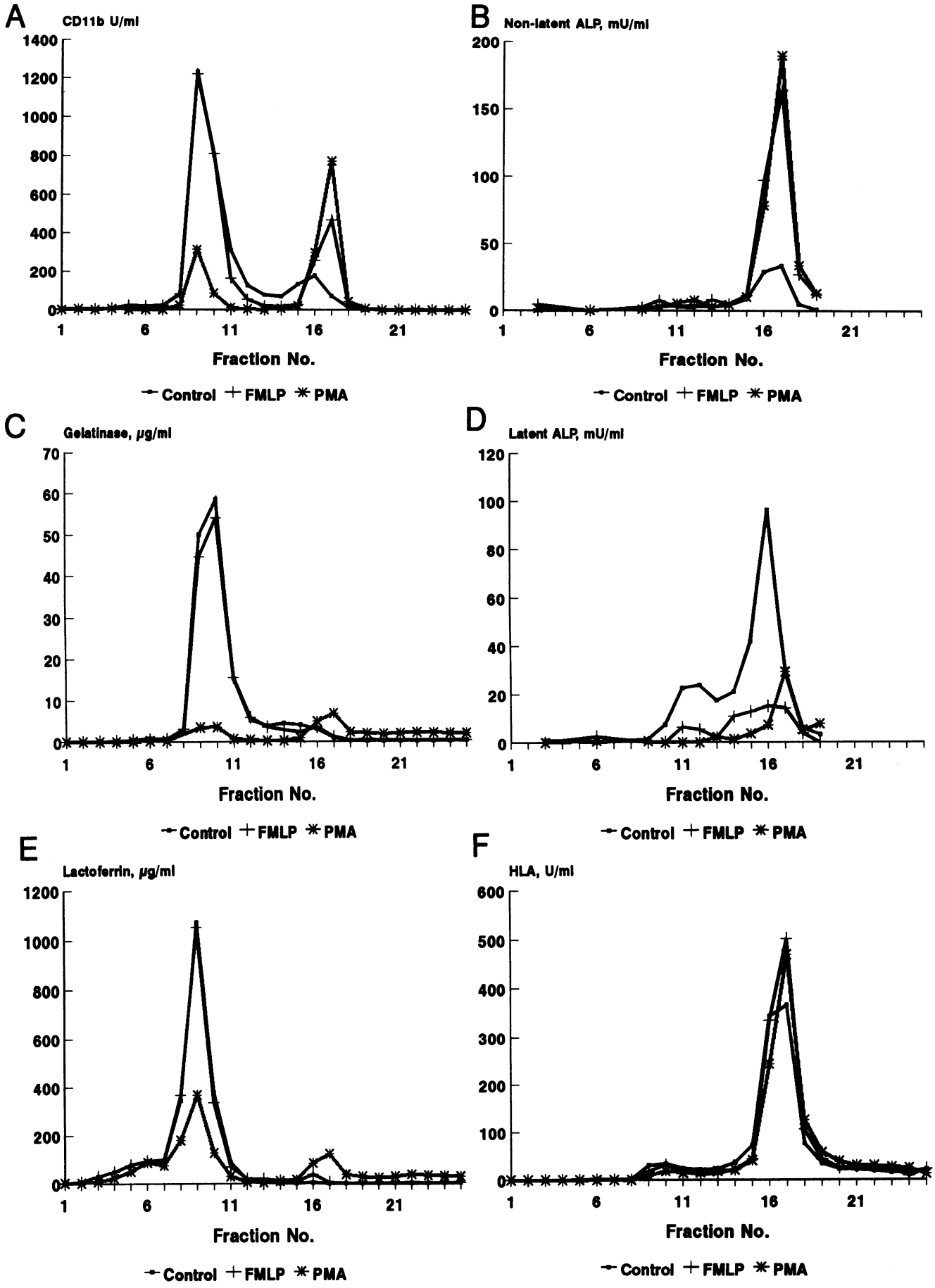


Figure 2. Subcellular profile of Mac-1. Subcellular distribution of CD11b and markers for specific granules (*A* and *B*), gelatinase granules (*C*), azurophil granules (*D*), secretory vesicles (*E* and *F*), and plasma membranes (*G*). Assays are from a single, representative fractionation (out of five) of unstimulated neutrophils held at 4°C. CD11b is shown for comparison in each profile (*gray line*) to other markers (*black line*).


A
CD11b U/ml
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Fraction No.
Control FMLP PMA
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Non-latent ALP, mU/ml
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Gelatinase, µg/ml
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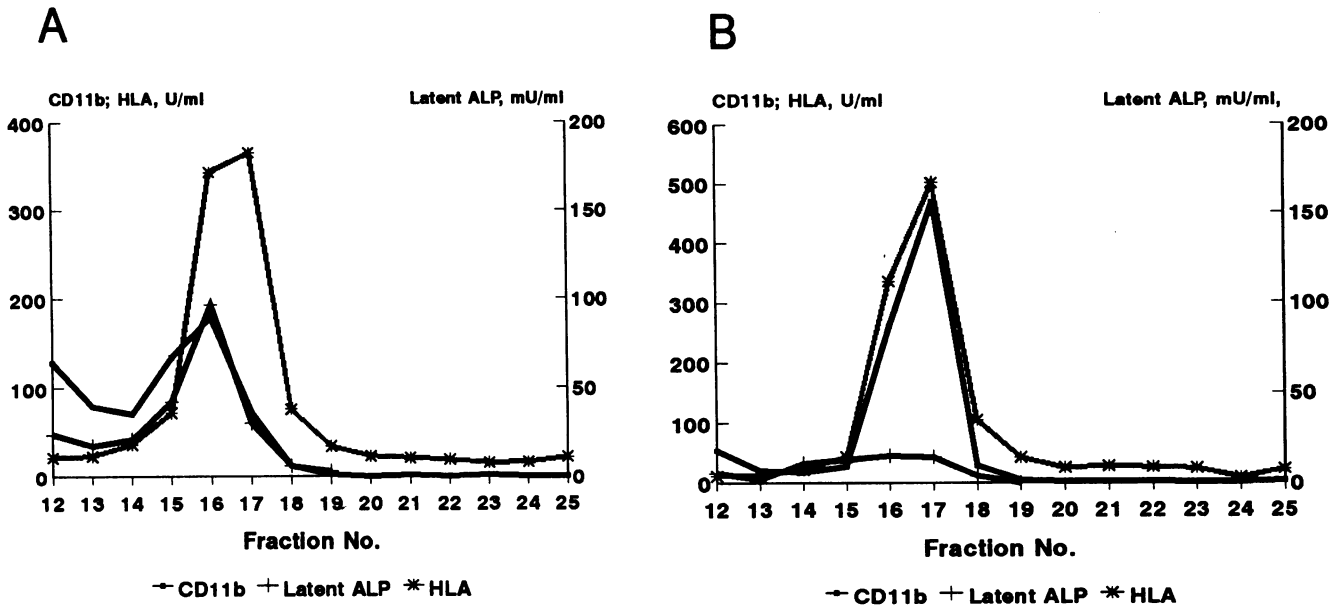

Figure 4. Mac-1 in light membranes. Subcellular localization of CD11b and markers for secretory vesicles (latent alkaline phosphatase) and plasma membranes (HLA) was determined in control cells (*A*) and FMLP-stimulated cells (*B*). Same data as in Fig. 3.

KCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) BSA (Sigma), 25 mM *N*-octyl glucoside, 0.2% CTAB, 8 mM Na_2HPO_4/KH_2PO_4, pH 7.2, incubated overnight in the cold and then applied to 96-well immunoplates (Nunc, Roskilde, Denmark) that had been coated with catching antibody and blocked as described below.

ELISA was performed using the same general procedure independent of the antibodies except as specified in previous publications regarding mixed ELISA (21) and lactoferrin (16) where avidin-biotin interaction was not used. Immunoplates were coated with 100 μl catching antibody, diluted in 50 mM Na_2HCO_3/NaH_2CO_3, pH 9.6, and incubated overnight at room temperature. The wells were washed in buffer A (500 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4/KH_2PO_4, pH 7.2, 1% Triton X-100). Additional binding sites were blocked by incubating with 200 μl buffer B (500 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4/KH_2PO_4, pH 7.2, 1% Triton X-100, 1% BSA) for 1 h. After washing three times in buffer A, 100 μl sample was applied along with serial dilutions of standard and incubated for 1 h. All samples and standards were diluted in buffer B (except for quantitation of CD11b as described above). After three additional washes in buffer A, 100 μl biotinylated catching antibody was applied, appropriately diluted in buffer B, and incubated for 1 h followed by washing three times in buffer A. 100 μl peroxidase-conjugated avidin (P347; Dakopatts) in buffer B was then added and incubated for 1 h followed by washing three times in buffer A and once in 100 mM Na_2HPO_4/NaH_2PO_4, 100 mM sodium citrate, pH 5.0. Color developed during a 30-min incubation in sodium phosphate citric acid buffer containing 0.04% (wt/vol) *o*-phenylenediamine and 0.03% H_2O_2, and was stopped by addition of 100 μl 1 M H_2SO_4. Absorbance was read at 492 nm in an automatic ELISA reader (Multiscan Plus; Labsystems, Helsinki, Finland).

FACS® analysis. Isolated neutrophils, either kept on ice, at 37°C or stimulated at 37°C as indicated, were fixed at 3×10^7 cells/ml in 2% paraformaldehyde, 0.05% glutaraldehyde in PBS for 15 min at 4°C, washed twice in PBS containing 0.5% (wt/vol) BSA. Labeling of cells was performed by incubating 25 μl cells at 3×10^7/ml in PBS containing 0.5% BSA with 50 μl monoclonal antibody (LM2/1 or anti-HLA class 1 (Dakopatts M736)) or murine preimmune IgG1 (Becton-Dickinson Immunocytometry Systems) for 1 h at 4°C. After washing twice, 50 μl fluorescein-conjugated rabbit anti-mouse antibody (Dakopatts) was added, and the cells were incubated for 30 min at 4°C. After two additional washes, the cells were resuspended in PBS containing 1% formaldehyde and analyzed in a FACScan® (Becton-Dickinson Immunocytometry Systems). Mean fluorescence intensity for specific antibodies was corrected for nonspecific fluorescence by subtracting mean fluorescence for preimmune IgG1.

Source of stimuli. FMLP (Sigma) 1 mM in ethanol. Granulocyte macrophage–colony stimulating factor (Sandoz Pharmaceuticals, East Hanover, NJ) 10^4 U/ml in H_2O. rIL-8 (a generous gift from Dr. K. Thestrup-Pedersen, Department of Dermatology, Marselisborg Hospital, Aarhus, Denmark) 10 μg/ml in PBS. Ionomycin (Calbiochem Corp., La Jolla, CA) 0.1 mM in DMSO. LTB_4 (Sigma) 1 mM in ethanol. Platelet-activating factor (Sigma) 1 mM in PBS 0.5% (wt/vol) HSA (Sigma). TNF (Amersham International, Amersham, United Kingdom) 10^4 U/ml in H_2O.

Results

A sandwich ELISA was developed for Mac-1 using two monoclonal antibodies that localized to epitopes on distinct regions of Mac-1 α chain (CD11b): CBRM1/23, which binds to the COOH-terminal segment, and LM2/1, which binds to the I domain (22, 23). Using this assay, we investigated the subcellular localization of Mac-1 in human neutrophils. To validate the specificity of the assay, CD11b was measured in fractions of

Figure 3. Dynamics of Mac-1. Effect of stimulation with FMLP (10^{-8} M) or PMA (2 μg/ml) on: the subcellular localization of CD11b (A), nonlatent alkaline phosphatase (membrane incorporated into the plasma membrane from secretory vesicles) (*B*), gelatinase granules (*C*), latent alkaline phosphatase (secretory vesicles) (D), lactoferrin (specific granules) (E), and HLA (plasma membrane) (*F*). Neutrophils from two normal donors were pooled, resuspended in KRP containing 1 mM sodium azide and 2,000 U/ml catalase (bovine liver; Sigma), and divided in three. One part was kept on ice (control) one part was stimulated with 10^{-8} M FMLP at 37°C, and one part was stimulated with 2 μg/ml PMA at 37°C before subcellular fractionation. The results of a single experiment, representative of three, is given.

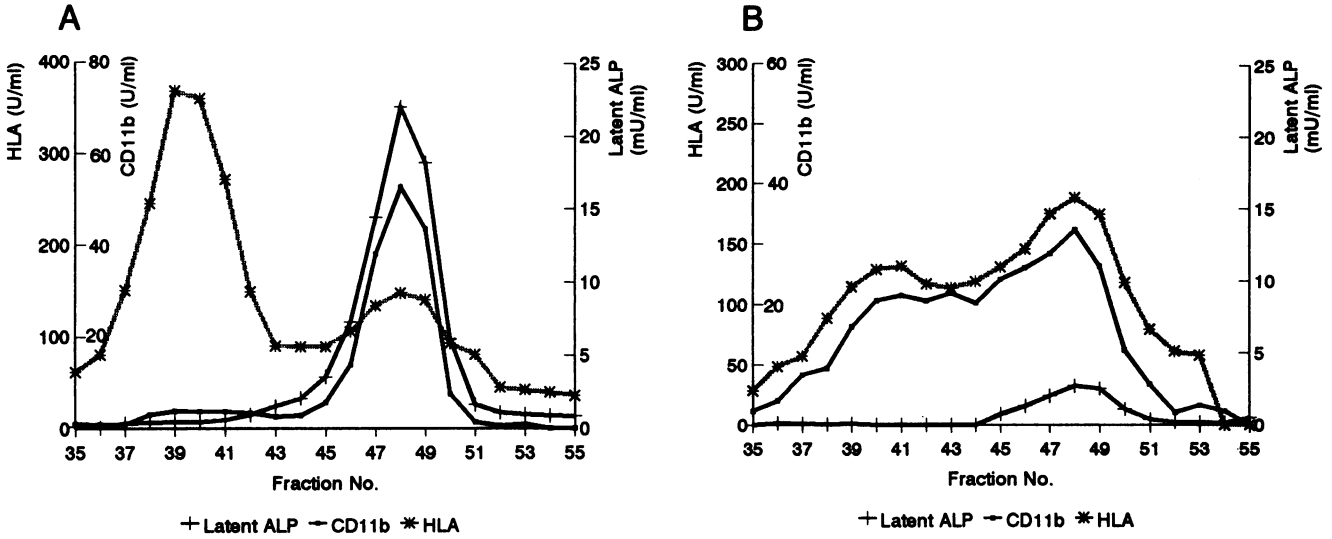

Figure 5. Free-flow electrophoresis. Membranes from the γ band of 4°C control neutrophils (*A*) and neutrophils stimulated with FMLP (10^{-8} M) at 37°C (*B*) were subjected to free flow electrophoresis. Material from 3×10^8 cells was used for each condition.

neutrophils from a patient with the less severe form of leukocyte adhesion deficiency (24–26). Fig. 1 A shows that in this patient, only 5% of the normal amount of CD11b was found, which agrees well with immunofluorescence studies (Fig. 1 *B*). Thus, the ELISA is specific for CD11b.

The major peak of Mac-1 is located in the β band, which contains both gelatinase and lactoferrin (Fig. 2). We confirm here the separation of peak lactoferrin and peak gelatinase that has been established before (16, 27). As seen in Fig. 2, we found that Mac-1 had an intermediate distribution among granules that contained lactoferrin and gelatinase, but mainly colocalized with lactoferrin in specific granules of the β band. In addition, Mac-1 was found in a second broad peak in the γ band colocalizing with latent alkaline phosphatase and albumin, both markers of the most rapidly and extensively mobilizable compartment, secretory vesicles (11), but with a different peak profile than the plasma membrane marker, HLA class I.

The resolution between organelles by subcellular fractionation is improved by examining the dynamics of the organelles in response to stimulation. Therefore, we studied the translocation of Mac-1 from intracellular membranes to the plasma membrane under conditions that discriminate between mobilization of secretory vesicles, gelatinase granules, and lactoferrin granules (Fig. 3). Mac-1 is translocated from intracellular membranes to the plasma membrane in response to FMLP, which mobilizes secretory vesicles almost completely, but only results in 15–25% release of gelatinase and < 3% release of lactoferrin (16), and in response to PMA, which results in complete mobilization of secretory vesicles and gelatinase granules, and almost complete mobilization of specific granules (1, 16, 20, 28). After stimulation with FMLP, Mac-1 redistributes within the region of the gradient that contains the light membranes (secretory vesicles and plasma membranes).

This is more easily observed in Fig. 4, which displays the fractions containing light membranes (fractions 12–25). In unstimulated cells, the Mac-1 profile colocalizes with latent alkaline phosphatase, the marker for secretory vesicles, in the light membrane region (Fig. 4 *A*), but not entirely with the profile of plasma membrane marker, HLA. FMLP stimulation promotes translocation of alkaline phosphatase from secretory vesicles to the plasma membrane with corresponding loss of latency (Fig. 3, *B* and *D*). Mac-1 also translocates in a corresponding manner and now colocalizes completely with the plasma membrane marker (Fig. 3 *A* and Fig. 4 *B*). The contribution of membrane from secretory vesicles to the plasma membrane is illustrated in Fig. 3 *B*, which gives the increase in nonlatent alkaline phosphatase present in the plasma membrane as a result of stimulation by FMLP and PMA. The alkaline phosphatase level in the plasma membrane increases fourfold after FMLP stimulation, and no further increase is observed after PMA stimulation, since secretory vesicles are almost fully mobilized by FMLP. Tailing of gelatinase (Fig. 3 *C*) was still present in fractions 13–15, even though Mac-1 had disappeared after stimulation by FMLP, excluding these granules as a major store of light membrane Mac-1. Thus, mobilization of secretory vesicles can account for most of the increase in plasma membrane CD11b content that is induced by stimulation with FMLP. The separation of secretory vesicles and plasma membranes can be greatly improved by combining density gradient centrifugation with high voltage free-flow electrophoresis. This modality takes advantage of differences in charge that can be selectively induced in plasma membrane vesicles by treatment with neuraminidase (12). Using this procedure, we obtained an almost total separation of plasma membranes and secretory vesicles (Fig. 5 *A*). It is clearly seen that very little Mac-1 is associated with the plasma membrane in unstimulated cells, and that the major part of Mac-1 colocalizes with latent alkaline phosphatase in secretory vesicles. In contrast, complete colocalization of the plasma membrane marker HLA with Mac-1 is seen in the γ band from FMLP-stimulated cells (Fig. 5 *B*).

To discriminate between colocalization of Mac-1 with gelatinase granules and lactoferrin granules, the translocation of Mac-1 to the plasma membrane in response to stimulation with PMA was determined (Fig. 3). The disappearance of Mac-1 from the β band (85%, median of three experiments, range 82–89%) was intermediate between the disappearance of lactoferrin (63%, median of three experiments, range 47–70%)

and the disappearance of gelatinase (93%, median of three experiments, range 89–95%). Not all Mac-1 that disappeared from the β band was recovered in the plasma membranes of the γ band. This may be caused by a change in the epitopes of the CD11b molecule by exposure to reactive oxygen species and proteases. However, inclusion of azide and catalase increased the recovery of CD11b in the plasma membrane considerably but was unable to completely protect the molecule. Ionomycin, another potent secretagogue, also induced translocation of Mac-1 from the β band to the γ band (data not shown). Although ionomycin does not activate the plasma membrane bound NADPH oxidase (29, 30), only 70% of Mac-1 that disappeared from the β band was recovered in the γ band. Other yet unclarified mechanisms must be responsible for the decrease in Mac-1 immunoreactivity after translocation to the neutrophil surface induced by potent stimuli. These observations on the subcellular localization and mobilization of Mac-1 were corroborated by flow cytometry as shown in Fig. 6, which gives the upregulation of Mac-1 in the surface membrane of intact cells in response to stimulation with a variety of inflammatory mediators. FMLP, GM-CSF, LTB_4, IL-8, PAF, TNF-α, and zymosan-activated serum (C5a) result in a 6–10-fold increase in the surface membrane content of Mac-1 and a corresponding almost complete mobilization of secretory vesicles (release of albumin), but little release of gelatinase and no release of lactoferrin. Ionomycin results in a 25-fold increase in the plasma membrane content of Mac-1 consistent with incorporation of the specific granule membrane pool of Mac-1 into the plasma membrane. In contrast, PMA stimulation did not significantly increase the amount of Mac-1 detectable on the surface of the cells beyond what is seen after FMLP stimulation, despite significant mobilization of specific granules. This is probably a consequence of both inactivation of Mac-1 as also observed in subcellular fractionation, and folding and invagination of the plasma membrane, which makes the epitopes inaccessible to the antibody. The latter is supported by the significant reduction in detectable HLA observed by FACS®, but not by ELISA after stimulation with PMA.

Discussion

Defense against microorganisms depends on the ability of neutrophils to stick to endothelium, migrate through it, phagocytose, and kill microorganisms by mechanisms that are both toxic and largely unspecific. On the other hand, integrity of the vascular bed depends on the ability of neutrophils to slide through the microcirculation in intimate contact with endothelium, without causing any damage to it. Storing proteins, active in mediating both adherence, phagocytosis, and generation of toxic oxygen species, in the membrane of mobilizable intracellular organelles, seems teleologically wise. The existence of four separate mobilizable intracellular organelles (10, 11, 16, 27), each with a distinct membrane composition, and with a strict hierarchy of mobilization (31), ties neutrophil activation tightly to control of exocytosis. Thus, it is important to elucidate both the mechanisms that mediate the selective mobilization of these granules and vesicles, and the contribution that each granule subset makes to the functional capacity of the plasma membrane. By combining subcellular fractionation of unstimulated cells with high voltage free-flow electrophoresis and with fractionation of cells that have been stimulated to selectively mobilize individual granule subsets, a high degree of resolution between granules with differences in density or mobilization can be achieved.

Using this approach, we have identified the intracellular location of Mac-1 as the membranes of specific granules, gelatinase granules, and secretory vesicles. Stimulation of neutrophils with inflammatory mediators results in incorporation of Mac-1 from secretory vesicles into the plasma membrane, and this accounts for most of the increase in plasma membrane Mac-1.

Clearly, not all intracellular Mac-1 is mobilized to the plasma membrane by FMLP stimulation of the cells. This is in contrast to data previously published by us based on FACS® analysis of fixed permeabilized versus unpermeabilized cells (8). The reason for this discrepancy most likely is the failure of the permeabilization procedure used to make the intragranular Mac-1 fully available to antibody. This was also an initial problem in this study, until the solubilization protocol combining *N*-octyl glucoside and CTAB was introduced. Failure to completely solubilize the membrane of specific granules may also have been a problem in other studies in which the subcellular localization of Mac-1 was determined by immunoprecipitation (32–36). Since secretory vesicles are more readily solubilized than specific granules (Sengeløv, H., unpublished observation), incomplete solubilization will result in relative overestimation of the Mac-1 present in the γ and pre-γ regions that contain secretory vesicles (32, 33).

The intracellular localization of Mac-1 has been the subject of much debate. The issue has been whether Mac-1 is localized in gelatinase, so called "tertiary," granules or in specific granules. Previous ultrastructural examination has demonstrated 80% colocalization of gelatinase and lactoferrin (14). However, the discovery of NGAL as a component of gelatinase casts some doubt on the specificity of the antibodies used to detect gelatinase. Subcellular fractionation studies reporting colocalization of Mac-1 and gelatinase have made use of a gelatinolytic assay for gelatinase (32–37). This assay, in which the enzyme has to be activated in the presence of other proteases, is problematic since it is not ascertained that all gelatinase is activated and none is inactivated. Using an ELISA for quantitation of gelatinase, these problems are avoided (16, 18). Furthermore, the above mentioned studies (32–37) have not taken into account the existence of secretory vesicles, and have therefore overlooked the most readily available pool of intracellular Mac-1.

It is apparent from our studies (Kjeldsen, L., D. F. Bainton, H. Sengeløv, and N. Borregaard, manuscript submitted for publication, and reference 16.) that gelatinase does not colocalize with lactoferrin. Our subcellular fractionation data indicate that Mac-1 is present in the membranes of both gelatinase and lactoferrin granules.

We conclude that secretory vesicles are the most important reservoir of Mac-1 that is mobilized to the plasma membrane during stimulation of neutrophils with inflammatory mediators. In the interplay between endothelium and neutrophils, which is most pronounced in venules (4), it is critical that mobilization of Mac-1 takes place rapidly, and before the neutrophil leaves this vascular bed, otherwise, the neutrophil will not adhere firmly to inflamed endothelium, but will arrive activated to the capillary bed of the lungs. Secretory vesicles, by far the most rapidly and extensively mobilized organelle of neutrophils, seem well-suited as a store of readily mobilizable Mac-1.

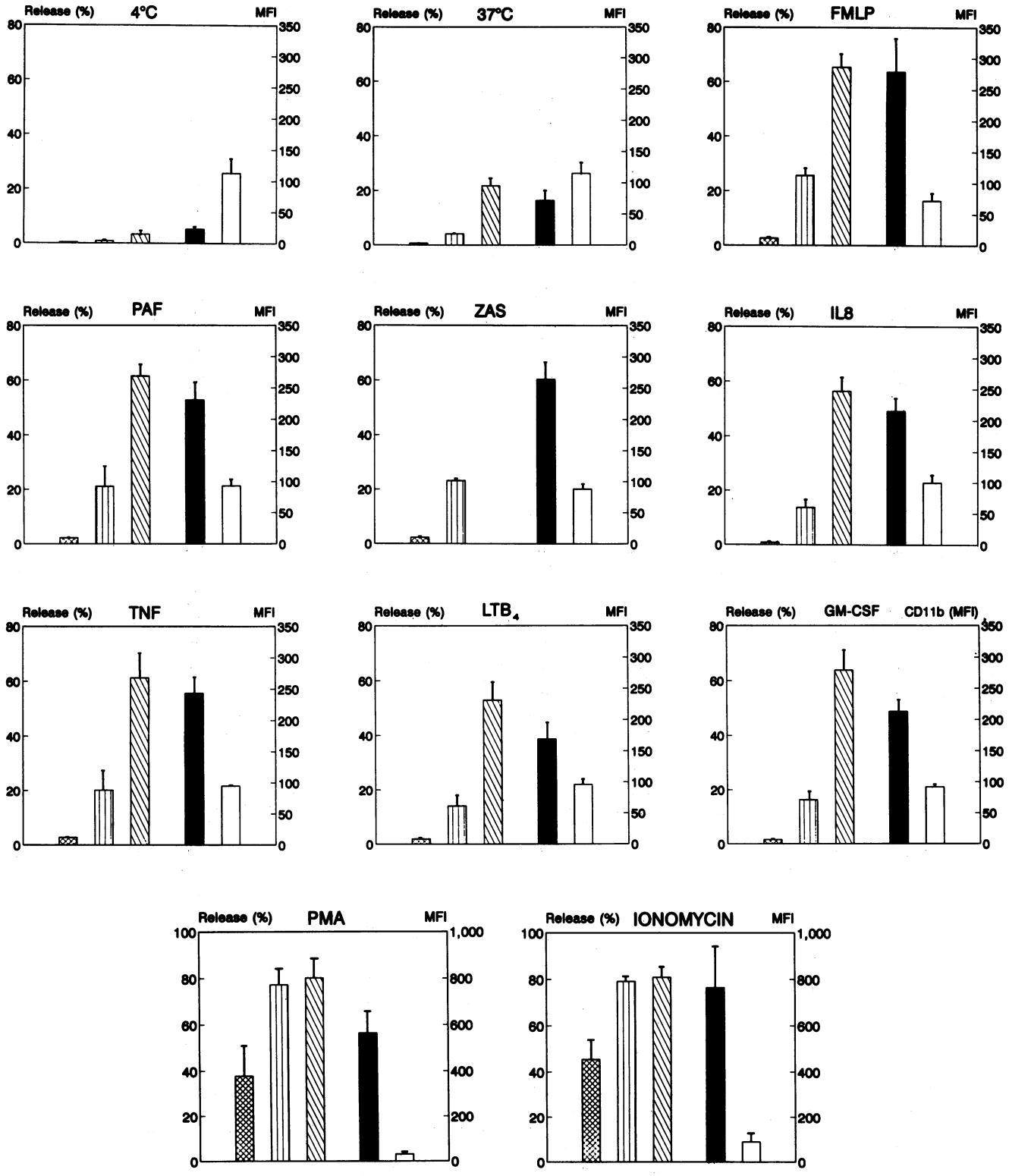

Figure 6. Upregulation of Mac-1 on intact neutrophils. Normal neutrophils were isolated, resuspended in KRP, and divided. Some were kept on ice (4°C) others incubated at 37°C with or without stimuli (10^{-8} M FMLP, 100 U/ml granulocyte macrophage–colony stimulating factor, 0.2 μg/ml IL-8, 1 μM Ionomycin, 10^{-8} M LTB_4, 10^{-8} M PAF, 2 μg/ml PMA, 500 U/ml TNF, and 5% zymosan-activated serum [*ZAS*]) as indicated. Upregulation of surface bound CD11b detected by LM2/1 was determined by FACS® analysis, and mean fluorescence intensity (*MFI*) is shown in combination with simultaneously determined release of granule markers. HLA class I was quantitated by FACS® on the same cells and given as MFI. Cells kept on ice displayed a small and variable subset of cells with apparent upregulation, observed only upon fixation. These were gated away before determination of MFI. *Double hatched bars,* lactoferrin release; *lined bars,* gelatinase release; *single-hatched bars,* albumin release; *solid bars,* MFI for CD11b; *open bars,* MFI for HLA. Albumin release could not be determined after ZAS stimulation because of the presence of albumin in ZAS. Note the different axis scale in the Ionomycin and PMA experiments. Data are given as mean (bar) + SD (error bar) of three to six experiments.

The exocytosis of gelatinase-rich granules does not contribute significantly to the enrichment of the plasma membrane in Mac-1 that takes place during stimulation with inflammatory mediators such as C5a, FMLP, IL-8, LTB_4, PAF, and TNF, but probably plays a major role during migration and diapedesis by virtue of the exocytosis of gelatinase. Two β_1 integrins (laminin receptor and fibronectin receptor), a β_2 integrin (Mac-1), and a β_3 integrin (vitronectin receptor) have been found only in specific granules by immunoelectron microscopy (38). However, Mac-1 is also present in secretory vesicles as shown in this study, and careful analysis of the surface upregulation of laminin receptors is suggestive of localization of laminin receptors in a more mobilizable compartment than specific granules (39).

In platelets, the β_3 integrin GPIIb/IIIa has been shown to be located intracellularly both in the α granules (40) and in vesicles which may be distinct from the open canalicular system (41). Translocation of GPIIb/IIIa to the platelet plasma membrane from α granules may facilitate the observed clustering of GPIIb/IIIa in areas of cell–cell interaction (42). In the platelet vesicles, GPIIb/IIIa is located together with GPIb (a β_1 integrin) (41). Interestingly, the residence and translocation of PGIIb/IIIa in platelet vesicles closely resembles the location of Mac-1 in secretory vesicles in neutrophils. This similarity probably reflects that both platelets and neutrophils are circulating cells with inherent extensive adhesive properties, which must be highly regulated.

The localization of Mac-1 in membranes of intracellular organelles with marked differences in mobilization may reflect the two different functions laid down in the same structure: the function as an integrin, important during chemotaxis and diapedesis, is best served by storing Mac-1 in the membrane of a highly mobilizable organelle as the secretory vesicles that responds to inflammatory mediators, whereas the function as receptor for C3bi is best served by storing Mac-1 in granules that are mobilized during phagocytosis, such as specific granules.

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